Benjamin Bonavida *Editor*

Nitric Oxide and Cancer: Pathogenesis and Therapy

Springer

Nitric Oxide and Cancer: Pathogenesis and Therapy

Benjamin Bonavida
Editor

Nitric Oxide and Cancer: Pathogenesis and Therapy

Springer

Editor
Benjamin Bonavida
Department of Microbiology, Immunology
and Molecular Genetics
University of California at Los Angeles
Los Angeles
California
USA

ISBN 978-3-319-13610-3 ISBN 978-3-319-13611-0 (eBook)
DOI 10.1007/978-3-319-13611-0

Library of Congress Control Number: 2015938700

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer is a brand of Springer International Publishing
Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The recent developments on understanding the challenging topic of nitric oxide (NO) and its derivatives in the field of cancer have yielded significant advances in the potential therapeutic use of NO-related donors in the fight against cancer, used either alone or in combination with other therapies to achieve synergy. The first book in this field "Nitric Oxide and Cancer: Prognosis, Prevention, and Therapy" was published in 2010 by Springer, New York. This book consisted of many reviews by authoritative scientists/ clinicians and included topics as follows: (1) The role of NO in the pathogenesis of cancer (2) The dual roles of NO in protecting or inducing cell death (3) The role of NO in metastasis (4) The chemo-immunosensitizing activities of NO (5) The prognostic significance of NO and (6) The therapeutic applications of NO. Hence, this first book provided a general introduction regarding the important role NO may play in cancer, a taboo subject that has not seriously been considered in the past.

This new book "Nitric Oxide and Cancer: Pathogenesis and Therapy" extends and adds several relevant advances that have been made in the last several years with a thorough understanding of the current status of NO in cancer and its potential therapeutic translational application in the clinic. This book has assembled contributions from experts in this field and reports on up to date reviews on novel findings on various topics of interest to both the scientific and non-scientific communities.

Part I deals with the "*Molecular cell signaling by NO in cancer*." Five contributions cover this topic. Doctors Du and Geller (University of Pittsburgh) reviewed the Wnt/β-catenin signaling pathways modulated iNOS/NO signaling in inflammation-induced carcinogenesis. They used transgenic animal models for their studies. Signals by iNOS/NO result in loss of heterozygosity of adenomatous polyposis colon (APC) and activate the Wnt/β-catenin signaling pathway and contribute to the development of cancer. In fact, inhibition of iNOS decreases the Wnt/β-catenin signaling and cancer growth. These investigators established three pathways for the interaction between iNOS and the Wnt signaling, namely (i) a positive feedback loop in which iNOS causes APC and β-catenin mutations and by Wnt-inducing iNOS expression (ii) a negative feedback between Wnt and Dichhoff-1 (DKK1) in which iNOS inhibits DKK1 gene expression and (iii) cross-regulation between the NF-κB and Wnt/β-catenin pathways through iNOS/NO genes. They suggested

that combining iNOS inhibitors with NSAIDs may synergize for more potent anti-cancer effects. Dr. Yakovlev (Virginia Commonwealth University) reviews "*Nitric Oxide and Genomic Stability*." It is well established that inflammation induces iNOS and results in the overproduction of NO and reactive nitrogen species (RNS) that participate in carcinogenesis by different mechanisms. Dr. Yakovlev discusses the NO/ RNS-dependent mechanisms of genomic instability and bystander effects. He reviews the mechanisms of "*Synthetic lethality*" of NO-RNS donor/PARP inhibitor combination in sensitizing cancer cells to DNA-mediated damage effects. Dr. Scicinski and colleagues (Mountainview, California) review "*Targeting hyponitroxia in cancer therapy.*" Hyponitroxia is a pro-neoplastic effector and they review strategies to reverse this effect by increasing NO and killing the tumor cells. NOS is inhibited due to hypoxia and stimulation under oxic conditions. They discuss attempts to manipulate hypoxia in cancer treatments and they postulate that manipulation of NO levels may represent a potential conversion form hypoxia to enoxia as a function of mutually reinforcing the relationship of NO and oxygen. Dr. Glynn and colleagues (National University, Ireland) discussed "*The role of NO in tumor invasion and metastasis*." They proposed that NOS expression in tumor epithelia has a tumor promoting activity, while NOS expression in tumor-associated macrophages has an anti-tumor activity. Hence, tumor progression/ regression depends on the balance between these two NO-associated activities. They present a challenging complexity of the cellular source of NO, the direct exposure and the amount of NO, all of which, form NO-mediated suppressive or stimulating activities in the tumor microenvironment. They proposed that more research is warranted to achieve a highly selective application to favor anti-tumor activity over pro-tumor activity by NO. Dr. Postovit (University of Alberta) reviews "*The role of NO in the regulation of the pro-tumorigenic and hypoxic phenotype.*" Clearly, tumor hypoxia correlates with poor clinical prognosis. Dr. Postovit discusses how NO can mimic and mitigate the effect of hypoxia in tumors as a function of the NO concentration. Several clinical trials have been used as examples in which NO-mediated anti-tumor effects correlated with inhibition of hypoxia. Furthermore, a report is discussed in which patients were treated with GTN and resulting in an increased response rate and decreased time to progression in stages IIIb/IV NSCLC treated with cis platinum and vinorelbine. Further, a retrospective study showed that GTN increases the response rate of patients with lung cancer treated with docetaxel and carboplatin. The patients treated with GTN showed decreased levels of VEGF and HIF-1α, corroborating the role of NO in mitigating the pro-tumorigenic effects of hypoxia. Dr. Postovit cautions of NO-mediated treatments to consider the paradoxical role of NO in the regulation of hypoxia-induced manifestations.

Part II covers three reviews on "S-nitrosylation and cancer." Dr. Brown and colleagues (Columbia State University) discussed "The signaling mediated by NO and through its S-nitrosylation of various proteins and their impact on the tumor cells." S-nitrosylation is reversible and involves the attachment of a nitroso moiety to the reactive thiol/cysteine residues and producing S-nitrothiol (SNO). Several proteins have been reported to be S-nitrosylated that are involved in transcription, DNA repair, and apoptosis, and also proteins involved in tumorigenesis. These investigators

have summarized in a table format several proteins that are S-nitrosylated and that are involved in either the progression or inhibition of cancer. Clearly, a better understanding of the mechanisms of signaling and consequences of S-nitrosylation is needed to enable the selective anti-tumorigenic activity over the pro-tumorigenic activity. Dr. Jeannin and colleagues (Burgundy University, Dijon, France) review "S-nitrosylation of cancer cells." They discussed several protein targets of chemotherapy that are S-nitrosylated. They discuss the potential role of activation of death receptor signaling pathways by NO to treat tumors. They elaborated on the role of NO in the regulation of death receptors, directly or indirectly, and how in combination with chemotherapeutics result in synergistic anti-tumor effects. Clearly, the potential clinical application of suitable NO donors in combination with chemotherapeutic drugs may result in an improved clinical response in cancer patients. Doctors Luanpitpong and Rojanasakul (West Virginia University) discussed "The role of S-nitrosylation in cancer metastasis." They reviewed the roles of S-nitrosylation in cancer focusing on anoikis, resistance, cell invasion, migration, and angiogenesis, all of which are key events in metastasis. In their review, they discuss the role of the constitutively activated PI3K-AKT signaling pathway, in turned on or off, via S-nitrosylation of the phosphatase PTEN. PTEN activity is inhibited by S-nitrosylation and, thus, enhances PI3K-AKT activity and cell survival. The mechanism of anoikis resistance in cancer and S-nitrosylation were well discussed. In addition, they also discuss the S-nitrosylation of various proteins involved in metastasis and apoptosis, including FLIP, Bcl2, cavolin 1, c-Src, EGFR, Ras, MMP-9, etc. These various S-nitrosylated proteins are shown to play an important role in the metastatic cascade and resistance to apoptosis.

Part III deals with the "*Modulation of anti-tumor immune responses by NO*." Three contributions are presented. Dr. Garban (University of California, Los Angeles) reviews "*The role of NO on the anti-tumor immune response*." He discusses the reported literature on the role of NO in the sensitization of drug-resistant tumor cells to immune-mediated cytotoxic activities. In addition, he discusses the role of NO-mediated modification of proteins that results in the potentiation of antigenicity. For instance, he refers on the role of NO in IL-2-mediated activation of anti-tumor response. He summarizes the role of NO on the inhibition of the constitutively activated NF-κB pathway and downstream inhibition of anti-apoptotic gene products as well as pro-inflammatory cytokines. In addition, he discusses the role of NO on the inhibition of the resistant factor Yin-Yang 1 (YY1) and FOXP3. Dr. Siesjo (Lund University, Sweden) reviews "*The regulation of anti-tumor immune response by NO.*" He discusses the contrasting roles of NO by direct cytotoxicity and by inhibition of anti-tumor immune reactivity. He elaborates on the role of NO on the regulation of both central and peripheral tolerance. Among the topics discussed, he reviews the regulation of T-cells activated by NO, the immune-suppressive role of NO, the potentiation of anti-tumor cytotoxic cells by NO, the role of dendritic cells and suppressor cells modulated by NO and the mechanism of NO-mediated immune suppression. He attributes the obstacle in manipulating NO in cancer therapy due to the lack of clinically approved NO donors or NO inhibitors. He also suggests the potential of combination of immunotherapy and NO-modulating agents

on the fight against cancer. Dr. Doctors Janakiram and Rao reviewed "*Nitric Oxide: Immune modulation of tumor growth*." It is well known that in the tumor microenvironment NO is generated by tumor cells, infiltrated cells, and tissue cells in the microenvironment. Hence, the generation of NO and its levels play a pivotal role in the regulation of tumor growth, both as an enhancer and as a repressor. Clearly, this complexity of the tumor microenvironment and the interaction of tumor cells with the infiltrating immune cells create a system that is not predictable and thus, difficult to establish the best approach to favor NO anti-cancer effects through the use of NO donors or NO inhibitors in clinical therapy.

Part IV deals with "*Therapeutics and overcoming resistance.*" Dr. Bonavida reviews the "*Role of NO in chemo-immune resistance*." In this review, he focused on the underlying mechanisms that regulate resistance and how NO treatment results in the reversal of resistance. Emphasis was placed on a dysregulated loop in cancer cells, namely, the NF-κB/Snail/YY1/RKIP loop, that was reported to regulate both drug and immune resistance. Each gene product in the loop was reported to regulate resistance as assessed by the use of specific inhibitors. Treatment with NO donors leads to the modification of the dysregulated loop resulting in the inhibition of NF-κB, Snail, and YY1 and concomitantly with the derepression and the upregulation of RKIP. The mechanism of inhibition of NO was examined and was found to be, in part, due to the direct S-nitrosylation of NF-κB (p50 and p65) and also by S-nitrosylation of Snail and YY1. The direct inhibition of NO as well as indirect inhibition of YY1 and Snail through NF-κB inhibition resulted in upregulation of RKIP and reversal of resistance. In addition, evidence was presented on the activity of NO donors in chemo-immunosensitization of resistant cells. A discussion was provided on the role of NO on inhibiting EMT via inhibition of the loop since inhibition of Snail, a metastasis inducer, was responsible, in part, to the inhibition of EMT and metastasis. Dr. McCarthy and McCrudder (University of Belfast, UK) discuss "*Emerging role of NO-mediated therapeutics*." They reviewed the emerging strategies of utilizing NO-mediated therapeutics for cancer. They also review the role of iNOS gene therapy and its limitations, which was not effective *in vivo*. These investigators have reported a novel inducible and tumor-specific activation of the *iNOS* gene for therapy. Using inducible promoters, they were able to deliver the *iNOS* gene for therapy and observed a delay in tumor growth. They pointed out that, in combination, treatments with high concentrations of NO may not result in antitumor activity. For example, NO can react with some chemo-therapeutic drugs such as etoposide and abolishes its activity. Other examples of iNOS upregulation for promoting tumor growth were presented. Doctors Rapozzi and Della Pietro (University of Udine, Italy) reviewed "*The role of NO in photodynamic therapy (PDT).*" PDT is clinically used therapeutically for treatment of early stages of cutaneous tumors. As PDT may induce apoptotic effects, these investigators have examined the role of NO in mediating PDT anti-tumor response. They discussed the induction of iNOS/NO by PDT and NO-mediated tumor cell death by PDT. They also discussed the alternatives to delivering NO-releasing compounds to enhance PDT anti-tumor response. They present the possibility of conjugating NO with a photosensitizer in PDT. This is a new application of PDT which has significant clinical ramifications.

Dr. Muntane and colleagues (University of Sevilla, Spain) reviewed "*The inhibition of cell death signaling by NO in cancer cells*." They discuss the role of NO in anti-tumor activity by the regulation of stress response mediated by HIF1-α and p53 that lead to cell growth arrest and apoptosis. They also discuss the induction of DNA damage by NO, the increase of p53 and cell death. They report that NO nitrosylates critical thiols in DNA repair enzymes in hepatoma cells that results in chemo-sensitization. Dr. Scicinski and colleagues (Mountainview, California) review "*Discovery and development of RRX-001*." They have developed a new compound, RRx-001, the first of a class of NO-mediated epigenetic anti-cancer agents. They reported that RRx-001 (designed by combining two structural components, a di-nitroazetidine derived from TNAZ [tri-nitroazetidine] and α-bromoacetate) was active as a single agent *in vitro* and *in vivo* against tumor cell lines. They describe the mechanisms by which RRx-001 mediates its activity via NO. They have completed a phase I study with RRx-001 and, aside from phase I end point, they also found clinical benefits in 70% of patients with multiple tumor types. Of interest, RRx-001 sensitized patients who previously failed therapy. Based on these positive findings, a phase II is being considered.

Part V deals with "*NO-mediated alterations in gene products*." Dr. Othman and colleagues (Northwestern University) review "*The role of NO in coagulation in cancer*." It is well known that NO is a rapid vasodilator and inhibitor of coagulation. Cancer patients are at high risk of developing venous and arterial thrombo-embolic events. They discuss the relationship of pro-coagulant factors and NO, the role of NO and global hemostasis, the link among hypoxia, NO, and coagulation, NO as a fibrinogen system, and NO and thrombosis. They also discuss both the pro and anti-tumorigenic effects of NO-mediated therapies. Doctors Mutus and Sin (University of Windsor, Canada) review "*The relationship between neutral sphingomyelase 2 and NO and their implications in cancer therapy*." Neutral sphingomyenase 2 is a regulator of ceramide and sphingolipid signals. Under oxidative stress, such as anti-cancer drugs, the level of SMase2 is upregulated resulting in increased levels of cellular ceramide, which lead to activate pathways that lead to apoptosis.

Clearly, the above contributions have added a new dimension in understanding the complex roles of NO in cancer and have presented several mechanisms of its multiple effects and its potential for therapy when used under optimal conditions. It is, noteworthy, that current studies are aimed at developing novel NO donors that will be effective in the treatment of highly resistant cancer as well as preventing metastasis, when used alone or in combination with sub-toxic therapeutics. In addition, current studies are also exploring the development of complexes consisting of NO and other agents for targeted delivery to enhance specificity and reduce toxicity.

Benjamin Bonavida

Acknowledgements

I'm grateful for all of the contributors in this book who have spent considerable time and effort in the preparation of their manuscript and who are responsible for the completion of this book. I wish to acknowledge the assistance and support provided by the Department of Microbiology Immunology and Molecular Genetics, David Geffen School of Medicine at UCLA and Jonsson Comprehensive Center at UCLA. The continuous advice, cooperation and consultation from Fiona Sarne, Joy Bramble, and Kelly Wilson from Springer have helped me throughout the preparation and editing of this book. I'm indebted to my assistants Daphne Liang, Melissa Cao, Kathy Nguyen, Suzie Vardanyan, and Anna Shvartsur for their dedication and secretarial assistance in the preparation of this book.

Contents

Part II S-Nitrosylation and Cancer

Contributors

Jose M. Álamo Department of General Surgery, Hospital Universitario Virgen del Rocío-Virgen Macarena/IBiS/CSIC/Universidad de Sevilla, Seville, Spain

Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREH o Ciberehd), Instituto de Salud Carlos III, Madrid, Spain

Instituto de Biomedicina de Sevilla (IBiS), IBiS/Hospital Universitario Virgen del Rocío-Virgen Macarena/Universidad de Sevilla/CSIC, Seville, Spain

Mark Bednarski Department of Radiology, Stanford University Medical Center, Stanford, CA, USA

Ali Bettaieb Immunology and Immunotherapy of Cancer Laboratory, Burgundy University, Dijon, France

EPHE, Paris, France

Benjamin Bonavida Department of Microbiology, Immunology & Molecular Genetics, David Geffen School of Medicine, Jonsson Comprehensive Cancer Center, University of California at Los Angeles, Los Angeles, CA, USA

Mark A. Brown Cell and Molecular Biology Program, Colorado State University, Fort Collins, CO, USA

Department of Clinical Sciences, Colorado State University, Fort Collins, CO, USA

Department of Ethnic Studies, Colorado State University, Fort Collins, CO, USA

Colorado School of Public Health, Fort Collins, CO, USA

Amy J. Burke Prostate Cancer Institute, School of Medicine, National University of Ireland, Galway, Ireland

Emilia Della Pietra Department of Medical and Biological Sciences, University of Udine, Udine, Italy

Ángela M. De los Santos Instituto de Biomedicina de Sevilla (IBiS), IBiS/Hospital Universitario Virgen del Rocío-Virgen Macarena/Universidad de Sevilla/CSIC, Seville, Spain

Benjamin A. Derman Northwestern University Feinberg School of Medicine, Chicago, USA

Qiang Du Department of Surgery, Starzl Transplantation Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Melissa A. Edwards Cell and Molecular Biology Program, Colorado State University, Fort Collins, CO, USA

Malak Elbatarny Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada

School of Baccalaureate Nursing, St Lawrence College, Kingston, ON, Canada

Gary Fanger EpicentRx, Inc, Mountain View, CA, USA

Gary R. Fanger EpicentRx, Inc, Mountain View, CA, USA

Francisco Gallardo-Chamizo Instituto de Biomedicina de Sevilla (IBiS), IBiS/Hospital Universitario Virgen del Rocío-Virgen Macarena/Universidad de Sevilla/CSIC, Seville, Spain

Hermes J. Garbán NantBioscience, Inc. NantWorks, LLC. California NanoSystems Institute (CnSI) at the University of California, Los Angeles (UCLA). Jonsson Comprehensive Cancer Center at UCLA. Department of Medicine. Division of Dermatology. David Geffen School of Medicine at UCLA., NantBioscience, Inc. NantWorks, LLC., Los Angeles, CA, USA

David A. Geller Department of Surgery, Starzl Transplantation Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Francis J. Giles Prostate Cancer Institute, School of Medicine, National University of Ireland, Galway, Ireland

Sharon A. Glynn Discipline of Pathology, School of Medicine, National University of Ireland, Galway, Ireland

Naveena B. Janakiram Center for Cancer Prevention and Cancer Drug Development, Department of Medicine, Hematology Oncology Section, PCS Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Dana M. Jarigese Department of Animal Sciences, Colorado State University, Fort Collins, CO, USA

Jean-François Jeannin Immunology and Immunotherapy of Cancer Laboratory, Burgundy University, Dijon, France

EPHE, Paris, France

Susan J. Knox Department of Radiation Oncology, Stanford University Medical Center, Stanford, CA, USA

Hau C. Kwaan Northwestern University Feinberg School of Medicine, Chicago, USA

Véronique Laurens Immunology and Immunotherapy of Cancer Laboratory, Burgundy University, Dijon, France

EPHE, Paris, France

Sudjit Luanpitpong Department of Pharmaceutical Sciences, West Virginia University, Morgantown, WV, USA

Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV, USA

Michelle Lybeck EpicentRx, Inc, Mountain View, CA, USA

Luís M. Marín Department of General Surgery, Hospital Universitario Virgen del Rocío-Virgen Macarena/IBiS/CSIC/Universidad de Sevilla, Seville, Spain

Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREH o Ciberehd), Instituto de Salud Carlos III, Madrid, Spain

Instituto de Biomedicina de Sevilla (IBiS), IBiS/Hospital Universitario Virgen del Rocío-Virgen Macarena/Universidad de Sevilla/CSIC, Seville, Spain

Helen O. McCarthy Experimental Therapeutics Research Group, School of Pharmacy, Queen's University Belfast, Belfast, UK

Cian M. McCrudden Experimental Therapeutics Research Group, School of Pharmacy, Queen's University Belfast, Belfast, UK

Tysha N. Medeiros Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, USA

Jordi Muntané Department of General Surgery, Hospital Universitario Virgen del Rocío-Virgen Macarena/IBiS/CSIC/Universidad de Sevilla, Seville, Spain

Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREH o Ciberehd), Instituto de Salud Carlos III, Madrid, Spain

Instituto de Biomedicina de Sevilla (IBiS), IBiS/Hospital Universitario Virgen del Rocío-Virgen Macarena/Universidad de Sevilla/CSIC, Seville, Spain

Bulent Mutus Department of Chemistry & Biochemistry, University of Windsor, Windsor, ON, Canada

Shoucheng Ning Department of Radiation Oncology, Stanford University Medical Center, Stanford, CA, USA

Bryan Oronsky EpicentRx, Inc, Mountain View, CA, USA

Neil Oronsky CFLS, San Jose, CA, USA

Maha Othman Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada

School of Baccalaureate Nursing, St Lawrence College, Kingston, ON, Canada

Francisco J. Padillo Department of General Surgery, Hospital Universitario Virgen del Rocío-Virgen Macarena/IBiS/CSIC/Universidad de Sevilla, Seville, Spain

Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREH o Ciberehd), Instituto de Salud Carlos III, Madrid, Spain

Instituto de Biomedicina de Sevilla (IBiS), IBiS/Hospital Universitario Virgen del Rocío-Virgen Macarena/Universidad de Sevilla/CSIC, Seville, Spain

Catherine Paul Immunology and Immunotherapy of Cancer Laboratory, Burgundy University, Dijon, France

EPHE, Paris, France

Sheila Pereira Instituto de Biomedicina de Sevilla (IBiS), IBiS/Hospital Universitario Virgen del Rocío-Virgen Macarena/Universidad de Sevilla/CSIC, Seville, Spain

Stéphanie Plenchette Immunology and Immunotherapy of Cancer Laboratory, Burgundy University, Dijon, France

EPHE, Paris, France

Lynne-Marie Postovit Department of Oncology, University of Alberta, Edmonton, AB, Canada

Chinthalapally V. Rao Center for Cancer Prevention and Cancer Drug Development, Department of Medicine, Hematology Oncology Section, PCS Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Valentina Rapozzi Department of Medical and Biological Sciences, University of Udine, Udine, Italy

Ángeles Rodríguez-Hernández Instituto de Biomedicina de Sevilla (IBiS), IBiS/Hospital Universitario Virgen del Rocío-Virgen Macarena/Universidad de Sevilla/CSIC, Seville, Spain

Yon Rojanasakul Department of Pharmaceutical Sciences, West Virginia University, Morgantown, WV, USA

Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV, USA

Health Sciences Center, West Virginia University, Morgantown, WV, USA

Sabrina Romagny Immunology and Immunotherapy of Cancer Laboratory, Burgundy University, Dijon, France

PHE, Paris, France

Aideen E. Ryan Discipline of Pharmacology and Therapeutics, School of Medicine, National University of Ireland, Galway, Ireland

Regenerative Medicine Institute, School of Medicine, National University of Ireland, Galway, Ireland

Jan Scicinski EpicentRx, Inc, Mountain View, CA, USA

Peter Siesjö Section of Neurosurgery, Department of Clinical Sciences, Lund University, Lund, Sweden

Department of Neurosurgery, Skane University Hospital, Lund, Sweden

Francis J. Sullivan Prostate Cancer Institute, School of Medicine, National University of Ireland, Galway, Ireland

Bei Lei Sun Department of Chemistry & Biochemistry, University of Windsor, Windsor, ON, Canada

Vasily A. Yakovlev Department of Radiation Oncology, VCU/Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA

Part I
Molecular Cell Signaling by NO in Cancer

Chapter 1
Inflammation-Associated Carcinogenesis Involves Interaction Between the iNOS/NO and Wnt/β-catenin Signaling Pathways

Qiang Du and David A. Geller

Abstract Wnt/β-catenin/TCF signaling is associated with carcinogenesis, and chronic inflammation activated NF-κB plays an essential role in the initiation of a cancer. Recent evidence shows that NF-κB constitutive activation synergizes with Wnt signaling. iNOS/NO signaling causes APC loss of heterozygosity and activates the Wnt/β-catenin signaling pathway, which contributes to oncogenic initiation and cancer development. In this chapter, we focus on the molecular interactions between iNOS and Wnt/β-catenin signaling for carcinogenesis. iNOS is induced in chronic inflammation by cytokines mainly through the NF-κB and STAT transcription pathways. The iNOS/NO signaling exerts key roles in driving carcinogenesis mediated by Wnt/β-catenin. iNOS/NO induces reactive oxygen and nitrogen species (RONS) and can cause APC or β-catenin mutation, resulting in β-catenin accumulation. However, chemical or genetic inhibition of iNOS can decrease Wnt/β-catenin signaling and carcinogenesis. At least three pathways are established for the interaction between iNOS and Wnt signaling: (1) a positive feedback loop established by iNOS causing APC or β-catenin mutation via reactive species, and by Wnt targeting iNOS gene expression; (2) a negative feedback mechanism between Wnt and Dickkofp-1 (DKK1) where iNOS inhibits DKK1 gene expression; and (3) cross-regulation between NF-κB and Wnt/β-catenin pathways mainly through iNOS/NO generating reactive species which induce β-catenin activation. We conclude that the iNOS/NO signaling plays a central role in the interaction between NF-κB and Wnt/β-catenin pathways that regulate inflammation-associated carcinogenesis.

D. A. Geller (✉)
Department of Surgery, Starzl Transplantation Institute, University of Pittsburgh School of Medicine, 3459 Fifth Avenue, UPMC Montefiore, 7 South, 15213-2582 Pittsburgh, PA, USA
e-mail: gellerda@upmc.edu

Q. Du
Department of Surgery, Starzl Transplantation Institute, University of Pittsburgh School of Medicine, 200 Lothrop Street, BST W1507, 15213-2582, Pittsburgh, PA, USA
e-mail: duq@upmc.edu

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_1

Keywords Carcinogenesis · Inflammation · INOS · NF-κB · Nitric oxide · Wnt/β-catenin

Abbreviations

AOM	Azoxymethane
APC	Adenomatous polyposis coli
β-TrCP1	β-Transducin-repeat-containing protein 1
CRC	Colorectal cancer
CAC	Colitis-associated CRC
COX-2	Cyclooxygenase 2
DKK1	Dickkopf-1
DSS	Dextran sulphate sodium
GSK3β	Glycogen synthase kinase 3β
IBD	Inflammatory bowel disease
IECs	Intestinal epithelial cells
IKKβ (2)	IκB kinaseβ (2)
iNOS	Inducible nitric oxide synthase
LOH	Loss of heterozygosity
LRP5/6	Lipoprotein receptor: related protein 5/6
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
RONS	Reactive oxygen and nitrogen species
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TCF	T cell factor
TNFα	Tumor necrosis factor-α
TLRs	Toll-like receptors
TGF-β	Transformed growth factor-β
VEGF	Vesicular epithelial growth factor

Overview of iNOS and Wnt/β-catenin in Carcinogenesis

Canonical Wnt signaling occurs when the oncoprotein β-catenin binds to nuclear partners (members of the T-cell factor (TCF)–lymphocyte enhancer factor (LEF) family) to create a transcription factor that regulates genes involved in cellular activation. Both Wnt/β-catenin and the inducible nitric oxide synthase (iNOS)/nitric oxide (NO) pathways have important roles in carcinogenesis [1–4], and both have been shown to be dysregulated in colon and breast carcinomas [5–9]. These observations led to explore the association of these two pathways in carcinogenesis, especially for understanding molecular mechanisms linking chronic inflammation and cancer initiation in their related microenvironment. By using transgenic animal models, recent studies have made great progress in intestinal tumorigenesis through the IKKβ/NF-κB/iNOS/NO and Wnt/β-catenin signaling pathways.

The oncogenic role of Wnt/β-catenin signaling in tumor formation has been known for more than 30 years [10–13]. The alteration of β-catenin cellular localization from membrane to cytosol or nucleus is a key step for it to be a co-transcription factor targeting gene expression. The target genes (the Wnt home page: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/) of the β-catenin/TCF signaling, such as c-MYC, cyclin D1 [14, 15] and human iNOS [16], have been shown to be carcinogenic. Either adenomatous polyposis coli (APC) or β-catenin gene mutation can cause β-catenin accumulation and translocation to the nucleus and in turn, constitutively activates oncogenic Wnt signaling which is regarded as the initiating event in colorectal cancer [10–12]. The β-catenin degradation complex not only controls the levels of β-catenin protein by proteolysis, but also inhibits its nuclear localization. APC is a component of this complex. The mutational inactivation of tumor suppressor genes, such as *APC*, *p53* and *TGF-β* (transforming growth factor β) are contributory steps in the pathophysiology of colorectal cancer [17]. Also somatic mutations and deletions that inactivate both copies of APC are present in many sporadic colorectal adenomas and cancers [12]. Chromosomal instability is an efficient mechanism for causing physical loss of a wild-type copy of a tumor-suppressor gene, such as *APC*, *p53*, and *SMAD* family member 4 (SMAD4), whose normal activities oppose the malignant phenotype [17].

Recently, progress has been made in understanding the role of inflammation related to carcinogenesis. One of the critical issues is that inflammation-related carcinogenesis is associated with Wnt/β-catenin signaling activation. Data obtained from animal models show that β-catenin/TCF oncogenic signaling activation mainly occurs through the IKKβ/NF-κB pathway. However, another mechanism identified is where some bacteria such as Fusobacterium nucleatum (Fn) can infect IECs which promote colorectal carcinogenesis by binding E-cadherin, activating β-catenin signaling and differentially regulating inflammatory and oncogenic responses via Fn FadA adhesion [18]. Fn infection modulates the tumor-immune microenvironment in $Apc^{Min/+}$ mouse models for intestinal tumorigenesis [19]. TLR4 activated by colonic bacteria can induce Wnt/β-catenin signaling in a PI3K-dependent manner to cause intestinal neoplasia in mice [20]. Pathogen-specific TLR2 protein activation by some bacterial infection programs macrophages to induce Wnt/β-catenin signaling in an iNOS/NO-dependent manner by using *C57BL/6* and $iNOS^{-/-}C57BL/6$ mice [21]. Additionally, in an in vitro study IL-1 and tumor associated macrophages, as well as TNFα can activate NF-κB-dependent PDK1/AKT signaling and thereby inactivate GSK-3β, thereby enhancing Wnt signaling and promoting growth of colon cancer cells. This is a novel molecular link between inflammation and tumor growth [22, 23]. DSS induced mouse models show that iNOS plays a critical role in mediating the inflammatory response during colitis [24], and also β-catenin in the nucleus and an early induction of proinflammatory factors were observed [25]. These analyses indicate that Wnt/β-catenin signaling is involved in inflammation-associated carcinogenesis.

As Clevers [26] histopathologically summarized, the sporadic colorectal cancer (CRC) follows “an adenoma to carcinoma sequence”. Neoplastic lesions typically present as aberrant crypt foci (ACF) or micro-adenomas and then develop through a large adenoma-stage into carcinoma in situ and eventually invasive adenocarcinoma, while ulcerative colitis-associated CRC take “inflammation to dysplasia

to carcinoma sequence". Despite these differences, the two forms of CRC share molecular mechanisms. It may be reasonable to consider both, once initiated, as a single disease entity [26]. A functional link between chronic inflammation and cancer has also long been suspected [26–28]. Population-based studies show that susceptibility to cancer increases when tissues are chronically inflamed; and long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of several cancers [29]. About 25% of human cancers are estimated to develop from chronic inflammation [30, 31]. At least two signaling pathways are involved in the carcinogenesis caused by activation of NF-κB [32, 33] and JAK/STAT [34, 35] signaling pathways. It is clear that Toll-like receptors (TLRs), TNFα receptor and others are involved in the activation of NF-κB and STAT [34, 36] in inflammation. NF-κB proteins and STAT3 are key players in inflammation related carcinogenesis. The activation of these signaling pathways either in cancer cells or in tumor-associated inflammatory cells promotes malignancy [35, 37], with dangerous implications for STAT3 and NF-κB collaboration or crosstalk in cancer cells [37]. It is interesting that both NF-κB and JAK/STAT pathways activate COX-2 and iNOS which are regarded as carcinogenesis-induced enzymes [32–35].

Transgenic mouse models have been very useful to demonstrate inflammation-associated cancer. Activated IKK2(β)/NF-κB signaling interacts with Wnt/β-catenin for the initiation of cancer. Importantly, iNOS plays a unique role in the regulation of Wnt/β-catenin and NF-κB pathways. Greten et al. found that NF-κB activation in both intestinal epithelia cells (IECs) and lamina propria macrophages play a critical role in the initiation of CRC [32]. In IECs, the pro-tumorigenic function of NF-κB appears to be mediated through its anti-apoptotic effect that prevents the elimination of premalignant cells, in which β-catenin signaling has been activated due to the mutations in the *CTTNB1* gene. NF-κB is a tumor promoter and the relationship between NF-κB and β-catenin oncogenic signal was proposed [32]. A subsequent study found that constitutive IκB kinase 2 (IKK2)/NF-κB activation in IECs induced mild inflammation in the colon and small intestine. Expression of constitutively active IκB kinase 2 (IKK2ca) in IECs strongly enhanced chemical carcinogen–induced intestinal tumor development, and promoted spontaneous tumor in the colon and small intestine of aged *IKK2caIEChom* mice. It is very interesting that constitutive IKK2/NF-κB activation strongly synergized with Wnt signaling to drive intestinal tumorigenesis by increasing β-catenin activity which might contribute to increase in proliferation, and elevated expression of genes encoding intestinal stem cell–associated factors including *Ascl2*, *Olfm4*, *DLK1*, and *Bmi*-1 [38]. iNOS/NO-induced reactive oxygen and nitrogen species (RONS) accelerate APC loss of heterozygosity and enhance Wnt β-catenin signaling for carcinogenesis [39]. TNFα increased β-catenin/TCF signaling activation through induction of NF-κB, which leads to recruitment of CBP and binding to β-catenin/TCF to enhance transcription of Wnt-dependent stem cell genes [40]. IKK2/IKKβ induced NF-κB activation triggers carcinogenesis and the functional inactivation of p53, and constitutive activation of the NF-κB pathway has been associated with several human cancers. p53 is a critical gatekeeper of cellular transformation. Data obtained from a recent study indicates that IKK2/IKKβ phosphorylates P53 at serines 362 and 366, which

leads to its recruitment and ubiquitination by β-transducin-repeat-containing protein 1 (β-TrCP1) for its degradation. Loss of P53 is very important for an inflamed cell to survive during the initiation of a cancer cell. Thus, IKK2/IKKβ and NF-κB pathways dominate anti-apoptotic and cellular transformation signaling for inflammation-associated carcinogenesis [41].

The major role of iNOS, when expressed in phagocytic cells upon activation of NF-κB and other transcription factors, is to fight pathogens during infection by catalyzing the production of toxic reactive nitrogen species (RNS) [42]. However, iNOS has also been implicated in the pathogenesis of inflammatory bowel disease (IBD), where it is expressed in IECs and contributes to tissue damage [43, 44]. High levels of NO during inflammation can cause DNA damage [45]. NO is known to cause DNA damage and nitrosylation of proteins [46, 47], and increased production in tumor cells would be expected to facilitate accumulation of sequential mutations [48]. NO activates diverse signaling pathways to regulate gene expression [49] and proliferation [50]. Recent findings have shown that iNOS expression is correlated with tumor growth and poor prognosis in patients with estrogen receptor-negative (ER-) breast cancer [8], melanoma [51, 52], glioma [53], and colon cancer [6, 54]. NO activates epithelial growth factor (EGF) receptor signaling pathway [55], p53, and vascular endothelial growth factor (VEGF) to promote angiogenesis of tumors [5]. NO and its related reactive species produced in oxidative and nitrosative stress are thought to be able to cause chromosomal instability, and may serve as signaling molecules that affect inflammatory epithelia cell carcinogenesis, tumor cell proliferation, survival, metabolism, angiogenesis, and metastasis [56, 57]. Excessive reactive oxygen species (ROS) and RNS production due to H. pylori infection in gastric epithelial cells cause DNA damage and are therefore mutagenic. In addition, they also suppress apoptosis and promote H. pylori-induced gastric carcinogenesis [58]. Understanding the molecular mechanism of carcinogenesis is important for considering two aspects of induced DNA damage [57] and interrupted DNA repair systems. The mismatch repair system [59] and RONS-induced DNA damage can be recognized by alkyladenine DNA glycosylase (Aag) to initiate base excision repair [58], especially during inflammation and the carcinogenesis microenvironment [60–62].

iNOS/NO is Associated with the Initiation of Intestinal Cancers

Established animal models have been used for the study of inflammation-associated colorectal cancer, and are mainly divided into two groups. One is where colitis is induced by chemical means, such as dextran sulphate sodium (DSS) treatment combined with a carcinogen such as azoxymethane (AOM), and one where carcinoma develops due to genetic alterations, such as gene *IKK2/IKKβ* [32, 38–40], *IL-10* gene knockout [63], and mutant mice for *IL-2* and *β macroglobulin* genes [64]. AOM treated mice have been reported as effective colon cancer models with iNOS

and COX-2 overexpression, and β-catenin activation [65]. Immunohistochemistry investigation of such dysplasia and neoplasms revealed that all lesions were positive for β-catenin, COX-2 and iNOS, but did not show P53 immunoreactivity in AOM and DSS treated mice [66]. Data obtained from rat colon adenocarcinomas induced by AOM, the frequent mutation and an altered cellular localization of β-catenin was described along with up-regulation of iNOS and COX-2 by analyzing the samples from hyperplastic and dysplastic type aberrant crypt, adenoma and adenocarcinoma [65]. Mutation analysis by the PCR-single strand conformation polymorphism method and direct sequencing demonstrated the β-catenin gene to be mutated in 2 of 3 dysplastic ACF, 2 of 6 adenomas and 20 of 26 adenocarcinomas, while immunohistochemical staining showed an alteration in cellular localization of β-catenin in all dysplastic ACF, adenomas and adenocarcinomas examined [65]. These important changes of β-catenin, iNOS and COX-2 gene expression were not only observed in carcinoma, but also in ACF and adenoma in AOM-induced mice [48, 67]. These results also show that β-catenin alterations might be related to the induction of iNOS expression, these being early events in carcinogen-induced colon tumorigenesis, which may play important roles in causing dysplastic changes [65]. Moreover, when 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine (PhIP) and DSS are used to induce rapid colon carcinogenesis in mice, co-expression of β-catenin and iNOS was also observed [68, 69]. iNOS was also observed to promote H. pylori-mediated gastric carcinogenesis [70]. Another interesting study showed that iNOS over-expression resulted in increased β-catenin expression in oral cancer cells [71]. The induced β-catenin mutation, which might be a co-transcription factor for the activation of β-catenin/TCF signaling, was suggested in mice and rats treated with AOM/DSS [65, 72]. Collectively, these findings indicate that the alterations of β-catenin, iNOS and COX-2 expressions are observed in the different phases of tumor formation, especially during the early stages of dysplastic changes. β-catenin/TCF signaling might be involved in the initiation of the experimental cancers, and the activation of oncogenic Wnt signaling contributes to iNOS expression.

In the models described by Nam et al. [70] and Handa et al. [56], H. pylori-infected $iNOS^{-/-}$ mice had gastric cancer induced by administration of N-methyl-N-nitrosourea. The overall incidence of gastric cancer after 50 weeks was significantly lower in $iNOS^{-/-}$ mice compared with their wild-type counterparts. An accumulation of intracellular ROS/RNS can induce DNA point mutations, thus disrupting the expression and function of several tumor-suppressing genes such as *P53*, which might contribute to the pathogenesis of gastric cancer [56, 70]. Data obtained from another important observation [73] showed that iNOS mRNA expression was related to P53 and APC mutation in primary colitis-associated colorectal (CAC) models. This might imply that iNOS/NO is a trigger to induce carcinogenic signaling p53 and APC mutation. In a recent study, chronic epithelial NF-κB activation accelerates APC loss of function and intestinal tumor initiation through iNOS up-regulation and further indicates that oxidative stress derived from iNOS/NO production contributes to APC mutation and β-catenin signaling activation for carcinogenesis

[39]. In the study, the authors generated *Ikkβ (EE)* ^{IEC} transgenic mice expressing constitutively active IκB kinase β (IKKβ) in IECs. Despite the absence of destructive colonic inflammation, *Ikkβ (EE)* ^{IEC} mice developed intestinal tumors after a long latency. However, when crossed to mice (*Apc*$^{+/\Delta IEC}$) with IEC-specific allelic deletion of the APC tumor suppressor locus, *Ikkβ (EE)* ^{IEC} mice exhibited more β-catenin (+) early lesions and visible small intestinal and colonic tumors relative to *Apc*$^{+/\Delta IEC}$ mice, and their survival was severely compromised. IEC of *Ikkβ (EE)* ^{IEC} mice expressed high amounts of iNOS and elevated DNA damage markers and contained more oxidative DNA lesions. Tumors in *Apc*$^{+/\Delta IEC}$ mice are formed when some cells randomly loose the WT Apc allele, thereby allowing β-catenin activation and clonal expansion, similarly to the *Apc*$^{+/Min}$ model. Treatment of *Ikkβ (EE)* ^{IEC}*/Apc*$^{+/\Delta IEC}$ mice with an iNOS inhibitor decreased DNA damage markers and reduced early β-catenin (+) lesions and tumor load. The results suggest that persistent NF-κB activation and iNOS expression in IECs may accelerate Apc loss of heterozygosity (LOH) by enhancing nitrosative DNA damage, thereby leading to accelerated formation of β-catenin+ lesions. This is an important study that established the molecular link between NF-κB (chronic inflammation) and Wnt/β-catenin (oncogenesis) pathways through accelerating loss of the wild type Apc allele by iNOS/NO induced reactive species [39].

Another experimental approach has been to use inhibitors or compounds to study the effect on carcinogenesis in AOM and other carcinogen-treated animal models.

The polyphenol curcumin inhibited carcinogenesis by interfering with Wnt/β-catenin and other oncogenic pathways by inhibiting iNOS, COX-2, β-catenin and other Wnt target genes in colitis-associated cancer [74]. Silibinin inhibited AOM-induced colon tumorigenesis in A/J mice [75] by decreasing iNOS expression and inactivating β-catenin [76]. Hydroxylated polymethoxy-flavones (PMFs) decreased the number of adenomatous polyposis coli tumors in colonic tissues of mice [77]. 2, 3', 4, 4', 5'-Pentamethoxy-trans-stilbene, a resveratrol derivative, inhibits colitis-associated colorectal carcinogenesis by significantly reducing the number of colonic neoplasms in mice [78]. Grape seed extracts (GSE) can decrease ACF through down-regulating NF-κB and inactivating β-catenin in AOM rats [79]. Licochalcone A (LicA) downregulates iNOS and COX-2 and inactivates β-catenin in the colon [80]. Dietary intake of pterostilbene, a constituent of blueberries, inhibits the β-catenin/p65 downstream signaling pathway and colon carcinogenesis in rats [81]. Taken together, these observations strongly imply that iNOS expression and β-catenin signaling exert key roles during intestinal carcinogenesis. Given inflammation-related iNOS activation and NO production can cause DNA damage in IECs in vivo, and considering the relationship among β-catenin, COX-2 and iNOS has been noticed in AOM models and the evidence from transgenic models [32, 39], iNOS/NO might be an effector for inducing carcinogenesis signaling. It is clear that activation of NF-κB pathway and expression of iNOS in epithelial cells contribute to inflammation-mediated oxidative and nitrosative stress, and along with NAPDH oxidase can induce genomic instability [82].

iNOS/NO Promotes β-catenin/TCF Signaling for Carcinogenesis

Several studies have investigated the causative link between iNOS/NO-mediated induction of β-catenin/TCF signaling and subsequent carcinogenesis. A research group studied IMCE ($Apc^{Min/+}$) cells, and a sister cell line of young adult mouse colon (YAMC) ($Apc^{+/+}$) with similar genetic background but differing in Apc genotype and consequently β-catenin levels. Unlike most colon cancer cell lines, this pair of cell lines has either non-detectable or low basal level of β-catenin when they are cultured under non-permissive and non-proliferative conditions. They found that IMCE cells, in comparison with YAMC cells, had markedly higher β-catenin/LEF-1 DNA complex formation under both resting conditions and after induction with NO. Using electrophoretic mobility shift assays, they described that both NOR-1 and S-nitro-N-acetyl-D L-penicillamine (SNAP) NO donors caused β-catenin/LEF-1 DNA complex formation in YAMC cells. Super-shift by anti-β-catenin antibody confirmed the presence of β-catenin in the complex. When they used the other NO-releasing agents (E)-methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexeneamide and SNAP, they also found NO greatly enhanced the formation of β-catenin/LEF-1 DNA binding complexes in a concentration- and time-dependent fashion in YAMC and IMCE cells [83]. Cell fractionation studies indicated that NO donors caused an increase in free soluble cytoplasmic β-catenin. This is further corroborated by the immunocytochemistry data showing the redistribution of β-catenin from the predominantly membrane localization into the cytoplasm to the nucleus after treatment with NO donors. Although the molecular mechanism is still under investigation, the effect of NO seems to increase free soluble β-catenin and the formation of nuclear β-catenin/LEF-1 DNA complex [83].

Significantly, IMCE cells showed a markedly greater amount of nuclear β-catenin/LEF-1 DNA binding complex in response to NO. This study indicates that the defective β-catenin degradation machinery attributable to $Apc^{Min/+}$ mutation in IMCE cells not only affects basal levels, but also contributes to NO-induced dysregulation of cytoplasmic β-catenin and nuclear β-catenin/LEF-1 DNA binding complex formation [84]. Another mechanism elucidated was that matrix metalloproteinases (MMPs) mediate NO-induced dissociation of β-catenin from membrane bound E-cadherin, and the formation of nuclear β-catenin/LEF-1 complex [85]. Consistent with the in vitro evidence from $Apc^{Min/+}$ cell lines [83–85], the results from $Apc^{Min/+}$ mice (with Apc mutation) [86, 87] treated with DSS show increased colonic neoplasms. These results imply that APC-related β-catenin/TCF signaling promotes tumorigenesis by enhancing iNOS/NO and β-catenin signals [88].

Despite being a mediator of pro-inflammatory responses in colitis, iNOS acts as a tumor promoter in colorectal cancer by modulating Apc/β-catenin/TCF signaling activation, thereby resulting in tumor multiplicity in min mice [88]. Furthermore, genetic disruption of iNOS reduces the incidences of gastric carcinogenesis induced

by H. pylori [70]. Grape seed extract prevents intestinal tumorigenesis in $Apc^{Min/+}$ mice by down-regulating iNOS and other proteins, as well as inactivating β-catenin signaling [89]. Moreover, Min mice that had chronic iNOS inhibition or double knockout mice with $Apc^{Min/+}/iNOS^{-/-}$ showed fewer intestinal tumors. These results imply that iNOS-derived NO contributes to tumor formation and promotes carcinogenesis by increasing Apc/β-catenin/TCF signaling [90].

Prior investigations show that APC and β-catenin mutations are frequently found in colon cancer, and that COX-2 and iNOS are commonly overexpressed. Since altered localization of β-catenin was apparent in lesions expressing iNOS, it might be possible that β-catenin alteration is related to the induction of iNOS expression. The evidence from these animal models strongly suggests that iNOS/NO contributes to β-catenin/TCF signaling by APC LOH and β-catenin mutation [32, 39, 65]. Results from the transgenic [32, 39] and Min mice [90] studies suggest that iNOS is a transcriptional target of Wnt/β-catenin signaling in cancer cells [16]. We previously proposed a positive feedback loop between iNOS and Wnt signaling pathways [91, 92], leading to β-catenin/TCF driven carcinogenesis (Fig. 1.1).

iNOS/NO Fine-Tunes β-catenin/TCF Signaling by Down-Regulating DKK1

The (Dickkopf) DKK family of glycoproteins encodes at least 4 members of secreted proteins in vertebrates. DKK1, 2, and 4 regulate Wnt signaling by binding the same receptor [93]. DKK1 was first identified as a Wnt antagonist and embryonic head inducer in Xenopus [94]. Wnts activates the canonical signaling pathway by binding the frizzled seven transmembrane receptor and coreceptor lipoprotein receptor-related protein 5/6 (LRP5/6) forming a ternary complex that stabilizes β-catenin. DKKs bind and modulate Wnt coreceptor of the LRP5/6 class, which are indispensable for routing Wnt signaling to the β-catenin pathway [95]. LRP5 and LRP6 are closely related type I transmembrane proteins and function as Wnt coreceptors whose activity is modulated by DKKs [95].

DKK1 is an endogenous antagonist of Wnt/β-catenin and plays a key role for the establishment of negative feedback mechanism to regulate Wnt/β-catenin through an autocrine loop. Interestingly, Wnt/β-catenin signaling transcriptionally targets both iNOS and DKK1 [16, 96–98]. We studied the relationship between iNOS/NO and DKK1 and found that iNOS/NO down-regulates DKK1 gene expression, thereby abrogating the negative feedback of DKK1 on Wnt/β-catenin signaling, and up-regulating Wnt/β-catenin signaling [99]. The net effect is a more powerful oncogenic Wnt/β-catenin pathway that promotes carcinogenesis. The genetic control mechanisms for the negative regulation of DKK1 might be defined as iNOS fine-tuning of β-catenin/TCF signaling (Fig. 1.1, 1.2).

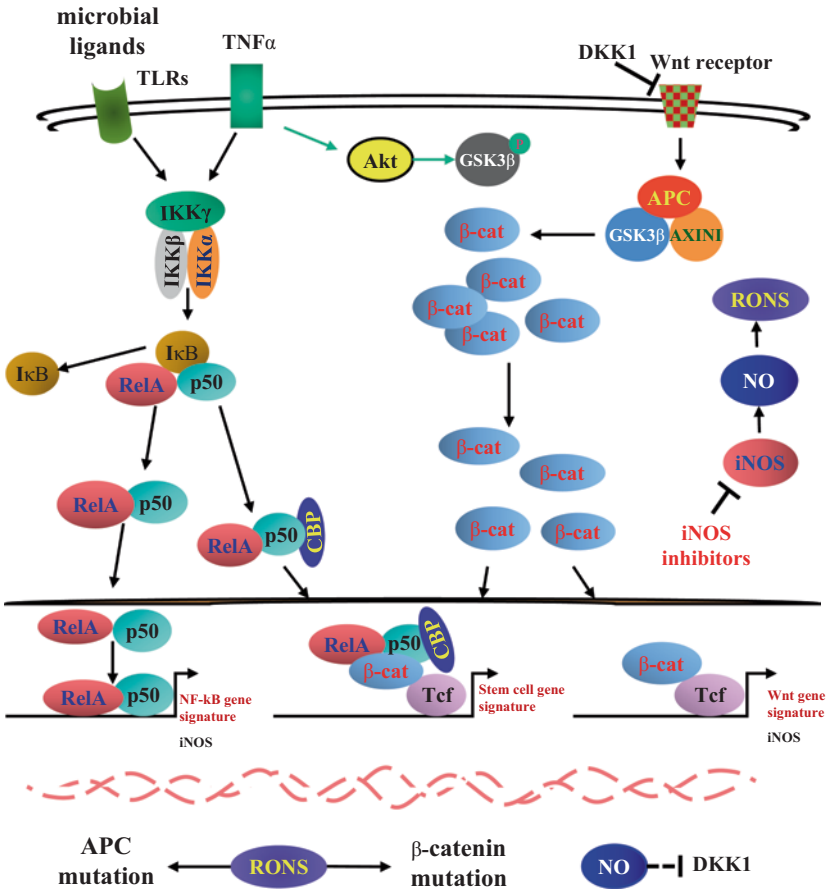


Fig. 1.1 Activated NF-κB/iNOS signaling induces carcinogenic signals through APC/β-catenin pathway. The model shows a mechanism where NF-κB/iNOS and Wnt/β-catenin signaling cross-regulate chronic inflammation-associated carcinogenesis [26, 40, 91]. Chronic inflammation activates NF-κB in intestinal epithelial cells through TNFα, Toll-like and other cytokine receptors, which causes iNOS over-expression and NO production. NO-related RONS can cause APC and/or β-catenin mutation, which leads to β-catenin accumulation in cytosol, and in turn, activated Wnt/β-catenin-TCF signaling, which contributes to intestinal carcinogenesis. iNOS is a transcriptional target of Wnt signaling and also down-regulates DKK1 gene expression. Thus, iNOS is a key mediator of both a positive feedback loop between inflammatory and Wnt signaling, or in a negative feedback loop between Wnt signaling and DKK1. iNOS/NO serves as a dominant switch in the regulation of Wnt/β-catenin and NF-κB pathways. An additional role of NF-κB/iNOS/NO is the anti-apoptotic properties, which prevents inflamed epithelial cells from apoptotic-mediated death. TNFα induced NF-κB binds with CBP and physically interacts with β-catenin/TCF to enhance the transcription of Wnt-dependent stem cell genes

iNOS/NO Regulates the Interaction Between β-catenin/TCF and NF-κB Pathways

Chronic inflammation-associated carcinogenesis involves two major pathways, NF-κB and JAK/STAT [32–34]. Data obtained from these studies demonstrate that iNOS and COX-2 are activated, resulting in p53 and β-catenin DNA mutations. Importantly, iNOS and NO can accelerate APC LOH [39], and cause APC [100] and β-catenin mutation [48, 101]. Activation of β-catenin/TCF signaling is crucial for the initiation of cancer. Based on the evidence from different animal models and clinical observations, we propose that interaction between inflammatory signaling such as constitutive NF-κB activation and β-catenin/TCF pathway contributes to carcinogenesis (Fig. 1.2). Although we previously reviewed the cross-regulation between Wnt and NF-κB signaling pathway [91], new studies have shed additional light on the complex mechanisms. Crosstalk can also occur in the tumor microenvironment between the inflammatory cells and cancer cells.

Macrophage-derived TNFα can induce β-catenin nuclear translocation and potentiates Wnt/β-catenin signaling during gastric carcinogenesis by phosphorylat-

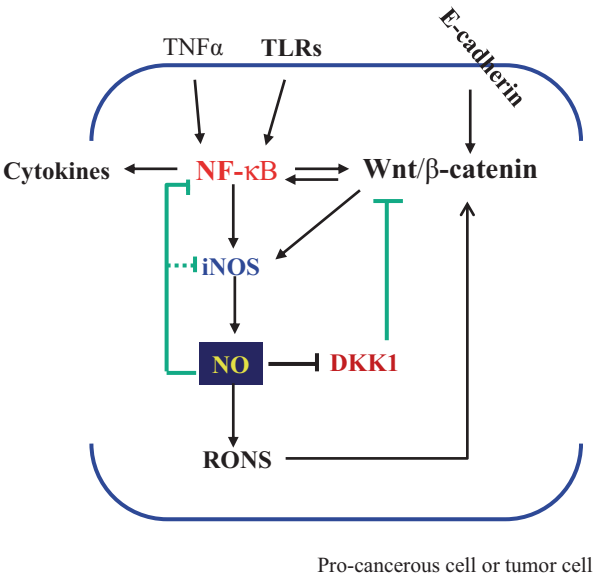


Fig. 1.2 Feedback loops between iNOS/NO, NF-κB and Wnt/β-catenin pathways. NF-κB pathway can be induced through activating TNFα, Toll-like and other cytokine receptors [40, 36]. NF-κB physically interacts with β-catenin/TCF to promote carcinogenesis. iNOS is transcriptionally targeted by both the NF-κB or Wnt/β-catenin pathways [16, 110]. RONS derived from iNOS/NO can cause APC or/and β-catenin mutation [39, 101] and enhance Wnt/β-catenin signaling for carcinogenesis. NO inhibits DKK1 gene expression, which is an antagonist of Wnt signaling [99]. NO production negatively regulates NF-κB pathway for iNOS gene expression and/or iNOS activity. iNOS inhibitors down-regulate Wnt/β-catenin signaling and inhibit carcinogenesis [39, 99]

ing glycogen synthase kinase 3β (GSK-3β) in gastric epithelial cells independently of the NF-κB pathway [23]. The NF-κB pathway indirectly interacts with Wnt/β-catenin between inflammatory cells and cancer cells, because the activation of NF-κB targets TNFα expression in inflammatory cells, which in turn, activates Wnt through NF-κB. By using a genetic model of IEC-restricted constitutive Wnt activation, NF-κB was shown to modulate Wnt signaling where IEC specific ablation of RelA/p65 retards crypt stem cell expansion. In contrast, elevated NF-κB signaling enhances Wnt activation and induces de-differentiation of non-stem cells that acquire tumor-initiating capacity. The data supports the concept of bidirectional conversion and highlights the importance of inflammatory signaling for de-differentiation and generation of tumor-initiating cells in vivo. One molecular mechanism shown was that NF-κB along with CBP physically binds to β-catenin/TCF and enhances Wnt signaling [40].

It is interesting that the *hiNOS* gene is transcriptionally regulated by the NF-κB [102] and Wnt/β-catenin pathways [16]. Recently, Du et al. [92] found that Wnt/β-catenin signaling regulated cytokine- or TNFα-induced hiNOS expression through interaction with NF-κB. SW480 cells stably transformed with wild-type APC showed decreased β-catenin protein and increased TNFα-induced p65 NF-κB binding as well as iNOS and Traf1 expression. β-catenin inversely correlated with iNOS and Fas expression in vivo in hepatocellular carcinoma (HCC) tumor samples. Our in vitro and in vivo data show that β-catenin signaling inversely correlates with cytokine-induced hiNOS and other NF-κB-dependent gene expressions. These findings underscore the complex role of Wnt/β-catenin, NF-κB, and iNOS signaling in the pathophysiology of inflammation-associated carcinogenesis.

Modulation of host cell death pathways induced by chronic inflammation has been recognized as another important aspect of tumorigenesis. For the initiation of cancer cells, one of the early events is to prevent the inflamed cells from death by anti-apoptotic mechanisms. Deng et al demonstrated cross-regulation between NF-κB and β-catenin by direct binding with both p65 and p50 [103]. By interacting with NF-κB subunits, β-catenin markedly attenuated the DNA binding ability of NF-κB complexes, transactivation activity, and target gene expression including Fas and Traf. Interestingly, a strong inverse correlation was also identified between the expression levels of β-catenin and Fas in colon and breast tumors, suggesting that β-catenin regulates NF-κB and its targets in vivo. The consequences of reduced NF-κB DNA-binding activity by β-catenin could promote oncogenesis [103]. It has long been noticed that high levels of induced NO can cause apoptosis, and iNOS and NO are observed to be elevated in chronic colonic inflammation. NO can stimulate apoptosis and, paradoxically, is implicated in the development of colon cancer. Resistance of colonic epithelial cells to the induction of apoptosis may contribute to tumor development. Using a β-catenin overexpression system that increased cytosolic β-catenin rendered colonic epithelial cells more resistant to NO-induced apoptotic cell death, independent of NO-induced accumulation of p53. Mechanisms involved were the inhibition of NO-induced release of cytochrome c from mitochondria, and blocking of NO-induced suppression of the anti-apoptotic protein, Bcl-xL, and phosphorylation of AKT [104]. The consequence is clonal expansion of these cells, which may undergo further transformation to cancer [104].

Crosstalk was also seen in colonic crypts. Binding to receptor ANXII at the plasma membrane, progastrine promotes intracellular signaling by up-regulating the IKKα, β/NF-κB and Wnt/β-catenin pathways in colonic crypts. β-catenin activation, however, was downstream to IKKα, β/NF-κB, because NEMO peptide inhibited β-catenin activation via activating Tyr216 phosphorylation of GSK-3β. NEMO peptide also blocked goblet cell hyperplasia implicating the IKKα, β/NF-κB and/or Wnt/β-catenin pathways in its regulation [105]. Normal intestinal stem cells reside at the bottom of the crypt, and the tumor microenvironment is a niche for gastrointestinal stem cells. The tumor-initiating cells require Wnt signaling, and activated NF-κB by TNFα enhances β-catenin signaling and transcription of the stem cell transcriptome [40, 60]. More evidence from Xenopus axis formation supports this notion. Sensitive axis formation assays in the embryo demonstrate that dorsalization by Wnt/β-catenin requires NF-κB protein activity, and vice versa. *Xenopus nodal-related 3 (Xnr3)* is one of the genes with dual β-catenin/NF-κB input, and a proximal NF-κB consensus site contributes to the regional activity of its promoter. Also in vitro binding of Xenopus β-catenin to several XRel proteins was observed. The interaction is observed in vivo upon Wnt-stimulation. These results suggest that β-catenin acts as a transcriptional co-activator of NF-κB-dependent transcription in frog primary embryonic cells, and imply that the interaction between these two pathways is a genetically conserved mechanism in development [106]. Figure 1.2 summarizes the essential interaction between Wnt and NF-κB signaling pathways in carcinogenesis, and it is clear to assume that iNOS/NO is an important mediator for the cross-regulation between Wnt and NF-κB signaling pathways.

Specific iNOS Inhibitors Down-Regulate β-catenin/TCF Signaling

Evidence from animal studies demonstrates that genetic inhibition of iNOS can decrease Apc/β-catenin signaling-induced carcinogenesis [90, 70]. Other studies have shown that agents that inhibit iNOS protein activity can block carcinogenesis and tumor development through down-regulating β-catenin/TCF signaling. The evidence from specific iNOS inhibitor assays implies that iNOS/NO plays key roles in cancer initiation. Considering the pathologic roles of COX-2 and iNOS for Wnt/β-catenin associated carcinogenesis, selective COX-2 and iNOS inhibitors might play critical roles in inhibiting carcinogenesis and cancer prevention.

Treatment of *Ikkβ (EE)* ^{IEC}*/Apc*$^{+/\Delta IEC}$ mice with the iNOS inhibitor, aminoguanidine hydrochloride (AG), decreased DNA damage markers and reduced early β-catenin (+) lesions and tumor load [39]. Other iNOS specific inhibitors 1400W and BYK191023 have also been shown to decrease carcinogenesis and tumor development in xenograft mouse models [99]. These results along with the observation that long term use of NSAIDs reduced the risk of several cancers [26, 27] may lead to strategies using next generation NSAIDs combined with either iNOS and/or COX-2 inhibitors for the prevention of chronic inflammation-related cancers.

iNOS/NO Activates Wnt/β-catenin Signaling Through Other Oncogenic Pathways

In addition to chronic inflammation associated-activation of Wnt/β-catenin signaling via NF-κB/iNOS-NO signaling, other pathways are also involved in iNOS/NO oncogenic signaling. A research team [107, 108] using ER- breast cancers found that NO play roles in inducing EGFR/Src-mediated activation of oncogenic signal transduction pathways by a mechanism inducing S-nitrosylation. They found that iNOS is associated with a basal-like transcription pattern in human breast cancer. NO-mediated nitrosation of thiols and nitration of tyrosines led to the activation of membrane receptors EGFR, and other proteins Src, Ras, and CD63. These events initiate oncogenic signaling pathways such as PI3K/AKT, RAS/ERK, β-catenin, NF-κB, and AP-1. The results suggest that iNOS can serve as a major "non-mutational driver" of ER- breast cancer [108].

Conclusions and Future Directions

In this chapter we describe iNOS/NO as a key mediator in the regulation of Wnt/β-catenin signaling and its crosstalk with NF-κB. iNOS/NO-induced RONS directly cause APC LOH and β-catenin mutation. Accumulated β-catenin in the cytosol then serves as an important source for β-catenin/TCF oncogenic signaling to interact with NF-κB to initiate intestinal tumor development. By down-regulating DKK1 gene expression, iNOS/NO controls the DKK1 and Wnt negative feedback loop to fine-tune β-catenin/TCF signaling and promotes carcinogenic properties. iNOS regulates Wnt/β-catenin signaling in carcinogenesis initiated by NF-κB activation. Chronic inflammation triggers TNFα, TLRs and other cytokine pathways to activate NF-κB in IECs. NF-κB serves as a central switch to block apoptotic signals in inflamed IECs, and induces iNOS expression and NO production that provoke oxidative and nitrosative stress via ROS and RNS, thereby causing DNA damage in IECs. iNOS/NO-related APC LOH or β-catenin mutation and inhibition of DKK1 expression constitutively activates β-catenin/TCF signaling for carcinogenesis in colon and small intestine (Fig. 1.1).

In this model we also describe that iNOS/NO plays as a dominant role in the crosstalk between the Wnt/β-catenin and NF-κB pathways. There is both a positive feedback loop between iNOS/NO and Wnt/β-catenin signaling pathways, and a negative feedback mechanism between DKK1 and Wnt/β-catenin signaling pathways (Fig. 1.2). Even though research progress has been made for elucidation of the link between inflammation and carcinogenesis, the determinants and detailed mechanisms involved in such divergent pathways are only beginning to be understood and represent a focus for future research.

Developing preventative and therapeutic cancer strategies is a major goal. It is possible that highly selective iNOS inhibitors may be effective for preventing or

treating certain kinds of cancers. Combining iNOS inhibitors with NSAIDs potentially may synergize for more effective anti-cancer effects. On the other hand, an important issue to consider is that treatment-induced inflammation may lead to resistance against an immunotherapy. Studies indicate that TNFα derived from activated macrophages can change the target expression for T cell therapy in melanoma cells and induce resistance [109]. Observation for unexpected consequences will have to be carried out. While iNOS/NO is involved in the regulation of gene expression for carcinogenesis mainly through modification of transcription, it will be essential to also consider epigenetic effects.

Conflict of Interest No conflict statement. No potential conflicts of interest exist.

Acknowledgements This work was supported by the NIH Grant GM52021 and NIH contract HHSN276201200017C (to D.A.G.), and by generous support from the Paul Esposito Foundation for Bile Duct/Liver Cancer Research (D.A.G.).

References

1. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. Nat Rev Cancer 2003;3:276–85.
2. Klaus A, Birchmeier W. Wnt signalling and its impact on development and cancer. Nat Rev Cancer. 2008;8:387–98.
3. Ridnour LA, Thomas DD, Switzer C, Flores-Santana W, Isenberg JS, Ambs S, Roberts DD, Wink DA. Molecular mechanisms for discrete nitric oxide levels in cancer. Nitric Oxide. 2008;19:73–6.
4. Wink DA, Ridnour LA, Hussain SP, Harris CC. The re-emergence of nitric oxide and cancer. Nitric Oxide. 2008;19:65–7.
5. Ambs S, Merriam WG, Bennett WP, Felley-Bosco E, Ogunfusika MO, Oser SM, Klein S, Shields PG, Billiar TR, Harris CC. Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. Cancer Res. 1998;58:334–41.
6. Ambs S, Merriam WG, Ogunfusika MO, Bennett WP, Ishibe N, Hussain SP, Tzeng EE, Geller DA, Billiar TR, Harris CC. p53 and vascular endothelial growth factor regulate tumor growth of NOS2-expressing human carcinoma cells. Nat Med. 1998;4:1371–6.
7. Ambs S, Glynn SA. Candidate pathways linking inducible nitric oxide synthase to a basal-like transcription pattern and tumor progression in human breast cancer. Cell Cycle. 2011;10:619–24.
8. Glynn SA, Boersma BJ, Dorsey TH, Yi M, Yfantis HG, Ridnour LA, Martin DN, Switzer CH, Hudson RS, Wink DA, Lee DH, Stephens RM, Ambs S. Increased NOS2 predicts poor survival in estrogen receptor-negative breast cancer patients. J Clin Invest. 2010;120:3843–54.
9. Jenkins DC, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S. Roles of nitric oxide in tumor growth. Proc Natl Acad Sci U S A. 1995;92:4392–6.
10. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H. Constitutive transcriptional activation by a beta-catenin-Tcf complex in *APC*$^{-/-}$ colon carcinoma. Science. 1997;275:1784–7.
11. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science. 1997;275:1787–90.
12. Goss KH, Groden J. Biology of the adenomatous polyposis coli tumor suppressor. J Clin Oncol. 2000;18:1967–79.

13. Nusse R, Varmus HE. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell. 1982;31:99–109.
14. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. Science. 1998;281:1509–1512.
15. Tetsu O, McCormick F. beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–426
16. Du Q, Park KS, Guo Z, He P, Nagashima M, Shao L, Sahai R, Geller DA, Hussain SP. Regulation of human nitric oxide synthase 2 expression by Wnt beta-catenin signaling. Cancer Res. 2006;66:7024–31.
17. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. N Engl J Med. 2009;361:2449–60.
18. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. Cell Host Microbe. 2013;14:195–206.
19. Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, Clancy TE, Chung DC, Lochhead P, Hold GL, El-Omar EM, Brenner D, Fuchs CS, Meyerson M, Garrett WS. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe. 2013;14:207–15.
20. Santaolalla R, Sussman DA, Ruiz JR, Davies JM, Pastorini C, Espana CL, Sotolongo J, Burlingame O, Bejarano PA, Philip S, Ahmed MM, Ko J, Dirsina R, Barrett TA, Shang L, Lira SA, Fukata M, Abreu MT. TLR4 activates the β-catenin pathway to cause intestinal neoplasia. PloS ONE. 2013;8:e6392.
21. Bansal K, Trinath J, Chakravortty D, Patil SA, Balaji KN. Pathogen-specific TLR2 protein activation programs macrophages to induce Wnt-β-catenin signaling. J Biol Chem. 2011;286:37032–37044.
22. Kaler P, Godasi BN, Augenlicht L, Klampfer L. The NF-kappaB/AKT-dependent Induction of Wnt Signaling in Colon Cancer Cells by Macrophages and IL-1beta. Cancer Microenviron. Cancer Microenviron. 2009;2:69–80.
23. Oguma K, Oshima H, Aoki M, Uchio R, Naka K, Nakamura S, Hirao A, Saya H, Taketo MM, Oshima M. Activated macrophages promote Wnt signalling through tumour necrosis factor-alpha in gastric tumour cells. EMBO J. 2008;27:1671–81.
24. Beck PL, Li Y, Wong J, Chen CW, Keenan CM, Sharkey KA, McCafferty DM. Inducible nitric oxide synthase from bone marrow-derived cells plays a critical role in regulating colonic inflammation. Gastroenterology. 2007;132:1778–90.
25. Talero E, Sánchez-Fidalgo S, Villegas I, de la Lastra CA, Illanes M, Motilva V. Role of different inflammatory and tumor biomarkers in the development of ulcerative colitis-associated carcinogenesis. Inflamm Bowel Dis. 2011;17:696–710.
26. Clevers H. At the crossroads of inflammation and cancer. Cell 2004;118:671–674.
27. Balkwill F, Coussens LM. Cancer: an inflammatory link. Nature 2004;431:405–6.
28. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010; 140:883–99.
29. Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomized trials. Lancet. 2011;377:31–41.
30. Hussain SP, Harris CC. Inflammation and cancer: an ancient link with novel potentials. Int J Cancer. 2007;121:2373–80.
31. Vendramini-Costa DB, Carvalho JE. Molecular link mechanisms between inflammation and cancer. Curr Pharm. 2012;18:3831–52.
32. Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF, Karin M. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell. 2004;118:285–296.

33. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature. 2004;431:461–466.
34. Hanada T, Kobayashi T, Chinen T, Saeki K, Takaki H, Koga K, Minoda Y, Sanada T, Yoshioka T, Mimata H, Kato S, Yoshimura A. IFNgamma-dependent, spontaneous development of colorectal carcinomas in SOCS1-deficient mice. J Exp Med. 2006;203:1391–7.
35. Bollrath J, Greten FR. IKK/NF-kB and STAT3 pathways: central signalling hubs in inflammation-mediated tumour promotion and metastasis. EMBO Rep. 2009;10:1314–9.
36. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004;118:229–41.
37. Grivennikov SI, Karin M. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. Cytokine Growth Factor Rev. 2010;21:11–9.
38. Vlantis K, Wullaert A, Sasaki Y, Schmidt-Supprian M, Rajewsky K, Roskams T, Pasparakis M. Constitutive IKK2 activation in intestinal epithelial cells induces intestinal tumors in mice. J Clin Invest. 2011;121:2781–93.
39. Shaked H, Hofseth LJ, Chumanevich A, Chumanevich AA, Wang J, Wang Y, Taniguchi K, Guma M, Shenouda S, Clevers H, Harris CC, Karin M. Chronic epithelial NF-kB activation accelerates APC loss and intestinal tumor initiation through iNOS up-regulation. Proc Natl Acad Sci U S A. 2012;109:14007–12.
40. Schwitalla S, Fingerle AA, Cammareri P, Nebelsiek T, Göktuna SI, Ziegler PK, Canli O, Heijmans J, Huels DJ, Moreaux G, Rupec RA, Gerhard M, Schmid R, Barker N, Clevers H, Lang R, Neumann J, Kirchner T, Taketo MM, van den Brink GR, Sansom OJ, Arkan MC, Greten FR. Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. Cell. 2013;152:25–38.
41. Xia Y, Padre RC, De Mendoza TH, Bottero V, Tergaonkar VB, Verma IM. Phosphorylation of p53 by IkappaB kinase 2 promotes its degradation by beta-TrCP. Proc Natl Acad Sci U S A. 2009;106:2629–34.
42. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol. 1997;15:323–50.
43. Beck PL, Xavier R, Wong J, Ezedi I, Mashimo H, Mizoguchi A, Mizoguchi E, Bhan AK, Podolsky DK. Paradoxical roles of different nitric oxide synthase isoforms in colonic injury. Am J Physiol Gastrointest Liver Physiol. 2004;286:G137–47.
44. Kolios G, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. Immunology. 2004;113:427–37.
45. Wink DA, Kasprzak KS, Maragos CM, Elespuru RK, Misra M, Dunams TM, Cebula TA, Koch WH, Andrews AW, Allen JS, et al. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science. 1991;254:1001–3.
46. Wink DA, Vodovotz Y, Laval J, Laval F, Dewhirst MW, Mitchell JB. The multifaceted roles of nitric oxide in cancer. Carcinogenesis. 1998;19:711–721.
47. Stamler JS, Simon DI, Jaraki O, Osborne JA, Francis S, Mullins M, Singel D, Loscalzo J. S-nitrosylation of tissue-type plasminogen activator confers vasodilatory and antiplatelet properties on the enzyme. Proc Natl Acad Sci U S A 1992;89:8087–91.
48. Cheng L, Lai MD. Aberrant crypt foci as microscopic precursors of colorectal cancer. World J Gastroenterol. 2003;9:2642–9.
49. Hemish J, Nakaya N, Mittal V, Enikolopov G. Nitric oxide activates diverse signaling pathways to regulate gene expression. J Biol Chem. 2003;278:42321–9.
50. Kuzin B, Roberts I, Peunova N, Enikolopov G. Nitric oxide regulates cell proliferation during Drosophila development. Cell. 1996;87:639–49.
51. Ekmekcioglu S, Ellerhorst JA, Prieto VG, Johnson MM, Broemeling LD, Grimm EA. Tumor iNOS predicts poor survival for stage III melanoma patients. Int J Cancer. 2006;119:861–6.
52. Ekmekcioglu S, Ellerhorst J, Smid CM, Prieto VG, Munsell M, Buzaid AC, Grimm EA. Inducible nitric oxide synthase and nitrotyrosine in human metastatic melanoma tumors correlate with poor survival. Clin Cancer Res. 2000;6:4768–75.

53. Eyler CE, Wu Q, Yan K, MacSwords JM, Chandler-Militello D, Misuraca KL, Lathia JD, Forrester MT, Lee J, Stamler JS, Goldman SA, Bredel M, McLendon RE, Sloan AE, Hjelmeland AB, Rich JN. Glioma stem cell proliferation and tumor growth are promoted by nitric oxide synthase-2. Cell. 2011;146:53–66.
54. Ambs S, Bennett WP, Merriam WG, Ogunfusika MO, Oser SM, Harrington AM, Shields PG, Felley-Bosco E, Hussain SP, Harris CC. Relationship between p53 mutations and inducible nitric oxide synthase expression in human colorectal cancer. J Natl Cancer Inst. 1999;91:86–8.
55. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. Nat Rev Cancer. 2006;6:521–34.
56. Halliwell B. Oxidative stress and cancer: have we moved forward? Biochem J. 2007;401:1–11.
57. Handa O, Naito Y, Yoshikawa T. Redox biology and gastric carcinogenesis: the role of Helicobacter pylori. Redox Rep. 2011;16:1–7.
58. Meira LB, Bugni JM, Green SL, Lee CW, Pang B, Borenshtein D, Rickman BH, Rogers AB, Moroski-Erkul CA, McFaline JL, Schauer DB, Dedon PC, Fox JG, Samson LD. DNA damage induced by chronic inflammation contributes to colon carcinogenesis in mice. J Clin Invest. 2008;118:2516–25.
59. Vilar E, Gruber SB. Microsatellite instability in colorectal cancer-the stable evidence. Nat Rev Clin Oncol. 2010;7:153–62.
60. Quante M, Varga J, Wang TC, Greten FR. The gastrointestinal tumor microenvironment. Gastroenterology. 2013;145:63–78.
61. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell. 2012;21:309–22.
62. Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. Nature. 2013;501:346–54.
63. Berg DJ, Davidson N, Kühn R, Müller W, Menon S, Holland G, Thompson-Snipes L, Leach MW, Rennick D. Enterocolitis and colon cancer in interleukin-10-deficient mice are associat with aberrant cytokine production and CD4(+) TH1-like responses. J Clin Invest. 1996;98:1010–20.
64. Shah SA, Simpson SJ, Brown LF, Comiskey M, de Jong YP, Allen D, Terhorst C. Development of colonic adenocarcinomas in a mouse model of ulcerative colitis. Inflamm Bowel Dis. 1998;4:196–202.
65. Takahashi M, Mutoh M, Kawamori T, Sugimura T, Wakabayashi K. Altered expression of a-catenin, inducible nitric oxide synthase and cyclooxygenase-2 in azoxymethane-induced rat colon carcinogenesis. Carcinogenesis. 2000;21:1319–27.
66. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Cancer Sci. 2003;94:965–73.
67. Tammali R, Reddy AB, Ramana KV, Petrash JM, Srivastava SK. Aldose reductase deficiency in mice prevents azoxymethane-induced colonic preneoplastic aberrant crypt foci formation. Carcinogenesis. 2009;30:799–807.
68. Cheung C, Loy S, Li GX, Liu AB, Yang CS. Rapid induction of colon carcinogenesis in CYP1A-humanized mice by 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine and dextran sodium sulfate. Carcinogenesis. 2011;32:233–9.
69. Tanaka T, Suzuki R, Kohno H, Sugie S, Takahashi M, Wakabayashi K. Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine and dextran sodium sulfate in male ICR mice possess beta-catenin gene mutations and increases immunoreactivity for beta-catenin, cyclooxygenase-2 and inducible nitric oxide synthase. Carcinogenesis. 2005;26:229–38.
70. Nam KT, Oh SY, Ahn B, Kim YB, Jang DD, Yang KH, Hahm KB, Kim DY. Decreased Helicobacter pylori associated gastric carcinogenesis in mice lacking inducible nitric oxide synthase. Gut. 2004;53:1250–5.

71. Suzuki R, Kohno H, Suzui M, Yoshimi N, Tsuda H, Wakabayashi K, Tanaka T. An animal model for the rapid induction of tongue neoplasms in human c-Ha-ras proto-oncogene transgenic rats by 4-nitroquinoline 1-oxide: its potential use for preclinical chemoprevention studies. Carcinogenesis. 2006;27:619–30.
72. Kohno H, Suzuki R, Sugie S, Tanaka T. Beta-Catenin mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate. Cancer Sci. 2005;96:69–76.
73. Fransén K, Dimberg J, Osterström A, Olsson A, Söderkvist P, Sirsjö A. Nitric oxide synthase 2 mRNA expression in relation to p53 and adenomatous polyposis coli mutations in primary colorectal adenocarcinomas. Surgery. 2002;131:384–92.
74. Villegas I, Sánchez-Fidalgo S, de la Lastra CA. Chemopreventive effect of dietary curcumin on inflammation-induced colorectal carcinogenesis in mice. Mol Nutr Food Res. 2011;55:259–67.
75. Ravichandran K, Velmurugan B, Gu M, Singh RP, Agarwal R. Inhibitory effect of silibinin against azoxymethane-induced colon tumorigenesis in A/J mice. Clin Cancer Res. 2010;16:4595–606.
76. Rajamanickam S, Kaur M, Velmurugan B, Singh RP, Agarwal R. Silibinin suppresses spontaneous tumorigenesis in *APC*$^{min/+}$ mouse model by modulating beta-catenin pathway. Pharm Res. 2009;26:2558–67.
77. Lai CS, Tsai ML, Cheng AC, Li S, Lo CY, Wang Y, Xiao H, Ho CT, Wang YJ, Pan MH. Chemoprevention of colonic tumorigenesis by dietary hydroxylated polymethoxyflavones in azoxymethane-treated mice. Mol Nutr Food Res. 2011;55:278–90.
78. Li H, Wu WK, Li ZJ, Chan KM, Wong CC, Ye CG, Yu L, Sung JJ, Cho CH, Wang M. 2, 3', 4, 4', 5'-Pentamethoxy-trans-stilbene, a resveratrol derivative, inhibits colitis-associated colorectal carcinogenesis in mice. Br J Pharmacol. 2010;160:1352–61.
79. Velmurugan B, Singh RP, Agarwal R, Agarwal C. Dietary-feeding of grape seed extract prevents azoxymethane-induced colonic aberrant crypt foci formation in fischer 344 rats. Mol Carcinog. 2010;49:641–52.
80. Kim JK, Shin EK, Park JH, Kim YH, Park JH. Antitumor and antimetastatic effects of licochalcone A in mouse models. J Mol Med (Berl). 2010;88:829–38.
81. Paul S, De Castro AJ, Lee HJ, Smolarek AK, So JY, Simi B, Wang CX, Zhou R, Rimando AM, Suh N. Dietary intake of pterostilbene, a constituent of blueberries, inhibits the beta catenin/p65 downstream signaling pathway and colon carcinogenesis in rats. Carcinogenesis. 2010;31:1272–8.
82. Wu Y, Antony S, Meitzler JL, Doroshow JH. Molecular mechanisms underlying chronic inflammation-associated cancers. Cancer Lett. 2014;345:164–73.
83. Mei JM, Hord NG, Winterstein DF, Donald SP, Phang JM. Differential formation of beta-catenin/lymphoid enhancer factor-1 DNA binding complex induced by nitric oxide in mouse colonic epithelial cells differing in adenomatous polyposis coli (Apc) genotype. Cancer Res. 2000;60:3379–83.
84. Mei JM, Hord NG, Winterstein DF, Donald SP, Phang JM. Expression of prostaglandin endoperoxide H synthase-2 induced by nitric oxide in conditionally immortalized murine colonic epithelial cells. FASEB J. 2000;14:1188–201.
85. Mei JM, Borchert GL, Donald SP, Phang JM. Matrix metalloproteinase(s) mediate(s) NO-induced dissociation of beta-catenin from membrane bound E-cadherin and formation of nuclear beta-catenin/LEF-1 complex. Carcinogenesis. 2002;23:2119–22.
86. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science 1990;247:322–4.
87. Luongo C, Moser AR, Gledhill S, Dove WF Loss of Apc+ in intestinal adenomas from Min mice. Cancer Res. 1994;54:5947–5952.

88. Tanaka T, Kohno H, Suzuki R, Hata K, Sugie S, Niho N, Sakano K, Takahashi M, Wakabayashi K. Dextran sodium sulfate strongly promotes colorectal carcinogenesis in *Apc*$^{(Min/+)}$ mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. Int J Cancer. 2006;118:25–34.
89. Velmurugan B, Singh RP, Kaul N, Agarwal R, Agarwal C. Dietary feeding of grape seed extract prevents intestinal tumorigenesis in *APC*$^{Min/+}$ mice. Neoplasia. 2010;12(1):95–102.
90. Ahn B, Ohshima H. Suppression of intestinal polyposis in Apc (Min/+) mice by inhibiting nitric oxide production. Cancer Res. 2001;61:8357–60.
91. Du Q, Geller DA. Cross-regulation between Wnt and NF-kB signaling pathways. Immunopathol Dis Therap. 2010;1:155–81.
92. Du Q, Zhang X, Cardinal J, Cao Z, Guo Z, Shao L, Geller DA. Wnt/beta-catenin signaling regulates cytokine-induced human inducible nitric oxide synthase expression by inhibiting nuclear factor-kappaB activation in cancer cells. Cancer Res. 2009;69:3764–71.
93. Niehrs C. Function and biological roles of the Dickkopf family of Wnt modulators. Oncogene. 2006;25:7469–81.
94. Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature. 1998;391:357–62.
95. He X, Semenov M, Tamai K, Zeng X. LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development. 2004;131:1663–77.
96. Chamorro MN, Schwartz DR, Vonica A, Brivanlou AH, Cho KR, Varmus HE. FGF-20 and DKK1 are transcriptional targets of beta-catenin and FGF-20 is implicated in cancer and development. EMBO J. 2005;24:73–84.
97. Gonzalez-Sancho JM, Brennan KR, Castelo-Soccio LA, Brown AM. Wnt proteins induce dishevelled phosphorylation via an LRP5/6-independent mechanism, irrespective of their ability to stabilize beta-catenin. Mol Cell Biol. 2004;24:4757–68.
98. Niida A, Hiroko T, Kasai M, Furukawa Y, Nakamura Y, Suzuki Y, Sugano S, Akiyama T. DKK1, a negative regulator of Wnt signaling, is a target of the betacatenin/TCF pathway. Oncogene. 2004;23:8520–6.
99. Du Q, Zhang X, Liu Q, Zhang X, Bartels CE, Geller DA. Nitric oxide production upregulates Wnt/β-catenin signaling by inhibiting Dickkopf-1. Cancer Res. 2013;73:6526–37.
100. Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW. APC mutations occur early during colorectal tumorigenesis Nature. 1992;359:235–7.
101. Takahashi M, Wakabayashi K. Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. Cancer Sci. 2004;95:475–80.
102. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem. 1994;269:4705–8.
103. Deng J, Miller SA, Wang HY, Xia W, Wen Y, Zhou BP, Li Y, Lin SY, Hung MC. Beta-catenin interacts with and inhibits NF-kappa B in human colon and breast cancer. Cancer Cell. 2002;2:323–34.
104. Wang H, MacNaughton WK. Overexpressed beta-catenin blocks nitric oxide-induced apoptosis in colonic cancer cells. Cancer Res. 2005;65:8604–7.
105. Umar S, Sarkar S, Wang Y, Singh P. Functional cross-talk between beta-catenin and NFkappaB signaling pathways in colonic crypts of mice in response to progastrin. J Biol Chem. 2009;284:22274–84.
106. Armstrong NJ, Fagotto F, Prothmann C, Rupp RA. Maternal Wnt/β-catenin signaling coactivates transcription through NF-kB binding sites during Xenopus axis formation. PLoS ONE. 2012;7:e36136.
107. Switzer CH, Glynn SA, Cheng RY, Ridnour LA, Green JE, Ambs S, Wink DA. S-nitrosylation of EGFR and Src activates an oncogenic signaling network in human basal-like breast cancer. Mol Cancer Res. 2012;10:1203–15.

108. Switzer CH, Ridnour LA, Cheng R, Heinecke J, Burke A, Glynn S, Ambs S, Wink DA. S-nitrosation mediates multiple pathways that lead to tumor progression in estrogen receptor-negative breast cancer. For Immunopathol Dis Therap. 2012;3:117–24.
109. Landsberg J, Kohlmeyer J, Renn M, Bald T, Rogava M, Cron M, Fatho M, Lennerz V, Wölfel T, Hölzel M, Tüting T. Melanomas resist T-cell therapy through inflammation-induced reversible dedifferentiation. Nature. 2012;490:412–6.
110. Taylor BS, de Vera ME, Ganster RW, Wang Q, Shapiro RA, Morris SM Jr, Billiar TR, Geller DA. Multiple NF-kappaB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. J Biol Chem. 1998;273:15148–56.

Chapter 2
Nitric Oxide and Genomic Stability

Vasily A. Yakovlev

Abstract Epidemiological evidence accumulating over the years has provided a positive correlation between cancer incidence and chronic inflammation. Regardless of etiology, inflammatory conditions are characterized by overexpression of inducible nitric oxide synthase (iNOS) and overproduction of nitric oxide/reactive nitrogen species (NO/RNS) in epithelial and inflammatory cells at the site of carcinogenesis. NO/RNS produced in infected and inflamed tissues can contribute to the process of carcinogenesis by different mechanisms. In this chapter, we discuss NO/RNS-dependent mechanisms of genomic instability (GI) and bystander effects. We explain the mechanism of "synthetic lethality" of the NO-donor/PARP-inhibitor combination and its role in sensitization of the cancer cells to DNA-damaging agents. We postulate the "mutator field" theory and the definition of mutagenesis efficacy.

Keywords BRCA1 · Bystander effect · Chronic inflammation · Genomic instability · Homologous recombination · Nitric Oxide · Non-homologous end joining · NOS · RAD51 · Synthetic lethality

Abbreviations

BDD	Bystander DNA damage
BER	Base excision repair
BRCA1	Breast cancer type 1 susceptibility protein;
DSB	Double-strand brake
EADC	Esophageal adenocarcinoma
eNOS	Endothelial nitric oxide synthase
GERD	Gastro-esophageal reflux disease
HCC	Hepatocellular carcinoma

V. A. Yakovlev (✉)
Department of Radiation Oncology, VCU/Massey Cancer Center, Virginia Commonwealth University, 401 College Street, Rm. 381, Richmond, VA 2398, USA
e-mail: vayakovlev@vcu.edu

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_2

HRND	High concentration range of NO-donors
IBDs	Inflammatory bowel diseases
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharides
MN	Micronuclei
MRND	Moderate concentration range of NO-donors
NHEJ	Non-homologous end joining
NHL	Non-Hodgkin lymphoma
NO/RNS	Nitric oxide/reactive nitrogen species
OLP	Oral lichen planus
OSCC	Oral squamous cell carcinoma
OV	Opisthorchis viverrini
PARP1	Poly(ADP-ribose) polymerase 1
PC	Prostate cancer
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphisms
SSB	Single-strand breaks
SSF	Stress signal factors

Inflammation and Carcinogenesis: Role of NO/RNS Generation

The link between inflammation and cancer was proposed more than 150 years ago when Virchow suggested that malignancies tend to arise at sites of chronic inflammation. Epidemiological evidence accumulating over the years has provided a positive correlation between cancer incidence and chronic inflammation [2], and it is now a well-recognized hallmark of cancer development [3–5]. Infection is proposed to contribute to carcinogenesis through inflammation- related mechanisms. Infection with hepatitis C virus, *Helicobacter pylori* and the liver fluke, Opisthorchis viverrini (OV), are important risk factors for hepatocellular carcinoma (HCC), gastric cancer, and cholangiocarcinoma, respectively. Inflammatory bowel diseases (IBDs) and oral diseases, such as oral lichen planus (OLP) and leukoplakia, are associated with colon carcinogenesis and oral squamous cell carcinoma (OSCC). Chronic gastro-esophageal reflux disease (GERD) underlies molecular progression to esophageal adenocarcinoma (EADC). Regardless of etiology, all these conditions are characterized by overexpression of inducible nitric oxide synthase (iNOS), overproduction of NO/RNS in epithelial and inflammatory cells at the site of carcinogenesis, and formation of DNA damages [6–16]. Antibacterial, antiviral, and antiparasitic drugs dramatically diminished the inflammatory stimulated iNOS expression and formation of the DNA lesions. iNOS inhibitors and NO/RNS-scavengers also significantly reduced DNA damages [13, 17, 18]. Therefore, it is considered that excessive amounts of reactive nitrogen species produced via iNOS during chronic

inflammation may play a key role in stimulation of DNA damages formation and activation of carcinogenesis.

Non-Inflammatory Stimulation of NO/RNS Generation and Its Role in Carcinogenesis

Not only inflammatory conditions can stimulate different types of NOSs. It would be logical to assume that any prolonged stimulation of NO/RNS production can lead to accumulation of DNA lesions and carcinogenesis. For example, the activity of NOSs can be stimulated by single nucleotide polymorphisms (SNPs). It was shown that SNP of the iNOS gene (*NOS2A* Ser608Leu) was responsible for a twofold risk increase for non-Hodgkin lymphoma (NHL) (OR = 2.2, 95 % CI = 1.1–4.4). This risk increase was consistent by cell lineage (B- and T-cell NHL) and pronounced for the two most common subtypes, diffuse large B-cell (OR = 3.4,95 % CI = 1.5–7.8) and follicular lymphomas (OR = 2.6, 95 % CI = 1.0-6.8) [19]. Another group of investigators revealed that the polymorphism in the promoter region of the *endothelial NOS (eNOS)* gene (786Thr > Cys) was the most important promoter alteration of the *eNOS* gene, which significanlty affected the prostate cancer (PC) progression. The incorporation of the Cys allele was associated with increased levels of eNOS transcripts and responsible for variations in the plasma NO, which may promote cancer progression by providing a selective growth advantage to a tumor. The authors suggested that NOS3 transcript level may be used as a biomarker together with the PCA3 marker for molecular staging of the PC [20].

NO/RNS: Different Concentrations, Different Effects

NO/RNS production is often associated with contradictory effects on cell proliferation and cytotoxicity, variably promoting and inhibiting apoptosis in normal and tumor cells [21–23]. Wink and coworkers have examined these contradictory observations and have proposed a set of five graduated levels of NO/RNS cellular responses that range from the promotion of cell survival and proliferation at low concentrations of NO/RNS to the promotion of cell cycle arrest and apoptosis at high concentrations of NO/RNS [21]. While high concentrations of NO/RNS can cause direct DNA damage and stimulate DNA double-strand brake (DSB), there is an emerging appreciation for determining the role of lower NO/RNS concentrations in signaling pathways related to apoptosis, cell cycle, and other facets of cell functions.

Previous reports show that NO/RNS production correlates with NO donor concentrations and time of incubation [24]. Measurements, using an NO-specific electrode, of actual NO concentrations during cell exposure to 125 to 500 μmol/L

of DETA indicated relatively constant NO concentrations in the 150–400 nmol/L range [24–26]. The NO concentrations produced *in vivo* at sites of colonic crypt chronic inflammation and airway inflammation were below 300 nmol/L and below 400 nmol/L, respectively [25, 27]. Many solid tumors grow in the inflammatory microenvironment [28–30], and it was demonstrated that the NO concentration was significantly higher than in the normal tissue. The NO concentration in the cutaneous melanoma reaches 200 nmol/L with the maximum on the periphery of the tumor [31]. In comparison, normal *in vivo* NO concentrations in the absence of inflammation are unlikely to exceed 50 nmol/L [8]. Hence, the amount of NO produced from ~ 100 to ~ 350 μmol/L of NO-donors (DETA NONOate or SNAP) *in vitro* represents the NO concentrations maintained *in vivo* at sites of the chronic inflammation and the growing edge of melanoma. This moderate concentration range of NO-donors (MRND) has a very interesting number of qualities (Fig. 2.1): A. It doesn't stimulate direct DNA damage and, as a result, doesn't affect ATM/ATR-dependent pathways [32]; B. It doesn't inhibit cell proliferation (in fact, 50–200 μmol/L of DETA NONOate or SNAP demonstrates a stimulatory effect on proliferation of the different cell lines); C. It significantly down-regulates error-free homologous

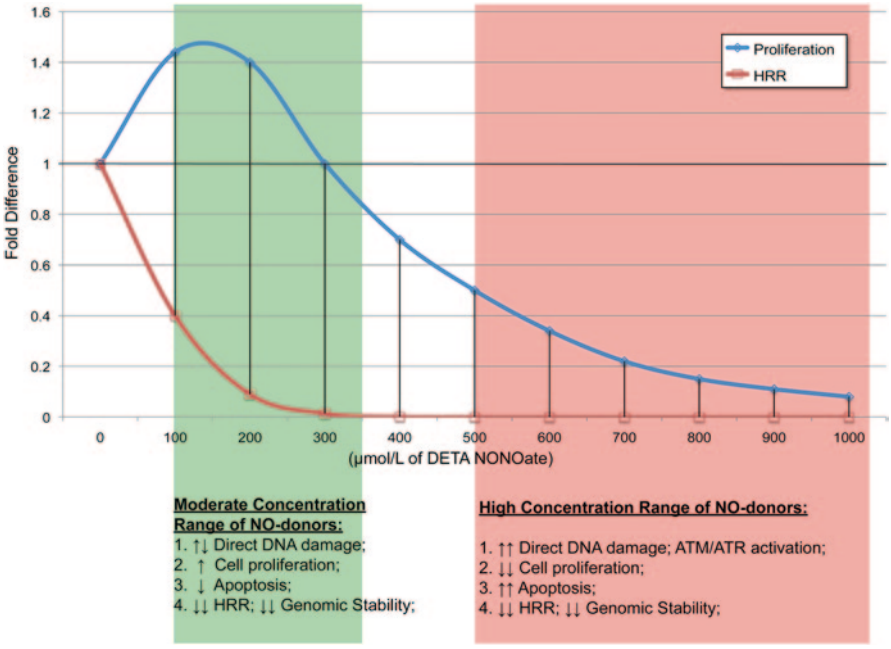


Fig. 2.1 Schematic representation of changes in the cell proliferation and DNA Homologous Recombination Repair (HRR) as a function of NO-donor (DETA NONOate) concentration. The *green zone* represents moderate concentration range of NO-donors in vitro and corresponds to NO/RNS concentrations under chronic inflammatory conditions and tumors inflammatory microenvironment in vivo. The *red zone* represents high concentration range of NO-donors, which is widely used for investigation of NO-dependent DNA damage

Table 2.1 The relative fold decrease of HRR by different concentrations of the NO-donors. A-549 (human lung adenocarcinoma epithelial cell line) and MCF-10A (immortalized human breast epithelial cell line) were incubated 18 h with indicated concentrations of NO-donors (SNAP or DETA NONOate). (HRR was measured by DRGFP reporter based assay [1])

NO-donor concentration (μM)	A549		MCF-10 A	
	SNAP	DETA	SNAP	DETA
0	1	1	1	1
50	1.84	1.39	1.54	2.06
100	2.29	2.73	1.95	3.36
200	5.08	20.89	7.4	24.67
300	37.6	188	74	74

recombination repair (HRR) of DNA DSBs (see Table 2.1) and substitutes it by error-prone non-homologous end joining (NHEJ), which stimulates the effect of genomic instability (NO/RNS-dependent mechanisms of HRR-NHEJ substitution will be discussed below). DSBs are serious genetic lesions that must be repaired to prevent catastrophic loss of chromosomes. In general, two classes of mechanisms exist for repairing DSBs, HRR or NHEJ [33]. HRR requires an identical (or nearly identical) template strand of DNA to mend a lesion whereas NHEJ repairs a double-strand gap in DNA without a homologous template. NHEJ is guided entirely by information in the lesion, which makes NHEJ error prone in comparison to HRR.

As it was shown by different groups of investigators, significant DNA damage as well as stimulation of DNA-damage signaling pathways (for example: ATM/ATR-dependent phosphorylation and activation of p53) can be achieved by using a high concentration range of NO-donors (HRND) > 500 μmol/L of NO-donor [34–36, 32]. The HRND is also characterized by significant inhibition of the cell proliferation and the activation of apoptosis (Fig. 2.1).

If we compare the efficacy of MRND and HRND with respect to mutagenesis, MRND allows the generation of DNA errors by switching from error free HRR to error-prone NHEJ. Also, stimulation of cell proliferation and downregulation of apoptosis by MRND of NO-donors facilitate the accumulation of the DNA errors in new generations of cells. On the other hand, HRND generates much more DNA errors by downregulation of HRR and by direct DNA damage. However, inhibition of the cell proliferation and the activation of apoptosis leads to elimination of most of the affected cells and prevents the accumulation of DNA mutations in the next cells generations. Hence, MRND, as well as the NO/RNS concentrations maintained *in vivo* at sites of chronic inflammation and growing edges of tumors, can be characterized as the ***most favorable environment for mutagenesis*** (MFEM) (see Fig. 2.1).

Mechanisms of NO/RNS-Dependent Mutagenesis

NO/RNS produced in infected and inflamed tissues can contribute to the process of carcinogenesis by different mechanisms. NO/RNS mediate cellular regulation through the posttranslational modifications of a number of regulatory proteins. The best studied of these modifications are S-nitrosylation (reversible oxidation of cysteine) [37–39] and tyrosine nitration [40–42]. NO/RNS-dependent posttranslational proteins modifications (Tyrosine nitration and S-nitrosylation) are well-accepted markers of the tissue inflammation, also stimulating attention by their significant impact to carcinogenesis and tumor growth. These modifications can up- or down-regulate functions of many proteins. The breast cancer type 1 susceptibility protein (BRCA1) contributes to cell viability in multiple ways, it plays a critical role in HRR of DNA DSBs, cell cycle checkpoint control, transcription, and regulation of chromosome segregation [43–45]. The loss of BRCA1 protein function predisposes to the development of breast and ovarian cancers [46].

BRCA1 expression is negatively regulated at the transcriptional level by the repressive complex of retinoblastoma-like protein 2 (RBL2) and E2F4. Formation of the repression RBL2/E2F4 complex can be accelerated by, for example, RBL2 dephosphorylation. Recently, protein phosphatase 2 A (PP2A), an enzyme responsible for RBL2 dephosphorylation, was shown to be activated by nitration on Tyr284 [47]. Inflammatory levels of NO/RNS, which don't induce significant DNA damage and maintains the ATM/ATR-dependent pathways intact, stimulate substantial dephosphorylation of RBL2. RBL2 dephosphorylation promotes a repressive RBL2/E2F4 complex formation with subsequent block of BRCA1 expression (Fig. 2.2) [1]. As result, BRCA1-dependent mechanisms of genomic stability can be significantly compromised. Interestingly, the same mechanism of BRCA1 downregulation takes place in the different types of cells under hypoxic condition [48]. That NOS activity and NO/RNS generations are stimulated under certain hypoxic conditions [49–51] suggest that some pro-carcinogenic effects of hypoxic microenvironment are also NO/RNS- dependent.

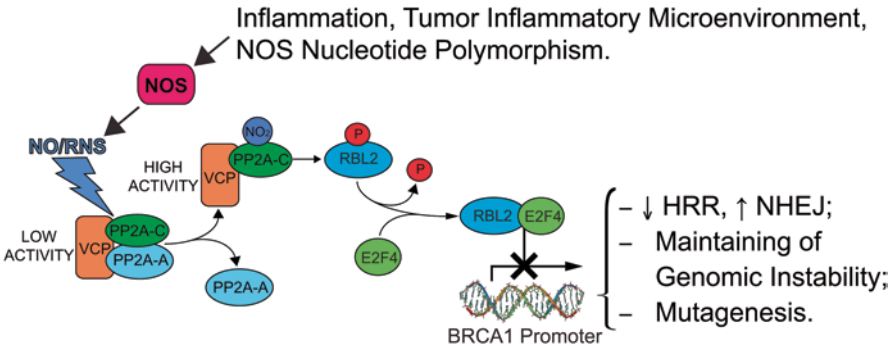


Fig. 2.2 Schematic representation of NO/RNS-dependent inhibition of BRCA1 protein expression

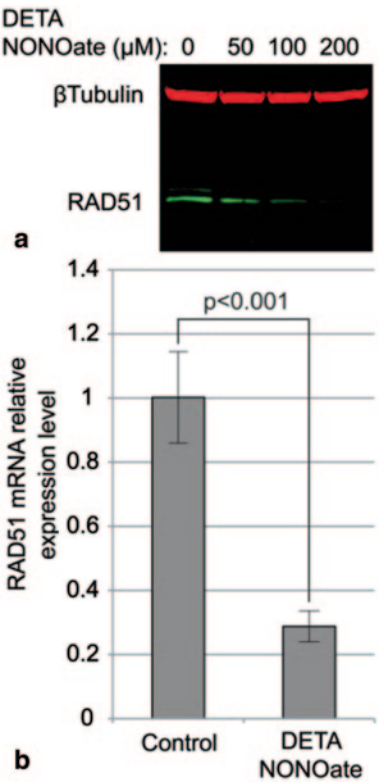


Fig. 2.3 NO/RNS-dependent down regulation of RAD51 protein expression in A549 cell line. A, Level of RAD51 protein after 18h of incubation with different doses of DETA NONOate. B, qPCR analysis of endogenous RAD51 expression after 18h of incubation with 200 μM of DETA NONOate. RAD51 expression was normalized to 18 S mRNA expression. Data are presented as the mean ± SD of 4 experiments. The P value was calculated with the Student t test. (unpublished data)

We recently found that protein RAD51, another critical mediator of HRR, is downregulated by NO/RNS (Fig. 2.3). Analyses of the RAD51 gene promoter activity and mRNA stability suggested that the NO/RNS-dependent regulation of this gene occurs via transcriptional repression (unpublished data). The E2F site in the *RAD51* promoter matches an identical sequence at a similar relative location within the *BRCA1* proximal promoter [52], and this *BRCA1* promoter element also mediates the repression of this gene in response to NO/RNS increase (Fig. 2.2). Hence, the expression of both proteins critical for HRR can be affected through the same signaling pathway: RNS/PP2A/RBL2/E2F4/proximal promoter of *BRCA1* and *RAD51*.

Synthetic Lethality: Combination of NO-Donors and PARP-Inhibitors

Poly(ADP-ribose) polymerase 1 (PARP1) is an abundant nuclear enzyme that synthesizes poly(ADP-ribose) polymer when activated by DNA nicks or breaks. Binding of PARP1 to DNA strand breaks is critical for resealing of the DNA sin-

gle-strand breaks (SSB) during base excision repair (BER) [53–57]. Loss of PARP activity results in accumulation of DNA SSBs, which are subsequently converted to DNA DSBs by the cellular replication and/or transcription machinery (see Fig. 2.4). In BRCA-positive cells, these DSBs are repaired by HRR, but they cannot be properly repaired in BRCA-deficient cells, leading to genomic instability, chromosomal rearrangements, and as a result—cell death [58, 59]. This effect is known as a synthetic lethality: when inhibition in either of the two signaling pathways (by gene mutation or chemical inhibitor) is present and remains independently viable, but when present together, the combination results in non-viability.

The role of PARP1 in the DNA damage response promoted the development of PARP inhibitors as chemo- and radio-sensitizers for the treatment of cancers harboring mutations in the *BRCA* genes [60]. Overall, it has been estimated that inherited *BRCA1* or *BRCA2* mutations account for 5–10 % of breast cancers and 10–15 % of ovarian cancers among white women in the United States [61]. However, a low frequency of *BRCA1* loss in non-hereditary tumors can limit the clinical use of this approach [58]. The possibility of applying the synthetic lethality in many types of cancers by lowering BRCA1 expression by pharmacological NO-donor treatments is very appealing (Fig. 2.4). In our recent work, we tested the effect of NO-donor/PARP-inhibitor combination on the sensitization of cancer cell lines to ionizing radiation (IR) (Fig. 2.5).

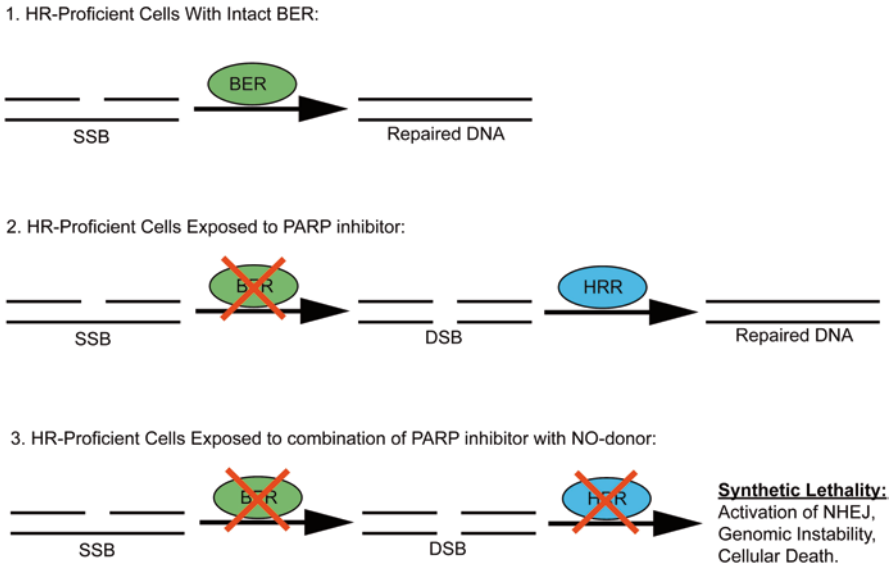


Fig. 2.4 A proposed stimulation of synthetic lethality by combination of PARP- inhibitor and NO-donor

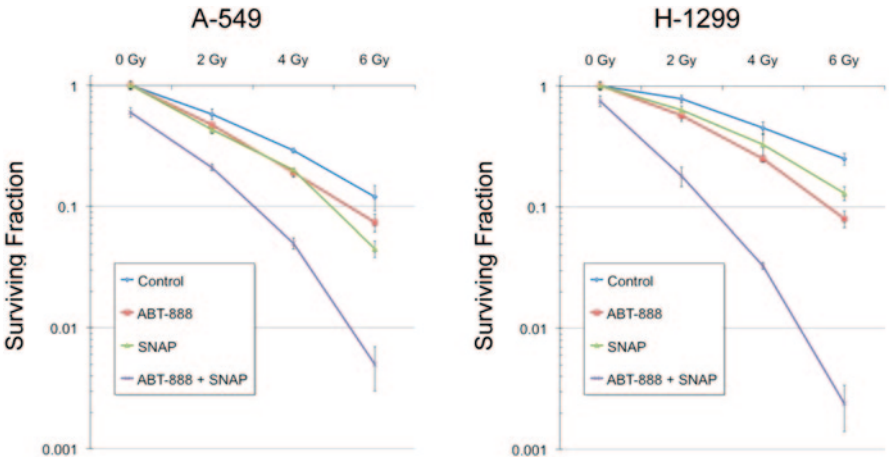


Fig. 2.5 Combined treatment with NO-donor SNAP (200 μM) and PARP inhibitor ABT-888 (10 μM) significantly increased sensitivity of A-549 and H-1299 (human non-small lung carcinoma cell line) to ionizing radiation. (unpublished data)

From Bystander Effect to Mutator Field

The radiation-induced bystander effect (RIBE) was studied widely in the past decades since the description of this phenomenon by Nagasawa and Little in 1992 [25]. It was shown that the irradiated cells release some "stress signal factors" (SSF) to affect the adjacent cells, or to affect the cells, which have received the medium, conditioned by the irradiated cells. These cells, which are not irradiated but are affected by the SSF, are called bystander cells. These SSF cause excess DNA damage, expression of DNA damage related proteins, chromosome aberration, mutations, and malignant transformation in the non- irradiated bystander cells [26].

The uniqueness of nitric oxide (NO•) as a redox signaling molecule resides in part in its relative stability and hydrophobic properties that permit its diffusion through the cytoplasm and plasma membranes over several cell diameter distances [62]. Hence, stimulation of NO• generation can affect different pathways within the cell in which it is produced or penetrates cell membranes to modulate signaling pathways in the bystander cells [63]. Recent studies have demonstrated the important role of NO• in mediating the bystander response induced by low-dose irradiation. Shao et al. [27, 31] presented evidence for a significant increase in the incidence of micronuclei (MN) in the non-irradiated bystander cells that were in the vicinity of ones irradiated through either the nuclei or the cytoplasm with a microbeam facility. Pre-treatment with a scavenger of NO• (c-PTIO) abolished excess of MN formation. Using the DAF-FM diacetate, NO-activated fluorescent probe, and targeted with a precision microbeam, Shao et al. [27] detected an increase in the number of fluorescent-positive cells than the actual number of directly irradiated cells. In another study, Han et al. revealed that the stimulated cell proliferation and the increased MN and DSB were observed simultaneously in the bystander cell

population, which were co-cultured with cells irradiated by low-dose α-particles (1–10 cGy) in a mixed system [64]. NO• played an essential role in the stimulation of these effects in the bystander cell population. Low concentrations of NO•, generated by spermidine (NO-donor), were proved to induce cell proliferation, DSB, and MN simultaneously.

Different factors can stimulate NO• production in target cells and increased DNA damage in bystander ones. Irradiation, as well as, stimulation of RAW 264.7 (mouse leukaemic monocyte macrophage cell line) by LPS induced an iNOS activity and NO• generation, which increased the DNA damage in bystander cells [65]. Pretreatment of target macrophages or bystander cells with the direct NOS inhibitor (L-NAME) significantly reduced the induction of gene expression and DNA damage in the bystander cells.

How NO• can stimulate DNA damage in the bystander cells? Some authors hypothesized that moderate concentrations of NO/RNS stimulate proliferation and shortening of the cell cycle in bystander cells, which gave them insufficient time to repair DSBs. The increased cell division might increase the probability of carcinogenesis in bystander cells since cell proliferation increased the probability of mutation from the mis-repaired DSBs [64]. However, another group of researchers demonstrated that accumulation of bystander DNA damages (BDD) is not dependent on the length of the cell cycle. Their results indicated that accumulation of BDD is possible in non-proliferative cells with a high transcription level [66, 67]. We know that NO/RNS concentrations, which stimulated cell proliferation, cannot damage DNA directly, but can block error-free HRR of DSB and lead to accumulation of mis-repaired DSB lesions. In our recent work, we demonstrated that NO•, generated in macrophages, initiated block of BRCA1 protein expression and subsequent inhibition of HRR in bystander cell lines (Fig. 2.6) [1]. Hence, NOS activation and overproduction of NO/RNS under chronic inflammation or tumor inflammatory microenvironment affect not only cells with activated NOS, but also bystander, not activated, cells. This effect can be determined as a "field effect" of genomic instability, maintained by the active inflammatory microenvironment. The similar "field effect" in cancer biology, related to the stromal production of reactive oxygen species (ROS) and NO/RNS, was recently proposed by another group of investigators [68]. They proposed that such "field effect" could be related not only to the immune cells, but also to the adjacent fibroblasts, as an additional source of ROS and NO/RNS. eNOS- expressing fibroblasts have the ability to downregulate Cav-1 (endogenous eNOS inhibitor) and induce mitochondrial dysfunction in adjacent fibroblasts that do not express eNOS. As such, the effects of stromal oxidative stress can be laterally propagated, amplified, and are effectively "contagious": spread from cell- to-cell like a virus, creating a ***"mutator field",*** promoting widespread genomic instability [68].

If we accept NO/RNS-depended mutagenesis as a completely stochastic process, we can postulate that the efficacy of mutagenesis (ME) and carcinogenesis in such "mutagenic filed" depends on the area of the field (FA), the strength of NO/RNS-maintained genomic instability (SGI), and the duration of this field maintenance (FD):

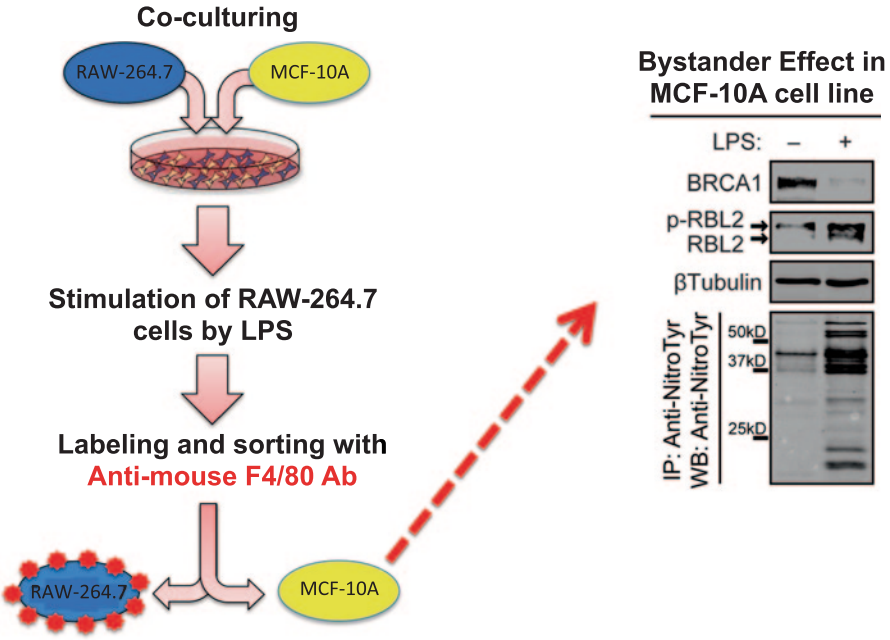


Fig. 2.6 NO-dependent BRCA1 down regulation in bystander MCF-10 A cell line. Stimulation of endogenous iNOS in RAW264.7 cell lines leads to stimulation of total Tyrosine nitration, RBL2 dephosphorylation, and BRCA1 downregulation in bystander MCF-10 A cells. Co-cultured MCF-10 A/RAW264.7 cell lines were incubated with 0.5 mg/mL of LPS for 18 h. (adopted from [1])

$$ME = FA^* \times FD \times SGI^{**}$$

We need to mention that this is a very simplified equation: *—it is obvious that FA cannot be always constant; **—SGI can demonstrate a different strength along the mutagenic field.

Acknowledgments The present work was supported by National Institutes of Health Grant R01 CA90881.

Conflict Statement No potential conflicts of interest were disclosed.

References

1. Yakovlev VA. Nitric oxide-dependent downregulation of BRCA1 expression promotes genetic instability. Cancer Res. 2013;73:706–15.
2. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Cogliano V. A review of human carcinogens–Part B: biological agents. Lancet Oncol. 2009;10:321–2.
3. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860–7.
4. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell. 2005;7:211–7.
5. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.

6. Wu X, Takenaka K, Sonoda E, Hochegger H, Kawanishi S, Kawamoto T, Takeda S, Yamazoe M. Critical roles for polymerase zeta in cellular tolerance to nitric oxide-induced DNA damage. Cancer Res. 2006;66:748–54.
7. Vane JR, Mitchell JA, Appleton I, Tomlinson A, Bishop-Bailey D, Croxtall J, Willoughby DA. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. Proc Natl Acad Sci U S A. 1994;91:2046–50.
8. Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. The Lancet Oncol. 2001;2:149–56.
9. Hussain SP, He P, Subleski J, Hofseth LJ, Trivers GE, Mechanic L, Hofseth AB, Bernard M, Schwank J, Nguyen G, Mathe E, Djurickovic D, Haines D, Weiss J, Back T, Gruys E, Laubach VE, Wiltrout RH, Harris CC. Nitric oxide is a key component in inflammation-accelerated tumorigenesis. Cancer Res. 2008;68:7130–6.
10. Vaninetti NM, Geldenhuys L, Porter GA, Risch H, Hainaut P, Guernsey DL, Casson AG. Inducible nitric oxide synthase, nitrotyrosine and p53 mutations in the molecular pathogenesis of Barrett's esophagus and esophageal adenocarcinoma. Mol Carcinog. 2008;47:275–85.
11. Chien YH, Bau DT, Jan KY. Nitric oxide inhibits DNA-adduct excision in nucleotide excision repair. Free Radic Biol Med. 2004;36:1011–7.
12. Jolly AJ, Wild CP, Hardie LJ. Sodium deoxycholate causes nitric oxide mediated DNA damage in oesophageal cells. Free Radic Res. 2009;43:234–40.
13. Ohshima H. Genetic and epigenetic damage induced by reactive nitrogen species: implications in carcinogenesis. Toxicol Lett. 2003;140–141:99–104.
14. Rajput S, Wilber A. Roles of inflammation in cancer initiation, progression, and metastasis. Fron Biosci. 2010;2:176–83.
15. McAdam E, Haboubi HN, Forrester G, Eltahir Z, Spencer-Harty S, Davies C, Griffiths AP, Baxter JN, Jenkins GJ. Inducible nitric oxide synthase (iNOS) and nitric oxide (NO) are important mediators of reflux-induced cell signalling in esophageal cells. Carcinogenesis. 2012;33:2035–43.
16. Lonkar P, Dedon PC. Reactive species and DNA damage in chronic inflammation: reconciling chemical mechanisms and biological fates. Int J Cancer. 2011;128:1999–2009.
17. Clemons NJ, McColl KE, Fitzgerald RC. Nitric oxide and acid induce double-strand DNA breaks in Barrett's esophagus carcinogenesis via distinct mechanisms. Gastroenterology. 2007;133:1198–209.
18. Shaked H, Hofseth LJ, Chumanevich A, Chumanevich AA, Wang J, Wang Y, Taniguchi K, Guma M, Shenouda S, Clevers H, Harris CC, Karin M. Chronic epithelial NF-kappaB activation accelerates APC loss and intestinal tumor initiation through iNOS up-regulation. Proc Natl Acad Sci U S A. 2012;109:14007–12.
19. Wang SS, Davis S, Cerhan JR, Hartge P, Severson RK, Cozen W, Lan Q, Welch R, Chanock SJ, Rothman N. Polymorphisms in oxidative stress genes and risk for non-Hodgkin lymphoma. Carcinogenesis. 2006;27:1828–34.
20. Marangoni K, Araujo TG, Neves AF, Goulart LR. The—786T>C promoter polymorphism of the NOS3 gene is associated with prostate cancer progression. BMC Cancer. 2008;8:273.
21. Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolocci N, Ambs S, Colton CA, Harris CC, Roberts DD, Wink DA. The chemical biology of nitric oxide: implications in cellular signaling. Free Radic Biol Med. 2008;45:18–31.
22. Wink DA, Ridnour LA, Hussain SP, Harris CC. The reemergence of nitric oxide and cancer. Nitric Oxide. 2008;19:65–7.
23. Wink DA, Mitchell JB. Nitric oxide and cancer: an introduction. Free Radic Biol Med. 2003;34:951–4.
24. Wei L, Gravitt PE, Song H, Maldonado AM, Ozbun MA. Nitric oxide induces early viral transcription coincident with increased DNA damage and mutation rates in human papillomavirus-infected cells. Cancer Res. 2009;69:4878–84.
25. Nagasawa H, Little JB. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res. 1992;52:6394–6.

26. Azzam EI, de Toledo SM, Little JB. Stress signaling from irradiated to non-irradiated cells. Curr Cancer Drug Targets. 2004;4:53–64.
27. Shao C, Stewart V, Folkard M, Michael BD, Prise KM. Nitric oxide- mediated signaling in the bystander response of individually targeted glioma cells. Cancer Res. 2003;63:8437–42.
28. Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. Crit Rev Oncol Hematol. 2008;66:1–9.
29. Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer Res. 2007;67:10019–26.
30. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene. 2008;27:5904–12.
31. Shao C, Folkard M, Michael BD, Prise KM. Targeted cytoplasmic irradiation induces bystander responses. Proc Natl Acad Sci U S A. 2004;101:13495–500.
32. Yakovlev VA, Bayden AS, Graves PR, Kellogg GE, Mikkelsen RB. Nitration of the tumor suppressor protein p53 at tyrosine 327 promotes p53 oligomerization and activation. Biochemistry. 2010;49:5331–9.
33. Shrivastav M, De Haro LP, Nickoloff JA. Regulation of DNA double- strand break repair pathway choice. Cell Res. 2008;18:134–47.
34. Hofseth LJ, Saito S, Hussain SP, Espey MG, Miranda KM, Araki Y, Jhappan C, Higashimoto Y, He P, Linke SP, Quezado MM, Zurer I, Rotter V, Wink DA, Appella E, Harris CC. Nitric oxide-induced cellular stress and p53 activation in chronic inflammation. Proc Natl Acad Sci U S A. 2003;100:143–8.
35. Schneiderhan N, Budde A, Zhang Y, Brune B. Nitric oxide induces phosphorylation of p53 and impairs nuclear export. Oncogene. 2003;22:2857–68.
36. Wang X, Zalcenstein A, Oren M. Nitric oxide promotes p53 nuclear retention and sensitizes neuroblastoma cells to apoptosis by ionizing radiation. Cell Death Differ. 2003;10:468–76.
37. Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ, Loscalzo J. S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. Proc Natl Acad Sci U S A. 1992;89:444–8.
38. Stamler JS, Meissner G. Physiology of nitric oxide in skeletal muscle. Physiol Rev. 2001;81:209–37.
39. Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene. 2003;22:5734–54.
40. Souza JM, Peluffo G, Radi R. Protein tyrosine nitration–functional alteration or just a biomarker? Free Radic Biol Med. 2008;45:357–66.
41. Radi R. Nitric oxide, oxidants, and protein tyrosine nitration. Proc Natl Acad Sci U S A. 2004;101:4003–8.
42. Yakovlev VA, Mikkelsen RB. Protein tyrosine nitration in cellular signal transduction pathways. J Recept Signal Transduct Res. 2010;30:420–9.
43. Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell. 2002;108:171–82.
44. Lou Z, Minter-Dykhouse K, Chen J. BRCA1 participates in DNA decatenation. Nat Struct Mol Biol. 2005;12:589–93.
45. Joukov V, Groen AC, Prokhorova T, Gerson R, White E, Rodriguez A, Walter JC, Livingston DM. The BRCA1/BARD1 heterodimer modulates ran- dependent mitotic spindle assembly. Cell. 2006;127:539–52.
46. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science. 1994;266:66–71.
47. Ohama T, Brautigan DL. Endotoxin conditioning induces VCP/p97- mediated and inducible nitric-oxide synthase-dependent Tyr284 nitration in protein phosphatase 2A. J Biol Chem. 2010;285:8711–8.
48. Bindra RS, Gibson SL, Meng A, Westermark U, Jasin M, Pierce AJ, Bristow RG, Classon MK, Glazer PM. Hypoxia-induced down-regulation of BRCA1 expression by E2Fs. Cancer Res. 2005;65:11597–604.

49. Mikula I, Durocher S, Martasek P, Mutus B, Slama-Schwok A. Isoform- specific differences in the nitrite reductase activity of nitric oxide synthases under hypoxia. Biochem J. 2009;418:673–82.
50. Strijdom H, Friedrich SO, Hattingh S, Chamane N, Lochner A. Hypoxia-induced regulation of nitric oxide synthase in cardiac endothelial cells and myocytes and the role of the PI3-K/PKB pathway. Mol Cell Biochem. 2009;321:23–35.
51. Feelisch M, Fernandez BO, Bryan NS, Garcia-Saura MF, Bauer S, Whitlock DR, Ford PC, Janero DR, Rodriguez J, Ashrafian H. Tissue processing of nitrite in hypoxia: an intricate interplay of nitric oxide-generating and -scavenging systems. J Biol Chem. 2008;283:33927–34.
52. Bindra RS, Glazer PM. Repression of RAD51 gene expression by E2F4/p130 complexes in hypoxia. Oncogene. 2007;26:2048–57.
53. Sakai A, Sakasai R, Kakeji Y, Kitao H, Maehara Y. PARP and CSB modulate the processing of transcription-mediated DNA strand breaks. Genes Genet Syst. 2012;87:265–72.
54. Wesierska-Gadek J, Zulehner N, Ferk F, Skladanowski A, Komina O, Maurer M. PARP inhibition potentiates the cytotoxic activity of C-1305, a selective inhibitor of topoisomerase II, in human BRCA1-positive breast cancer cells. Biochem Pharmcol. 2012;84:1318–31.
55. Drew Y, Plummer R. PARP inhibitors in cancer therapy: two modes of attack on the cancer cell widening the clinical applications. Drug Resist Updat. 2009;12:153–6.
56. Peralta-Leal A, Rodriguez-Vargas JM, Aguilar-Quesada R, Rodriguez MI, Linares JL, de Almodovar MR, Oliver FJ. PARP inhibitors: new partners in the therapy of cancer and inflammatory diseases. Free Radic Biol Med. 2009;47:13–26.
57. Rouleau M, Patel A, Hendzel MJ, Kaufmann SH, Poirier GG. PARP inhibition: PARP1 and beyond. Nat Rev Cancer. 2010;10:293–301.
58. Daemen A, Wolf DM, Korkola JE, Griffith OL, Frankum JR, Brough R, Jakkula LR, Wang NJ, Natrajan R, Reis-Filho JS, Lord CJ, Ashworth A, Spellman PT, Gray JW, van't Veer LJ. Cross-platform pathway-based analysis identifies markers of response to the PARP inhibitor olaparib. Breast Cancer Res Treat. 2012;135:505–17.
59. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC, Ashworth A. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005;434:917–21.
60. Kummar S, Chen A, Parchment RE, Kinders RJ, Ji J, Tomaszewski JE, Doroshow JH. Advances in using PARP inhibitors to treat cancer. BMC Med. 2012;10:25.
61. Campeau PM, Foulkes WD, Tischkowitz MD. Hereditary breast cancer: new genetic developments, new therapeutic avenues. Hum Genet. 2008;124:31–42.
62. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996;271:C1424–37.
63. Murad F. Nitric oxide signaling: would you believe that a simple free radical could be a second messenger, autacoid, paracrine substance, neurotransmitter, and hormone? Recent Prog Horm Res. 1998;53:43–59; discussion 59–60.
64. Han W, Chen S, Yu KN, Wu L. Nitric oxide mediated DNA double strand breaks induced in proliferating bystander cells after alpha-particle irradiation. Mutat Res. 2010;684:81–9.
65. Ghosh S, Maurya DK, Krishna M. Role of iNOS in bystander signaling between macrophages and lymphoma cells. Int J Radiat Oncol Biol Phys. 2008;72:1567–74.
66. Dickey JS, Baird BJ, Redon CE, Avdoshina V, Palchik G, Wu J, Kondratyev A, Bonner WM, Martin OA. Susceptibility to bystander DNA damage is influenced by replication and transcriptional activity. Nucleic Acids Res. 2012;40:10274–86.
67. Dickey JS, Baird BJ, Redon CE, Sokolov MV, Sedelnikova OA, Bonner WM. Intercellular communication of cellular stress monitored by gamma-H2AX induction. Carcinogenesis. 2009;30:1686–95.
68. Martinez-Outschoorn UE, Balliet RM, Rivadeneira DB, Chiavarina B, Pavlides S, Wang C, Whitaker-Menezes D, Daumer KM, Lin Z, Witkiewicz AK, Flomenberg N, Howell A, Pestell RG, Knudsen ES, Sotgia F, Lisanti MP. Oxidative stress in cancer associated fibroblasts drives tumor-stroma co- evolution: a new paradigm for understanding tumor metabolism, the field effect and genomic instability in cancer cells. Cell Cycle. 2010;9:3256–76.

Chapter 3
Targeting Hyponitroxia in Cancer Therapy

Bryan Oronsky, Neil Oronsky, Michelle Lybeck, Gary Fanger and Jan Scicinski

Abstract It was Oscar Wilde who said "nothing succeeds like excess." Tumors, in particular, subscribe to this creed of greed, avidly consuming glucose, glutamine and lipids while simultaneously overexpressing and stimulating signal transduction pathways in order to accelerate the rate of proliferation and progression. Nitric oxide (NO) is the rare exception to the success of excess in tumors since, in this case, less is more: at low "hyponitroxic" levels NO promotes proliferation while at high levels NO suppresses it.

Keywords Hyponitroxia · Nitric oxide · NO donors

Abbreviations

DeoxyHb	Deoxygenated Hemoglobin
FOLFIRI	Chemotherapy regimen for colorectal cancer: Folinic acid/ Fluorouracil/irinotecan
GC	guanyl cyclase
GTN	Glyceryl trinitrate
HIF-1alpha	hypoxia-inducible factor-1alpha
iNOS	Inducible Nitric Oxide Synthase
L-NNA	N-nitro-l-arginine
NF-κB	nuclear factor-kappaB
NO	Nitric oxide
NOS	Nitric Oxide Synthase
NOx	Nitrogen oxides
NSCLC	Non Small Cell Lung Cancer
RNS	Reactive Nitrogen Species
RONS	Reactive Oxygen and Nitrogen Species
ROS	Reactive Oxygen Species

J. Scicinski (✉) · B. Oronsky · M. Lybeck · G. Fanger
EpicentRx, Inc, 800 W El Camino Real, Suite 180, Mountain View, CA 94040, USA
e-mail: jscicinski@epicentrx.com

N. Oronsky
CFLS, 560 South Winchester Blvd, San Jose, CA, USA

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_3

Introduction

The aim of this chapter is to explore and discuss the two sides of nitric oxide (NO) in cancer biology, which can function as a dose-dependent tumor promoter or protector. Tumor promotion is greatest at persistently low NO concentrations, which we refer to as hyponitroxia. This chapter will also discuss the potential role of hyponitroxia as a novel therapeutic target to treat cancer; tumors thrive in a Goldilocks zone—"not too much, not too little, but just right"—of NO concentration and excursions outside of this zone of nitric oxide signal strength by the application of NO donors and inhibitors result in cell death, thus, providing new opportunities for pharmacological intervention in cancer.

Synergistic interactions between tumor hypoxia and hyponitroxia or low levels of nitric oxide (NO) influence and contribute to upregulation of signaling pathways mediated by nuclear factor-kappaB (NF-κB) and hypoxia-inducible factor-1alpha (HIF-1alpha) [1] that drive metastatic progression, drug resistance and post-treatment relapses.

This chapter highlights the function of hyponitroxia as a pro-neoplastic effector at concentrations that are present in tumor tissues, summarizes pharmacologic strategies to force the tumor out of its NO comfort zone into an NO kill zone and makes the case for hyponitroxia as a high priority and accessible target for intervention in cancer therapy.

The Hyponitroxia and Hypoxia Axis

Tumors are adapted to thrive within a narrow 'comfort' zone of free radical stress [2]. They have the ability to insulate themselves with ARE-mediated antioxidant production and cope with redox stress but only to an extent; few tumors will thrive when the free radical burden deviates significantly and persistently beyond that comfort zone. As a free radical, nitric oxide is a double edged prooxidant sword that benefits tumors at low doses and inflicts harm to them at higher concentrations; risk reduction in tumors is mediated through hyponitroxia: endogenous production of low levels of nitric oxide (< 100 nmol/L) [3, 4] are catalyzed by three nitric oxide synthase (NOS) enzymes, two of which are constitutive (neuronal or brain NOS and endothelial NOS), and the third is inducible (iNOS) [5, 6] associated with the oxidative burst of macrophages. At these low concentrations, nitric oxide mediates redox signaling pathways and promotes malignant conversion, tumor progression [6] and resistance to therapy in multiple cancers including prostate [7], colonic adenocarcinomas, lung adenocarcinomas [8] and mammary adenocarcinomas [8, 9]. Nitric oxide can also act as an oncogenic agent through activities that include cell proliferation, invasion, metastasis and stem cell renewal [3]. This biphasic relationship, characterized by a beneficial effect at low doses and a detrimental effect at high doses, represents a modified form of hormesis [10], a U-shaped or curvilinear dose response model.

Hypoxia interacts and synergizes with the oncogenicity of nitric oxide: molecular O_2 is an essential substrate for the activity of nitric oxide synthases and oxygen insufficiency limits endogenous NO production by these enzymes [11, 12]. As the hypoxia gradient increases, NO synthesis in tumors decreases, however, since total anoxia is rarely, if ever, reached, NO synthesis is only downregulated [12], resulting in the continued constitutive induction of the enzyme guanyl cyclase (GC) [13], the accumulation of cGMP and the stimulation of cGMP-dependent kinases. Hypoxia also increases arginase activity [14], an enzyme, which converts L-arginine to urea, limiting arginine availability to act as a substrate for NO production. Thus, as a downregulating mechanism for endogenous NO production hypoxia leads to hyponitroxia [15] promoting a protumorogenic status.

Conversely, hyponitroxia can heighten hypoxia via nitric oxide modulation of blood flow and increased mitochondrial oxygen utilization [16, 17]. From this perspective, hypoxia and hyponitroxia are two halves of a coupled axis that mutually influence each other and each other's downstream targets.

The "Goldilocks Zone" and Nitric Oxide

Since its discovery, NO has been implicated in a wide and dizzying variety of potential effects and mechanisms, both physiologic and pathological. Frustratingly, NO is highly dependent on context and modulatory milieu, that is, the effects differ in normal vs neoplastic tissue and according to the concentration—cytotoxicity at high doses and mitogenicity at low doses [18]—and for these reasons NO is a moving target that defies easy generalization or descriptions of function. By general consensus it seems that NO is characterized as a riddle, wrapped in a mystery, inside an enigma; its biology is fickle, unpredictable and nearly impossible to pin down to the point of hand-wringing exasperation; and more than occasionally that frustration with its seemingly irrational biologic complexity boils over and manifests in the normally staid scientific literature as name-calling—"two-faced" [19], "flip-flopper" [20], "foe" [21], and "enemy" [22] are some of the opprobria applied to nitric oxide.

The way out of this conundrum may be to consider NO in its context with O_2—as partners in crime or two sides of the same coin, with the perturbation of the one feeding back on the other in mutually reinforcing, interconnected vicious or virtuous circles (Fig. 3.1). Hyponitroxia fuels hypoxia in a vicious cycle and vice versa while euoxia drives nitroxia and vice versa. As oxygen decreases so does its accomplice nitric oxide, which is favorable for tumor proliferation and progression and, conversely, to the detriment of the tumor, increased O_2 drives a virtuous circle of increased NO and tumor perfusion. In tumors, hypoxia is a product of aberrant vasculature with poor blood flow that hyponitroxia compounds. NO, then, is the 'power behind the O_2 throne', a kind of puppet master, that pulls the strings and wields a considerable influence on oxygen levels.

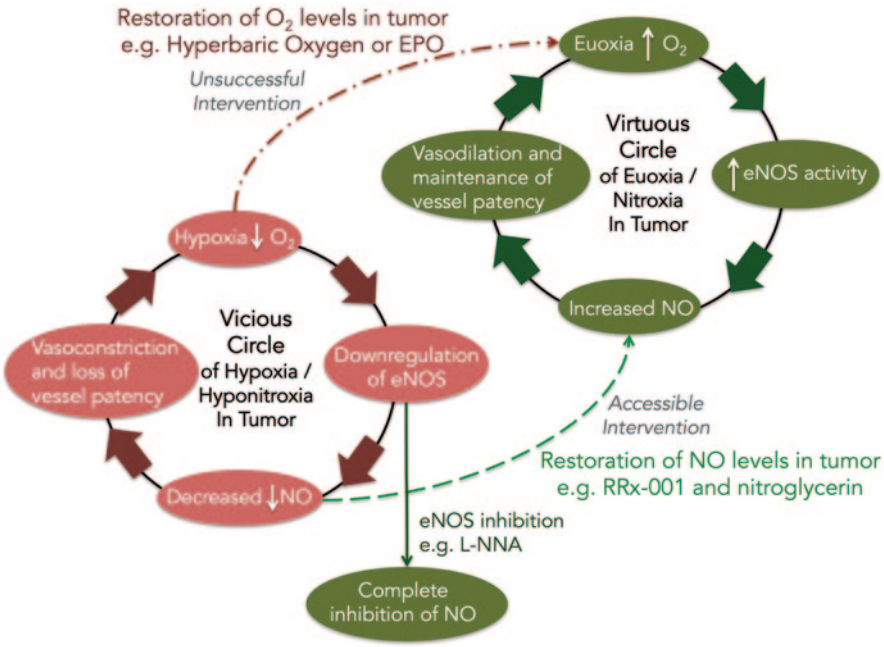


Fig. 3.1 The mutually reinforcing vicious cycle of hypoxia/hyponitroxia drives tumor progression. Whereas attempts to treat hypoxic tumors via improved oxygenation, for example with hyperbaric oxygen or erythropoetin, have by and large been unsuccessful, total inhibition or restoration of nitric oxide levels is an accessible pharmacologic lever by which to reoxygenate the tumor and transform the vicious cycle of hypoxia/hyponitroxia into a virtuous cycle of euoxia/nitroxia

Compared with tumor oxygenation, NO is a relatively accessible target. The reason is that tumors are limited to a relatively narrow bandwidth or threshold of NO tolerance, a concentration range that could be called the "Goldilocks zone;" this term borrowed from astronomy refers to the perfectly aligned location of the Earth precisely in the habitable region or zone of the sun [23, 24] wherein temperatures and conditions are manifestly compatible with life, as we know it. This razor thin margin between harm and benefit of prooxidant NO makes the tumor highly vulnerable to changes in NO levels. If the concentration is shifted even slightly in an up or down direction, the 'safe zone' becomes a 'kill zone' for the tumor, as discussed below (Fig. 3.2).

The Dose and Cytotoxicity Threshold

As a radical with a free electron capable of interacting with reactive oxygen species such as the superoxide anion, NO is associated with a rogue's gallery of highly reactive nitrogen oxides (NOx). The term nitrosative stress refers to the formation

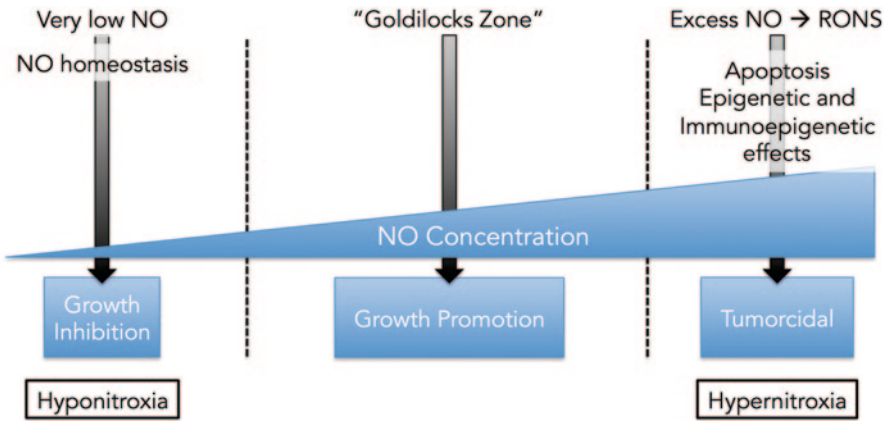


Fig. 3.2 Tumor response to hyponitroxia and hypernitroxia. Tumors require an optimal concentration of nitric oxide for proliferation. Very low levels of intratumoral levels of nitric oxide inhibit tumor growth, while very high levels induce tumor cell death

of this poisonous brew of NOx, such as peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), and dinitrogen trioxide (N_2O_3), responsible for cytotoxic nitration and oxidation reactions [23, 25, 26]. In particular, the formation of peroxynitrite, which follows first order kinetics [23], is dependent on the concentrations of NO and superoxide anion.

Xie et al [27] demonstrated that high levels of NO from transfection of murine K-1735 melanoma cells with iNOS resulted in suppression of tumorigenicity and metastasis. The cytotoxicity of higher concentrations of NO is related to the formation of NOx and peroxynitrite formation, in light of severely impaired antioxidant machinery in tumors compared with healthy cells [28].

However, cytotoxicity may not require super elevated doses but approximate restoration to physiologic levels [29] with correction of hyponitroxia: Frederiksen et al demonstrated that NO enrichment with low concentrations of the NO mimetics glyceryl trinitrate (GTN) and isosorbide dinitrate attenuated growth of doxorubicin-resistant tumors in prostate cancer mouse models.

At the other end of the spectrum, Kashiwagi and Jain [30] described radiosensitization in glioma xenografts with NOS inhibition; lower concentrations of NO reduce signal transduction below a physiologic baseline, leading to a loss of the aberrant induction of proangiogenic [31] signaling [32] networks that promote malignant progression.

Against this emerging background of conflicting preclinical evidence that both pro- and anti-NO cancer therapies are therapeutically effective, human clinical trials with both nitric oxide donors and nitric oxide inhibitors such as A) nitroglycerin (NTG) B) L-NNA and C) RRx-001 to modulate the hyponitroxia tumor advantage and, thereby, push the tumor out of its hormetic comfort zone have been initiated.

A. Nitroglycerin

Nitroglycerin is an approved anti-anginal NO-donating nitrate ester [33] repositioned as an anticancer single agent and a chemosensitizer in late stage cancer clinical trials.

Monotherapy

In a phase II study treatment with low-dose, sustained delivery of glyceryl trinitrate to the prostate after failure of primary therapy in prostate cancer resulted in a significant decrease in PSA. The authors suggested that, although low-dose NO had no direct cytotoxic effect, NO decreased invasiveness and metastatic potential [20, 34], potentially by raising levels of nitric oxide to physiologic ranges. A second explanation is the possibility of tachyphylaxis (decreased responsiveness after repeated doses) with the inhibition of guanyl cyclase due to the sustained delivery of NO [35].

Chemosensitization

In a double blind phase II randomized study, 120 patients with stage IIIB/IV NSCLC [36] were randomized to a polychemotherapeutic regimen of vinorelbine and cisplatin with a nitroglycerin patch (Arm A) or with a placebo patch (Arm B). The rates of overall response and time to disease progression were significantly increased in the nitroglycerin arm; the explanation may be related to elevation of NO levels into a normal physiologic range in the tumor or NO-induced feedback inhibition with disruption of the proangiogenic redox signaling circuitry.

B. L-NNA

Given the reliance of tumors [37] on NO, N-nitro-l-arginine (L-NNA), a competitive inhibitor of nitric oxide synthase with selectivity for the neuronal and endothelial isoforms of the enzyme, was investigated in a phase 1 NSCLC. Serial assessment with dynamic contrast-enhanced computed tomography demonstrated a sustained decrease in tumor perfusion by 40% up to 24 h post-treatment [38]. It is unknown whether the decreased blood volume induced tumor shrinkage.

The extrapolation from these data suggests that tumors thrive within a hyponitroxic “sweet spot” of signaling cell strength; excursions below and above this narrow range result in cell death. The inhibition of NO synthesis leads to catastrophic shutdown of vascular function, with inhibition of NO-dependent angiogenesis and vascular patency.

The sustained disruption of the tumor vasculature was preceded by a mild transient increase in systemic blood pressure due to a differential dependence on NO in healthy and cancerous tissues [39]. Unlike the tightly regulated homeostatic control of the cardiovascular system, the patency of vessels within these tumors is maintained by increased expression of NO. Therefore, the consequence of NO inhibition was a net vasoconstrictor effect with a collapse of blood flow.

C. RRx-001

RRx-001 [40] is a novel aerospace-industry derived Phase 2 molecule with epigenetic and NO-donating properties. The molecule is characterized by a marked lack of systemic toxicity, related to selective modification of hemoglobin in the red blood cell, resulting in a catalytic, hypoxia-driven overproduction of nitrogen oxides (NOx), free radicals, diffusible metabolites, chemokines and cytokines, which are preferentially toxic and selectively targeted to the tumor and the tumor vasculature. One basis for therapeutic selectivity is oxidative inactivation of DNA methyltransferases and histone deacetylases in the tumor [41].

RRx-001-modified red blood cells are long-lived, biocompatible, circulating couriers, a kind of FedEx for cancer therapy, that deliver their free radical kiss of death directly to the tumor "zip code" in the presence of low oxygen, leading to selective tumor cytotoxicity. In effect, these red blood cells are 'reprogrammed' to generate NOx with increased catalytic efficiency, amplifying oxidative stress under low oxygen conditions specific to the tumor microenvironment.

As an NO donor, RRx-001 covalently binds to and allosterically modifies its target, deoxygenated hemoglobin (deoxyHb) [42]. DeoxyHb has an enzymatic function as a nitrite reductase, which reduces or converts the inorganic anion nitrite into nitric oxide under hypoxic conditions. The binding of RRx-001 to deoxyhemoglobin, therefore, converts red blood cells into circulating NO bioreactors with a tropism for hypoxic tumors [43]. This in situ generation of ROS/RNS shifts the biocharacter of the tumor microenvironment from habitable to inhabitable. The ultrashort lifetime of ROS and RNS confines their activity to the tumor, sparing normal tissues from toxicity. In this way, the adaptation of tumor cells is prevented, the proangiogenic signaling equilibrium responsible for the maintenance of the oncogenic phenotype is prevented and a free radical storm is selectively released in the tumor.

The highlights from a completed Phase I trial in multiple types of cancer included prolonged disease stabilization > 9 months and resensitization of chemoresistant colorectal tumors to treatments such as FOLFIRI that were formerly tried and failed via epigenetic modulation linked to nitro-oxidative stress [44, 45]. At the time of this book chapter, a Phase 3 trial in hepatocellular carcinoma vs sorafenib is planned.

Conclusions and Future Directions

A search on clinicaltrials.gov revealed over a hundred studies involving cancer and hypoxia. By contrast, there is less than a handful involving cancer and NO, a reflection perhaps of the frustration with its unpredictable and contradictory behavior.

The solution to this problem may be to consider NO as one part of an integrated whole, of which the other half is oxygen: NOS is inhibited under hypoxia and stimulated under oxic conditions, while NO interferes with mitochondrial respiration and increases oxygen availability. In addition, NO and superoxide anion scavenge each other [46]. In this coupled control, modulation of one element of the axis should induce modulation of the other in the same direction i.e. up or down.

This is a potentially promising and exciting anticancer strategy since direct approaches to boost oxygenation and correct tumor hypoxia for therapeutic advantage with hyperbaric oxygen, erythropoietin administration or methods of enhanced delivery have by and large been unsuccessful [47]. By contrast, hyponitroxia may provide indirect access to the stubbornly resistant problem of tumor hypoxia.

The reliance of the tumor on a narrow hyponitroxic range conducive to invasion, angiogenesis and metastasis and the sensitivity to perturbation of NO concentration represents a tipping point of vulnerability and fragility that is relatively easily exploitable; any significant alteration in NO levels in either direction, below or above, is likely to elicit a pivotal antitumor response especially in combination with chemotherapy or ionizing radiation.

The central challenge to the adoption of NO manipulation above or below hyponitroxic values is the prevailing mindset that hypoxia is not only a separate therapeutic target from NO but unequal in its importance given the latter's predictably unpredictable properties. However, this perception of NO as entirely untamable and unreliable may need to be revised in light of the single-agent activity of RRx-001, the red blood cell-mediated NO-donor sourced from the aerospace industry. With RRx-001, the therapeutic gauntlet has been thrown down to challenge conventional models of drug development and the importance of hyponitroxia as a target for treatment.

A universally recognized hallmark of solid tumors is hypoxia, which contributes to cancer cell survival, angiogenesis and chemo/radioresistance. Despite the acceptance of its presence and the identification of its importance in tumors, efforts to eradicate it generally have been unsuccessful. However, the ability to manipulate NO levels with GTN (and other organic nitrates), RRx-001 and L-NNA above and below the "Goldilocks zone" represents a potential pharmacologic entry point or lever to influence the oxygenation status in tumors and tip the balance from hypoxia to euoxia due to the mutually reinforcing relationship of NO and oxygen. A nitroxic-based strategy will hopefully help both identify prospective patients and develop more efficacious but less toxic treatment options.

Acknowledgments The authors would like to acknowledge collaboration with ATK Aerospace Systems on the discovery and development of RRx-001.

Conflict Statement All authors are either employees of RadioRx, Inc (1) or are advisors and consultants to RadioRx, Inc (2).

References

1. Culver C, Sundqvist A, Mudie S, et al. Mechanism of hypoxia-induced NF-kappaB. Mol Cell Biol. 2010;30:4901–21.
2. Schulze A, Harris AL. How cancer metabolism is tuned for proliferation and vulnerable to disruption. Nature. 2012;491:364–73.
3. Ambs S, Glynn SA. Candidate pathways linking inducible nitric oxide synthase to a basal-like transcription pattern and tumor progression in human breast cancer. Cell Cycle. 2011;10:619–24.
4. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. Cardiovasc Res. 1999;43:521–31.
5. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev. 1991;43:109–42.
6. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. Nat Rev Cancer. 2006;6:521–34.
7. Klotz T, Bloch W, Volberg C, et al. Selective expression of inducible nitric oxide synthase in human prostate carcinoma. Cancer. 1998;82:1897–903.
8. Fujimoto H, Ando Y, Yamashita T, et al. Nitric oxide synthase activity in human lung cancer. Jpn J Cancer Res. 1997;88:1190–8.
9. Lala PK. Significance of nitric oxide in carcinogenesis, tumor progression and cancer therapy. Cancer Metastasis Rev. 1998;17:1–6.
10. Calabrese EJ. Hormesis is central to toxicology, pharmacology and risk assessment. Hum Exp Toxicol. 2010;29:249–61.
11. Kim N, Vardi Y, Padma-Nathan H, et al. Oxygen tension regulates the nitric oxide pathway. Physiological role in penile erection. J Clin Invest. 1993;91:437–42.
12. Matthews NE, Adams MA, Maxwell LR, et al. Nitric oxide-mediated regulation of chemosensitivity in cancer cells. J Natl Cancer Inst. 2001;93:1879–85.
13. Lucas KA, Pitari GM, Kazerounian S, et al. Guanylyl cyclases and signaling by cyclic GMP. Pharmacol Rev. 2000;52:375–414.
14. Prieto CP, Krause BJ, Quezada C, et al. Hypoxia-reduced nitric oxide synthase activity is partially explained by higher arginase-2 activity and cellular redistribution in human umbilical vein endothelium. Placenta. 2011;32:932–40.
15. Brantley EC, Guo L, Zhang C, et al. Nitric oxide-mediated tumoricidal activity of murine microglial cells. Transl Oncol. 2010;3:380–8.
16. Jordan BF, Sonveaux P, Feron O, et al. Nitric oxide as a radiosensitizer: evidence for an intrinsic role in addition to its effect on oxygen delivery and consumption. Int J Cancer. 2004;109:768–73.
17. Oronsky BT, Scicinski JJ, Reid T, Knox S. Beyond antiangiogenesis: vascular modulation as an anticancer therapy-a review. Transl Oncol. 2012;5:133–40.
18. Mocellin S, Bronte V, Nitti D. Nitric oxide, a double edged sword in cancer biology: searching for therapeutic opportunities. Med Res Rev. 2007;27:317–52.
19. Thippeswamy T, McKay JS, Quinn JP, Morris R. Nitric oxide, a biological double-faced janus–is this good or bad? Histol Histopathol. 2006;21:445–58.
20. Oronsky BT, Knox SJ, Scicinski JJ. Is Nitric Oxide (NO) the Last Word in Radiosensitization? A review. Transl Oncol. 2012;5:66–71.
21. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996;271:C1424–37.
22. Rogalska J. Nitric oxide in pathology of hypoxia–ally or enemy. Postepy Hig Med Dosw. 2000;54:669–86.

23. Squadrito GL, Pryor WA. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. Free Radic Biol Med. 1998;25:392–403.
24. von Braun K. Astrophysical parameters and Habitable Zone of the Exoplanet Hosting Star GJ 581. Astrophys J Lett. 2011;729:L26.
25. Hirst DG, Robson T. Nitrosative stress in cancer therapy. Front Biosci 2007;12:3406–18.
26. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87:315–424.
27. Xie K, Huang S, Dong Z, et al. Transfection with the inducible nitric oxide synthase gene suppresses tumorigenicity and abrogates metastasis by K-1735 murine melanoma cells. J Exp Med. 1995;181:1333–43.
28. Casaril M, Corso F, Bassi A, et al. Decreased activity of scavenger enzymes in human hepatocellular carcinoma, but not in liver metastases. Int J Clin Lab Res 1994;24:94–7.
29. Frederiksen LJ, Sullivan R, Maxwell LR, et al. Chemosensitization of cancer in vitro and in vivo by nitric oxide signaling. Clin Cancer Res. 2007;13:2199–206.
30. Kashiwagi S, Tsukada K, Xu L, et al. Perivascular nitric oxide gradients normalize tumor vasculature. Nat Med. 2008;14:255–7.
31. Ridnour LA, Isenberg JS, Espey MG, et al. Nitric oxide regulates angiogenesis through a functional switch involving thrombospondin-1. Proc Natl Acad Sci U S A. 2005;102:13147–52.
32. Rudic RD, Shesely EG, Maeda N, et al. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. J Clin Invest. 1998;101:731–6.
33. Ignarro LJ, Napoli C, Loscalzo J. Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide: an overview. Circ Res. 2002;90:21–8.
34. Heaton JP, Adams MA, Graham CH, et al. Evidence for the use of low dose nitric oxide in the treatment of rising PSA associated with biochemical failure following radical prostatectomy. J Urol. 2003;169:279 (1082 Suppl. S).
35. Agvald P, Adding LC, Gustafsson LE, Persson MG. Nitric oxide generation, tachyphylaxis and cross-tachyphylaxis from nitrovasodilators in vivo. Eur J Pharmacol. 1999;385:137–45.
36. Yasuda H, Yamaya M, Nakayama K, et al. Randomized phase II trial comparing nitroglycerin plus vinorelbine and cisplatin with vinorelbine and cisplatin alone in previously untreated stage IIIB/IV non-small-cell lung cancer. J Clin Oncol. 2006;24:688–94.
37. Xu W, Liu LZ, Loizidou M, et al. The role of nitric oxide in cancer. Cell Res. 2002;12:311–20.
38. Cardnell RJ, Mikkelsen RB. Nitric oxide synthase inhibition enhances the antitumor effect of radiation in the treatment of squamous carcinoma xenografts. PLoS One. 2011;6:e20147.
39. Ng QS, Goh V, Milner J, et al. Effect of nitric-oxide synthesis on tumour blood volume and vascular activity: a phase I study. Lancet Oncol. 2007;8:111–8.
40. Ning S, Bednarski M, Oronsky B, et al. Dinitroazetidines are a novel class of anticancer agents and hypoxia-activated radiation sensitizers developed from highly energetic materials. Cancer Res. 2012;72:2600–8.
41. RadioRx Inc, unpublished data.
42. Scicinski J, Oronsky B, Taylor M, et al. Preclinical evaluation of the metabolism and disposition of RRx-001, a novel investigative anticancer agent. Drug Metab Dispos. 2012;40:1810–6.
43. Fens M, Larkin S, Morris C, et al. NO or No NO, increased reduction of Nitrite to Nitric Oxide by modified red blood cells. Blood (ASH Annual Meeting Abstracts). 2011;118:2125.
44. Reid T, Dad S, Korn R, et al. Two case reports of resensitization to previous chemotherapy with the novel hypoxia-activated hypomethylating anticancer agent RRx-001 in metastatic colorectal cancer patients. Case Rep Oncol. 2014;7:79–85.
45. Reid T, Oronsky B, Infante J, et al. A phase 1 trial and pharmacokinetic study of RRx-001, a novel ROS-mediated pan-epigenetic agent. J Clin Oncol. 2014;32:5s(suppl; abstr 2578).
46. Tritto I, Ambrosio G. The multi-faceted behavior of nitric oxide in vascular “inflammation”: catchy terminology or true phenomenon? Cardiovasc Res. 2004;63:1–4.
47. Oronsky BT, Knox SJ, Scicinski J. Six degrees of separation: the oxygen effect in the development of radiosensitizers. Transl Oncol. 2011;4:189–98.

Chapter 4
Mechanisms of Nitric Oxide-Dependent Regulation of Tumor Invasion and Metastasis

Aideen E. Ryan, Amy J. Burke, Francis J. Giles, Francis J. Sullivan and Sharon A. Glynn

Abstract The nitric oxide synthases (NOS) are key enzymes activated as part of the wound healing and host immune response. Their product nitric oxide (NO) is a short-lived, pleiotropic molecule that is a key physiological signaling molecule. Emerging evidence suggests that the NOS may be key regulators of tumor invasion and metastasis. However, there are markedly conflicting findings in the literature regarding NO and its role in carcinogenesis and tumor progression, and in particular its effect on tumor invasion and metastasis. In this review, we present the evidence for the roles played by the NOS in particular focusing on their cellular sources, which include but are not limited to tumor epithelial cells and tumor-associated macrophages. We propose that NOS expression in tumor epithelia cells tends towards activation of tumor-promoting effects, while NOS expression in tumor-associated macrophages has a tumor-inhibitory effect. The balance between the two as such determines whether a tumor progresses or regresses. This presents a therapeutic opportunity to target tumor invasion and metastasis by either disrupting tumor epithelial NOS expression by NOS inhibition, or by introducing NOS vectors or NO-donating drugs to increase the levels of intratumor NO to those that induce DNA damage and apoptosis.

Keywords Nitric oxide · Metastasis · Tumour associated macrophages · Immune infiltration · Pro- & anti-tumour immune responses

S. A. Glynn (✉)
Discipline of Pathology, School of Medicine, National University of Ireland, Galway, Ireland
e-mail: sharon.glynn@nuigalway.ie

A. E. Ryan
Discipline of Pharmacology and Therapeutics, School of Medicine, National University of Ireland, Galway, Ireland

Regenerative Medicine Institute, School of Medicine, National University of Ireland, Galway, Ireland

A. J. Burke · F. J. Giles · F. J. Sullivan
Prostate Cancer Institute, School of Medicine, National University of Ireland, Galway, Ireland

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_4

Abbreviations

ADAM	A Desintegrin And Metalloproteinase
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase
GEMM	genetically engineered mouse model
IFN-α	interferon alpha
IFN-γ	interferon gamma
iMCs	immature myeloid cells
iNOS	inducible nitric oxide synthase
LDI	low dose irradiation
LPS	lipoploysaccaride
MLF2	myeloid leukemia factor 2
MMP	matrix metalloproteinase
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells NO- nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NO2	Nitrogen Dioxide, ROS- reactive oxygen species
NSCLC	non small cell lung carcinoma
RNS	reactive nitrogen species
SNO	S nitrosation, RPL39- ribosomal protein L39TAM—tumor associated macrophages
TGF-β	transforming growth factor beta
TLRx	toll like receptor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

Introduction

There is strong evidence of a role for nitric oxide (NO) in the regulation of tumor invasion and metastasis. NO can be generated by three different isoforms of NO-Synthase (NOS)—neuronal (nNOS/NOS1), inducible (iNOS/NOS2) and endothelial (eNOS/NOS3). The NOS are dimeric enzymes, each monomer consisting of two distinct catalytic domains: an NH2-terminal oxygenase domain and a COOH-terminal reductase domain, which catalyze NO synthesis from L-arginine using NADPH and molecular oxygen as co-substrates [1, 2]. NO synthesis is a two-step process with NOS hydroxylating L-arginine to N-hydroxy-L-arginine, which is then oxidized to L-citrulline and NO [3, 4]. The unpaired electron in NO enables reaction with an array of substrates including inorganic molecules (e.g. oxygen, superoxide or transition metals), structures in deoxyribonucleic acid (DNA), prosthetic groups (e.g. heme) or proteins, facilitating extensive biological effects [5]. NOS1 and NOS3 generate short nanomolar bursts of NO, while NOS2 produces micromolar concentrations of NO over sustained periods [6]. In this review, we discuss the

implications of NOS expression in the tumor microenvironment and the cell-type specific effects of NO production on tumor growth and metastatic potential.

Concentration-Dependent Effects of NO

Before we can consider the impact of NO on tumor biology and metastasis, one needs to understand the concentration effects of NO on the cell. At low concentrations, NO acts as a signal transducer and affects many physiological processes including blood flow regulation, iron homeostasis and neurotransmission. Conversely, at high concentrations it exerts cytotoxic protective effects, e.g. the immune response against infections and potentially for the elimination of tumors [7]. In addition to its direct biological effects, NO can exert indirect effects through reaction with reactive oxygen species such as superoxide radicals, to generate reactive nitrogen species (RNS), nitrogen dioxide (NO2) and peroxynitrite ($ONOO^{-}$) [8]. Peroxynitrite promotes cellular transformation by functioning as a powerful antioxidant and interacting with or oxidizing kinases and transcription factors, perturbing the cellular signaling network [9], in addition to tyrosine nitration of proteins [10]. Nitrites, nitrates, s-nitrosothiols and nitrosamines are metabolites of NO and mediators of its cytotoxic/cytoprotective effects, namely, inhibition of mitochondrial respiration, protein and DNA damage leading to gene mutation, loss of protein function, necrosis and apoptosis [11].

It is well established that NO operates in a bimodal fashion. The dichotomous effects of NO on cancer arise from its ability to positively or negatively regulate crucial processes including proliferation, migration, invasion, survival, angiogenesis, and metastasis, depending on the concentration of NO involved. An additional layer of complexity arises when one includes the impact of NO flux, and the duration of NO exposure [8, 12, 13]. NO effects will also be dependent on tissue oxygen tension and local superoxide concentrations [14], and therefore are influenced by tissue oxygenation/hypoxia and the composition of the tumor microenvironment.

Evidence for the Effects of Tumor Epithelial-Associated NOS Expression in the Development of Human Metastatic Disease

NO and NOS2 are associated with numerous tumor types including lung [15, 16], colon [17, 18], breast [19–21], melanoma [22] and pancreatic cancers [23]. For the purposes of this review, we focus on the impact of NO and NOS on tumor invasion and metastasis in the best characterized cancers which include breast cancer, melanoma, colorectal cancer, pancreatic cancer, prostate cancer and to a lesser extent lung cancer. The ability of the neoplastic cell to activate invasion and metastasis is one of the major hallmarks of cancer as recognized in the 2000 landmark paper by Hanahan and Weinberg [24]. In their 2011 update, they identified the ability of

the tumor to avoid immune detection, in addition to tumor-promoting inflammation as being emerging hallmarks of cancer [25]. NOS are key inflammatory mediators induced as part of the wound healing response, and as such are potential candidate regulators of the 3 hallmarks of cancer listed above.

NOS2 is frequently expressed in the tumor epithelia of breast tumors [19–21], [26, 27]. Its expression correlates with high tumor grade [19, 20, 26], increased angiogenesis [19, 26] and decreased breast cancer specific survival [19, 21, 27], suggesting that it may play a role in breast cancer metastasis. The NOS2 association with poor outcome in breast cancer seems to be largely associated with estrogen receptor (ER) negative breast tumors [19], and of limited prognostic potential in ER positive tumors. Clinical data show that NOS2 expression in ER-negative breast tumors correlate with tumor vascularization, accumulations of p53 mutations, and activated EGFR [19]. NO has also been shown to induce CD44 and c-Myc, linked to stem cell-like phenotype in breast cancer [28]. This mechanism of action by NO is mediated in part by the Ets-1 transcription factor in a Ras-dependent manner [29]. A recent study demonstrated that polymorphisms in the NOS2A gene are associated with the development of ER/progesterone receptor (PR)-negative breast cancer [30]. One potential mechanism by which NOS2 may influence breast cancer metastasis is via S-nitrosation (SNO) of the EGFR and c-Src leading to enhanced signaling by these proteins [31]. This includes activation of β-catenin, leading to translocation to the nucleus and TCF transcriptional activity leading to activation of epithelial mesenchymal transition (EMT) [31]. NOS2 has also been shown to activate β-catenin signaling through inhibition of Dickkopf-1, a negative regulation of β-catenin signaling [32]. Another mechanism by which NOS2 promotes invasion and metastasis in breast cancer may be through the up-regulation of the matrix metalloproteinases (MMP)-2 [33] and MMP-9 [34] which aid in tumor invasion and extravasation. Indeed, anti-metastatic agents have been shown to exert their effects through the downregulation of NOS2, MMP-2 and MMP-9 [35]. A Desintegrin And Metalloproteinase 23 (ADAM23) intratumoral heterogeneity was recently shown to promote tumor metastasis in breast cancer [36]. $ADAM23^-$ tumor cells tend to display enhanced cell migration and invasion. Intriguingly, $ADAM23^-$ tumor cells promoted the invasion of $ADAM23^+$tumor cells. This was though increased expression of NOS3 in the $ADAM23^-$ tumor cells. ShRNA inhibition of NOS3 led to reduced invasive capacity and ablated the pro-invasive effects of $ADAM23^-$ tumor cells on $ADAM23^+$tumor cells [36]. Dave et al identified ribosomal protein L39 (RPL39) and myeloid leukemia factor 2 (MLF2) as candidates for the regulation of breast cancer stem cell self-renewal. ShRNA directed against RPL39 and MLF2 resulted in reduced tumor volume, tumor metastasis and improved survival. Overexpression of RPL39 and MLF2 resulted in increased expression of NOS2 and NOS3, but not NOS1. The NOS inhibitor, L-NAME, significantly reduced RPL39 and MLF2-mediated cell migration indicating that NOS plays a major role in RPL39 and MLF2-associated cell migration [37].

High levels of NOS2 in primary ulveal [38] and stage III [39, 40] melanoma lesions are predictive of poor outcome and risk of metastasis. NOS2 and nitrotyrosine formation in metastatic melanoma are also associated with poor outcome whether

measured from date of diagnosis or date of treatment [41]. Ahmed and Van Den Oord reported that NOS2 may be more critical in the early invasive radial growth phase by stimulation of neo-angiogenesis, than the later vertical phase of metastatic melanoma development [42]. Studies suggest that NOS2 expression in melanoma is associated with the development of blood and lymphatic microvessel formations that may support the development of metastatic disease [43]. Conversely, induction of NOS2 in the murine melanoma K-1735 using retroviral mediated transfer resulted in reduced tumor growth and decreased lung metastasis compared to controls [44]. This brings to question whether introduction of high levels of NOS2 in non-adapted tumor cells may have inhibitory effects as the cells may tolerate high loads of nitrosative stress, compared to tumor cells that naturally express NOS2. Similar effects are seen with the induction of NOS2 in prostate cancer cells [45]. In addition to NOS2, melanoma cells also express NOS3, which correlates with the expression of VEGF and increased microvessel density. This may be another way by which NO induces neovascularization in melanoma [46]. NOS3 may also play a role in in lymphatic microvessel formation in melanoma [47]. NOS1 expression in melanoma regulates suppression of immune surveillance. Amplification of the NOS1 locus correlates with aberrant IFN signaling. In this instance, NOS1 appears to act as an inhibitor of phospho-STAT1 induction following IFN-α stimulation in CD4+ and CD8+ T cells and monocytes suppressing immune response post IFN stimulation leading to immune dysfunction [48]. Another potential mechanism of action in melanoma is through repression of CXCL10. NOS2 expressing melanoma cells exhibit decreased CXCL10 levels which are associated with poor outcome [49]. Treatment of NOS2 negative melanoma cell lines with NO suppresses the expression of CXCL10 which acts as a T-cell attractant. Scavenging of NO in NOS2 overexpressing melanoma cells with cPTIO leads to the down regulation of IL8 and the upregulation of CXCL10. cPTIO-treated melanoma cells induce increased migration of plasmacytoid dendritic cells, which is reversible with the addition of SNAP (NO-donor). This effect was reversed with the addition of a CXCL10 blocking antibody [49].

Colorectal cancers stain positively for NOS2 in both tumor epithelia and infiltrating mononuclear cells [18]. NOS2 expression is highest in adenoma [18] and then subsequently decreases with advancing stage [18, 50] while the lowest levels were seen in metastatic lesions [18]. Further research showed that high levels of NOS2 are predictive of poor outcome and the development of metastasis [51]. A key feature for developing metastasis appears to be maintenance of enzymatically active NOS2 [51]. NOS2 is a particularly good predictor of outcome in patients diagnosed with stage II and stage III disease, and correlates with node positive disease at diagnosis [52]. NOS2 also correlates positively with lymph node metastasis and lymphatic invasion in colorectal carcinoma [53]. NOS2 expression in adenoma and colorectal cancer correlates with the accumulation of p53 mutations in adenoma (34 %) and colorectal cancer (48 %), with the predominant mutation being G:C to A:T transitions at CpG dinucleotides [17]. This indicates that early acquisition of NOS2-associated p53 mutations in adenomas may be a driver of progression from adenoma to carcinoma. Ulcerative colitis, an inflammatory bowel disease, is

known to predispose patients to the development of colorectal cancer. p53 mutation positive ulcerative colitis lesions also correlated with the expression of NOS2 [54]. Similar to melanoma, NOS2 correlates with VEGF expression and microvessel density, implicating it in tumor angiogenesis [55]. NOS2 is also a driver of cell invasion in colorectal cancer cells. NOS2 expressing cell lines exhibit increased invasive capacity compared to NOS2 negative cell lines, which is repressed upon NOS2 inhibition. Exogenous NO treatment of NOS2 negative colorectal cancer cell lines increases their invasive capacity [56]. Investigations into the mechanisms by which NOS2 increases invasive capacity in colorectal cancer cells show that NO increases the expression of the cell migratory proteins RhoB and Rac1, in addition to increases in MMP-2 and MMP-9 [57].

NOS3 appears to play a dominant role in prostate cancer outcome. The NOS3 4a/b polymorphism is associated with the detection of circulating tumor cells in prostate cancer patients which may be a risk factor for the development of micrometastasis [58]. Subsequently, Sanli et al reported an association between the NOS3 4a/b polymorphism and increased plasma nitrate/nitrite (NO_x) levels, and an increased risk of diagnosis with late stage disease and for the development of bone metastasis in prostate cancer patients [59]. A separate study also showed a positive correlation between serum and tissue levels of NO_x, and Gleason Score and pathological stage at diagnosis [60]. Conversely, the nitrate levels were inversely associated with advanced-stage prostate cancer in the Health professional study [61]. A second polymorphism in NOS3 (Glu-Asp298) is also associated with advanced disease and the development of bone metastasis [62]. In addition to polymorphism in NOS3, tumor expression of NOS3 correlates with poor outcome in prostate cancer patients, in particular when nuclear NOS3 staining is detectible. Nuclear NOS3 staining was associated with the formation of nuclear ER?/NOS3 complexes [63]. NOS2 may also play a role in prostate cancer. Aaltomaa et al examined the association of NOS2 with prostate cancer diagnosis. While NOS2 was associated with diagnosis of advanced stage disease, it was not predictive of biochemical recurrence. No data were available for the risk of metastasis or overall survival [64]. In a second study by the same authors, strong NOS2 expression in tumor cells was again positively associated with advanced stage disease ($p = 0.001$), in addition to high Gleason score and risk of metastasis. While strong NOS2 expression was associated with patient outcome in the univariate analysis, it was not found to be an independent predictor of outcome in prostate cancer [65].

The role for NOS2 in lung cancer is less clear. Ambs et al demonstrated that NOS1 and NOS3 activities are low in non-small cell lung carcinoma (NSCLC). NOS2 expression was also low in NSCLC, except for in the squamous cell carcinoma (SCC) subtype, where tumor epithelial and tumor infiltrating macrophages staining were observed [16]. Marrogi et al reported that NOS2 expression in NSCLC correlated with increase microvessel density, but not with patient outcome [66]. Puhakka et al reported that NOS2 expression was relatively low compared to NOS1 or NOS3 in lung cancer [67]. In tumors negative for NOS2, strong NOS2 positivity of intra-alveolar macrophages cells was observed including NOS2 positive macrophages infiltration of tumor tissue. This was accompanied by increased

apoptosis index compared to NOS2 positive tumors. No association of NOS2 with outcome was observed in this dataset ($n=89$). While the evidence provided here for a role of tumor epithelial associated NOS in tumor invasion and metastasis is compelling, it is important to recognize and place this in the context of an increasing body of evidence that also supports an anti-tumor effect of NOS2 expression in tumor infiltrating macrophages. Much of this emerging data have been demonstrated in experimental tumor models, but nonetheless provide convincing evidence that the role of NOS2 in the tumor microenvironment is more complex than previously envisaged. In the following section, we present this body of evidence, including the first group of confirmatory studies from human cancer patient cohorts.

Evidence for the Effects of Macrophage-Associated NOS2 Expression in Experimental Tumor Models

Tumor-associated macrophages (TAMs) constitute a large proportion of the inflammatory cells that infiltrate human solid tumors, including breast [68], colon [69], gastric [70] and ovary [71], and represents one of the hallmarks of cancer-associated inflammation [72]. Many epidemiological studies have demonstrated correlations between TAM infiltration and poor prognosis [68, 70, 71]. TAMs are heterogeneous, with distinct phenotypes that are influenced by the tumor microenvironment. In tumors, macrophages can be recruited to suppress T cells both by promotion of abnormal and dysfunctional blood vessels and also by inhibitory effects on extravasated T cells [73]. Additionally, TAMs can influence many hallmarks of cancer progression including tumor cell survival, invasion, metastasis and inflammation [69]. Recent studies however, have provided evidence that macrophages can be involved in both tumor promotion and tumor cell killing, depending on macrophage activation. Macrophage activation results in two broad but distinct macrophage phenotypes, M1 and M2. M1-like macrophages are induced by exposure to interferon (IFN)-γ or LPS and M2-like macrophages, which can be further subdivided into 3 groups; M2a, induced with IL-4 and IL-13; M2b, which are stimulated by immune complexes and Toll-like receptor (TLR) agonists; and exert host defensive function against viral and microbial infections through producing large amounts of inflammatory cytokines and nitric oxide (NO). On the other hand, M2 macrophages function in the scavenging of debris, angiogenesis, remodeling, and repair of wounded/damaged tissues. M2 macrophages produce arginase, resulting in the generation of ornithine and polyamines [74] and have been also shown to facilitate tumor progression by various different mechanisms [75–79]. M2 macrophages can also suppress adaptive tumor specific immune responses [80]. In contrast, the anti-tumor effects of M1 macrophages have been largely attributed to the production of NO [81–84]. It is well recognized that NOS2 dominates arginine metabolism in M1 macrophages to convert into nitric oxide that combining with oxygen radicals leads to the formation of cytotoxic peroxynitrite [85].

Although NOS2 has been previously associated with immunosuppressive functions through its ability to suppress T cell function [86, 87], several studies now exist that demonstrate enhanced anti-tumor immune responses and subsequent tumor rejection can be mediated by macrophage production of NO in experimental tumor models. Klug and colleagues recently showed that induction of an $NOS2^{+}$M1-like macrophage phenotype is required and sufficient to mediate effector T cell recruitment into tumor tissue [83]. Using a genetically engineered mouse model (GEMM) (RIP1-Tag5) of pancreatic islet carcinoma and a xenotransplanted model of human melanoma, the authors could show that low dose irradiation (LDI) induced a differentiation switch resulting in enhanced tumor infiltration by CD8+ T cells. This increased infiltration of CD8+ T cells, the primary effectors of anti-tumor immunity, was dependent on tumor infiltrating macrophage NOS2 expression and NO secretion and was abrogated following pharmacological inhibition of NOS2 in this model. This effect of NO is supported by previous studies showing that NO is important in the process of CD8+ T cell differentiation [88, 89]. Additionally, both LDI and T cell transfer converted the dysfunctional tumor blood vessels into a more 'normalized' vascular network, which likely facilitate the homing and extravasation of transferred CD8+ cells into the tumors. In another study, using $Stat6^{-/-}$ tumor bearing mice, Sinha et al. showed convincingly that following surgical resection of primary mammary gland tumors (4T1), >60% mice survive indefinitely compared to <5% of their $Stat6^{+/+}$ counterparts [90]. Macrophages from Stat6-/- tumor bearing mice displayed an M1-like phenotype with a low level expression of arginase and high level of NO, which the authors showed was cytotoxic for 4T1 tumor cells and largely responsible for the dramatic effect on tumor regression in $Stat6^{-/-}$ mice. Importantly, Sinha and colleagues suggest that because NO preferentially induces type I T cell differentiation [81], CD8+ T cells of $Stat6^{-/-}$ may be more efficacious because $Stat6^{-/-}$ macrophages are polarized towards NO production, which they showed to be dependent on IFN-γ in their model [90].

Furthermore, Sica and colleagues, showed that blocking activation of the IL-10 pathway in tumor infiltrating macrophages resulted in the restoration of NF-κB-dependent inflammatory functions, including the expression of NO and TNF-α, thereby inducing an M1-like inflammatory phenotype and intratumoral cytotoxicity in models of fibrosarcoma and melanoma [91]. Similarly, in a model of peritoneal metastasis, we recently demonstrated that the presence of an NOS2 expressing tumor infiltrating macrophage, in tumors lacking NF-κB activity, resulted in significantly increased survival in tumor bearing mice [82]. The presence of intratumoral NOS2 expressing macrophages was associated with increased $CD8^{+}$ T cell activation, higher levels of intratumoral apoptosis and decreased micro-vessel density [82]. These effects could be reversed following macrophage depletion in vivo, demonstrating the anti-tumor, cytotoxic effects of M1-like macrophages in NF-κB deficient tumors. In another study, Guiducci and colleagues demonstrated that inducing a switch in macrophage polarization using the TLR9 ligand, CpG, in addition to anti-IL-10 receptor antibody triggered the subsequent rejection of pre-established colon tumors [92]. Interestingly, two other studies demonstrated that strategies to manipulation of macrophage phenotype, to promote the expression

of NOS2 and other M1-like cytokines, including IL-12, TNF-α and IL-1β [93], or suppress an M2-like macrophage phenotype, can suppress both tumor angiogenesis and tumor growth and metastasis [94]. Using another strategy, Luo et al. demonstrated a dramatic reduction in pro-angiogenic factors released by TAMs, including VEGF and MMP-9 using a legumian-based DNA vaccine that successfully induced a $CD8^+$ T cell response against TAMs. Importantly, the success of this strategy was demonstrated in murine models of metastatic breast, colon, and non-small cell lung cancers, where 75 % of vaccinated mice survived lethal tumor cell challenges and 62 % were completely free of metastases.

As shown here, although tumor epithelial NOS2 expression has been associated with poor prognosis in several human tumors including breast [19], pancreas and lung [11], localization of expression varies in the diverse compartments of the complex tumor microenvironment. In support of the experimental tumor studies summarized above, several recent reports on human tumor have provided evidence that NOS2 is expressed in infiltrating macrophages, and the subsequent production of nitric oxide is positively associated with tumor rejection and survival time in non-small cell lung cancer (NSLC) [95, 96], gastric [70] and pancreatic cancers [83]. A study by Pantano et al. showed that M1 macrophage density, as determined by CD68 and NOS2 staining, was a prognostic factor in multivariate analysis in predicting patients survival time after radical surgery in gastric cancer patients [70]. In an earlier study, Ma and colleagues, found that M1 densities in tumor islets and/or stroma are positively associated with patients survival time [95].More recently, in a comprehensive study, Klug et al. demonstrated in a retrospective analysis of human pancreatic adenocarcinoma previously treated by LDI in a neoadjuvant setting, that NOS2-expressing macrophages and CD8+ T cells were significantly increased [83]. Increased intraepithelial T cell infiltrates are correlated with improved survival in patients with pancreatic cancer. Crucially, the authors showed that NOS2 expression in human tumor infiltrating macrophages was induced by local low dose irradiation, which can be easily applied clinically [73].

Conclusion

Although considerable progress has been made in understanding NOS2/NO signaling, the NOS2-targeted cancer therapy is complicated by many factors, not least by the complexity of the tumor microenvironment. Although NOS2 up regulation has been associated previously with immunosuppressive functions in stromal cells, including immature myeloid cells (iMCs) [97], and mesenchymal stromal cells [87], recent studies reviewed here reveal an unexpected role for NOS2 in promotion of an effective anti-tumor CD8-mediated immune response [83], normalization of tumor vasculature [83, 98, 99] and induction of cytotoxicity in tumor cells [82, 90, 91, 100]. These findings indicate that the diverse role of NO in the tumor microenvironment needs to be investigated in more detail. Most likely, the cellular source, the duration of exposure and the amount of NO are critical for its suppressive as opposed

to its stimulatory capacity, particularly in the complex microenvironment of solid tumors. As suggested recently by De Palma and colleagues [73], these apparently contradictory roles for NO may reflect a mutual inhibitory interplay between NOS2 tumor epithelial cells, NOS2 macrophages and other tumor stromal cells, including mesenchymal stromal cells and myeloid derived suppressor cells in the complex tumor microenvironment. How these effects can be selectively manipulated and achieved, in the context of targeted cancer therapy, will undoubtedly be the focus of future research efforts.

Acknowledgements Dr. Aideen Ryan is a recipient of an Irish Cancer Society Research Fellowship. Dr. Sharon Glynn is funded by the Galway University Foundation, Ireland; Breast Cancer Campaign, UK; and the Irish Cancer Society, Ireland.

Conflict Statement No potential conflicts of interest were disclosed.

References

1. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001;357:593–615.
2. Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J. 2012;33:829–37, 37a-37d.
3. Noble MA, Munro AW, Rivers SL, Robledo L, Daff SN, Yellowlees LJ, Shimizu T, Sagami I, Guillemette JG, Chapman SK. Potentiometric analysis of the flavin cofactors of neuronal nitric oxide synthase. Biochemistry. 1999;38:16413–8.
4. Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. J Biol Chem. 2001;276:14533–6.
5. Singh S, Gupta AK. Nitric oxide: role in tumor biology and iNOS/NO-based anticancer therapies. Cancer Chemother Pharmacol. 2011;67:1211–24.
6. Michel T, Feron O. Nitric oxide synthases: which, where, how, and why? J Clin Invest. 1997;100:2146–52.
7. Ignarro LJ. Nitric oxide: biology and pathobiologyed. Burlington: Academic; 2000.
8. Mocellin S, Bronte V, Nitti D. Nitric oxide, a double edged sword in cancer biology: searching for therapeutic opportunities. Med Res Rev. 2007;27:317–52.
9. Kundu JK, Surh YJ. Emerging avenues linking inflammation and cancer. Free Radic Biol Med. 2012;52:2013–37.
10. Ischiropoulos H, Zhu L, Chen J, Tsai M, Martin JC, Smith CD, Beckman JS. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Arch Biochem Biophys. 1992;298:431–7.
11. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumor progression. Nat Rev Cancer. 2006;6:521–34.
12. Ridnour LA, Thomas DD, Donzelli S, Espey MG, Roberts DD, Wink DA, Isenberg JS. The biphasic nature of nitric oxide responses in tumor biology. Antioxid Redox Signal. 2006;8:1329–37.
13. Villalobo A. Enhanced cell proliferation induced by nitric oxide. Dyn Cell Biol. 2007;1:60–4.
14. Wink DA, Mitchell JB. Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Radic Biol Med. 1998;25:434–56.
15. Okayama H, Saito M, Oue N, Weiss JM, Stauffer J, Takenoshita S, Wiltrout RH, Hussain SP, Harris CC. NOS2 enhances KRAS-induced lung carcinogenesis, inflammation and microRNA-21 expression. Int J Cancer. 2013;132:9–18.

16. Ambs S, Bennett WP, Merriam WG, Ogunfusika MO, Oser SM, Khan MA, Jones RT, Harris CC. Vascular endothelial growth factor and nitric oxide synthase expression in human lung cancer and the relation to p53. Br J Cancer. 1998;78:233–9.
17. Ambs S, Bennett WP, Merriam WG, Ogunfusika MO, Oser SM, Harrington AM, Shields PG, Felley-Bosco E, Hussain SP, Harris CC. Relationship between p53 mutations and inducible nitric oxide synthase expression in human colorectal cancer. J Natl Cancer Inst. 1999;91:86–8.
18. Ambs S, Merriam WG, Bennett WP, Felley-Bosco E, Ogunfusika MO, Oser SM, Klein S, Shields PG, Billiar TR, Harris CC. Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. Cancer Res. 1998;58:334–41.
19. Glynn SA, Boersma BJ, Dorsey TH, Yi M, Yfantis HG, Ridnour LA, Martin DN, Switzer CH, Hudson RS, Wink DA, Lee DH, Stephens RM, Ambs S. Increased NOS2 predicts poor survival in estrogen receptor-negative breast cancer patients. J Clin Invest. 2010;120:3843–54.
20. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S. Nitric oxide synthase activity in human breast cancer. Br J Cancer. 1995;72:41–4.
21. Bulut AS, Erden E, Sak SD, Doruk H, Kursun N, Dincol D. Significance of inducible nitric oxide synthase expression in benign and malignant breast epithelium: an immunohistochemical study of 151 cases. Virchows Arch. 2005;447:24–30.
22. Grimm EA, Ellerhorst J, Tang CH, Ekmekcioglu S. Constitutive intracellular production of iNOS and NO in human melanoma: possible role in regulation of growth and resistance to apoptosis. Nitric Oxide. 2008;19:133–7.
23. Xiong Q, Shi Q, Le X, Wang B, Xie K. Regulation of interleukin-8 expression by nitric oxide in human pancreatic adenocarcinoma. J Interferon Cytokine Res. 2001;21:529–37.
24. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57–70.
25. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
26. Vakkala M, Kahlos K, Lakari E, Paakko P, Kinnula V, Soini Y. Inducible nitric oxide synthase expression, apoptosis, and angiogenesis in in situ and invasive breast carcinomas. Clin Cancer Res. 2000;6:2408–16.
27. Loibl S, Buck A, Strank C, von Minckwitz G, Roller M, Sinn HP, Schini-Kerth V, Solbach C, Strebhardt K, Kaufmann M. The role of early expression of inducible nitric oxide synthase in human breast cancer. Eur J Cancer. 2005;41:265–71.
28. Ambs S, Glynn SA. Candidate pathways linking inducible nitric oxide synthase to a basal-like transcription pattern and tumor progression in human breast cancer. Cell Cycle. 2011;10:619–24.
29. Switzer CH, Cheng RY, Ridnour LA, Glynn SA, Ambs S, Wink DA. Ets-1 is a transcriptional mediator of oncogenic nitric oxide signaling in estrogen receptor-negative breast cancer. Breast Cancer Res. 2012;14:R125.
30. Slattery ML, John EM, Torres-Mejia G, Lundgreen A, Lewinger JP, Stern MC, Hines L, Baumgartner KB, Giuliano AR, Wolff RK. Angiogenesis genes, dietary oxidative balance and breast cancer risk and progression: the breast cancer health disparities study. Int J Cancer. 2014;134:629–44.
31. Switzer CH, Glynn SA, Cheng RY, Ridnour LA, Green JE, Ambs S, Wink DA. S-nitrosylation of EGFR and Src activates an oncogenic signaling network in human basal-like breast cancer. Mol Cancer Res. 2012;10:1203–15.
32. Du Q, Zhang X, Liu Q, Bartels CE, Geller DA. Nitric oxide production upregulates Wnt/beta-catenin signaling by inhibiting Dickkopf-1. Cancer Res. 2013;73:6526–37.
33. Zhang HJ, Zhao W, Venkataraman S, Robbins ME, Buettner GR, Kregel KC, Oberley LW. Activation of matrix metalloproteinase-2 by overexpression of manganese superoxide dismutase in human breast cancer MCF-7 cells involves reactive oxygen species. J Biol Chem. 2002;277:20919–26.
34. Ridnour LA, Windhausen AN, Isenberg JS, Yeung N, Thomas DD, Vitek MP, Roberts DD, Wink DA. Nitric oxide regulates matrix metalloproteinase-9 activity by guanylyl-cyclase-dependent and -independent pathways. Proc Natl Acad Sci U S A. 2007;104:16898–903.

35. Al Dhaheri Y, Attoub S, Arafat K, Abuqamar S, Viallet J, Saleh A, Al Agha H, Eid A, Iratni R. Anti-metastatic and anti-tumor growth effects of Origanum majorana on highly metastatic human breast cancer cells: inhibition of NFkappaB signaling and reduction of nitric oxide production. PLoS One. 2013;8:e68808.
36. Costa ET, Barnabe GF, Li M, Dias AA, Machado TR, Asprino PF, Cavalher FP, Ferreira EN, Del Mar Inda M, Nagai MH, Malnic B, Duarte ML, Leite KR, de Barros AC, Carraro DM, Chammas R, Armelin HA, Cavenee W, Furnari F, Camargo AA. Intratumoral heterogeneity of ADAM23 promotes tumor growth and metastasis through LGI4 and nitric oxide signals. Oncogene. 2014;0. doi:10.1038/onc.2014.70.
37. Dave B, Granados-Principal S, Zhu R, Benz S, Rabizadeh S, Soon-Shiong P, Yu KD, Shao Z, Li X, Gilcrease M, Lai Z, Chen Y, Huang TH, Shen H, Liu X, Ferrari M, Zhan M, Wong ST, Kumaraswami M, Mittal V, Chen X, Gross SS, Chang JC. Targeting RPL39 and MLF2 reduces tumor initiation and metastasis in breast cancer by inhibiting nitric oxide synthase signaling. Proc Natl Acad Sci U S A. 2014;111:8838–43.
38. Johansson CC, Mougiakakos D, Trocme E, All-Ericsson C, Economou MA, Larsson O, Seregard S, Kiessling R. Expression and prognostic significance of iNOS in uveal melanoma. Int J Cancer. 2010;126:2682–9.
39. Ekmekcioglu S, Ellerhorst JA, Prieto VG, Johnson MM, Broemeling LD, Grimm EA. Tumor iNOS predicts poor survival for stage III melanoma patients. Int J Cancer. 2006;119:861–6.
40. Johansson CC, Egyhazi S, Masucci G, Harlin H, Mougiakakos D, Poschke I, Nilsson B, Garberg L, Tuominen R, Linden D, Stolt MF, Hansson J, Kiessling R. Prognostic significance of tumor iNOS and COX-2 in stage III malignant cutaneous melanoma. Cancer Immunol Immunother. 2009;58:1085–94.
41. Ekmekcioglu S, Ellerhorst J, Smid CM, Prieto VG, Munsell M, Buzaid AC, Grimm EA. Inducible nitric oxide synthase and nitrotyrosine in human metastatic melanoma tumors correlate with poor survival. Clin Cancer Res. 2000;6:4768–75.
42. Ahmed B, Van Den Oord JJ. Expression of the inducible isoform of nitric oxide synthase in pigment cell lesions of the skin. Br J Dermatol. 2000;142:432–40.
43. Massi D, De Nisi MC, Franchi A, Mourmouras V, Baroni G, Panelos J, Santucci M, Miracco C. Inducible nitric oxide synthase expression in melanoma: implications in lymphangiogenesis. Mod Pathol. 2009;22:21–30.
44. Juang SH, Xie K, Xu L, Wang Y, Yoneda J, Fidler IJ. Use of retroviral vectors encoding murine inducible nitric oxide synthase gene to suppress tumorigenicity and cancer metastasis of murine melanoma. Cancer Biother Radiopharm. 1997;12:167–75.
45. Coulter JA, Page NL, Worthington J, Robson T, Hirst DG, McCarthy HO. Transcriptional regulation of inducible nitric oxide synthase gene therapy: targeting early stage and advanced prostate cancer. J Gene Med. 2010;12:755–65.
46. Tu YT, Tao J, Liu YQ, Li Y, Huang CZ, Zhang XB, Lin Y. Expression of endothelial nitric oxide synthase and vascular endothelial growth factor in human malignant melanoma and their relation to angiogenesis. Clin Exp Dermatol. 2006;31:413–8.
47. Lahdenranta J, Hagendoorn J, Padera TP, Hoshida T, Nelson G, Kashiwagi S, Jain RK, Fukumura D. Endothelial nitric oxide synthase mediates lymphangiogenesis and lymphatic metastasis. Cancer Res. 2009;69:2801–8.
48. Liu Q, Tomei S, Ascierto ML, De Giorgi V, Bedognetti D, Dai C, Uccellini L, Spivey T, Pos Z, Thomas J, Reinboth J, Murtas D, et al. Melanoma NOS1 expression promotes dysfunctional IFN signaling. J Clin Invest. 2014;124:2147–59.
49. Tanese K, Grimm EA, Ekmekcioglu S. The role of melanoma tumor-derived nitric oxide in the tumor inflammatory microenvironment: its impact on the chemokine expression profile, including suppression of CXCL10. Int J Cancer. 2012;131:891–901.
50. Ropponen KM, Kellokoski JK, Lipponen PK, Eskelinen MJ, Alanne L, Alhava EM, Kosma VM. Expression of inducible nitric oxide synthase in colorectal cancer and its association with prognosis. Scand J Gastroenterol. 2000;35:1204–11.
51. Lagares-Garcia JA, Moore RA, Collier B, Heggere M, Diaz F, Qian F. Nitric oxide synthase as a marker in colorectal carcinoma. Am Surg. 2001;67:709–13.

52. Zafirellis K, Zachaki A, Agrogiannis G, Gravani K. Inducible nitric oxide synthase expression and its prognostic significance in colorectal cancer. APMIS. 2010;118:115–24.
53. Nozoe T, Yasuda M, Honda M, Inutsuka S, Korenaga D. Immunohistochemical expression of cytokine induced nitric oxide synthase in colorectal carcinoma. Oncol Rep. 2002;9:521–4.
54. Hussain SP, Amstad P, Raja K, Ambs S, Nagashima M, Bennett WP, Shields PG, Ham AJ, Swenberg JA, Marrogi AJ, Harris CC. Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. Cancer Res. 2000;60:3333–7.
55. Cianchi F, Cortesini C, Fantappie O, Messerini L, Schiavone N, Vannacci A, Nistri S, Sardi I, Baroni G, Marzocca C, Perna F, Mazzanti R, Bechi P, Masini E. Inducible nitric oxide synthase expression in human colorectal cancer: correlation with tumor angiogenesis. Am J Pathol. 2003;162:793–801.
56. Siegert A, Rosenberg C, Schmitt WD, Denkert C, Hauptmann S. Nitric oxide of human colorectal adenocarcinoma cell lines promotes tumor cell invasion. Br J Cancer. 2002;86:1310–5.
57. Babykutty S, Suboj P, Srinivas P, Nair AS, Chandramohan K, Gopala S. Insidious role of nitric oxide in migration/invasion of colon cancer cells by upregulating MMP-2/9 via activation of cGMP-PKG-ERK signaling pathways. Clin Exp Metastasis. 2012;29:471–92.
58. Medeiros R, Morais A, Vasconcelos A, Costa S, Carrilho S, Oliveira J, Lopes C. Endothelial nitric oxide synthase gene polymorphisms and the shedding of circulating tumor cells in the blood of prostate cancer patients. Cancer Lett. 2003;189:85–90.
59. Sanli O, Kucukgergin C, Gokpinar M, Tefik T, Nane I, Seckin S. Despite the lack of association between different genotypes and the presence of prostate cancer, endothelial nitric oxide synthase a/b (eNOS4a/b) polymorphism may be associated with advanced clinical stage and bone metastasis. Urol Oncol. 2011;29:183–8.
60. Dillioglugil MO, Mekik H, Muezzinoglu B, Ozkan TA, Demir CG, Dillioglugil O. Blood and tissue nitric oxide and malondialdehyde are prognostic indicators of localized prostate cancer. Int Urol Nephrol. 2012;44:1691–6.
61. Wu T, Wang Y, Ho SM, Giovannucci E. Plasma levels of nitrate and risk of prostate cancer: a prospective study. Cancer Epidemiol Biomarkers Prev. 2013;22:1210–8.
62. Medeiros RM, Morais A, Vasconcelos A, Costa S, Pinto D, Oliveira J, Ferreira P, Lopes C. Outcome in prostate cancer: association with endothelial nitric oxide synthase Glu-Asp298 polymorphism at exon 7. Clin Cancer Res. 2002;8:3433–7.
63. Nanni S, Benvenuti V, Grasselli A, Priolo C, Aiello A, Mattiussi S, Colussi C, Lirangi V, Illi B, D'Eletto M, Cianciulli AM, Gallucci M, et al. Endothelial NOS, estrogen receptor beta, and HIFs cooperate in the activation of a prognostic transcriptional pattern in aggressive human prostate cancer. J Clin Invest. 2009;119:1093–108.
64. Aaltomaa SH, Lipponen PK, Viitanen J, Kankkunen JP, Ala-Opas MY, Kosma VM. The prognostic value of inducible nitric oxide synthase in local prostate cancer. BJU Int. 2000;86:234–9.
65. Aaltoma SH, Lipponen PK, Kosma VM. Inducible nitric oxide synthase (iNOS) expression and its prognostic value in prostate cancer. Anticancer Res. 2001;21:3101–6.
66. Marrogi AJ, Travis WD, Welsh JA, Khan MA, Rahim H, Tazelaar H, Pairolero P, Trastek V, Jett J, Caporaso NE, Liotta LA, Harris CC. Nitric oxide synthase, cyclooxygenase 2, and vascular endothelial growth factor in the angiogenesis of non-small cell lung carcinoma. Clin Cancer Res. 2000;6:4739–44.
67. Puhakka A, Kinnula V, Napankangas U, Saily M, Koistinen P, Paakko P, Soini Y. High expression of nitric oxide synthases is a favorable prognostic sign in non-small cell lung carcinoma. APMIS. 2003;111:1137–46.
68. Mahmoud SM, Lee AH, Paish EC, Macmillan RD, Ellis IO, Green AR. Tumor-infiltrating macrophages and clinical outcome in breast cancer. J Clin Pathol. 2012;65:159–63.
69. Bingle L, Brown NJ, Lewis CE. The role of tumor-associated macrophages in tumor progression: implications for new anticancer therapies. J Pathol. 2002;196:254–65.

70. Pantano F, Berti P, Guida FM, Perrone G, Vincenzi B, Amato MM, Righi D, Dell'aquila E, Graziano F, Catalano V, Caricato M, Rizzo S, Muda AO, Russo A, Tonini G, Santini D. The role of macrophages polarization in predicting prognosis of radically resected gastric cancer patients. J Cell Mol Med. 2013;17:1415–21.
71. He YF, Zhang MY, Wu X, Sun XJ, Xu T, He QZ, Di W. High MUC2 expression in ovarian cancer is inversely associated with the M1/M2 ratio of tumor-associated macrophages and patient survival time. PLoS One. 2013;8:e79769.
72. Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. Crit Rev Oncol Hematol. 2008;66:1–9.
73. De Palma M, Coukos G, Hanahan D. A new twist on radiation oncology: low-dose irradiation elicits immunostimulatory macrophages that unlock barriers to tumor immunotherapy. Cancer Cell. 2013;24:559–61.
74. Morris SM, Jr, Kepka-Lenhart D, Chen LC. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. Am J Physiol. 1998;275:E740–7.
75. Mills CD. Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. Crit Rev Immunol. 2001;21:399–425.
76. Colvin EK. Tumor-associated macrophages contribute to tumor progression in ovarian cancer. Front Oncol. 2014;4:137.
77. Bronte V, Serafini P, De Santo C, Marigo I, Tosello V, Mazzoni A, Segal DM, Staib C, Lowel M, Sutter G, Colombo MP, Zanovello P. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. J Immunol. 2003;170:270–8.
78. Van Overmeire E, Laoui D, Keirsse J, Van Ginderachter JA. Hypoxia and tumor-associated macrophages: a deadly alliance in support of tumor progression. Oncoimmunology. 2014;3:e27561.
79. Van Overmeire E, Laoui D, Keirsse J, Van Ginderachter JA, Sarukhan A. Mechanisms driving macrophage diversity and specialization in distinct tumor microenvironments and parallelisms with other tissues. Front Immunol. 2014;5:127.
80. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol. 2011;11:723–37.
81. Niedbala W, Wei XQ, Campbell C, Thomson D, Komai-Koma M, Liew FY. Nitric oxide preferentially induces type 1T cell differentiation by selectively up-regulating IL-12 receptor beta 2 expression via cGMP. Proc Natl Acad Sci U S A. 2002;99:16186–91.
82. Ryan AE, Colleran A, O'Gorman A, O'Flynn L, Pindjacova J, Lohan P, O'Malley G, Nosov M, Mureau C, Egan LJ. Targeting colon cancer cell NF-kappaB promotes an anti-tumor M1-like macrophage phenotype and inhibits peritoneal metastasis. Oncogene. 2014;0. doi:10.1038/onc.2014.86.
83. Klug F, Prakash H, Huber PE, Seibel T, Bender N, Halama N, Pfirschke C, Voss RH, Timke C, Umansky L, Klapproth K, Schäkel K, Garbi N, Jäger D, Weitz J, Schmitz-Winnenthal H, Hämmerling GJ, Beckhove P. Low-dose irradiation programs macrophage differentiation to an iNOS(+)/M1 phenotype that orchestrates effective T cell immunotherapy. Cancer Cell. 2013;24:589–602.
84. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol. 2009;27:451–83.
85. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. J Immunol. 2005;175:342–9.
86. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumors. Nat Rev Immunol. 2012;12:253–68.
87. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell. 2008;2:141–50.
88. Rodriguez PC, Zea AH, Culotta KS, Zabaleta J, Ochoa JB, Ochoa AC. Regulation of T cell receptor CD3zeta chain expression by L-arginine. J Biol Chem. 2002;277:21123–9.

89. Rodriguez PC, Zea AH, DeSalvo J, Culotta KS, Zabaleta J, Quiceno DG, Ochoa JB, Ochoa AC. L-arginine consumption by macrophages modulates the expression of CD3 zeta chain in T lymphocytes. J Immunol. 2003;171:1232–9.
90. Sinha P, Clements VK, Ostrand-Rosenberg S. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. J Immunol. 2005;174:636–45.
91. Sica A, Saccani A, Bottazzi B, Polentarutti N, Vecchi A, van Damme J, Mantovani A. Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. J Immunol. 2000;164:762–7.
92. Guiducci C, Vicari AP, Sangaletti S, Trinchieri G, Colombo MP. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer Res. 2005;65:3437–46.
93. Rauh MJ, Sly LM, Kalesnikoff J, Hughes MR, Cao LP, Lam V, Krystal G. The role of SHIP1 in macrophage programming and activation. Biochem Soc Trans. 2004;32:785–8.
94. Luo Y, Zhou H, Krueger J, Kaplan C, Lee SH, Dolman C, Markowitz D, Wu W, Liu C, Reisfeld RA, Xiang R. Targeting tumor-associated macrophages as a novel strategy against breast cancer. J Clin Invest. 2006;116:2132–41.
95. Ma J, Liu L, Che G, Yu N, Dai F, You Z. The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. BMC Cancer. 2010;10:112.
96. Ohri CM, Shikotra A, Green RH, Waller DA, Bradding P. Macrophages within NSCLC tumor islets are predominantly of a cytotoxic M1 phenotype associated with extended survival. Eur Respir J. 2009;33:118–26.
97. Pahler JC, Tazzyman S, Erez N, Chen YY, Murdoch C, Nozawa H, Lewis CE, Hanahan D. Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response. Neoplasia. 2008;10:329–40.
98. Huang Y, Snuderl M, Jain RK. Polarization of tumor-associated macrophages: a novel strategy for vascular normalization and antitumor immunity. Cancer Cell. 2011;19:1–2.
99. Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, Squadrito ML, Segura I, Li X, Knevels E, Costa S, Vinckier S, et al. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. Cancer Cell. 2011;19:31–44.
100. Farias-Eisner R, Sherman MP, Aeberhard E, Chaudhuri G. Nitric oxide is an important mediator for tumoricidal activity in vivo. Proc Natl Acad Sci U S A. 1994;91:9407–11.

Chapter 5
Role of Nitric Oxide in the Regulation of the Pro-tumourigenic Hypoxic Phenotype: From Instigation to Mitigation

Lynne-Marie Postovit

Abstract Low oxygen levels (hypoxia) are a biophysical consequence of tumour growth that exceeds vascular capacity. Hence, hypoxia is commonly present in solid tumours. Many studies, spanning decades, have demonstrated that the extent of tumour hypoxia is positively correlated with a poor clinical prognosis. These clinical findings have been corroborated with laboratory-based studies demonstrating that hypoxia induces hallmarks of cancer such as metabolic reprogramming, metastatic potential, immune cell evasion and angiogenesis. Accordingly, there has been a great emphasis placed on the need to understand the mechanisms by which hypoxia promotes tumourigenic phenotypes; so that interventional therapeutics can be developed. One candidate modulator of the hypoxic response is Nitric Oxide (NO). In this review, we describe how NO can mimic or mitigate the effects of hypoxia in tumours in a context and concentration-dependent manner. We further reveal emerging research suggesting that NO signalling may be harnessed to prevent tumour progression.

Keywords Cancer · cGMP · Hypoxia · Hypoxia inducible factors · Nitric oxide

Abbreviations

5'UTR	5'Untranslated Region
ADAM10	A Disintegrin And Metalloprotease Domain 10
ADMA	Asymmetrical DiMethylArginine (ADMA)
AMP	Adenosine Monophosphate
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
CA9	Carbonic Anhydrase IX
CCL28	CC-chemokine ligand 28
cAMP	cyclin Adenosine MonoPhosphate
cGMP	cyclic Guanosine MonoPhosphate
DETA/NO	Diethylenetriamine/nitric oxide adduct

L.-M. Postovit (✉)
Department of Oncology, University of Alberta, 5-142E Katz Group Building, 114th St and 87th Ave, Edmonton, AB T6G 2E1, Canada
e-mail: postovit@ualberta.ca

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_5

ECM	ExtraCellular Matrix
EMT	Epithelial-to-Mesenchymal Transition
eNOS	Endothelial Nitric Oxide Synthase
EPO	Erythropoietin
ERK	Extracellular Regulated Kinase
ET-1	Endothelin-1
Glut1	Glucose Transporter 1
GMP	Guanosine Monophosphate
GTP	Guanosine 5-TriPhosphate
GTN	Glyceryl TriNitrate
H_2O_2	Hydrogen Peroxide
HIF	Hypoxia Inducible Factor
HRE	Hypoxia Responsive Element
HUVEC	Human Umbilical Cord Vein Cells
iNOS	Inducible Nitric Oxide Synthase
JNK	c-Jun-NH2-Terminal Kinase
L-NAME	L-NG-Nitroarginine Methyl Ester
L-NMMA	NG-MonoMethyl-l-Arginine
LOX	Lysyl Oxidase
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
MAPK	Mitogen Activate Protein Kinase
MKP-1	MAPK Phosphatase 1
MICA	MHC Class I Chain Related Molecule
NSCLC	Non-Small Cell Lung Cancer
NK	Natural Killer
O_2	Oxygen
$ONOO^-$	Peroxynitrite
ODD	Oxygen Dependent Degradation Domain
PDE	PhosphoDiEsterase
PDL-1	Programmed Cell Death Ligand-1
PHDs	Prolyl Hydroxylases
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKG	cGMP-dependent Protein Kinase
PSA	Prostate Specific Antigen
pVHL	von Hippel-Lindau tumour suppressor protein
sGC	soluble Guanylyl Cyclase
SOD	Superoxide Dismutase
TNFα	Tumour Necrosis Factor alpha
uPAR	Urokinase Plasminogen Activator Receptor
VEGF	Vascular Endothelial Growth Factor

Introduction

Low oxygen tensions (hypoxia) are a common feature of solid tumours [1]. Indeed, depending on the size and type of tumour, between 1 and 50% of cancer cells are exposed to less than 1.3% O_2 [2]. One of the first models of tumour hypoxia, proposed by Thomlinson and Gray in 1955, posited that cells located farther than 70–150 μm away from a blood supply experience hypoxia due to the inability of oxygen to diffuse beyond this distance. Notably, this study, and several others that followed, showed that unlike many normal cells, cancer cells survive in chronic levels of hypoxia for hours to a few days [3–5]. More recently, measuring blood flow and oxygen levels in xenografts revealed the phenomenon of perfusion-limited hypoxia (acute or fluctuating hypoxia), in which perfusion of blood vessels is inefficient and dynamic, due to rapid and pathological angiogenesis. This type of perfusion defect results in transient periods of severe hypoxia within the tumour [6–8]. Hence, solid tumours commonly experience hypoxia, due to both diffusion limitations and abnormally formed blood vessels [9].

Several studies have demonstrated that hypoxia causes tumour cells to acquire and sustain more aggressive phenotypes. For example, hypoxia induces the expression of proteins such as the urokinase receptor (uPAR) [10], which promote invasion and metastasis and enhance immune tolerance by inducing the expression of the chemokine CC-chemokine ligand 28 (CCL28) which promotes the recruitment of regulatory T cells [11]. In addition, through the up-regulation of glucose transporters such as GLUT1, hypoxia promotes glycolytic metabolism [12] and by inducing VEGF it facilitates angiogenesis [13, 14]. Finally, hypoxia allows cells to resist apoptosis and induces the expression of multidrug resistance proteins, such as the P-glycoprotein that allow the efflux of drugs [15, 16]. Hence, hypoxia is able to potently activate many of the hallmarks of cancer, making it an important target for anti-neoplastic treatments [1, 14, 17, 18].

In contrast to hypoxia, which is almost entirely cancer-promoting, Nitric Oxide (NO) plays a controversial role in cancer, stimulating progression in some cases and mitigating it in others. These apparently paradoxical roles for NO in cancer have been reviewed extensively by others and are due largely to context and concentration-dependent effects [19, 20]. This review will further highlight the importance of context by describing how NO signalling can both activate and mitigate the hypoxic response in cancer cells. It will further highlight emerging studies indicating that NO signalling may be targeted to prevent hypoxia-induced cancer hallmarks.

Hypoxia: A Microenvironmental Driver of Tumour Progression

Oxygen levels play a pivotal role in regulating gene expression and cellular behaviour. This phenomenon is particularly apparent when oxygen availability decreases, leading to a state of low oxygen or hypoxia [9, 21]. The levels of oxygen

in healthy tissues vary greatly with physiological "normoxia" ranging from ~20% in the upper airways to ~0.5% in tissues such as the retina [22, 23]. In contrast, growing tumours are characterized by "hypoxia" wherein oxygen tensions fall below physiological levels. For example, hypoxia is common within breast cancers which have a median O_2 concentration of 1.3%, as compared to ~8.5% for normal breast tissue [24]. In response to hypoxia, cells express genes that are essential for their survival. In tumour cells, this O_2-regulated gene expression leads to more aggressive phenotypes, including those that increase the ability of cells to resist therapy, recruit a vasculature, and metastasize [10, 21, 25–27]. Accordingly, there is a growing body of evidence correlating tumour hypoxia with a poor clinical outcome for patients with a variety of cancers [25, 28–31].

Hypoxia initiates complex and specialized responses at the molecular, cellular, and organismal levels to re-establish oxygen homeostasis and minimize injury [32]. For example, in response to hypoxia, there is reduced oxidative phosphorylation, and repression of energy-demanding processes, such as translation of mRNA [33]. Moreover, glycolysis is upregulated to compensate for reduced ATP production [33]. Most transcription is reduced during times of hypoxia; however, a subset of genes is increased dramatically, including Vascular Endothelial Growth Factor *(VEGF),* Erythropoietin *(EPO),* and Glucose Transporters 1 and 3 *(SLC2A1* and *SLC2A3)* [32–34]. These alterations allow cells to adapt to the low oxygen conditions, and as a consequence also promote tumour progression. Hypoxia-induced VEGF expression increases angiogenesis, enabling further growth and providing a vascular conduit for metastasis [14]. Moreover, the induction of extracellular matrix (ECM)-associated proteins such as Lysyl Oxidase (LOX), and certain matrix metalloproteinases allow cells to invade from the primary tissue site and to colonize secondary metastatic sites such as the lung [35–38]. Critical drug resistance proteins, such as P-glycoprotein, are induced by hypoxia, facilitating therapy resistance [39]. Finally, hypoxia promotes immune escape by causing the shedding of immune stimulating MICA from the cell surface and by up-regulating the expression of the immune inhibitory factor, Programmed Cell Death Ligand-1 (PD-L1) [40, 41]. Indeed, the pro-tumourigenic effects of hypoxia are vast, and have been the subject of several excellent reviews [1, 14, 17, 18].

Tumour hypoxia can also induce and/or support cancer stem cells [9]. For example, hypoxia promotes $CD133^+$ cancer stem cell populations in glioblastomas [42] and hypoxia can induce the expression of a hESC − associated gene signature, inclusive of *POU5F1* and *NANOG,* in a number of cancer cell types, including prostate cancer and brain cancer [43]. Several studies have also suggested that hypoxia can induce cancer stem cell-like phenotypes in breast cancers. Well-differentiated T47D, MCF-7 and CAMA breast cancer cells dedifferentiate following 3 days of exposure to 1% O_2, characterized by a decrease in estrogen receptor expression, and an increase in cytokertain 19 (an epithelial stem cell marker) [44]. Moreover, several studies have used archival breast cancer samples to demonstrate that $CD44^+$/$CD24^-$ breast cancer stem cells are enriched in hypoxic tumours, delineated by the expression of Hypoxia Inducible Factor-1 (HIF-1) or Carbonic Anhydrase IX (CA9) [45, 46]. Finally, exposure to low O_2 increases epithelial-to mesenchymal transition

(EMT) in breast cancer cells concomitant with a marked enhancement of tumour initiation and metastatic potential [47–51]. Thus, in addition to driving classical hallmarks of cancer, hypoxia may actually promote the very seed of this disease.

Hypoxia Inducible Factors: Master Transcriptional Regulators of the Hypoxic Response

Approximately 1–1.5% of the genome is transcriptionally regulated by hypoxia, either through Hypoxia Inducible Factor (HIF)-dependent or HIF-independent pathways [52–54]. The HIF pathway is present in nearly all eukaryotic cells wherein it dominates the cellular response to hypoxia [54]. HIFs are heterodimeric transcription factors composed of an α(HIF-α) and a β(HIF-β/aryl hydrocarbon receptor nuclear translocator (ARNT)) subunits, both of which belong to the basic helix-loop-helix per-arnt-sim protein family [54]. HIF-1β is constitutively expressed while the α subunit is tightly regulated at the levels of protein stability and transactivational activity. Under normal physiological conditions, HIF-1 activity is virtually absent. However, following a reduction in O_2, there is a marked increase in HIF-1 DNA-binding and functional activity. Under normal conditions, HIF-1α is a substrate for the von Hippel-Lindau tumour suppressor protein (pVHL) ubiquitylation complex, and is consequentially targeted for degradation by the 26 S proteosome. Recognition by VHL is dependent on hydroxylation of prolyl residues in the O_2-dependent degradation domain of HIF-1α. The enzymes that catalyze this event, the HIF-1α prolyl-hydroxylases (PHDs), have an absolute requirement for molecular O_2 as a co-substrate and for iron as a co-factor; hence, disruptions in O_2 availability or iron homeostasis similarly result in HIF-1α accumulation and activity [28, 55–57]. In hypoxia, HIF-α accumulates in the cytoplasm as it is no longer targeted for degradation [54, 58, 59]. HIF-α then translocates to the nucleus where it heterodimerizes with HIF-β/ARNT and binds to hypoxia responsive element (HRE) regions in target genes. There are three known HIF-α proteins (HIF-1, HIF-2 and HIF-3) of which HIF-1 and HIF-2 are best characterised. HIF-1α and HIF-2α display a high sequence similarity and are both able to heterodimerize with ARNT and bind to HREs [57, 59].

Nitric Oxide

Nitric oxide plays a pivotal role in regulating multiple biological functions. For example, it induces vasodilatation, regulates platelet adhesion, acts as a neurotransmitter, and mediates cell growth and apoptosis [60–64]. Because NO is a small molecule without any charge, it can diffuse freely across cell membranes without the need for a transport system. Furthermore, NO is not very reactive, hence it is able to diffuse from adjacent cells without being altered [65, 66]. Accordingly, NO is a prevalent signalling molecule in the tumour microenvironment. NO also shares

a number of features that can allow it to interact with and even mimic some of the functions of oxygen. In particular, the electronic structure of NO makes it an outstanding ligand for heme moieties. Therefore, NO can bind to proteins such as prolyl hydroxylases at low concentrations [65–67].

Cellular Nitric Oxide Production

Nitric oxide is produced by nitric oxide synthases (NOS). Three isoforms of NOS have been discovered: neuronal (nNOS or NOS I), inducible (iNOS or NOS II), and endothelial (eNOS or NOS III) [68–71]. The levels of NO produced by each NOS isoform vary between 5 nM and 4 μM [72, 73], with NOS III and NOS I generating the lower basal levels and NOS II contributing to the more transient micromolar concentrations. The catalysis of NO involves the conversion of l-Arginine and O_2 into NO and citrulline [68–70]. This reaction is dependent on the availability of several co-factors and co-substrates, including NADPH, FAD, and tetrahydrobiopterin [68–74]. Interestingly, cells are also capable of synthesizing endogenous NOS inhibitors such as NG-monomethyl-l-arginine (l-NMMA) and asymmetrical dimethylarginine (ADMA) [68, 75]. These methylated l-arginine analogues, which competitively inhibit NOS by mimicking l-Arginine, allow for the endogenous regulation of NO synthesis. Although constitutive in many tissues, the expression of NOS is regulated by several factors. For example, shear stress has been shown to increase eNOS levels in endothelial cells, and studies have demonstrated that cytokines such as Tumour Necrosis Factor alpha (TNF) promote iNOS expression [76, 77]. Oxygen levels also regulate NOS levels in a cell type and isoform specific manner [21, 71] by altering enzyme expression and by limiting the availability of O_2, a key substrate for NO synthesis.

Nitric Oxide Production in Hypoxia

Like most phenomena associated with NO, oxygen regulates the expression of NOS in a context and isoform specific manner. Indeed, hypoxia has been shown to increase, decrease, or not affect cellular NOS levels [71, 78–82], depending on what model is used and what isoform is measured. For example, the *iNOS* gene can be significantly up-regulated by hypoxia in many cell types [80, 81]. Conversely, hypoxia has been shown to inhibit the transactivation of the iNOS gene in cells exposed to certain cytokines, and exposure of B16F10 murine melanoma cells to low levels of O_2 results in decreased iNOS protein levels [21, 78]. Moreover, a recent study using endothelial cells suggests that HIF-1 induces iNOS-dependent NO whereas HIF-2 reduces it [83]. In contrast to iNOS, studies generally demonstrate that nNOS is induced by hypoxia [71, 84, 85]. One mechanism underlying this induction involves the up-regulation of a nNOS transcript with a short 5' Untranslated Region

(5'UTR). This short UTR greatly enhances translational efficiency, allowing higher levels of nNOS protein to be made, even as hypoxia generally reduces translation [85]. In contrast to nNOS, the majority of studies suggest that hypoxia reduces the expression of eNOS [71, 86]. This down-regulation in expression occurs due to several factors including reduced transcription and mRNA stability [82], increased expression of an antisense transcript [87], and epigenetic modifications [88].

Given the variations in how hypoxia may affect NOS levels, it is difficult to predict NO production in the tumour microenvironment, based on the expression of one isoform. This is further compounded by how oxygen availability may affect NOS activity, based on its requirement as a substrate. For example, the Kms of oxygen are 350, 130 and 4 mM, for nNOS, iNOS and eNOS, respectively [71, 85]. Hence, NO production by nNOS and iNOS would be greatly limited in the hypoxic tumour microenvironment. While the effects on eNOS activity would be minimal, the general down-regulation in the expression of this enzyme in hypoxia would likely result in a net reduction in NO availability. Indeed, low levels of O_2 (1–3 %) inhibit NO production by up to 90 % in macrophages and endothelial cells [21, 89–91]. Hypoxia also affects NOS activity by regulating the expression of endogenous antagonists. For example, conditions associated with hypoxia have been shown to up-regulate the expression of the NOS antagonist ADMA [21, 89, 90, 92]. In further support of the concept that hypoxia decreases NOS activity, several studies have shown that hypoxia and NOS inhibition can commonly affect downstream signalling. Both hypoxia and NOS inhibition decrease cellular cGMP levels [10, 93, 94]. Culture of MDA-MB-231 breast carcinoma cells in 0.5 % O_2 results in a reduction in cGMP accumulation , an effect that can be rescued by the addition of NO donors [10]. Furthermore, inhibition of the NO-activated cGMP signalling pathway can elicit responses that are similar to those induced by hypoxia. NOS inhibition and hypoxia similarly increase uPAR expression, ET-1 production, chemoresistance, cellular invasiveness, and metastatic potential [21, 26, 95, 94]. In addition, prolonged exposure to L-NAME or knock down of eNOS increases migration and VEGF production in human umbilical cord vein (HUVEC) cells, concomitant with stabilized HIF. These effects of NOS inhibition can be mitigated by relatively low levels (500 nM) of the NO donor, DETA/NO, suggesting that a lack of NO can mimic certain aspects of hypoxia [97]. Thus, when considering the role of NO in cancer, one must account for oxygen availability as well as NOS isoform expression within the tumour microenvironment.

Nitric Oxide Signalling

NO signalling encompasses three broad categories of molecular modifications: (i) the S-nitrosylation of thiol groups; (ii) peroxynitrite ($ONOO^-$) generation, leading to nitrotyrosine formation; and (iii) the donation of electrons to transition metals such as copper, zinc and iron [21, 70]. The path by which NO mediates its effects depends on the local concentrations of NO as well as the microenvironmental context.

For example, nitrosylation and nitrotyrosine formation occur when NO concentrations are high (>1 μM) whereas interactions with transition metals, such as the iron moiety of soluble guanylyl cyclase (sGC), require relatively low (<1 μM) levels of NO [21, 70, 98, 99].

When NO concentrations exceed 1 μM, the autooxidation of NO to nitrogen dioxide (NO_2) can occur [100]. NO_2 subsequently oxidises additional NO molecules to dinitrogen trioxide (N_2O_3), which rapidly decomposes into a nitrite ion (NO_2^-) and a nitrosonium ion (NO^+). The latter (NO^+) nitrosylates electrophilic compounds and deaminates DNA [70, 101]. Another high concentration effect of NO is peroxynitrite (ONOO−) generation, a process that leads to peroxynitrous acid formation and tyrosine nitration [70, 102]. This phenomenon occurs when NO reacts with a superoxide radical (O_2^-) and is, therefore, dependent on the redox state of a cell and the activity of copper/zinc superoxide dismutase (SOD), the enzyme which catalyses the reduction of O_2^- to hydrogen peroxide (H_2O_2) [70, 103]. Furthermore, due to its dependence on O_2^- availability, endogenous scavengers such as glutathione (GSH) can regulate the rate of peroxynitrite formation [104]. Peroxynitrite is a highly reactive oxidant and a strong nitrating agent. As such, it rapidly reacts with proteins, lipids, sulphydryl groups and DNA, and is believed to be a significant mediator of damage by inflammation, ischemia/reperfusion and atherosclerosis [102, 105]. Like nitrosylation, ONOO− has been shown to activate signalling factors, such as the c-Jun-NH2-terminal kinase (JNK), that are similarly induced by hypoxia [106, 107].

When NO concentrations are relatively low, interactions with heme-containing proteins, such as sGC, predominate. SGC is a heterodimeric protein composed of two subunits (one alpha and one beta) [108]. There are at least two isoforms of each subunit (α1, α2, β1, and β2). However, the β2 subunit does not seem to contribute to a NO-sensing protein [109] and the α1β1 combination is the most ubiquitously expressed [21, 108–111]. NO binds to a protoporphyrin IX type heme moiety in sGC, severing the bond between the heme iron and its ligating histidine residue, resulting in the activation of the enzyme [112, 113]. SGC amplifies the effects of NO by catalyzing the second messenger cyclic guanosine monophosphate (cGMP) from guanosine 5-triphosphate (GTP) [114, 115].

Several proteins interact with cGMP to mediate alterations in gene expression and cell phenotype. These include the cGMP-dependent protein kinase (PKG), cGMP activated phosphodiesterases (PDEs), and cGMP-gated ion channels [108, 114]. Of these, PKG, a serine/threonine kinase that is activated following cGMP binding, is responsible for the many of the cellular effects of cGMP [108, 116]. There are two isoforms of PKG (PKG I and PKG II), however PKG I is the most ubiquitously expressed [117, 118]. Upon activation, PKG phosphorylates many intracellular targets, often resulting in alterations in gene expression. For example, PKG can inhibit the Ras/MAPK pathway by mitigating the activation of cRaf kinase and by inducing the expression of MAPK phosphatase 1 (MKP-1), an enzyme responsible for MAPK inactivation [119]. The Ras/MAPK pathway triggers the activation of the extracellular regulated kinases (ERK 1 and 2) which can activate proliferation, survival and invasion-associated genes [120]. Therefore, by promoting

MKP-1 expression, and preventing cRaf activation, PKG may play an important role in regulating cellular adaptations to changes in the microenvironment.

The levels of cyclic nucleotides within a cell are controlled by a large superfamily of enzymes known as the phosphodiesterases (PDEs) [121–123]. These enzymes catalyse the hydrolysis of the 3-phosphodiester bond of cyclic adenosine monophosphate (cAMP) and cGMP to yield AMP and GMP, respectively. There are currently 11 PDE families, distinguished by unique combinations of enzymatic characteristics and pharmacological inhibitory profiles [123]. Thus far, studies have revealed that PDEs 1, 2, 5, 6, 9, and 11 preferentially degrade cGMP, and that cGMP can bind to and increase the activities of PDE 2 and PDE 5, thereby providing a mechanism to control endogenous cGMP signalling [121, 123, 124].

NO as A Hypoxia-Mimetic

NO can activate signalling pathways and phenotypes commonly associated with hypoxia. These effects of NO are largely attributable to high concentration events such as nitrosylation. For example, nitrosylation causes the activation of Ras [125, 126]. Consequences of Ras activation include the stimulation of the MAPK and PI3K pathways, both of which modulate hypoxia induced responses [68, 127]. NO donors can also promote HIF-1 accumulation via an increase in HIF-1transcription and translation. This effect of NO on HIF-1 expression is due to the sequential activation of Ras and PI3K [128]. Finally, stresses to the cell, other than hypoxia, can also induce HIF in an NO-dependent manner. Indeed, a recent study showed that doxorubicin increases HIF-1 in breast cancer cells via a process dependent on NO production by iNOS [129]. NO has been shown to activate phenotypes, such as invasion and stemness, which are similarly induced by hypoxia. Melanoma cell invasion and lung colonization is increased in BRAF mutant cells, where PDE5A is down-regulated. The effect of PDE5A down-regulation on invasion is mediated by cGMP [130]. NO has also been shown to signal in a cGMP/PKG-dependent manner to drive Notch signalling in PDGF-induced gliomas; leading to the enhancement of cancer stem cells and increased tumourigenesis [131]. In agreement with these findings, iNOS is associated with a poor prognosis in patients with glioma and inhibition of this NOS isoform inhibits glioma growth in animal models [132].

NO can also act directly on the HIF regulatory machinery to enhance the stabilization and/or activity of this important transcription factor. High doses of GSNO and other NO donors enhance S-nitrosylation of prolyl hydroxylase, leading to HIF-1α accumulation [133, 134] and binding of HIF to DNA [135]. Furthermore, NO generated by iNOS S-Nitrosylates Cys 533 in the ODD of HIF-1, decreasing pVHL binding and enabling HIF accumulation [136]. VHL activity can also be inhibited by S-Nitrosylation [137]. NO has also been shown to directly enhance HIF activity through the nitrosylation of a cysteine residue (C800) in the C terminal activation domain of HIF-1 [138]. This nitrosylation increases the association of HIF-1 with the p300 co-activator, thereby enhancing HIF-1 transactivation

and transcriptional activity [138] Finally, NO may even enhance HIF-1 levels in hypoxia. For example, NO produced endogenously by HCT116 human colon cancer cells cultured in mild hypoxia (3 % O_2) enhanced the accumulation of HIF by promoting S-Nitrosylation of PHD2 [139]. Hence, in the right context, NO can instigate hypoxia-associated phenotypes by inducing similar signalling pathways and stabilizing HIF.

NO in the Mitigation of Hypoxia-Induced Phenotypes

The ability of NO to bind heme proteins in a manner similar to O_2 suggests that it may be able to modulate hypoxia-induced phenotypes by mimicking O_2. Indeed, NO can inhibit the hypoxic up-regulation of genes including *EPO*, *ET-1* and *VEGF* and NO donors diminish the hypoxic accumulation of HIF-1 [108, 140–145]. Accordingly, NO mitigates the hypoxic induction of phenotypes such as cellular invasiveness, metastatic potential, immune escape and resistance to therapy [10, 26, 40, 41, 146]. SNP decreases hypoxia-induced invasion and HIF-1 levels in PC3 prostate cancer and T24 bladder cancer cells [147]. Importantly, GTN and DETA/NO can also prevent the hypoxic up-regulation of B16F10 murine melanoma growth in the lung, suggesting that NO donors could be used to prevent or treat metastatic disease [21]. As mentioned above, immune escape or evasion has emerged as an important pro-tumourigenic hypoxia-induced phenotype. Hypoxia increases immune escape from innate immunity in breast and prostate cancer cells by increasing ADAM10 in a HIF1-dependent manner. ADAM10 cleaves the MHC Class I chain related molecule, MICA, so that it can be shed from the cell surface. As MICA triggers cytolytic function of immune effectors like natural killer (NK) cells, the hypoxic induction of ADAM10 allows cancer cells to better evade the immune system and survive [40]. NO donors or 8Bromo-cGMP impair the hypoxic induction of HIF, thereby decreasing immune escape. Indeed, GTN blunted the growth of DU145 prostate tumours in mice in a NK-cell dependent manner [40]. GTN can also allay the hypoxic induction of immune escape from adaptive immunity by preventing the up-regulation of PDL1, a ligand that reduces the ability of cytotoxic T lymphocytes to kill cancer cells [41]. Finally, a growing body of evidence demonstrates that NO can prevent hypoxia-induced therapy resistance. NO mimetics significantly inhibit hypoxia-induced resistance to doxorubicin and 5-flourouracil in a variety of cell types [26, 146]. In PC-3 prostate cancer cells, NO-Sulindac reduces the hypoxic signature by mitigating HIF-1 levels and this effect of NO results in a greater sensitivity to radiation [148, 149]. Collectively, these studies reveal that NO is able to regulate an array of O_2-sensitive phenotypes.

NO can also act directly on the HIF regulatory machinery to decrease the hypoxia-induced stabilization and/or activity of this important transcription factor. NO decreases PHD activity in normoxia, leading to increased HIF-1 levels but increases it in hypoxia, thereby mitigating the hypoxic induction of HIF-1 [150]. Mechanistically, NO may mimic O_2 at the Fe^{2+} catalytic site [67]. Alternatively, NO may

impede mitochondrial oxygen consumption by inhibiting cytochrome C, allowing the repurposing of oxygen toward PHDs, increasing their activity and reducing HIF levels [141].

Mechanistically, it is likely that many of the anti-tumourigenic effects of NO in hypoxia are mediated via cGMP-dependent pathways. For example, a non-hydrolysable cGMP analogue (8-BrcGMP) can inhibit uPAR expression and *in vitro* invasion through the ECM in a manner similar to NO-mimetic treatment [10] and can abrogate the hypoxic up-regulation of B16F10 murine melanoma cell metastasis [21]. It is likely that this effect of cGMP is mediated by PKG, as treatment with a selective inhibitor (KT5823) blocks the anti-tumourigenic effects of 8-BrcGMP. These findings are in agreement with several other studies demonstrating that NO inhibits the hypoxic induction of genes including *VEGF, EPO* and *ET-1* by activating sGC and increasing cGMP levels [142, 151, 152]. More recently, Murad and colleagues have shown that increasing cGMP, either by overexpressing a constitutively active sGC or by treating with PDE inhibitors, decreases glioma cell growth both *in vitro* and in murine xenograft models [108, 153]. PKG2 activity, downstream of cGMP , has similarly been shown to inhibit glioma proliferation, and studies indicate that it may also induce the differentiation of stem cell–like cells in this cancer [154]. This phenomenon may not be specific to brain cancers, as a recent study showed that by inhibiting cGMP and PKG signalling, PDE 10 A promotes cancer-associated β-catenin signalling and increases proliferation in colon cancer cells [155]. Moreover, cGMP, induced following binding of to a 67 kDa laminin receptor and/or treatment with a PDE5 inhibitor, can inhibit the growth of multiple cancer cell types, including primary multiple myelomas [156]. Finally, treatment of DU-145 prostate cancer cells with PDE inhibitors, which prevent the degradation of cGMP, reduced the hypoxic acquisition of resistance to Doxorubicin, and reversed the hypoxic induction of MICA, leading to an NK-dependent attenuation of tumour growth in mice [157]. Collectively, these findings indicate that, through the sequential activation of sGC and PKG, NO is able to mitigate the hypoxic up-regulation of phenomena such as HIF accumulation, invasion and metastatic potential.

Conclusions: Moving toward the Clinical Use of NO for the Treatment of Cancer

NO is emerging as a potential therapy for the treatment of cancers, with positive results in several clinical trials. In a recent phase II clinical trial on patients with non-small cell lung cancer (NSCLC), GTN was shown to improve the efficacy of cisplatin plus vinorelbine and radiotherapy [158]. A previous study similarly demonstrated that GTN could increase response rates and decrease time to progression in Stage IIIb/IV NSCLC patients treated with a cisplatin and vinorelbine regimen [159]. Moreover, a retrospective study showed that GTN increases the response rate in lung cancer patients treated with docetaxel and carboplatin [159]. In this latter

study, VEGF and HIF-1 levels were lower in patients treated with GTN, suggesting that NO could be mitigating the pro-tumourigenic effects of hypoxia [160]. In another phase II study, GTN significantly reduced prostate specific antigen (PSA) doubling time in patients with primary treatment failure, suggesting that this NO donor can diminish progression [161]. It is tempting to speculate that the effects of NO in these trials are due to the inhibition of a hypoxia-induced program. Based on the confounding roles of NO presented in this review, great caution must be used when interpreting results, and choosing patients for future trials. For example, it may be wise to ensure that patients have hypoxic tumours, where the ability of cells to endogenously produce NO is limited. This is particularly important, as providing more NO to well oxygenated tumours may initiate, rather than mitigate, hypoxia-associated gene signatures. Hence, as we forge forward with new treatments that target NO signalling, we must always consider the paradoxical role of NO in the regulation of hypoxia-induced phenomena, wherein it can both instigate and mitigate pro-tumourigenic phenotypes.

Acknowledgments This work was conducted with funds from the Canadian Institutes of Health Research (CIHR) awarded to LMP (MOP 89714, MOP 119589 and PLS 95381). LMP is the recipient of the Premier New Investigator Award from the CIHR and is the Sawin-Baldwin Chair in Ovarian Cancer & Dr. Anthony Noujaim Legacy Oncology Chair and the Alberta Innovates Health Solutions Translational Chair in Cancer.

Conflict of Interest Statement No conflicts of interest are disclosed

References

1. Keith B, Johnson RS, Simon MC. HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. Nat Rev Cancer. 2012;12(1):9–22.
2. Bennewith KL, Dedhar S. Targeting hypoxic tumour cells to overcome metastasis. BMC cancer. 2011;11:504.
3. Thomlinson RH, Gray LH. The histological structure of some human lung cancers and the possible implications for radiotherapy. BrJCancer. 1955;9(4):539–549.
4. Franko AJ, Sutherland RM. Rate of death of hypoxic cells in multicell spheroids. RadiatRes. 1978;76(3):561–72.
5. Durand RE, Raleigh JA. Identification of nonproliferating but viable hypoxic tumor cells in vivo. Cancer Res. 1998;58(16):3547–3550.
6. Bennewith KL, Durand RE. Quantifying transient hypoxia in human tumor xenografts by flow cytometry. Cancer Res. 2004;64(17):6183–9.
7. Lanzen J, Braun RD, Klitzman B, Brizel D, Secomb TW, Dewhirst MW. Direct demonstration of instabilities in oxygen concentrations within the extravascular compartment of an experimental tumor. Cancer Res. 2006;66(4):2219–23.
8. Brurberg KG, Thuen M, Ruud EB, Rofstad EK. Fluctuations in pO2 in irradiated human melanoma xenografts. Radiat Res. 2006;165(1):16–25.
9. Quail DF, Taylor MJ, Postovit LM. Microenvironmental regulation of cancer stem cell phenotypes. Curr Stem Cell ResTher. 2012;7(3):197–216.

10. Postovit LM, Adams MA, Lash GE, Heaton JP, Graham CH. Oxygen-mediated regulation of tumor cell invasiveness. Involvement of a nitric oxide signaling pathway. J Biol Chem. 2002;277(38):35730–7.
11. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang LP, et al. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. Nature. 2011;475(7355):226–30.
12. Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol. 2005;202(3):654–62.
13. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature. 1992;359(6398):843–5.
14. Lu X, Kang Y. Hypoxia and hypoxia-inducible factors: master regulators of metastasis. Clin Cancer Res. 2010;16(24):5928–35.
15. Wartenberg M, Ling FC, Muschen M, Klein F, Acker H, Gassmann M, et al. Regulation of the multidrug resistance transporter P-glycoprotein in multicellular tumor spheroids by hypoxia-inducible factor (HIF-1) and reactive oxygen species. FASEB J Off Publ Fed Am Soc Exp Biol. 2003;17(3):503–5.
16. Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res. 2002;62(12):3387–94.
17. Ruan K, Song G, Ouyang G. Role of hypoxia in the hallmarks of human cancer. J Cell Biochem. 2009;107(6):1053–62.
18. Mucaj V, Shay JE, Simon MC. Effects of hypoxia and HIFs on cancer metabolism. Int J Hematol. 2012;95(5):464–70.
19. Burke AJ, Sullivan FJ, Giles FJ, Glynn SA. The yin and yang of nitric oxide in cancer progression. Carcinogenesis. 2013;34(3):503–12.
20. Cheng H, Wang L, Mollica M, Re AT, Wu S, Zuo L. Nitric oxide in cancer metastasis. Cancer Lett. 2014;353(1):1–7.
21. Postovit LM, Adams MA, Lash GE, Heaton JP, Graham CH. Nitric oxide-mediated regulation of hypoxia-induced B16F10 melanoma metastasis. IntJCancer. 2004;108(1):47–53.
22. Nathan AT, Singer M. The oxygen trail: tissue oxygenation. BrMedBull. 1999;55(1):96–108.
23. Birol G, Wang S, Budzynski E, Wangsa-Wirawan ND, Linsenmeier RA. Oxygen distribution and consumption in the macaque retina. Am J Physiol Heart Circ Physiol. 2007;293(3):H1696–H704.
24. Vaupel P, Schlenger K, Knoop C, Hockel M. Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O_2 tension measurements. Cancer Res. 1991;51(12):3316–22.
25. Chaudary N, Hill RP. Hypoxia and metastasis. Clin Cancer Res. 2007;13(7):1947–9.
26. Matthews NE, Adams MA, Maxwell LR, Gofton TE, Graham CH. Nitric oxide-mediated regulation of chemosensitivity in cancer cells. J Natl Cancer Inst. 2001;93(24):1879–85.
27. Young SD, Marshall RS, Hill RP. Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci USA. 1988;85(24):9533–7.
28. Le QT, Denko NC, Giaccia AJ. Hypoxic gene expression and metastasis. Cancer Metastasis Rev. 2004;23(3–4):293–310.
29. Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, et al. Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res. 1996;56(5):941–3.
30. Brizel DM, Sibley GS, Prosnitz LR, Scher RL, Dewhirst MW. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. Int J Radiat Oncol Biol Phys. 1997;38(2):285–9.
31. Lartigau E, Randrianarivelo H, Avril MF, Margulis A, Spatz A, Eschwege F, et al. Intratumoral oxygen tension in metastatic melanoma. Melanoma Res. 1997;7(5):400–406.
32. Wenger RH. Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. FASEB J Off Publ Fed Am Soc Exp Biol. 2002;16(10):1151–62.

33. Hochachka PW, Lutz PL. Mechanism, origin, and evolution of anoxia tolerance in animals. Comp Biochem Physiol B BiochemMolBiol. 2001;130(4):435–59.
34. Marin-Hernandez A, Gallardo-Perez JC, Ralph SJ, Rodriguez-Enriquez S, Moreno-Sanchez R. HIF-1alpha modulates energy metabolism in cancer cells by inducing over-expression of specific glycolytic isoforms. Mini Rev Med Chem. 2009;9(9):1084–101.
35. Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell. 2009;15(1):35–44.
36. Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature. 2006;440(7088):1222–6.
37. Postovit LM, Abbott DE, Payne SL, Wheaton WW, Margaryan NV, Sullivan R, et al. Hypoxia/reoxygenation: a dynamic regulator of lysyl oxidase-facilitated breast cancer migration. J Cell Biochem. 2008;103(5):1369–78.
38. Finger EC, Giaccia AJ. Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. Cancer Metastasis Rev. 2010;29(2):285–93.
39. Sullivan R, Pare GC, Frederiksen LJ, Semenza GL, Graham CH. Hypoxia-induced resistance to anticancer drugs is associated with decreased senescence and requires hypoxia-inducible factor-1 activity. Mol Cancer Therapeutics. 2008;7(7):1961–73.
40. Barsoum IB, Hamilton TK, Li X, Cotechini T, Miles EA, Siemens DR, Hypoxia induces escape from innate immunity in cancer cells via increased expression of ADAM10: role of nitric oxide. Cancer Res. 2011;71(24):7433–41.
41. Barsoum IB, Smallwood CA, Siemens DR, Graham CH. A mechanism of hypoxia-mediated escape from adaptive immunity in cancer cells. Cancer Res. 2014;74(3):665–74.
42. Soeda A, Park M, Lee D, Mintz A, ndroutsellis-Theotokis A, McKay RD, et al. Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha. Oncogene. 2009;28(45):3949–59.
43. Mathieu J, Zhang Z, Zhou W, Wang AJ, Heddleston JM, Pinna CM, et al. HIF Induces Human Embryonic Stem Cell Markers in Cancer Cells. Cancer Res. 2011;71(13):4640–52.
44. Helczynska K, Kronblad A, Jogi A, Nilsson E, Beckman S, Landberg G, et al. Hypoxia promotes a dedifferentiated phenotype in ductal breast carcinoma in situ. Cancer Res. 2003;63(7):1441–4.
45. Oliveira-Costa JP, Zanetti JS, Silveira GG, Soave DF, Oliveira LR, Zorgetto VA, et al. Differential expression of HIF-1alpha in CD44 + CD24-/low breast ductal carcinomas. Diagn Pathol. 2011;6:73.
46. Currie MJ, Beardsley BE, Harris GC, Gunningham SP, Dachs GU, Dijkstra B, et al. Immunohistochemical analysis of cancer stem cell markers in invasive breast carcinoma and associated ductal carcinoma in situ: relationships with markers of tumor hypoxia and microvascularity. Hum Pathol. 2013;44(3):402–11.
47. Lock FE, McDonald PC, Lou Y, Serrano I, Chafe SC, Ostlund C, et al. Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. Oncogene. 2013;32(44):5210–9.
48. Conley SJ, Gheordunescu E, Kakarala P, Newman B, Korkaya H, Heath AN, et al. Antiangiogenic agents increase breast cancer stem cells via the generation of tumor hypoxia. Proc Natl Acad Sci USA. 2012;109(8):2784–9.
49. Louie E, Nik S, Chen JS, Schmidt M, Song B, Pacson C, et al. Identification of a stem-like cell population by exposing metastatic breast cancer cell lines to repetitive cycles of hypoxia and reoxygenation. Breast Cancer Res. 2010;12(6):R94.
50. Xing F, Okuda H, Watabe M, Kobayashi A, Pai SK, Liu W, et al. Hypoxia-induced Jagged2 promotes breast cancer metastasis and self-renewal of cancer stem-like cells. Oncogene. 2011;30(39):4075–86.
51. Schwab LP, Peacock DL, Majumdar D, Ingels JF, Jensen LC, Smith KD, et al. Hypoxia-inducible factor 1alpha promotes primary tumor growth and tumor-initiating cell activity in breast cancer. Breast Cancer Res. 2012;14(1):R6.

52. Harris AL. Hypoxia–a key regulatory factor in tumour growth. Nat Rev Cancer. 2002;2(1):38–47.
53. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 2003;3(10):721–32.
54. Semenza GL. Hypoxia-inducible factor 1 (HIF-1) pathway. SciSTKE5. 2007;2007(407):cm8.
55. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature. 2006;441(7092):437–443.
56. Postovit LM, Sullivan R, Adams MA, Graham CH. Nitric oxide signalling and cellular adaptations to changes in oxygenation. Toxicology. 2005;208(2):235–248.
57. Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. Cell. 2007;129(3):465–72.
58. Coleman ML, Ratcliffe PJ. Oxygen sensing and hypoxia-induced responses. Essays Biochem. 2007;43:1–15.
59. Brahimi-Horn C, Pouyssegur J. The role of the hypoxia-inducible factor in tumor metabolism growth and invasion. Bull du Cancer. 2006;93(8):E73–80.
60. Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature. 1990;347(6295):768–0.
61. de Graaf JC Banga JD Moncada S Palmer RM de Groot PG Sixma JJ. Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. Circulation. 1992;85(6):2284–90.
62. Garg UC, Hassid A. Nitric oxide-generating vasodilators inhibit mitogenesis and proliferation of BALB/C 3T3 fibroblasts by a cyclic GMP-independent mechanism. Biochem Biophys Res Commun. 1990;171(1):474–9.
63. Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature. 1988;333(6174):664–6.
64. Nakaki T, Kato R. Nitric oxide in vascular remodeling. Jpn Heart J. 1996;37(4):431–45.
65. Fukuto JM. Chemistry of nitric oxide: biologically relevant aspects. Adv Pharmacol (San Diego, Calif). 1995;34:1–15.
66. Kerwin JF, Jr., Lancaster JR, Jr., Feldman PL. Nitric oxide: a new paradigm for second messengers. J Med Chem. 1995;38(22):4343–62.
67. Berchner-Pfannschmidt U, Tug S, Kirsch M, Fandrey J. Oxygen-sensing under the influence of nitric oxide. Cell Signal. 2010;22(3):349–56.
68. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001;357(Pt 3):593–615.
69. Griffith OW, Stuehr DJ. Nitric oxide synthases: properties and catalytic mechanism. Annu Rev Physiol. 1995;57:707–36.
70. Hanafy KA, Krumenacker JS, Murad F. NO, nitrotyrosine, and cyclic GMP in signal transduction. Med Sci Monit Int J Exp Clin Res. 2001;7(4):801–19.
71. Ho JJ, Man HS, Marsden PA. Nitric oxide signaling in hypoxia. J Mol Med (Berlin, Germany). 2012;90(3):217–31.
72. Kiechle FL, Malinski T. Nitric oxide. Biochemistry, pathophysiology, and detection. Am J Clin Pathol. 1993;100(5):567–75.
73. Malinski T, Bailey F, Zhang ZG, Chopp M. Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. J Cereb Blood Flow Metab Off J Int Soc Cereb Blood Flow Metab. 1993;13(3):355–8.
74. Stuehr DJ. Mammalian nitric oxide synthases. Biochimica Et biophysica Acta. 1999;1411(2–3):217–30.
75. Leiper J, Vallance P. Biological significance of endogenous methylarginines that inhibit nitric oxide synthases. Cardiovasc Res. 1999;43(3):542–348.
76. Koide M, Kawahara Y, Tsuda T, Yokoyama M. Cytokine-induced expression of an inducible type of nitric oxide synthase gene in cultured vascular smooth muscle cells. FEBS Lett. 1993;318(3):213–7.
77. Ziegler T, Silacci P, Harrison VJ, Hayoz D. Nitric oxide synthase expression in endothelial cells exposed to mechanical forces. Hypertension. 1998;32(2):351–5.

78. Inoue T, Kwon AH, Oda M, Kaibori M, Kamiyama Y, Nishizawa M, et al. Hypoxia and heat inhibit inducible nitric oxide synthase gene expression by different mechanisms in rat hepatocytes. Hepatology. 2000;32(5):1037–44.
79. Kashiwagi K, Iizuka Y, Mochizuki S, Tsumamoto Y, Mishima HK, Araie M, et al. Differences in nitric oxide production: a comparison of retinal ganglion cells and retinal glial cells cultured under hypoxic conditions. Brain Res Mol Brain Res. 2003;112(1–2):126–34.
80. Palmer LA, Semenza GL, Stoler MH, Johns RA. Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. Am J Physiol. 1998;274(2 Pt 1):L212–9.
81. Zulueta JJ, Sawhney R, Kayyali U, Fogel M, Donaldson C, Huang H, et al. Modulation of inducible nitric oxide synthase by hypoxia in pulmonary artery endothelial cells. Am J Respir Cell Mol Biol. 2002;26(1):22–230.
82. McQuillan LP, Leung GK, Marsden PA, Kostyk SK, Kourembanas S. Hypoxia inhibits expression of eNOS via transcriptional and posttranscriptional mechanisms. The American journal of physiology. 1994;267(5 Pt 2):H1921–927.
83. Branco-Price C, Zhang N, Schnelle M, Evans C, Katschinski DM, Liao D, et al. Endothelial cell HIF-1alpha and HIF-2alpha differentially regulate metastatic success. Cancer Cell. 2012;21(1):52–65.
84. Axelson H, Fredlund E, Ovenberger M, Landberg G, Pahlman S. Hypoxia-induced dedifferentiation of tumor cells–a mechanism behind heterogeneity and aggressiveness of solid tumors. Semin Cell Dev Biol. 2005;16(4–5):554–63.
85. Ward ME, Toporsian M, Scott JA, Teoh H, Govindaraju V, Quan A, et al. Hypoxia induces a functionally significant and translationally efficient neuronal NO synthase mRNA variant. J Clin Investig. 2005;115(11):3128–39.
86. Ostergaard L, Stankevicius E, Andersen MR, Eskildsen-Helmond Y, Ledet T, Mulvany MJ, et al. Diminished NO release in chronic hypoxic human endothelial cells. Am J Physiol Heart Circ Physiol. 2007;293(5):H2894–903.
87. Fish JE, Matouk CC, Yeboah E, Bevan SC, Khan M, Patil K, et al. Hypoxia-inducible expression of a natural cis-antisense transcript inhibits endothelial nitric-oxide synthase. J Biol Chem. 2007;282(21):15652–66.
88. Fish JE, Yan MS, Matouk CC, St Bernard R, Ho JJ, Gavryushova A, et al. Hypoxic repression of endothelial nitric-oxide synthase transcription is coupled with eviction of promoter histones. J Biol Chem. 2010;285(2):810–26.
89. Aneman A, Backman V, Snygg J, von Bothmer C, Fandriks L, Pettersson A. Accumulation of an endogenous inhibitor of nitric oxide synthase during graded hemorrhagic shock. Circ Shock. 1994;44(3):111–4.
90. McCormick CC, Li WP, Calero M. Oxygen tension limits nitric oxide synthesis by activated macrophages. Biochem J. 2000;350(Pt 3):709–16.
91. Whorton AR, Simonds DB, Piantadosi CA. Regulation of nitric oxide synthesis by oxygen in vascular endothelial cells. Am J Physiol. 1997;272(6 Pt 1):L1161–6.
92. Kantrow SP, Huang YC, Whorton AR, Grayck EN, Knight JM, Millington DS, et al. Hypoxia inhibits nitric oxide synthesis in isolated rabbit lung. Am J Physiol. 1997;272(6 Pt 1):L1167–73.
93. Adachi T, Shinomura T, Nakao S, Kurata J, Murakawa M, Shichino T, et al. Chronic treatment with nitric oxide synthase (NOS) inhibitor profoundly reduces cerebellar NOS activity and cyclic guanosine monophosphate but does not modify minimum alveolar anesthetic concentration. Anesth Analg. 1995;81(4):862–5.
94. Taylor CT, Lisco SJ, Awtrey CS, Colgan SP. Hypoxia inhibits cyclic nucleotide-stimulated epithelial ion transport: role for nucleotide cyclases as oxygen sensors. J Pharmacol Exp Therapeutics. 1998;284(2):568–75.
95. Faller DV. Endothelial cell responses to hypoxic stress. Clin Exp Pharmacol Physiol. 1999;26(1):74–84.
96. Tharaux PL, Chatziantoniou C, Casellas D, Fouassier L, Ardaillou R, Dussaule JC. Vascular endothelin-1 gene expression and synthesis and effect on renal type I collagen synthesis and nephroangiosclerosis during nitric oxide synthase inhibition in rats. Circulation. 1999;99(16):2185–91.

97. Cattaneo MG, Cappellini E, Benfante R, Ragni M, Omodeo-Salè F, Nisoli E, et al. Chronic deficiency of nitric oxide affects hypoxia inducible Factor-1a (HIF-1a) stability and migration in human endothelial cells. PloS One. 2011;6(12):e29680.
98. Bellamy TC, Garthwaite J. Sub-second kinetics of the nitric oxide receptor, soluble guanylyl cyclase, in intact cerebellar cells. J Biol Chem. 2001;276(6):4287–92.
99. Kalra D, Baumgarten G, Dibbs Z, Seta Y, Sivasubramanian N, Mann DL. Nitric oxide provokes tumor necrosis factor-alpha expression in adult feline myocardium through a cGMP-dependent pathway. Circulation. 2000;102(11):1302–07.
100. Ford PC, Wink DA, Stanbury DM. Autoxidation kinetics of aqueous nitric oxide. FEBS Lett. 1993;326(1–3):1–3.
101. Martínez-Ruiz A, Lamas S. S-nitrosylation: a potential new paradigm in signal transduction. Cardiovasc Res. 2004;62(1):43–52.
102. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996;271(5 Pt 1):C1424–37.
103. Urushitani M, Shimohama S. The role of nitric oxide in amyotrophic lateral sclerosis. Amyotrophic lateral sclerosis and other motor neuron disorders: official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases. 2001;2(2):71–81.
104. Hendrix MJ, Seftor EA, Meltzer PS, Gardner LM, Hess AR, Kirschmann DA, et al. Expression and functional significance of VE-cadherin in aggressive human melanoma cells: role in vasculogenic mimicry. Proc Natl Acad Sci USA. 2001;98(14):8018–23.
105. Szabo C. Multiple pathways of peroxynitrite cytotoxicity. Toxicol Lett. 2003;140–141:105–12.
106. Clancy R, Rediske J, Koehne C, Stoyanovsky D, Amin A, Attur M, et al. Activation of stress-activated protein kinase in osteoarthritic cartilage: evidence for nitric oxide dependence. Osteoarthr Cartil/OARS, Osteoarthr Res Soc. 2001;9(4):294–9.
107. Scott PH, Paul A, Belham CM, Peacock AJ, Wadsworth RM, Gould GW, et al. Hypoxic stimulation of the stress-activated protein kinases in pulmonary artery fibroblasts. Am J Respir Crit Care Med. 1998;158(3):958–62.
108. Bian K, Murad F. sGC-cGMP signaling: Target for anticancer therapy. Adv Exp Med Biol. 2014;814:5–13.
109. Russwurm M, Behrends S, Harteneck C, Koesling D. Functional properties of a naturally occurring isoform of soluble guanylyl cyclase. Biochem J. 1998;335 (Pt 1):125–30.
110. Hobbs AJ. Soluble guanylate cyclase: the forgotten sibling. Trend Pharmacol Sci. 1997;18(12):484–91.
111. Koesling D. Studying the structure and regulation of soluble guanylyl cyclase. Methods (San Diego, Calif). 1999;19(4):485–93.
112. Denninger JW, Marletta MA. Guanylate cyclase and the NO/cGMP signaling pathway. Biochimica Et Biophysica Acta. 1999;1411(2–3):334–50.
113. Zhao Y, Brandish PE, Ballou DP, Marletta MA. A molecular basis for nitric oxide sensing by soluble guanylate cyclase. Proc Natl Acad Sci USA. 1999;96(26):14753–8.
114. Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, et al. Guanylyl cyclases and signaling by cyclic GMP. Pharmacol Rev. 2000;52(3):375–414.
115. Nakane M, Murad F. Cloning of guanylyl cyclase isoforms. Adv Pharmacol (San Diego, Calif). 1994;26:7–18.
116. Lincoln TM, Dey N, Sellak H. Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. J Appl Physiol (Bethesda, Md: 1985). 2001;91(3):1421–30.
117. Lohmann SM, Vaandrager AB, Smolenski A, Walter U, De Jonge HR. Distinct and specific functions of cGMP-dependent protein kinases. Trend Biochem Sci. 1997;22(8):307–12.
118. Tsukamoto Y, Kuwabara K, Hirota S, Ikeda J, Stern D, Yanagi H, et al. 150-kD oxygen-regulated protein is expressed in human atherosclerotic plaques and allows mononuclear phagocytes to withstand cellular stress on exposure to hypoxia and modified low density lipoprotein. J Clin Investig. 1996;98(8):1930–41.
119. Suhasini M, Li H, Lohmann SM, Boss GR, Pilz RB. Cyclic-GMP-dependent protein kinase inhibits the Ras/Mitogen-activated protein kinase pathway. Mol Cell Biol. 1998;18(12):6983–94.

120. Deschenes-Simard X, Kottakis F, Meloche S, Ferbeyre G. ERKs in cancer: friends or foes? Cancer Res. 2014;74(2):412–9.
121. Maurice DH, Palmer D, Tilley DG, Dunkerley HA, Netherton SJ, Raymond DR, et al. Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. Mol Pharmacol. 2003;64(3):533–46.
122. Soderling SH, Beavo JA. Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. Curr Opin Cell Biol. 2000;12(2):174–9.
123. Maurice DH, Ke H, Ahmad F, Wang Y, Chung J, Manganiello VC. Advances in targeting cyclic nucleotide phosphodiesterases. Nat Rev. 2014;13(4):290–314.
124. Conti M, Jin SL. The molecular biology of cyclic nucleotide phosphodiesterases. Prog Nucleic Acid Res Mol Biol. 1999;63:1–38.
125. Lander HM, Ogiste JS, Pearce SF, Levi R, Novogrodsky A. Nitric oxide-stimulated guanine nucleotide exchange on p21ras. J Biol Chem. 1995;270(13):7017–20.
126. Williams JG, Pappu K, Campbell SL. Structural and biochemical studies of p21Ras S-nitrosylation and nitric oxide-mediated guanine nucleotide exchange. Proc Natl Acad Sci U S A. 2003;100(11):6376–81.
127. Alvarez-Tejado M, Naranjo-Suarez S, Jimenez C, Carrera AC, Landazuri MO, del Peso L. Hypoxia induces the activation of the phosphatidylinositol 3-kinase/Akt cell survival pathway in PC12 cells: protective role in apoptosis. J Biol Chem. 2001;276(25):22368–74.
128. Kasuno K, Takabuchi S, Fukuda K, Kizaka-Kondoh S, Yodoi J, Adachi T, et al. Nitric oxide induces hypoxia-inducible factor 1 activation that is dependent on MAPK and phosphatidylinositol 3-kinase signaling. J Biol Chem. 2004;279(4):2550–8.
129. Sun J, Liu S-z, Lin Y, Cao X-p, Liu J-m. TGF-β promotes glioma cell growth via activating Nodal expression through Smad and ERK1/2 pathways. Biochem Biophys Res Commun. 2014;443(3):1066–72.
130. Arozarena I, Bischof H, Gilby D, Belloni B, Dummer R, Wellbrock C. In melanoma, beta-catenin is a suppressor of invasion. Oncogene. 2011;30(45):4531–43.
131. Charles N, Ozawa T, Squatrito M, Bleau AM, Brennan CW, Hambardzumyan D, et al. Perivascular nitric oxide activates notch signaling and promotes stem-like character in PDGF-induced glioma cells. Cell Stem Cell. 2010;6(2):141–52.
132. Eyler CE, Wu Q, Yan K, MacSwords JM, Chandler-Militello D, Misuraca KL, et al. Glioma stem cell proliferation and tumor growth are promoted by nitric oxide synthase-2. Cell. 2011;146(1):53–66.
133. Sumbayev VV, Budde A, Zhou J, Brune B. HIF-1 alpha protein as a target for S-nitrosation. FEBS Lett. 2003;535(1–3):106–12.
134. Metzen E, Zhou J, Jelkmann W, Fandrey J, Brune B. Nitric oxide impairs normoxic degradation of HIF-1alpha by inhibition of prolyl hydroxylases. Mol Biol Cell. 2003;14(8):3470–81.
135. Palmer LA, Gaston B, Johns RA. Normoxic stabilization of hypoxia-inducible factor-1 expression and activity: redox-dependent effect of nitrogen oxides. Mol Pharmacol. 2000;58(6):1197–203.
136. Li F, Sonveaux P, Rabbani ZN, Liu S, Yan B, Huang Q, et al. Regulation of HIF-1alpha stability through S-nitrosylation. Mol Cell. 2007;26(1):63–74.
137. Palmer LA, Doctor A, Chhabra P, Sheram ML, Laubach VE, Karlinsey MZ, et al. S-nitrosothiols signal hypoxia-mimetic vascular pathology. J Clin Investig. 2007;117(9):2592–601.
138. Yasinska IM, Sumbayev VV. S-nitrosation of Cys-800 of HIF-1alpha protein activates its interaction with p300 and stimulates its transcriptional activity. FEBS Lett. 2003;549(1–3):105–9.
139. Chowdhury R, Godoy LC, Thiantanawat A, Trudel LJ, Deen WM, Wogan GN. Nitric oxide produced endogenously is responsible for hypoxia-induced HIF-1alpha stabilization in colon carcinoma cells. Chem Res Toxicol. 2012;25(10):2194–2202.
140. Ghiso N, Rohan RM, Amano S, Garland R, Adamis AP. Suppression of hypoxia-associated vascular endothelial growth factor gene expression by nitric oxide via cGMP. Investig Ophthalmol Vis Sci. 1999;40(6):1033–9.

141. Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. Science. 2003;302(5652):1975–1878.
142. Huang LE, Willmore WG, Gu J, Goldberg MA, Bunn HF. Inhibition of hypoxia-inducible factor 1 activation by carbon monoxide and nitric oxide. Implications for oxygen sensing and signaling. J Biol Chem. 1999;274(13):9038–44.
143. Junbao D, Jianfeng J, Wanzhen L, Bin Z, Heping Z. Nitric oxide impacts endothelin-1 gene expression in intrapulmonary arteries of chronically hypoxic rats. Angiology. 1999;50(6):479–485.
144. Kourembanas S, McQuillan LP, Leung GK, Faller DV. Nitric oxide regulates the expression of vasoconstrictors and growth factors by vascular endothelium under both normoxia and hypoxia. J Clin Investig. 1993;92(1):99–104.
145. Sogawa K, Numayama-Tsuruta K, Ema M, Abe M, Abe H, Fujii-Kuriyama Y. Inhibition of hypoxia-inducible factor 1 activity by nitric oxide donors in hypoxia. Proc Natl Acad Sci USA. 1998;95(13):7368–73.
146. Frederiksen LJ, Siemens DR, Heaton JP, Maxwell LR, Adams MA, Graham CH. Hypoxia induced resistance to doxorubicin in prostate cancer cells is inhibited by low concentrations of glyceryl trinitrate. J Urol. 2003;170(3):1003–1007.
147. Wang F, Zhang R, Xia T, Hsu E, Cai Y, Gu Z, et al. Inhibitory effects of nitric oxide on invasion of human cancer cells. Cancer Lett. 2007;257(2):274–82.
148. Stewart GD, Nanda J, Katz E, Bowman KJ, Christie JG, Brown DJ, et al. DNA strand breaks and hypoxia response inhibition mediate the radiosensitisation effect of nitric oxide donors on prostate cancer under varying oxygen conditions. Biochem Pharmacol. 2011;81(2):203–10.
149. Stewart GD, Nanda J, Brown DJ, Riddick AC, Ross JA, Habib FK. NO-sulindac inhibits the hypoxia response of PC-3 prostate cancer cells via the Akt signalling pathway. Int J Cancer. 2009;124(1):223–32.
150. Berchner-Pfannschmidt U, Yamac H, Trinidad B, Fandrey J. Nitric oxide modulates oxygen sensing by hypoxia-inducible factor 1-dependent induction of prolyl hydroxylase 2. J Biol Chem. 2007;282(3):1788–96.
151. Liu Y, Christou H, Morita T, Laughner E, Semenza GL, Kourembanas S. Carbon monoxide and nitric oxide suppress the hypoxic induction of vascular endothelial growth factor gene via the 5' enhancer. J Biol Chem. 1998;273(24):15257–62.
152. Mitsutomi N, Akashi C, Odagiri J, Matsumura Y. Effects of endogenous and exogenous nitric oxide on endothelin-1 production in cultured vascular endothelial cells. Eur J Pharmacol. 1999;364(1):65–73.
153. Zhu CS, Pinsky PF, Kramer BS, Prorok PC, Purdue MP, Berg CD, et al. The prostate, lung, colorectal, and ovarian cancer screening trial and its associated research resource. J Natl Cancer Inst. 2013;105(22):1684–93.
154. Swartling FJ, Ferletta M, Kastemar M, Weiss WA, Westermark B. Cyclic GMP-dependent protein kinase II inhibits cell proliferation, Sox9 expression and Akt phosphorylation in human glioma cell lines. Oncogene. 2009;28(35):3121–31.
155. Li N, Lee K, Xi Y, Zhu B, Gary BD, Ramirez-Alcantara V, et al. Phosphodiesterase 10 A: a novel target for selective inhibition of colon tumor cell growth and beta-catenin-dependent TCF transcriptional activity. Oncogene. 2014. doi:10.1038/onc.2014.94. [Epub ahead of print]
156. Kumazoe M, Sugihara K, Tsukamoto S, Huang Y, Tsurudome Y, Suzuki T, et al. 67-kDa laminin receptor increases cGMP to induce cancer-selective apoptosis. J Clin Investig. 2013;123(2):787–99.
157. Hamilton TK, Hu N, Kolomitro K, Bell EN, Maurice DH, Graham CH, et al. Potential therapeutic applications of phosphodiesterase inhibition in prostate cancer. World J Urol. 2013;31(2):325–30.
158. Arrieta O, Blake M, de la Mata-Moya MD, Corona F, Turcott J, Orta D, et al. Phase II study. Concurrent chemotherapy and radiotherapy with nitroglycerin in locally advanced non-small cell lung cancer. Radiother Oncol J Eur Soc Therapeutic Radiol Oncoly. 2014;111(2):311–5.

159. Yasuda H, Yamaya M, Nakayama K, Sasaki T, Ebihara S, Kanda A, et al. Randomized phase II trial comparing nitroglycerin plus vinorelbine and cisplatin with vinorelbine and cisplatin alone in previously untreated stage IIIB/IV non-small-cell lung cancer. J Clin Oncol Off J Am Soc Clin Oncol. 2006;24(4):688–94.
160. Yasuda H, Nakayama K, Watanabe M, Suzuki S, Fuji H, Okinaga S, et al. Nitroglycerin treatment may enhance chemosensitivity to docetaxel and carboplatin in patients with lung adenocarcinoma. Clin Cancer Res. 2006;12(22):6748–57.
161. Siemens DR, Heaton JP, Adams MA, Kawakami J, Graham CH. Phase II study of nitric oxide donor for men with increasing prostate-specific antigen level after surgery or radiotherapy for prostate cancer. Urology. 2009;74(4):878–83.

Part II
S-Nitrosylation and Cancer

Chapter 6
Impacts of S-Nitrosylation in Cancer

Tysha N. Medeiros, Dana M. Jarigese, Melissa A. Edwards and Mark A. Brown

Abstract From its diminutive size and ephemeral nature to its lipophilicity and ability to rapidly diffuse across cell membranes, nitric oxide (NO) is a highly effective signal molecule. In this capacity, NO regulates the activity of a wide range of target proteins through its central function in S-nitrosylation. In the past decade, S-nitrosylation signaling events have garnered an increasing amount of interest with regard to their impacts on malignant neoplasms. In this chapter, we review both the pathogenic and prospective therapeutic roles of S-nitrosylation in cancer biology.

Keywords S-nitrosylation · Nitric oxide · Cancer · Post-translational modifications

Abbreviations

AGT	O^6-alkylguanine-DNA alkyltransferase
EGFR	Epithelial growth factor receptor
eNOS	Endothelial Nitric Oxide Synthase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

M. A. Brown (✉) · M. A. Edwards
Cell and Molecular Biology Program, Colorado State University, Fort Collins, CO, USA

T. N. Medeiros
Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, USA

D. M. Jarigese
Department of Animal Sciences, Colorado State University, Fort Collins, CO, USA

M. A. Brown
Department of Clinical Sciences, Colorado State University, Fort Collins, CO, USA
e-mail: M.Brown@colostate.edu

Department of Ethnic Studies, Colorado State University, Fort Collins, CO, USA

Colorado School of Public Health, Fort Collins, CO, USA

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_6

HIF-1	Hypoxia-inducible factor 1
iNOS	Inducible Nitric Oxide Synthase
NOS	Nitric Oxide Synthase
nNOS	Neuronal Nitric Oxide Synthase
SNO	S-nitrosothiol

Introduction

NO-mediated signal transduction is central to many cell regulatory pathways [1, 2] and is essential in some immunological response factors such as the superoxide-mediated cytotoxicity of leukocytes [3–6]. The physiological impacts of NO signaling vary contingent upon the rate and concentration at which it is delivered [6–9]. For example, although NO-mediated cytotoxicity is induced by extreme, often precipitous levels of NO [6], platelet accretion is controlled by localization of relatively low concentrations of nitric oxide [8, 9]. NO signaling is responsible for the regulation of several immunological pathways targeting cancer [10–12] and has also been implicated in the initiation of apoptosis in tumors by compromising the integrity of the mitochondrial membrane resulting in the discharge of cytochrome c oxidase [13, 14].

Three principal isoforms of NO synthase (NOS) are responsible for the synthesis of nitric oxide under a broad range of conditions. Acting on a common substrate, L-arginine, these isoforms include neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The nNOS and eNOS isoforms are calcium-dependent and provide signaling within and outside the central nervous system, respectively. The iNOS isoform is calcium-independent and is capable of rapidly synthesizing high concentrations of NO in response to immunological stimuli [15, 16].

An important mechanism of NO-mediated signaling occurs via post-translational modifications to key regulatory proteins. The reversible S-nitrosylation reaction (Fig. 6.1) involves the attachment of a nitroso moiety to the reactive thiol of cysteine residues thereby producing S-nitrosothiol (SNO) [17–19]. To date, a number

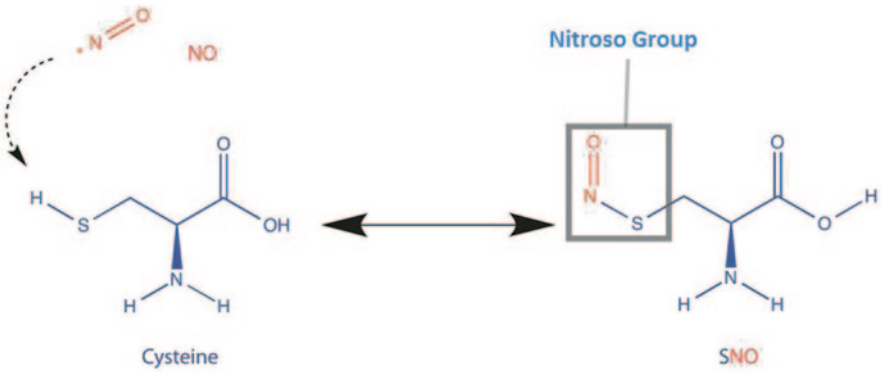


Fig. 6.1 Overview of the S-nitrosylation reaction

of specific protein targets have been identified by which S-nitrosylation is known to regulate processes involved in transcription, DNA repair, and apoptosis [20–22]. Unfortunately, aberrant S-nitrosylation is also known to occur and has been implicated in tumorigenic processes. The S-nitrosylation event is dependent upon the redox state at the site of the reaction. Selectivity for the sulfhydryl group of thiols is regulated by protein-protein interactions and co-localization with NOS [23]. Cellular SNO concentration is governed by denitrosylases such as GSNO reductase [24], thioredoxin [25], and xanthine oxidoreductase [26].

S-Nitrosylation and Cancer

All three NOS isoforms have been shown to be upregulated in a wide range of cancers [27]. For example, in breast carcinomas, interaction between STAT3 and the nuclear epithelial growth factor receptor (EGFR) activates transcription of iNOS [28]. In turn, activation of iNOS in tumor cells is known to induce upregulation of VEGF resulting in angiogenesis and tumor growth [29, 30]. Increasing evidence now implicates S-nitrosylation of VEGF transcriptional regulators in the induction of angiogenesis in tumor tissues (bentham).

Protein nitrosylation often triggers conformational changes (Fig. 6.2) that either enhance or inhibit protein function depending on the target molecule. In some cases, as with TRX-1, nitrosylation and denitrosylation allow proteins to switch between two independent functions. S-nitrosylation of a growing list of proteins has been

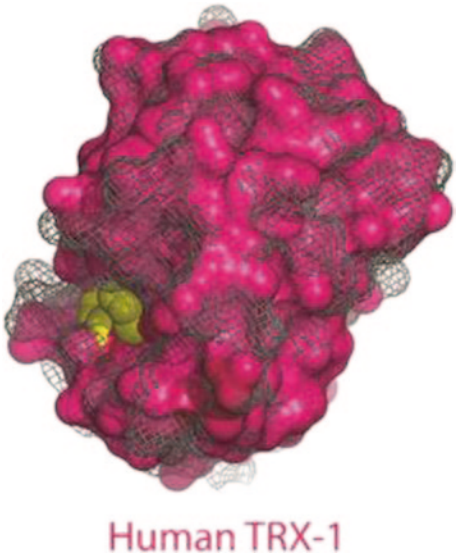


Fig. 6.2 The non-modified TRX-1 protein (shown in *gray mesh*) has been aligned with its nitrosylated counterpart to provide a representation of nitrosylation-induced conformational changes. Nitrosylated cysteine residues are shown in yellow space fill

Table 6.1 S-Nitrosylated Proteins Involved in the progression of cancer

Protein	Impacts of S-Nitrosylation	Reference(s)
HIF-1α	Stabilization of HIF-1α leading to radiotherapy resistance and promotion of angiogenesis	[31–35]
Bcl-2	Stabilization of Bcl-2 leading to inhibition of apoptosis	[36]
AGT	Degradation of AGT leading to increased tumor incidence	[37]
PTEN	Inhibition of PTEN phosphatase activity leading to promotion of angiogenesis	[38]
p53	Loss of p53 function leading to inhibition of apoptosis	[39]
MKP-7	Promotion of angiogenesis	[40]
C-Src	Progression of tumor invasion and metastasis	[41]

linked to tumor progression and metastasis. A list of S-nitrosylated proteins and their role in the progression of cancer is provided in Table 6.1 and detailed below.

Hypoxia-inducible factor 1 (HIF-1) governs the transcription of hypoxia-responsive genes including key regulators of angiogenesis, cell cycle, and apoptosis [42]. The heterodimeric complex of HIF-1 contains two subunits including HIF-1α and HIF-1β. Under normal circumstances, HIF-1α is destroyed via the ubiquitin-proteasomal degradation pathway. During hypoxia, however, this degradation is inhibited [31–33]. Likewise, S-nitrosylation at C533 in the degradation domain of HIF-1α is known to inhibit its degradation. This, in turn, has been shown to promote both angiogenesis as well as resistance to radiotherapy [34, 35].

Bcl-2 is well documented for its role in inhibiting apoptosis by interfering with pathways that result in the release of mitochondrial cytochrome c oxidase [43]. Nitrosylation of Bcl-2 at C158 and C229 stabilizes the protein and prevents its ubiquitin-proteasomal pathway-mediated degradation [36]. This allows the anti-apoptotic protein to accumulate in tumor cells where it facilitates evasion of apoptotic signals. The nitrosylation of Bcl-2 is particularly problematic for cancer patients undergoing treatment with cytotoxic agents such as Cisplatin which are designed, in part, to target Bcl-2 for destruction [44].

O^6-alkylguanine-DNA alkyltransferase (AGT) is a DNA repair enzyme which is central to the prevention of tumorigenesis caused by alkylating carcinogens. S-nitrosylation of AGT at C145 has been shown to destabilize the protein and induce its ubiquitin-proteasomal pathway-mediated degradation [37]. This, in turn, leads to a loss of critical repair mechanisms associated with alkylating carcinogens, thereby driving a gradual increase in genetic instability and ultimately, tumorigenesis.

The tumor suppressor PTEN is a protein phosphatase which acts on a number of substrates to inhibit the angiogenic pathways induced by PI3K/Akt signaling [45]. S-nitrosylation of PTEN has been shown to induce structural changes which result in a loss of phosphatase activity. This loss of PI3K/Akt signaling inhibition leads to enhanced angiogenesis and rapid growth in tumor tissues [38].

Other proteins which are known to be modified by S-nitrosylation resulting in the progression of cancer include p53, MKP-7, and C-Src. MKP-7 dephosphorylation is known to inactivate JNK3, thereby inhibiting endothelial cell migration.

Table 6.2 S-Nitrosylated proteins involved in the inhibition of cancer

Protein	Impacts of S-Nitrosylation	Reference(s)
FasR	Induction of apoptosis and increased sensitivity to chemotherapeutics	[47]
Hdm2	Induction of apoptosis	[46]
GAPDH	Increased sensitivity to chemotherapeutics	[48, 49]
NF-kappaB	Reduction of cell proliferation and induction of apoptosis	[50–54]

However, S-nitrosylation of MKP-7 has been shown to eliminate its phosphatase activity resulting in prolonged JNK3-mediated enhancement of angiogenesis [40]. S-nitrosylation of p53 results in catastrophic structural changes and consequent loss of tumor suppression activity [39]. Finally, S-nitrosylation of C-Src significantly enhances its kinase activity and corresponding impacts on tumor metastasis [41].

While the S-nitrosylation of proteins is often a factor in the progression of tumors, as illustrated above, there are also cases in which nitrosylation functions to inhibit the development and progression of cancer. For example, the nitrosylation of Hdm2 has been shown to prevent its inactivation of p53 [46]. A list of S-nitrosylated proteins and their role in the inhibition of cancer is provided in Table 6.2 and detailed below.

Interaction of the Fas ligand with its receptor, FasR, leads to the induction of apoptosis [55]. This response represents one of two primary pathways for initiating programmed cell death. Unfortunately, this event is often circumvented in a wide range of cancers. S-nitrosylation of FasR at C304, however, compensates for such Fas resistance by escalating the process of Fas accumulation at the plasma membrane and facilitating the formation of the death-inducing signaling complex [47].

Hdm2 is known to be a key regulator of p53. Hdm2 binding of p53 facilitates both the inhibition and ubiquitination of the tumor suppressor [56]. Ubiquitination of p53 mediates destabilization and proteasomal degradation. However, S-nitrosylation of Hdm2 interferes with the ability of Hdm2 to bind p53, thereby preventing Hdm2-faciliated inhibition and ubiquitination of p53 [46].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is classically recognized for its role in glycolysis. However, it has more recently been shown to play a distinct signaling role in the process of apoptosis. Under conditions of cell stress, apoptotic initiation induces S-nitrosylation of GAPDH at C150 [57]. This induces recruitment, binding, and stabilization of the E3 ubiquitin ligase, Siah1. Subsequent nuclear translocation of the GAPDH-Siah1 complex is mediated via the nuclear localization signal of Siah1. In the nucleus, Siah1 ubiquitination of nuclear proteins induces degradation, thereby advancing the apoptotic cascade [48, 49].

Misregulation of the transcriptional regulator NF-kappaB is involved in a broad range of cancers. The central role of this protein in the regulation of key pathways related to cell proliferation and survival has made it a common target for prospective therapeutics. S-nitrosylation of NF-kappaB has been shown to reduce its DNA binding potential and thereby inhibit activation of its target genes [50–52].

The list of pro- and anti-cancer protein nitrosylation events described in this section represents the few about which we have the clearest understanding. However, this list is far from complete. There are new nitrosylation events discovered on a regular basis and many of these have a role in cancer. Among the targets are proteins such as MKP-1 [58], dynamin [59, 60], Caveolin-1 [61], and β-catenin [62] which, when nitrosylated, appear to have roles in the progression of cancer. Others, such as HDAC2, SIRT1, and DNA-PK, which are nitrosylated as part of the GAPDH nitrosylation cascade, likely play a role in tumor suppression [48]. A more complete understanding of nitrosylation events involved in cancer and their role in the progression or inhibition of tumors will likely lead to new avenues in the clinical management of cancer.

Conclusions

The capacity of cancer cells to cultivate increasing resistance to cytotoxic therapeutics has been a key challenge in the clinical management of tumors. This is further complicated by the dose-limiting toxicity associated with such therapeutics. Nitric oxide has emerged as a potential adjuvant with an ability to sensitize tumors to traditional therapies [63]. However, conflicting reports continue to point to the capacity of NO to actually enhance the development and progression of cancer. An evaluation of the biological pathways impacted by NO, the levels and localization of NO required to elicit those impacts, and an understanding of the sensitivity of these attributes to timing is necessary to therapeutically direct the signaling potential of NO.

The role of S-nitrosylation in the signaling of NO has garnered increasing interest as many targets of this post-translational modification are involved in critical pathways of the cell. Of particular interest is a growing list of proteins whose impacts on cancer biology are modified as a result of S-nitrosylation. In some cases, S-nitrosylation appears to serve as a regulatory mechanism to inhibit cancer. However, as with all signal events, S-nitrosylation can be manipulated in tumorigenic processes toward the misregulation of proteins which are critical in the governance of cell proliferation and survival. Whether involved in the progression or inhibition of cancer, an understanding of the signal capacities of S-nitrosylation can lead to a powerful new approach to enhancing anti-cancer mechanisms while inhibiting misregulated pathways.

Acknowledgements MAB thankfully acknowledges support from the National Science Foundation (1060548), the CVMBS College Council, and the Flint Animal Cancer Center.

Conflicts of Interest The authors declare no conflicts of interest.

References

1. Osorio J, Recchia F. The role of nitric oxide in metabolism regulation: from basic sciences to the clinical setting. Intensiv Care Med. 2000;26(9):1395–98.
2. Moncada S. Nitric oxide: discovery and Impact on clinical medicine. J Royal Soc Med. 1999;92(4):164–9.
3. Umansky V, Shirrmacher V. Nitric oxide-induced apoptosis in Tumor Cells. Adv Cancer Res. 2001;82:107–31.
4. Albina J, Reichner J. Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. Cancer Metastasis Rev. 1998;17(1):39–53.
5. Li H, Hu J, Xin W, Zhao B. Production and interaction of oxygen and nitric oxide free radicals in PMA stimulated macrophages during the respiratory burst. Redox Rep. 2000;5(6):353–8.
6. Leiro J, Iglesias R, Paramá A, Sanmartin ML, Ubeira FM. Respiratory burst responses of rat macrophages to microsporidian spores. Exp Parasitol. 2001;98(1):1–9.
7. Reynolds MM, Witzeling SD, Damodaran VB, et al. Applications for nitric oxide in halting proliferation of tumor cells. Biochem Biophys Res Commun. 2013;431(4):647–51.
8. Gkaliagkousi E, Ferro A. Nitric oxide signalling in the regulation of cardiovascular and platelet function. Front Biosci. 2011;16:1873–97.
9. Somers M, Harrison D. Reactive oxygen species and the control of vasomotor tone. Curr Hypertens Rep. 1999;1(1):102–8.
10. Wink D, Hines H, Cheng R, et al. Nitric oxide and the redox mechanisms in the immune response. J Leukocyte Biol. 2011;89(6):873–91.
11. Binder C, Schulz M, Hiddemann W, Oellerich M. Induction of inducible nitric oxide synthase is an essential part of tumor necrosis factor-alpha-induced apoptosis. Lab Investig. 1999;79(12):1703–12.
12. Farias-Eisner R, Sherman MP, Aeberhard E, Chaudhuri G. Nitric oxide is an important mediator for tumoricidal activity in vivo. Proc Natl Acad Sci. 1994;91(20):9407–11.
13. Sarti P, Forte E, Mastronicola D, Giuffrè A, Arese M. Cytochrome c oxidase and nitric oxide in action: molecular mechanisms and pathophysiological implications. Biochimica et Biophysica Acta (BBA)–Bioenerg. 2012;1817(4):610–19.
14. Boyd CS, Cadenas E. Nitric oxide and cell signaling pathways in mitochondrial-dependent apoptosis. Biol Chem. 2002;383:411.
15. Hughes MN. Chemistry of nitric oxide and related species. In: Robert KP, editor. Methods in enzymology. Vol. 436. Academic; 2008. pp. 3–19. (Is available for purchase from Elsevier Academic).
16. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. Cardiovasc Res. 1999;43(3):521–31.
17. Foster MW, Hess DT, Stamler JS. Protein S-nitrosylation in health and disease: a current perspective. Trend Mol Med. 15(9):391–404.
18. Stamler JS, Simon DI, Osborne JA, et al. S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. Proc Natl Acad Sci. 1992;89(1):444–8.
19. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nat Cell Biol. 2001;3(2):193–7.
20. Mannick JB. Regulation of apoptosis by protein S-nitrosylation. Amino Acids. 2007;32(4):523–6.
21. Benhar M, Stamler JS. A central role for S-nitrosylation in apoptosis. Nat Cell Biol. 2005;7(7):645–6.
22. Melino G, Bernassola F, Knight R, Corasaniti M, Nistico G, Finazzi-Agro A. S-Nitrosylation regulates apoptosis. Nature. 1997;388:432–3.
23. Martínez-Ruiz A, Lamas S. Two decades of new concepts in nitric oxide signaling: from the discovery of a gas messenger to the mediation of nonenzymatic posttranslational modifications. IUBMB Life. 2009;61(2):91–8.

24. Liu L, Yan Y, Zeng M, et al. Essential roles of S-Nitrosothiols in vascular homeostasis and endotoxic shock. Cell. 2004;116(4):617–28.
25. Benhar M, Forrester MT, Hess DT, Stamler JS. Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. Science. 2008;320(5879):1050–4.
26. Trujillo M, Alvarez MaN, Peluffo G, Freeman BA, Radi R. Xanthine oxidase-mediated decomposition of S-Nitrosothiols. Jf Biol Chem. 1998;273(14):7828–34.
27. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. Nat Rev Cancer. 2006;6(7):521–34.
28. Lo H-W, Hsu S-C, Ali-Seyed M, et al. Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. Cancer Cell. 2005;7(6):575–89.
29. Ambs S, Merriam WG, Ogunfusika MO, et al. p53 and vascular endothelial growth factor regulate tumor growth of NOS2-expressing human carcinoma cells. Nat Med. 1998;4(12):1371–6.
30. Jenkins DC, Charles IG, Thomsen LL, et al. Roles of nitric oxide in tumor growth. Proc Natl Acad Sci. 1995;92(10):4392–6.
31. Cockman ME, Masson N, Mole DR, et al. Hypoxia inducible factor-α binding and ubiquitylation by the von hippel-lindau tumor suppressor protein. J Biol Chem. 2000;275(33):25733–41.
32. Jaakkola P, Mole DR, Tian Y-M, et al. Targeting of HIF-α to the von hippel-lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science. 2001;292(5516):468–72.
33. Maxwell PH, Wiesener MS, Chang G-W, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 1999;399(6733):271–5.
34. Lima B, Lam GKW, Xie L, et al. Endogenous S-nitrosothiols protect against myocardial injury. Proc Natl Acad Sci. 2009;106(15):6297–302.
35. Palmer LA, Gaston B, Johns RA. Normoxic stabilization of hypoxia-inducible factor-1 expression and activity: redox-dependent effect of nitrogen oxides. Mol Pharmacol. 2000;58(6):1197–203.
36. Azad N, Vallyathan V, Wang L, et al. S-Nitrosylation of Bcl-2 inhibits its ubiquitin-proteasomal degradation: a novel antiapoptotic mechanism that suppresses apoptosis. J Biol Chem. 2006;281(45):34124–34.
37. Liu L, Xu-Welliver M, Kanugula S, Pegg AE. Inactivation and degradation of O6-Alkylguanine-DNA alkyltransferase after reaction with nitric oxide. Cancer Res. 2002;62(11):3037–43.
38. Yu C-X, Li S, Whorton AR. Redox regulation of PTEN by S-Nitrosothiols. Mol Pharmacol. 2005;68(3):847–54.
39. Calmels S, Hainaut P, Ohshima H. Nitric oxide induces conformational and functional modifications of wild-type p53 tumor suppressor protein. Cancer Res. 1997;57(16):3365–9.
40. Pi X, Wu Y, Ferguson JE, Portbury AL, Patterson C. SDF-1α stimulates JNK3 activity via eNOS-dependent nitrosylation of MKP7 to enhance endothelial migration. Proc Natl Acad Sci. 2009;106(14):5675–80.
41. Rahman MA, Senga T, Ito S, Hyodo T, Hasegawa H, Hamaguchi M. S-nitrosylation at cysteine 498 of c-Src tyrosine kinase regulates nitric oxide-mediated cell invasion. J Biol Chem. 2010;285(6):3806–14.
42. Sharp FR, Bernaudin M. HIF1 and oxygen sensing in the brain. Nat Rev Neurosci. 2004;5(6):437–48.
43. Martinou J-C, Youle Richard J. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev Cell. 2011;21(1):92–101.
44. Iyer AKV, Azad N, Wang L, Rojanasakul Y. Role of S-nitrosylation in apoptosis resistance and carcinogenesis. Nitric Oxide. 2008;19(2):146–51.
45. Hollander MC, Blumenthal GM, Dennis PA. PTEN loss in the continuum of common cancers, rare syndromes and mouse models. Nat Rev Cancer. 2011;11(4):289–301.
46. Schonhoff CM, Daou M-C, Jones SN, Schiffer CA, Ross AH. Nitric oxide-mediated inhibition of Hdm2−p53 binding†. Biochemistry. 2002;41(46):13570–4.

47. Leon-Bollotte L, Subramaniam S, Cauvard O, et al. S-Nitrosylation of the death receptor fas promotes fas ligand-mediated apoptosis in cancer cells. Gastroenterology. 2011;140(7):2009-2018. (e2004).
48. Kornberg MD, Sen N, Hara MR, et al. GAPDH mediates nitrosylation of nuclear proteins. Nat Cell Biol. 2010;12(11):1094–100.
49. Sen N, Hara MR, Kornberg MD, et al. Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis. Nat Cell Biol. 2008;10(7):866–73.
50. Marshall HE, Hess DT, Stamler JS. S-nitrosylation: physiological regulation of NF-κB. Proc Natl Acad Sci USA. 2004;101(24):8841–2.
51. Marshall HE, Stamler JS. Nitrosative stress-induced apoptosis through inhibition of NF-κB. J Biol Chem. 2002;277(37):34223–8.
52. Kelleher ZT, Matsumoto A, Stamler JS, Marshall HE. NOS2 regulation of NF-κB by S-nitrosylation of p65. J Biol Chem. 2007;282(42):30667–72.
53. Bonavida B, Baritaki S. Dual role of NO donors in the reversal of tumor cell resistance and EMT: downregulation of the NF-κB/Snail/YY1/RKIP circuitry. Nitric Oxide. 2011;24(1):1–7.
54. Huerta-Yepez S, Vega M, Jazirehi A, et al. Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-[kappa]B and inhibition of Bcl-xL expression. Oncogene. 2004;23(29):4993–5003.
55. Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the fas ligand, a novel member of the tumor necrosis factor family. Cell. 1993;75(6):1169–78.
56. Pant V, Lozano G. Limiting the power of p53 through the ubiquitin proteasome pathway. Gene Dev. 2014;28(16):1739–51.
57. Hara MR, Agrawal N, Kim SF, et al. S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. Nat Cell Biol. 2005;7(7):665–74.
58. Guan W, Sha J, Chen X, Xing Y, Yan J, Wang Z. S-Nitrosylation of mitogen activated protein kinase phosphatase-1 suppresses radiation-induced apoptosis. Cancer Lett. 2012;314(2):137–46.
59. Kang-Decker N, Cao S, Chatterjee S, et al. Nitric oxide promotes endothelial cell survival signaling through S-nitrosylation and activation of dynamin-2. J Cell Sci. 2007;120(3):492–501.
60. Wang G, Moniri NH, Ozawa K, Stamler JS, Daaka Y. Nitric oxide regulates endocytosis by S-nitrosylation of dynamin. Proc Natl Acad Sci USA. 2006;103(5):1295–300.
61. Chanvorachote P, Nimmannit U, Lu Y, Talbott S, Jiang B-H, Rojanasakul Y. Nitric oxide regulates lung carcinoma cell anoikis through inhibition of ubiquitin-proteasomal degradation of Caveolin-1. J Biol Chem. 2009;284(41):28476–84.
62. Nath N, Chattopadhyay M, Pospishil L, et al. JS-K; a nitric oxide-releasing prodrug, modulates β-catenin/TCF signaling in leukemic jurkat cells: evidence of an S-nitrosylated mechanism. Biochem Pharmacol. 2010;80(11):1641–49.
63. Bonavida B, Baritaki S, Huerta-Yepez S, Vega MI, Chatterjee D, Yeung K. Novel therapeutic applications of nitric oxide donors in cancer: roles in chemo- and immunosensitization to apoptosis and inhibition of metastases. Nitric Oxide. 2008;19(2):152–7.

Chapter 7
S-Nitrosylation in Cancer Cells: To Prevent or to Cause?

Ali Bettaieb, Stéphanie Plenchette, Catherine Paul, Véronique Laurens, Sabrina Romagny and Jean-François Jeannin

Abstract The purpose of this review is to point out some important proteins targeted by chemotherapy in cancer patients as well as by NO (S-nitrosylation) in tumor cells. We, therefore, confronted data from clinical and preclinical studies and discussed their respective anti-tumor effects to determine whether the associations of chemotherapy with NO donor therapy may be considered as novel therapeutic approaches (considered as rational therapeutic interventions).

Keywords Cancer · Chemotherapy · NO donors · S-nitrosylation · Signaling

Abbreviations

AR	Androgen receptor
CCL4	(C-C motif) ligand 4
DETANO	Diethylenetriamine/nitric oxide
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
Fas (*APO-1, CD95*)	Apoptosis antigen 1
GSNO	S-nitrosoglutatione
GTN	Glyceryltrinitrate
HER	Human epidermal growth factor receptor
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein

A. Bettaieb (✉) · S. Plenchette · C. Paul · V. Laurens · S. Romagny · J.-F. Jeannin
Immunology and Immunotherapy of Cancer Laboratory, Burgundy University, EA 7269, EPHE, 7 Bd Jeanne d'Arc, 21079 Dijon, France EPHE, 75014 Paris, France
e-mail: ali.bettaieb@u-bourgogne.fr

J.-F. Jeannin
e-mail: jean-francois.jeannin@u-bourgogne.fr

A. Bettaieb · S. Plenchette · C. Paul · V. Laurens · S. Romagny · J.-F. Jeannin
EPHE, 75014 Paris, France

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_7

IKK	IκB kinase
JNK	c-jun NH2-terminal kinase
N_2O_3	Nitrogen trioxide
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NO_2	Nitrites
NO-NSAIDs	NO-donating non-steroidal anti-inflammatory drugs
PKB	Protein kinase B
PTEN	Phosphatase and TENsin homolog
SM	Smac mimetic
SNO	S-nitrosothiol
STAT3	Signal transduction and activator of transcription 3
TKI	Tyrosine kinase inhibitor
TRAIL	Tumor-necrosis-factor related apoptosis inducing ligand

Introduction

The physiological role of cysteines is important. Cysteine residues can be modified by different post translational modifications controlling the conformation and fate of target proteins. Thus, (1) by forming disulfide bonds, cysteines contribute to protein folding (2) their S-palmitoylation and S-acylation are involved in protein location, stability and activity in membranes and (3) their S-sulfhydration or S-gluthionylation have been shown to be involved in redox sensing and signaling. Cysteines by themselves also coordinate metal binding and catalysis, providing protein stability and activity [1].

S-nitrosylation defines the non-enzymatic binding of a nitroso group to a sulfhydryl group of cysteine residues to form S-nitrosocysteine and appears to be the principal mechanism by which NO signals. NO can mediate this post-translational modification either by direct interaction or through related Reactive Nitrogen Species (RNS) such as peroxinitrite ($ONOO^-$), nitrogen dioxide (NO_2) or dinitrogen trioxide (N_2O_3) [2].

Similar to protein phosphorylation, protein S-nitrosylation is a reversible process that exerts many effects on protein function, by modulating protein activity, expression, protein-protein interactions and protein localization.

Until 2013, 171 S-nitrosylated proteins were identified from physiological conditions, 28 under various pathological conditions, and 34 under either physiological or pathological conditions [1] and at least around twenty in cancer [3]. The number of S-nitrosylated proteins identified remains small and might be underestimated due to the difficulties to put into evidence S-nitrosylation. In the present review, we will shed light on proteins that are targeted in current chemotherapy trials and that are at the same time shown to be S-nitrosylated in preclinical studies upon NOS activation or using NO donors. The objective is to determine if S-nitrosylation can induce an antitumor effect as chemotherapy, targeting the same protein but following another pathway. This could be a rationale to associate chemotherapy and S-nitrosylation in clinical trials to prevent tumor resistance.

Transcription Factors

NF-κB

NO exerts a dual role on NF-κB activity. NO-mediated activation of NF-κB can result from upstream stimulation of Ras activity via an S-nitrosylation-dependent mechanism [4]. On the other hand, S-nitrosylation-mediated inhibition can act on several targets along the NF-κB signal transduction pathway. The IKK complex containing IKKα, IKKβ and NEMO (IKKγ) is a central regulator of the classical NF-κB pathway activation [5]. IKKβ phosphorylates IκBα on serine residues 32 and 36, a prerequisite for its polyubiquitination and subsequent degradation by the proteasome. NF-κB homo- or heterodimers (for example p50/p65) retained in the cytoplasm by interaction with IκB, thereby, translocate into the nucleus for transcriptional activation of targeted genes [6]. Reynaert and colleagues have reported that the S-nitrosylation of IKK at cysteine residue 179 can suppress its kinase activity and further downstream signaling [7]. In addition, both NF-κB p50 and p65 monomers can also be S-nitrosylated at a cysteine in the DNA-binding region of the Rel homology domain (RHD) which is highly conserved among the NF-κB proteins [8, 9]. Indeed, S-nitrosylation of NF-κB p50 (cysteine 62) and p65 (cysteine 38) is correlated with decreased DNA binding and the inhibition of targeted gene transcription [8, 9]. The classical NF-κB signaling pathway is active in a number of tumor cell types, conferring survival advantages by promoting cell proliferation and cell survival [10]. Recently, NO-donating NSAIDs, known to induce apoptosis in many cancer cells, have unveiled a new mode of action. Indeed, NO-NSAIDs mediate S-nitrosylation of NF-κB p65 that, in turn, inhibits cell growth in human colon cancer cells [11]. For several years, IKK has been exploited as a therapeutic target for the development of selective inhibitors of the NF-κB pathway [12, 13]. Preclinical studies of BMS-345541, a selective inhibitor of the catalytic subunit of IKK, have shown anti-cancer activity in human xenograft tumor models including breast and melanoma [13, 14]. Selective inhibitors of NF-κB activation, however, have not reached clinical trials as yet. Interestingly, clinical trials evaluating non-selective NF-κB inhibitors such as Bortezomibe and Curcumin in combination with chemotherapeutic agents are currently ongoing (clinical trials.gov).

STAT 3

Signal transduction and activator of transcription 3 (STAT3) protein is an emerging target in cancer therapy. Stimulation by extracellular ligands such as cytokines (IL-6) or growth factors (EGF) results in the tyrosine 705 phosphorylation of STAT3 proteins via the activation of the upstream tyrosine kinase JAK2. STAT3 regulates cellular functions that lead to oncogenesis, and constitutive activation of STAT3 is observed in various cancers. Two inhibitors of STAT3 phosphorylation are now evaluated in clinical trials, OPB-31121 [15, 16] and OPB-51602 (clinicaltrials.gov) and STAT3 antisense-based drugs are also emerging. This is the case of

ISIS 481464, a modified antisense oligonucleotide [17], now being evaluated in phase I/II and of a cyclic STAT3 decoy oligonucleotide [18] currently in phase zero. Recently, it was reported that STAT3 is regulated by S-nitrosylation on cysteine 259 [19]. The NO donor GSNO inhibits IL-6 microglial proliferation via S-nitrosylation of STAT3 and inhibition of STAT3 phosphorylation. Thus, targeting STAT3 with this NO donor could represent another way to inhibit STAT3 phosphorylation. Indeed, it was shown that STAT3 phosphorylation in metastatic colorectal cancer cells was correlated with a decrease in clinical efficacy of anti-EGFR (cetuximab) therapy [20]. Therefore, STAT3, as a downstream mediator of EGFR, constitutes a critical molecular target to optimize anti-tumoral therapies.

Receptors

Androgen Receptor

The androgen receptor (AR) is a well-known therapeutic target in prostate cancer. The hormonal therapy strategy often associates androgen deprivation therapy with the use of AR antagonists. Blocking AR activation is temporary efficient, but the disease finally progresses to castration-resistant prostate cancer within 2–3 years. If the AR signaling cascade is implicated in the initiation it is also implicated in the progression of the disease in castration-resistant cancers [21]. AR functions as a ligand-dependent transcription factor and is regulated by its partners and post-translational modifications. Recently, inactivation of AR function by S-nitrosylation has been described and characterized on the single cysteine residue 601 of the DNA-binding domain [22]. It was specified that HSP90, a partner of AR in the cytoplasm and a key target of S-nitrosylation, transnitrosylates AR. This decreases AR ability to bind AR-responsive elements in the promoter region of target genes and to inhibit AR transcriptional activity. Preclinical data indicate that GSNO inhibits prostate cancer cell growth in xenograft models. Moreover, a phase II clinical trial has shown an inhibition of disease progression and a decrease in PSA doubling time in prostate cancer patients treated with low dose of the NO donor GTN patches [23]. A phase III is currently in progress evaluating the efficacy of two doses of GTN therapy and the impact of GTN treatment on biomarkers of immune escape. The tumor microenvironment that is known to influence tumor progression can also be regulated by AR. In prostate cancer, macrophages appear to promote tumorigenesis via the CCL4/AR signaling axis and STAT3 phosphorylation. This can be prevented using an enhancer of AR degradation, ASC-J9 [24]. Evaluating the impact of inactivation by NO of AR function as well as STAT3 phosphorylation on the immune anti-tumoral response in prostate cancer could be interesting.

Cell Death Receptors

Some members of the tumor necrosis factor receptor family have been described to be S-nitrosylated by NO donors. Indeed, our laboratory has shown that the S-nitrosylation of Fas on its cysteine 304 promotes FasL-mediated apoptosis in cancer cells [25]. TRAIL-R1 (DR4), is also S-nitrosylated by nitrosylcobalamin (an analogue of vitamin B12 that delivers NO), on its cysteine 336 and promotes TRAIL-mediated apoptosis in cancer cells [26]. These data uncover S-nitrosylation as a new mechanism to render some death receptors more sensitive to their ligands and leading to an increase of cancer cell death. Considering the selectivity of TRAIL against tumor cells [27], several TRAIL-based anticancer drugs have been tested during the last years. Agonistic antibodies recognizing either TRAIL-R1 or -R2 (DR5) and recombinant forms of TRAIL have been developed and tested in clinics. Despite the anti-tumor activity shown in cell lines and xenograft models with these molecules, the reported results of clinical trials in monotherapy have been disappointing. Recent clinical trials indicate that the combination of DR4/DR5 agonists with other antitumor agents yields better response rates [28]. Thus, it would be interesting to evaluate the anti-tumor activity of the combination of recombinant forms of TRAIL or agonistic antibodies against DR4 and DR5 with NO donors such as GTN in xenograft models and their safety in clinical trials.

Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor belonging to the ErbB family. These receptors are expressed in a large variety of cells and are key players in development, proliferation and differentiation. Inappropriate activation of EGFR is often found in various tumor types, resulting in unregulated growth stimulation and tumorigenesis [29]. Therefore, EGFR and particularly its inhibition represent a key target for cancer therapy. The last 30 years have led to the development of several therapeutic agents targeting EGFR including monoclonal antibodies, tyrosine-kinase inhibitors (TKIs), antibody based immunoconjugates (Trastuzumab-Emtansine, EQ75-ADR), antisense oligodeoxynucleotides (GEM 231) or other novel agents [30]. Currently, Cetuximab and Panitumumab are the only monoclonal antibodies approved in several countries for the treatment of colon cancer, as well as head and neck cancer [31]. These antibodies bind the extracellular domain of EGFR, thereby blocking the ligand-binding activity unlike the EGFR TKIs that directly bind and inactivate the kinase domain [32]. Two generations of TKI have been developed and tested in clinical trials, such as erlotinib, gefitinib, afatinib and lapatinib [31]. However, the response of a single agent such as EGFR antibodies or TKI is relatively low and extensive preclinical studies suggest that using two different antibodies or an antibody and a tyrosine kinase inhibitor has additive or synergistic anti-tumor activity [31].

Another strategy to inhibiting EGFR consists of S-nitrosylation. In fact, few reports suggest that S-nitrosylation of EGFR, at cysteines 166 or 305, results in kinase inhibition [33, 34]. However, another report demonstrated that, in human basal-like breast cancer, DETANO or NO produced by IFN/LPS stimulated-macrophages, induces S-nitrosylation of EGFR resulting in reduced signaling and STAT-3 phosphorylation [35].

Enzymes

Ras

Ras proteins is a family of small GTPases that transduce signals after their activation by phosphorylation [36] or S-nitrosylation. Indeed, S-nitrosylation of cysteine 118 in p21ras stimulates guanine nucleotide exchange leading to activation of the downstream Ras pathway [4]. Even if the activity of Ras is induced by NO, several kinases downstream of the Ras effector pathways are targeted by NO. The best understood and most studied Ras effector pathway is the Raf-MEK-ERK cascade [37]. Recently, it has been reported that ERK undergoes S-nitrosylation at cysteine residue 183 which inhibits its phosphorylation and triggers the apoptotic program [38]. ERK1/2 are the only known substrates of MEK1/2 for which a number of potent and selective MEK1 and MEK2 inhibitors have been developed and are currently under clinical evaluation. Among them, selumetinib (AZD6244). Selumetinib is an orally bio-available benzimidazole derivative known to potently inhibit MEK1/2 in vitro and in cell-based assays [39]. Preclinical studies showed selumetinib's antitumor activity in several human xenograft models including colon, pancreas, breast, lung cancers and melanoma and led to further clinical development. Cell based assays suggest that MEK inhibitors may be effective against BRAF but not Ras mutant cancer cells [40]. Preliminar results of a phase I study concluded that AZD6244 was well tolerated [41]. Currently, there are up to 70 completed or ongoing Phase I/II clinical trials evaluating AZD6244 as monotherapy or in combination with conventional cytotoxic drugs (clinicaltrials.gov). Another kinase downstream of the Ras pathway, JNK2, is S-nitrosylated and leading to the suppression of its activity [42]. Although JNK is involved in apoptosis, it has been recently shown that JNK is required for Ras-dependent lung tumor development [43]. Altogether, these data suggest that, despite the fact that NO activates the Ras pathway, it could also represent an interesting alternative to target the Ras downstream signaling pathway.

Akt

Akt/PKB, commonly activated in human cancer, controls cell survival and is involved in resistance to cancer therapy [44]. Akt/PKB is activated upon cell stimulation by many different growth factors. In cancer, this kinase is activated as

a result of mutation-dependent activation of EGFR, PI3K or Akt gene amplification (HER2) and deletion or reduction of PTEN function [44, 45]. This has led to a major effort to develop inhibitors of PI3K/Akt and other components of the pathway [46]. Currently, there are up to 50 completed or ongoing Phase I/II clinical trials evaluating an Akt inhibitor MK-2206 as monotherapy or in combination with conventional cytotoxic drugs (clinicaltrials.gov). However, aside from their anti-tumor effects, Akt inhibitors cause a marked rise of the expression of EGFR, HER3 and HER4 (activated and over-expressed in many types of cancer) and increase the HER3 phosphorylation [47]. This may attenuate its anti-tumor activity and, thus, may limit its use in clinics as a single therapy. Yasukawa et al have shown that NO donors inactivated Akt/PKB both in acellular preparations and in cell based assays with simultaneous S-nitrosylation of the kinase at cysteine 224 [48]. Therefore, NO could be used to inhibit the activation of Akt and to evaluate whether it increases the expression and activation of proteins of the EGFR pathway.

The Inhibitor of Apoptosis Protein

The inhibitor of apoptosis protein (IAP) family members are frequently over expressed in human cancers and confer drug resistance, disease progression and poor prognosis [49]. XIAP, cIAP1 and cIAP2 have been well-studied and validated as important cancer targets [50]. Physiologically, IAPs can be antagonized by Smac. The interaction of Smac with XIAP, disables its inhibitory effect on caspases and promotes apoptosis [51]. Therapeutic approaches for targeting IAP proteins were built around XIAP inhibition at its gene expression and functional levels. AEG35156 is a second-generation XIAP antisense oligonucleotide evaluated in the clinic for several years [52]. Generally very well-tolerated, AEG35156 as monotherapy has demonstrated some signs of anti-cancer activity on advanced-stage refractory cancer [52, 53]. Over the past decade, particular interests have been focused on pan-IAP inhibitors such as Smac mimetics (SMs) designed to inhibit XIAP anti-caspase activity but with the greatest effect on cIAP1/2 proteasomal degradation [54]. SMs display potent antitumor activity in both human cancer cells and tumor xenograft murine models including breast and melanoma [54, 55]. Several studies have reported a synergistic activity in combination with TNFα or TRAIL [56–58]. Six SMs have reached clinical trials and are currently being tested [59]. The first phase I clinical study of LCL161 (Novartis Pharmaceuticals) demonstrated a good tolerance and target inhibition [60, 61]. Clinical development of LCL161 is ongoing in solid tumors and haematological malignancies in combination with conventional therapies (clinicaltrials.gov).

A different approach to antagonize XIAP has taken into account the contribution of S-nitrosylation. Two recent independent studies have shown that NO, in the process of neurodegeneration, can react with XIAP, thus, forming S-nitrosylated XIAP that accumulates in the brain of patients [62, 63]. Particularly, XIAP S-nitrosylation compromises XIAP antiapoptotic function in neuronal cells and is associated with elevated levels of active caspase-3 that further activate caspase-dependent

cell death [62, 63]. Although these two studies support a role for SNO-XIAP in neurodegenerative disorders, the molecular mechanism through which S-nitrosylation impairs XIAP anticaspase function is somewhat controversial. Tsang and colleagues' study suggests that XIAP is S-nitrosylated at the BIR domains which impairs its inhibitory effect on caspase-3 [62]. On the other and, Nakamura and colleagues' study demonstrated that NO S-nitrosylates the RING domain at cysteine 450 which impairs XIAP ubiquitin E3 ligase activity from degrading caspase-3 [63]. Furthermore, Nakamura and colleagues demonstrated that the S-nitrosylation of XIAP occurred through a transnitrosylation from SNO-caspase-3 to XIAP. NO-mediated S-nitrosylation is likely to be a useful approach to overcoming XIAP. Whether or not NO could S-nitrosylate XIAP and enhance tumor cell response to chemotherapeutic agents remains to be determined.

Chaperone Proteins

Heat Shock Protein 90

HSP90 belongs to the well-known family of Heat Shock Proteins (HSP). These proteins are molecular chaperones that protect cellular proteins from degradation under stress conditions such as elevated temperature, oxidative stress or hypoxia. HSP90 is a ubiquitous protein that acts as an ATP-dependent molecular chaperone. HSP90 is frequently up-regulated in cancer cells, stabilizing many important oncogenic proteins such as EGFR, HER2, BRAF, Akt, leading to increased proliferation, survival and metastasis [64].

HSP90 inhibitors show great promise in cancer treatment since they have the ability to inhibit multiple oncogenic signaling pathways simultaneously, thus reducing the possibility of tumor resistance [65]. These inhibitors act mainly at the N-terminal ATP-binding domain of HSP90, inducing the degradation of the target protein by the ubiquitin-proteasome pathway [66]. HSP90 inhibitors include natural products such as geldanamycin (ansamycin antibiotic) and radicicol as well as theirs derivatives. More recently, several small synthetic inhibitors with different characteristics were developed [64, 67]. Many HSP90 inhibitors are tested in phase I or II and only two molecules are being tested in phase III studies for patients with non-small-cell lung cancer or gastrointestinal stromal tumors. Another approach to inhibit HSP90 consists in modulating its post-translational modifications. Indeed, the chaperone cycle of HSP90 is regulated by phosphorylation and acetylation processes [68, 69]. Martinez-Ruiz and collaborators reported the S-nitrosylation at cysteine 597 of human HSP90 in endothelial cells, thereby inhibiting its chaperone activity [70]. More recently, Retzlaff and colleagues demonstrated that S-nitrosylation at the C-terminal domain inhibits the ATPase activity that is present in the N-terminal domain of HSP90 [71]. At this stage, we can speculate that the antitumor activity of NO may result, at least in part, from NO-mediated HSP90 inhibition in cancer cells [72].

Discussion and Conclusion

This review intended to highlight proteins targeted both by current chemotherapies and by NO donors in preclinical studies leading to their S-nitrosylation, thereby, for the majority of them, inhibiting their protumor properties.

It is important to note that NO plays a controversial role in cancer biology. Indeed and as shown previously, while the S-nitrosylation of Ras protein (p21 for example) increases its activity and indirectly increases the synthesis of NF-κB subunits, the S-nitrosylation of downstream proteins (such as ERK1/2 or IKKβ respectively) inhibits their activity and, thus, inhibits protumor signaling pathways. In this particular case, the result is an anti-tumor effect, however, due to possible tumor escape, NO is not an appropriate anti-cancer molecule.

One strategy to kill cancer cells is to activate their death receptor signaling pathways. Intending to do this, some chemotherapies use agonist ligands to activate the cancer cell death program, but still, resistance emerges. We know that NO can activate these receptors both at the transcriptional level (e.g. inhibition of YY1) or post-translationnal level (e.g. S-nitrosylation of Fas receptor). These open the opportunity to combine chemotherapeutic drugs with NO donors to (1) increase the activation of death receptors and (2) bypass drugs resistance.

The conditions of specificity of S-nitrosylation with NO donors would be important to know to in order to develop these molecules as therapies. Most importantly, to get suitable concentrations and low side effects, it is important to target specifically tumor cells in vivo with NO donors. Although progress has been made in these fields, two clinical trials that combined chemotherapy and NO donors [73] or radiotherapy and NO donors [23] have yielded positive responses.

Acknowledgments No conflict statement: “any potential conflicts of interest were disclosed.”

References

1. Gould N, Doulias PT, Tenopoulou M, Raju K, Ischiropoulos H. Regulation of protein function and signaling by reversible cysteine S-nitrosylation. J Biol Chem. 2013;288:26473–9.
2. Martinez-Ruiz A, Araujo IM, Izquierdo-Alvarez A, Hernansanz-Agustin P, Lamas S, Serrador JM. Specificity in S-nitrosylation: a short-range mechanism for NO signaling? Antioxid Redox Signal. 2013;19:1220–35.
3. Aranda E, Lopez-Pedrera C, De La Haba-Rodriguez JR, Rodriguez-Ariza A. Nitric oxide and cancer: the emerging role of S-nitrosylation. Current Molecular Medicine. 2012;12:50–67.
4. Lander HM, Ogiste JS, Pearce SF, Levi R, Novogrodsky A. Nitric oxide-stimulated guanine nucleotide exchange on p21ras. J Biol Chem. 1995;270:7017–20.
5. Israël A. The IKK complex, a central regulator of NF-kappaB activation. Cold Spring Harb Perspect Biol. 2010;2:a000158.
6. Napetschnig J, Wu H. Molecular basis of NF-kB signaling. Annu Rev Biophys. 2013;42: 443–68.
7. Reynaert NL, Ckless K, Korn SH, Vos N, Guala AS, Wouters EF, van der Vliet A, Janssen-Heininger YM. Nitric oxide represses inhibitory kappaB kinase through S-nitrosylation. Proc Natl Acad Sci USA. 2004;101:8945–50.

8. Kelleher ZT, Matsumoto A, Stamler JS, Marshall HE. NOS2 regulation of NF-kappaB by S-nitrosylation of p65. J Biol Chem. 2007;282:30667–72.
9. Marshall HE, Stamler JS. Inhibition of NF-kappa B by S-nitrosylation. Biochemistry. 2001;40:1688–93.
10. Prasad S, Ravindran J, Aggarwal BB. NF-kappaB and cancer: how intimate is this relationship. Mol Cell Biochem. 2010;336:25–37.
11. Chattopadhyay M, Goswami S, Rodes DB, Kodela R, Velazquez CA, Boring D, Crowell JA, Kashfi K. NO-releasing NSAIDs suppress NF-kB signaling in vitro and in vivo through S-nitrosylation. Cancer Lett. 2010;298:204–11.
12. May MJ, D'Acquisto F, Madge LA, Glöckner J, Pober JS, Ghosh S. Selective inhibition of NF-kappaB activation by a peptide that blocks the interaction of NEMO with the IkappaB kinase complex. Science. 2000;289:1550–4.
13. Yang J, Amiri KI, Burke JR, Schmid JA, Richmond A. BMS-345541 targets inhibitor of kappaB kinase and induces apoptosis in melanoma: involvement of nuclear factor kappaB and mitochondria pathways. Clin Cancer Res. 2006;12:950–60.
14. Wu L, Shao L, Li M, Zheng J, Wang J, Feng W, Chang J, Wang Y, Hauer-Jensen M, Zhou D. BMS-345541 sensitizes MCF-7 breast cancer cells to ionizing radiation by selective inhibition of homologous recombinational repair of DNA double-strand breaks. Radiat Res. 2013;179:160–70.
15. Hayakawa F, Sugimoto K, Harada Y, Hashimoto N, Ohi N, Kurahashi S, Naoe T. A novel STAT inhibitor, OPB-31121, has a significant antitumor effect on leukemia with STAT-addictive oncokinases. Blood Cancer J. 2013;3:e166.
16. Kim MJ, Nam HJ, Kim HP, Han SW, Im SA, Kim TY, Oh DY, Bang YJ. OPB-31121, a novel small molecular inhibitor, disrupts the JAK2/STAT3 pathway and exhibits an antitumor activity in gastric cancer cells. Cancer Lett. 2013;335:145–52.
17. Burel SA, Han SR, Lee HS, Norris DA, Lee BS, Machemer T, Park SY, Zhou T, He G, Kim Y, et al. Preclinical evaluation of the toxicological effects of a novel constrained ethyl modified antisense compound targeting signal transducer and activator of transcription 3 in mice and cynomolgus monkeys. Nucleic Acid Ther. 2013;23:213–27.
18. Sen M, Thomas SM, Kim S, Yeh JI, Ferris RL, Johnson JT, Duvvuri U, Lee J, Sahu N, Joyce S, et al. First-in-human trial of a STAT3 decoy oligonucleotide in head and neck tumors: implications for cancer therapy. Cancer Discov. 2012;2:694–705.
19. Kim J, Won JS, Singh AK, Sharma AK, Singh I. STAT3 regulation by S-Nitrosylation: implication for inflammatory disease. Antioxid Redox Signal. 2014;20:2514–27.
20. Dobi E, Monnien F, Kim S, Ivanaj A, N'Guyen T, Demarchi M, Adotevi O, Thierry-Vuillemin A, Jary M, Kantelip B, et al. Impact of STAT3 phosphorylation on the clinical effectiveness of anti-EGFR-based therapy in patients with metastatic colorectal cancer. Clin Colorectal Cancer. 2013;12:28–36.
21. Augello MA, Den RB, Knudsen KE. AR function in promoting metastatic prostate cancer. Cancer Metastasis Rev. 2014;3:399–411.
22. Qin Y, Dey A, Purayil HT, Daaka Y. Maintenance of androgen receptor inactivation by S-nitrosylation. Cancer Res. 2013;73:6690–9.
23. Siemens DR, Heaton JP, Adams MA, Kawakami J, Graham CH. Phase II study of nitric oxide donor for men with increasing prostate-specific antigen level after surgery or radiotherapy for prostate cancer. Urology. 2009;74:878–83.
24. Izumi K, Chang C. Targeting inflammatory cytokines-androgen receptor (AR) signaling with ASC-J9(®) to better battle prostate cancer progression. Oncoimmunology. 2013;2:e26853.
25. Leon-Bollotte L, Subramaniam S, Cauvard O, Plenchette-Colas S, Paul C, Godard C, Martinez-Ruiz A, Legembre P, Jeannin JF, Bettaieb A. S-nitrosylation of the death receptor fas promotes fas ligand-mediated apoptosis in cancer cells. Gastroenterology. 2011;140:2009–18,18e1–4.
26. Tang Z, Bauer JA, Morrison B, Lindner DJ. Nitrosylcobalamin promotes cell death via S nitrosylation of Apo2L/TRAIL receptor DR4. Mol Cell Biol. 2006;26:5588–94.

27. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med. 1999;5:157–63.
28. Martinez-Lostao L, Marzo I, Anel A, Naval J. Targeting the Apo2L/TRAIL system for the therapy of autoimmune diseases and cancer. Biochem Pharmacol. 2012;83:1475–83.
29. Yarden Y, Pines G. The ERBB network: at last, cancer therapy meets systems biology. Nat Rev Cancer. 2012;12:553–63.
30. Yewale C, Baradia D, Vhora I, Patil S, Misra A. Epidermal growth factor receptor targeting in cancer: a review of trends and strategies. Biomaterials. 2013;34:8690–707.
31. Tebbutt N, Pedersen MW, Johns TG. Targeting the ERBB family in cancer: couples therapy. Nat Rev Cancer. 2013;13:663–73.
32. Mendelsohn J, Baselga J. Epidermal growth factor receptor targeting in cancer. Semin Oncol. 2006;33:369–85.
33. Estrada C, Gómez C, Martín-Nieto J, De Frutos T, Jiménez A, Villalobo A. Nitric oxide reversibly inhibits the epidermal growth factor receptor tyrosine kinase. Biochem J. 1997;326:369–76.
34. Murillo-Carretero M, Torroglosa A, Castro C, Villalobo A, Estrada C. S-Nitrosylation of the epidermal growth factor receptor: a regulatory mechanism of receptor tyrosine kinase activity. Free Radic Biol Med. 2009;46:471–9.
35. Switzer CH, Glynn SA, Cheng RY, Ridnour LA, Green JE, Ambs S, Wink DA. S-nitrosylation of EGFR and Src activates an oncogenic signaling network in human basal-like breast cancer. Mol Cancer Res. 2012;10:1203–15.
36. McCubrey JA, Milella M, Tafuri A, Martelli AM, Lunghi P, Bonati A, Cervello M, Lee JT, Steelman LS. Targeting the Raf/MEK/ERK pathway with small-molecule inhibitors. Curr Opin Investig Drugs. 2008;9:614–30.
37. Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene. 2007;26:3291–310.
38. Feng X, Sun T, Bei Y, Ding S, Zheng W, Lu Y, Shen P. S-nitrosylation of ERK inhibits ERK phosphorylation and induces apoptosis. Sci Rep. 2013;3:1814.
39. Davies BR, Logie A, McKay JS, Martin P, Steele S, Jenkins R, Cockerill M, Cartlidge S, Smith PD. AZD6244 (ARRY-142886), a potent inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 kinases: mechanism of action in vivo, pharmacokinetic/pharmacodynamic relationship, and potential for combination in preclinical models. Mol Cancer Ther. 2007;6:2209–19.
40. Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A, Ye Q, Lobo JM, She Y, Osman I, et al. BRAF mutation predicts sensitivity to MEK inhibition. Nature. 2006;439:358–62.
41. Adjei AA, Cohen RB, Franklin W, Morris C, Wilson D, Molina JR, Hanson LJ, Gore L, Chow L, Leong S, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral, small-molecule mitogen-activated protein kinase kinase 1/2 inhibitor AZD6244 (ARRY-142886) in patients with advanced cancers. J Clin Oncol. 2008;26:2139–46.
42. So HS, Park RK, Kim MS, Lee SR, Jung BH, Chung SY, Jun CD, Chung HT. Nitric oxide inhibits c-Jun N-terminal kinase 2 (JNK2) via S-nitrosylation. Biochem Biophys Res Commun. 1998;247:809–13.
43. Cellurale C, Sabio G, Kennedy NJ, Das M, Barlow M, Sandy P, Jacks T, Davis RJ. Requirement of c-Jun NH(2)-terminal kinase for Ras-initiated tumor formation. Mol Cell Biol. 2011;31:1565–76.
44. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer. 2002;2:489–501.
45. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer. 2005;5:341–54.
46. Courtney KD, Corcoran RB, Engelman JA. The PI3K pathway as drug target in human cancer. J Clin Oncol. 2010;28:1075–83.

47. Chandarlapaty S, Sawai A, Scaltriti M, Rodrik-Outmezguine V, Grbovic-Huezo O, Serra V, Majumder PK, Baselga J, Rosen N. AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell. 2011;19:58–71.
48. Yasukawa T, Tokunaga E, Ota H, Sugita H, Martyn JA, Kaneki M. S-nitrosylation-dependent inactivation of Akt/protein kinase B in insulin resistance. J Biol Chem. 2005;280:7511–8.
49. LaCasse EC, Mahoney DJ, Cheung HH, Plenchette S, Baird S, Korneluk RG. IAP-targeted therapies for cancer. Oncogene. 2008;27:6252–75.
50. Hunter AM, LaCasse EC, Korneluk RG. The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis. 2007;12:1543–68.
51. Holcik M, Gibson H, Korneluk RG. XIAP: apoptotic brake and promising therapeutic target. Apoptosis. 2001;6:253–61.
52. LaCasse EC. Pulling the plug on a cancer cell by eliminating XIAP with AEG35156. Cancer Lett. 2013;332:215–24.
53. Dean E, Jodrell D, Connolly K, Danson S, Jolivet J, Durkin J, Morris S, Jowle D, Ward T, Cummings J, et al. Phase I trial of AEG35156 administered as a 7-day and 3-day continuous intravenous infusion in patients with advanced refractory cancer. J Clin Oncol. 2009;27:1660–6.
54. Lu J, Bai L, Sun H, Nikolovska-Coleska Z, McEachern D, Qiu S, Miller RS, Yi H, Shangary S, Sun Y, et al. SM-164: a novel, bivalent Smac mimetic that induces apoptosis and tumor regression by concurrent removal of the blockade of cIAP-1/2 and XIAP. Cancer Res. 2008;68:9384–93.
55. Krepler C, Chunduru SK, Halloran MB, He X, Xiao M, Vultur A, Villanueva J, Mitsuuchi Y, Neiman EM, Benetatos C, et al. The novel SMAC mimetic birinapant exhibits potent activity against human melanoma cells. Clin Cancer Res. 2013;19:1784–94.
56. Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, Zobel K, Dynek JN, Elliott LO, Wallweber HJ, et al. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell. 2007;131:669–81.
57. Cheung HH, Mahoney DJ, Lacasse EC, Korneluk RG. Down-regulation of c-FLIP Enhances death of cancer cells by smac mimetic compound. Cancer Res. 2009;69:7729–38.
58. Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG. A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. Science. 2004;305:1471–4.
59. Dubrez L, Berthelet J, Glorian V. IAP proteins as targets for drug development in oncology. Onco Target Ther. 2013;9:1285–304.
60. Infante JR DE, Burris HA, Zawel L, Sager JA, Stevenson C, Clarke K, Dhuria S, Porter D, Sen SK, et al. Paper presented at: 101st Annual meeting of the American association for cancer research. 2010, and Washington DP, PA. Abstract nr 2775.; 2010.
61. Houghton PJ, Kang MH, Reynolds CP, Morton CL, Kolb EA, Gorlick R, Keir ST, Carol H, Lock R, Maris JM, et al. Initial testing (stage 1) of LCL161, a SMAC mimetic, by the pediatric preclinical testing program. Pediatr Blood Cancer. 2012;58:636–9.
62. Tsang AH, Lee YI, Ko HS, Savitt JM, Pletnikova O, Troncoso JC, Dawson VL, Dawson TM, Chung KK. S-nitrosylation of XIAP compromises neuronal survival in Parkinson's disease. Proc Natl Acad Sci USA. 2009;106:4900–5.
63. Nakamura T, Wang L, Wong CC, Scott FL, Eckelman BP, Han X, Tzitzilonis C, Meng F, Gu Z, Holland EA. Transnitrosylation of XIAP regulates caspase-dependent neuronal cell death. Mol Cell. 2010;39:184–95.
64. Pillai RN, Ramalingam SS. Heat shock protein 90 inhibitors in non-small-cell lung cancer. Curr Opin Oncol. 2014;26:159–64.
65. De Mattos-Arruda L, Cortes J. Breast cancer and HSP90 inhibitors: is there a role beyond the HER2-positive subtype? Breast. 2012;21:604–7.
66. Prodromou C, Pearl LH. Structure and functional relationships of Hsp90. Curr Cancer Drug Target. 2003;3:301–23.
67. Zagouri F, Sergentanis TN, Chrysikos D, Papadimitriou CA, Dimopoulos MA, Psaltopoulou T. Hsp90 inhibitors in breast cancer: a systematic review. Breast. 2013;22:569–78.

68. Scroggins BT, Neckers L. Just say NO: nitric oxide regulation of Hsp90. EMBO Rep. 2009;10:1093–4.
69. Wandinger SK, Suhre MH, Wegele H, Buchner J. The phosphatase Ppt1 is a dedicated regulator of the molecular chaperone Hsp90. EMBO J. 2006;25:367–76.
70. Martínez-Ruiz A, Villanueva L, González de Orduña C, López-Ferrer D, Higueras MA, Tarín C, Rodríguez-Crespo I, Vázquez J, Lamas S. S-nitrosylation of Hsp90 promotes the inhibition of its ATPase and endothelial nitric oxide synthase regulatory activities. Proc Natl Acad Sci USA. 2005;102:8525–30.
71. Retzlaff M, Stahl M, Eberl HC, Lagleder S, Beck J, Kessler H, Buchner J. Hsp90 is regulated by a switch point in the C-terminal domain. EMBO Rep. 2009;10:1147–53.
72. Mollapour M, Neckers L. Post-translational modifications of Hsp90 and their contributions to chaperone regulation. Biochim Biophys Acta. 2012;1823:648–55.
73. Yasuda H, Yamaya M, Nakayama K, Sasaki T, Ebihara S, Kanda A, Asada M, Inoue D, Suzuki T, Okazaki T, et al. Randomized phase II trial comparing nitroglycerin plus vinorelbine and cisplatin with vinorelbine and cisplatin alone in previously untreated stage IIIB/IV non-small-cell lung cancer. J Clin Oncol. 2006;24:688–94.

Chapter 8
The Emerging Role of Protein *S*-Nitrosylation in Cancer Metastasis

Sudjit Luanpitpong and Yon Rojanasakul

Abstract Nitric oxide (NO) has increasingly been recognized as an important cell signaling molecule that controls various steps of cancer development and metastasis. NO regulates a wide range of tumor-associated proteins through *S*-nitrosylation, a reversible coupling of a nitroso moiety to a reactive cysteine thiol (SH) group to form an *S*-nitrosothiol (SNO). In this article, we discuss the various roles of protein *S*-nitrosylation in cancer development with a focus on anoikis resistance, cell invasion and angiogenesis, which are key determinants of cancer metastasis. We specially address the effect of *S*-nitrosylation on protein function and discuss how this post-translational modification affects the aggressive and metastatic behaviors of cancer cells. We propose that dysregulated NO signaling is common in many, if not most, metastatic cancers and that understanding the *S*-nitrosylation process will facilitate the development of novel therapeutic and preventive strategies against cancers.

Keywords Anoikis · Cancer · Invasion · Metastasis · Migration · Nitric Oxide · *S*-nitrosylation

Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
c-Src	cellular Src
DETA	diethylenetriamine

Y. Rojanasakul (✉) · S. Luanpitpong
Department of Pharmaceutical Sciences, West Virginia University, P.O. Box 9530, 26506 Morgantown, WV, USA
e-mail: yrojan@hsc.wvu.edu

Mary Babb Randolph Cancer Center, West Virginia University, 26506 Morgantown, WV, USA

S. Luanpitpong
e-mail: suidjit@gmail.com

Y. Rojanasakul
Health Sciences Center, West Virginia University, P.O. Box 9530, 26506 Morgantown, WV, USA

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_8

DISC	death-inducing signaling complex
DPTA	dipropylenetriamine
DR	death receptor
DTT	dithiothreitol
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
eNOS (NOS3)	endothelial nitric oxide synthase
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FLIP	FLICE-inhibitory protein
FLIP2CM	FLIP double-cysteine mutant
JNK	Jun N-terminal kinases
IGF	insulin-like growth factor
iNOS (NOS2)	inducible nitric oxide synthase
MKP7	MAP kinase phosphatase 7
MMP	matrix metalloproteinase
NO	nitric oxide
NOS	nitric oxide synthase
nNOS (NOS1)	neuronal NOS
PI3K	phosphoinositide-3-kinase
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PTM	post-translational modification
RNOS	reactive nitrogen-oxygen species
ROS	reactive oxygen species
SDF-1α	stromal cell-derived factor-1α
SH	cysteine thiol
SNAP	*S*-nitroso-*N*-acetylpenicillamine
SNO	*S*-nitrosothiol
SNOC	*S*-nitrosocysteine
VEGF	vascular endothelial growth factor

Introduction

Nitric oxide (NO, formula N=O) is an important signaling molecule that functions as a messenger or effector in various biological processes [1]. NO is synthesized by the metabolism of L-arginine to L-citrulline through a complex reaction catalyzed by NADPH-dependent enzymes called nitric oxide synthases (NOS), which exist in three isoforms, namely, neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3) [2]. Expression of NOS and NO activities are involved in the pathophysiology of cancers, particularly in tumorigenesis and metastasis in various tissues including brain, breast, lung, prostate, and

pancreas [3–6]. With regards to cancers, NO is derived either from tumor cells or neighboring cells, e.g. endothelial cells in the microvasculature and immune and stromal cells in the tumors [6, 7]. NO with its lipophilic nature could diffuse freely across cellular membranes, i.e. of neighboring cells and ultimately exerts its effect on tumor cells. Depending on (i) its activity and cellular sources (tumor or neighboring cells) (ii) localization of NOS (iii) concentration and duration of NO exposure (iv) cellular context and sensitivity to NO and (v) tumor stage, NO appears to exert dichotomous roles (promotion or inhibition) in cancers [8–10].

With its unique chemistry, the reactivity of NO varies under different biological and pathological conditions. The chemical biology of NO is generally classified into direct and indirect effects [11]. Direct effects are defined as those of direct interactions between NO, generally at a low level, and specific molecular targets, e.g. metals, lipids and DNA through free radical reactions. Indirect effects are those mediated by reactive nitrogen-oxygen species (RNOS) derived from the reaction of NO, generally at a high level, with various reactive oxygen species (ROS) leading to nitrosative or oxidative stress. For instance, NO reacts with superoxide anion ($O_2^{\bullet -}$) in the inner-membrane environment that results in the generation of (i) peroxynitrite ($ONOO^-$) in the case of equal concentrations of NO and $O_2^{\bullet -}$ or (ii) dinitrogen trioxide (N_2O_3) in the case of excess NO. The reaction of NO and O_2 (auto-oxidation) yields a nitrogen dioxide (NO_2) intermediate that forms N_2O_3. N_2O_3 (major species) and $ONOO^-$ are endogenous *S*-nitrosylating agents that lead to *S*-nitrosylation of proteins with reactive sulfhydryl groups.

In cancers, *S*-nitrosylation is an important post-translational protein modification (PTM) process that affects virtually all cancer cell phenotypes including cell growth and differentiation, apoptosis, migration and invasion, and angiogenesis [12]. The principal target of protein *S*-nitrosylation is the thiol group of protein's cysteine residues. Not all cysteine residues, however, are susceptible for *S*-nitrosylation and/or responsible for the alteration of protein functions, which depend largely on the degree of hydrophobicity, electrostatic environment, orientation of aromatic residues and proximity of target thiols to redox center, and protein-protein interactions [13–15]. In this article, we will review current findings on NO signaling and its role in cancer with a focus on protein *S*-nitrosylation and its effect on the various steps of cancer progression and metastasis.

Cancer Metastasis

Neoplastic transformation is an early cellular event leading to carcinogenesis. Neoplastic transformation of normal cells is typically a result of chronic or persistent inflammation of tissues in response to stresses or a result of genetic mutations caused by carcinogens, or both [12]. For example, NO has been shown to mediate the neoplastic effect of the carcinogenic metal chromium (VI) on human lung epithelial cells through NO-mediated *S*-nitrosylation of the Bcl-2 protein [16, 17].

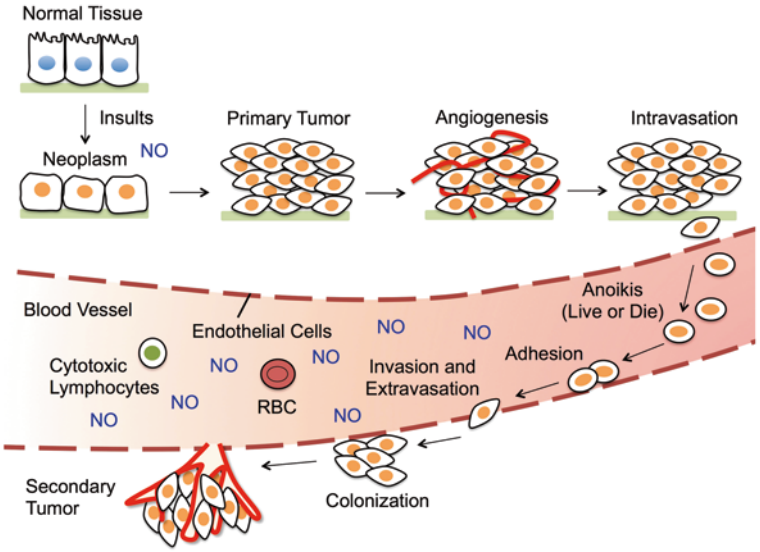


Fig. 8.1 Diagrammatic representation of major steps involved in cancer metastasis. Increased NO production has been associated with many human metastatic tumors. Its effects span from neoplastic transformation to tumor colonization at distant sites

The continuous expansion and progression of a primary tumor frequently leads to metastasis, a process in which a restricted proportion of tumor cells spreads from the primary tumor to form secondary tumors at distant sites. Because metastatic cells are generally resistant to radiation and chemotherapy, they are a major cause of cancer-related death and prime targets for novel cancer therapies [18]. To metastasize, tumor cells must acquire or possess the following properties [19, 20]: (i) unlimited or enhanced proliferative capacity (ii) vascularization within the surrounding host tissues through the synthesis and secretion of angiogenesis factors (iii) local invasion of the host stroma by tumor cells into the blood and/or lymphatic circulation (intravasation) (iv) survival of tumor cells in the circulation (anoikis resistance) (v) adhesion to the capillary wall (vi) invasion and penetration of the cells out of the circulation (extravasation) and (vii) colonization, proliferation, and angiogenesis of tumor cells at distant sites (Fig. 8.1). NO has been shown to participate in all of these steps, which are further discussed below.

S-nitrosylation and Anoikis

Apoptosisor programmed cell death is a tightly regulated process characterized by shrinkage of cells, blebbing of plasma membranes, and condensation and fragmentation of chromatin. Acquired apoptosis resistance is a hallmark of most, if not all, types of cancer that is implicated in the neoplastic evolution of pre-malignant cells

and in cancer metastasis [21]. With regards to metastasis, the loss of cell interaction with neighboring cells and the extracellular matrix (ECM) following intravasation into the circulation triggers apoptosis referred to as *anoikis* [22]. Anoikis prevents detached tumor cells from colonizing elsewhere, thereby, it is a critical step in determining cancer metastasis. Surviving anoikis facilitates subsequent reattachment and colonization of tumor cells at distant sites [23]. Clinical evidence has demonstrated a strong correlation between anoikis resistancein advanced stage cancers and poor survival of patients, strengthening the notion that anoikis resistance is a prerequisite for cancer metastasis [23, 24].

Anoikis is regulated by many signaling pathways, notably by the pro-survival signals phosphoinositide-3-kinase (PI3K)/Akt, extracellular signal-regulated kinases (ERK), Jun N-terminal kinases (JNK), and apoptosis-regulatory signals as well as certain membrane microdomains and oncogenes. A number of direct and indirect evidence suggests that increased NO production suppresses anoikis through *S*-nitrosylation of several target proteins described below.

S-Nitrosylation and Pro-Survival Signals

Abnormal regulation of the phosphatases/kinases including PI3K/Akt activates pro-survival signaling and suppresses anoikis [25]. Numajiri et al. demonstrated that PI3K/Akt on-off signaling was regulated through *S*-nitrosylation of phosphatase and tensin homolog deleted on chromosome ten (PTEN) [26]. *S*-nitrosylation of PTEN by a low level ($\leq$10 μM) of *S*-nitrosocysteine (SNOC) was shown to inhibit its phosphatase activity and subsequently increases Akt phosphorylation, kinase activity, and cell survival. In many cancers such as glioblastoma, prostate, lung and breast carcinoma, loss of PTEN confers resistance to anoikis [27, 28]. Kwak et al. demonstrated that *S*-nitrosylation of PTEN correlated with its ubiquitin-proteasomal degradation [29]. Although this *S*-nitrosylation-based regulation of PTEN was shown in the experimental model of neurons not cancers, it demonstrates a regulatory mechanism that might account for the loss of PTEN in aggressive tumors.

S-Nitrosylation and Apoptosis-regulatory Proteins

As a form of apoptotic cell death, anoikis is regulated through the common death receptor and mitochondrial apoptosis pathways (Fig. 8.2). The extrinsic death receptor pathway is activated through the cell surface death receptors (DRs) upon binding with specific death ligands such as Fas (CD95) ligand, tumor necrosis factor-α (TNF-α), and TNF-related apoptosis-inducing ligand (TRAIL). The death-inducing signaling complex (DISC) then assembles, activates initiator caspases (caspase-8 or FLICE and caspase-10), which subsequently activate effector caspases (caspase-3, caspase-6 and caspase-7) to cleave cellular substrates. FLIP (FLICE-inhibitory protein) has a higher affinity for the DISC than caspase-8, thus inhibiting caspase-8

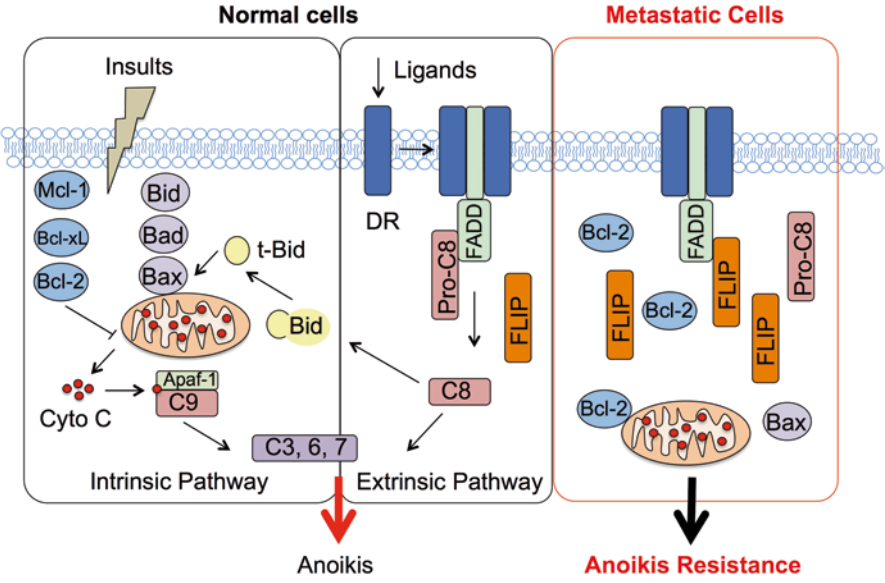


Fig. 8.2 Diagrammatic representation of the intrinsic (mitochondrial) and extrinsic (death receptor) pathway of apoptosis and anoikis subtype. In metastatic cancer cells, increased Bcl-2 and FLIP expression promote anoikis resistance

processing and apoptosis induction [30]. The intrinsic mitochondrial pathway is activated in response to various death signals, e.g. DNA damage, ROS/RNS stress and cytotoxic agents, leading to mitochondrial membrane depolarization, which is controlled by the balance of Bcl-2 family proteins including the anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1, and the pro-apoptotic proteins Bax, Bak, Bok, Bim, Bik, Bad and Bid. The subsequent released cytochrome C binds to the caspase adaptor molecule Apaf-1 and recruits the initiator procaspase-9 to form a molecular complex called the apoptosome, which functions to recruit effector caspases to induce apoptosis [31].

Several studies have demonstrated that metastatic malignant cells acquire anoikis resistance through an upregulation of anti-apoptotic proteins such as FLIP [32, 33] and Bcl-2 [34, 35] (Fig. 8.2). NO has been shown to suppress apoptosis induced by various agents including Fas ligand, chemotherapeutic agents, and heavy metals through *S*-nitrosylation of FLIP and Bcl-2 [36–38]. *S*-nitrosylation of these proteins at their cysteine residues prevents their degradation through the ubiquitin-proteasome pathway. We have recently shown that *S*-nitrosylation of FLIP also mediates apoptosis resistance by disrupting its own interaction with an NF-κB adaptor molecule, receptor-interacting protein 1 (RIP1), which results in NF-κB activation [39]. Figure 8.3a illustrates that FLIP binds to RIP1 in the absence of the death ligand TNF-α in HEK293 cells, and that this complex is disrupted by TNF-α treatment, which results in the translocation of RIP1 to the cell membrane. Lack of

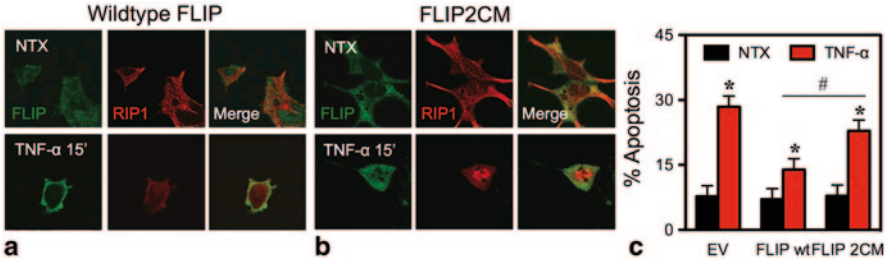


Fig. 3 *S*-nitrosylation of FLIP mediates its interaction with RIP1 and subsequent anti-apoptotic function. **A, B,** HEK-293 cells were transfected with wild-type FLIP **a** or FLIP2CM mutant. **b** Together with RIP1 plasmids. Cells were then treated with TNF-α (50 ng/mL) for 15 min and analyzed for FLIP/RIP1 colocalization by confocal microscopy. **c** Effect of *S*-nitrosylation on anti-apoptotic activity of FLIP. MCF-7 cells were transfected with empty vector (EV), wild-type FLIP or FLIP2CM mutant plasmid, after which they were treated with TNF-α (50 ng/mL) for 16 h. Apoptosis was then determined by flow cytometry using annexin V and propidium iodide assays. Both early and late apoptosis were combined and plotted. *$p < 0.05$ versus non-treated EV control. #$p < 0.05$ versus treated FLIP wild-type cells

FLIP *S*-nitrosylation in a FLIP double-cysteine mutant (FLIP2CM) inhibits the RIP1 translocation (Fig. 8.3b). The FLIP-RIP1 complex is believed to contribute to the reversed anti-apoptotic effect of FLIP in human breast carcinoma MCF-7 cells (Fig. 8.3c). Accordingly, it is postulated that NO might exert its anti-anoikis effect through *S*-nitrosylation of FLIP and Bcl-2.

S-Nitrosylation and Caveolin-1

Caveolin-1 is an essential constituent of caveolae, the flask-shaped membrane invaginations that occupy about 20% of the cell membrane [40]. Such invaginations provide a platform for various signaling mechanisms, where caveolin-1 interacts with signaling molecules and controls their subcellular distributions and functions. Caveolin-1 has been shown to play a role in the multidrug resistance of cancer cells partly through its interaction and regulation of multidrug resistance ATP-binding cassette sub-family G member 2 (ABCG2) transporter [41]. In the past decade, the role of caveolin-1 in the regulation of anoikis has gained increasing attention. Caveolin-1 expression has been shown to be associated with poor prognosis and metastasis of several types of cancer, including lung cancer, prostate cancer, renal cell carcinoma, hepatocellular carcinoma, and melanoma [42–44]. Ectopic expression of caveolin-1 was shown to prevent anoikis through various mechanisms, including p53 inactivation, upregulation of insulin-like growth factor (IGF)-I receptor, activation of Akt, and Mcl-1 stabilization in cancer cells [45–48]. A previous study by our group has shown that caveolin-1 expression is downregulated during anoikis through ubiquitin-proteasomal degradation and that NO inhibits this process by inducing *S*-nitrosylation of the protein, thus providing a mechanism by which cancer

cells acquire anoikis resistance [49]. In this study, caveolin-1 was shown to be nitrosylated and resistant to proteasomal degradation upon treatment with NO donors such as sodium nitroprusside (SNP) and diethylenetriamine (DETA) NONOate. Such treatments also inhibited anoikis, the effect that can be reversed by blocking caveolin-1 *S*-nitrosylation, thus supporting the role of *S*-nitrosylation in anoikis resistance of cancer cells.

S-Nitrosylation and Cell Migration and Invasion

Cell migration and invasion are the critical steps of cancer metastasis. To intravasate into the blood or lymphatic circulation and to extravasate out of the circulation, primary tumor cells must migrate and invade through the epithelial and vascular basement membranes and surrounding extracellular matrix [50]. It has been established that only a small fraction of primary tumor cells becomes invasive and eventually metastatic at any given time. NO has been reported to have both promoting and inhibitory effects on tumor cell mobility through the regulation of multiple proteins depending on its concentration. The role of *S*-nitrosylation of specific proteins on cell motility is discussed below.

S-Nitrosylation and Caveolin-1

As mentioned above, caveolin-1 is subjected to *S*-nitrosylation and is associated with metastasis and poor patient survival. In human lung carcinoma cells, we previously reported that NO promoted malignant transformation of the cells through a caveolin-1-dependent mechanism [49]. Caveolin-1 was also shown to promote both cell migration and invasion in human lung cancer and melanoma cells as indicated by their increased motility upon caveolin-1 overexpression and by decreased motility upon caveolin-1 knockdown [51]. A recent study by Sanuphan et al. demonstrated that prolonged exposure of human lung cancer cells, e.g. up to 14 days, to non-cytotoxic concentrations of DPTA NONOate increased cell motility through both caveolin-1-dependent and independent pathways [52]. In the caveolin-1-dependent pathway, caveolin-1 was found to activate focal adhesion kinase (FAK) and its downstream target Akt, whereas in the caveolin-1-independent pathway, Cdc42 and filopodia were activated. It was postulated that *S*-nitrosylation of caveolin-1 might regulate an on-off pattern that controls the FAK-Akt signaling.

S-Nitrosylation and c-Src

c-Src (cellular Src) is a tyrosine kinase that promotes cell invasion and metastasis in many human cancers, including colon, breast, pancreatic and brain cancer [53].

A previous study by Rahman et al. demonstrated that *S*-nitrosylation of c-Src at cysteine 498 in breast cancer MCF-7 cells is critical for its activation and cell invasion induced by SNAP and β-estradiol [54]. In breast cancer cells that have estrogen receptors with minimal invasive property such as MCF-7 cells, the promoting effect of β-estradiol is dependent on NO through eNOS induction. Further, FAK was found to be a substrate of c-Src since c-Src activation led to tyrosine phosphorylation of FAK. The authors suggested that FAK might be subjected to *S*-nitrosylation since it has the cysteine residue that corresponds to cysteine 498 of c-Src.

S-Nitrosylation and EGFR

Epidermal growth factor receptor (EGFR) contributes to the aggressive nature of basal-like subtype of breast cancer and colon cancers [55]. Previous studies have shown that iNOS expression is associated with EGFR phosphorylation and poor disease outcome of estrogen receptor negative (ER−) breast cancer patients [56]. Likewise, NO, at physiological concentrations, promoted ER—cell migration, thus suggesting that iNOS/NO signaling is involved in the cell aggressiveness [56]. *S*-nitrosylation of EGFR (and c-Src) in ER— breast cancer MDA-MB-468 cells induced by DETA/NO resulted in the activation of EGFR/c-Src kinases, which led to the induction of oncogenic c-Myc, Akt, STAT3, and β-catenin signaling pathways as well as the inhibition of tumor suppressor PPA2 [57]. One of the clinically relevant phenotypes of basal-like breast cancer is the CD44−/CD24+ cancer stem cell subpopulation, which requires STAT3 signaling for its proliferation. NO signaling via *S*-nitrosylation of EGFR was shown to upregulate CD44 expression concomitantly with STAT3 phosphorylation, suggesting its role in cancer stem cell regulation [57].

S-Nitrosylation and Ras

The Ras superfamily of small GTPase consists of many subfamilies, including Ras, Rho, and Rab. Among these, H-Ras, N-Ras, and K-Ras are the clinically most notable members because of their implication in cancers and their role as proto-oncogenes [58]. Mutations and activation of Ras proto-oncogenes have been found in about 30% of all human cancers. Ectopic expression of human or rodent H-Ras in noncancerous cells leads to increased invasiveness and acquisition of the metastatic phenotype [59]. Interestingly, these Ras proteins contain redox active residues that are sensitive to NO modifications [60]. Lim et al. reported that *S*-nitrosylation of H-Ras is required for its tumor promoter function [61]. Knockdown of wild-type H-Ras in the oncogenic K-Ras-driven pancreatic tumor CFPac1 cells reduced tumor xenograft growth in immunocompromised mice, the effect that can be reversed by re-expression of the wild-type H-Ras but not the H-Ras mutant lacking *S*-nitrosylation at cysteine 118. However, Raines et al. reported the suppressive role of *S*-nitrosylation on the tumorigenic effect of H-Ras in N293 cells (HEK-293

ectopically transfected with nNOS) [62]. It was suggested that the difference in NO sources, e.g. nNOS or iNOS, may attribute to the differential tumorigenic response.

K-Ras was reported to regulate colon cancer cell migration through a caveolin-1-dependent mechanism [63]. Ectopic expression of K-Ras in colon cancer HCT116 cells upregulated the expression of caveolin-1 through the Akt pathway, and caveolin-1 was, in turn, required for K-Ras signaling in promoting the HCT116 cell migration. Although the role of NO in the K-Ras/caveolin-1 regulatory axis has not been firmly established, it is likely that NO regulates this axis as both K-Ras and caveolin-1 are known targets for *S*-nitrosylation.

S-Nitrosylation and FLIP

FLIP is a key anti-apoptotic protein involved in the regulation of cell death. It also plays a role in cancer cell motility [64]. Downregulation of FLIP in human cervical cancer HeLa cells by siRNA impaired cell motility by enhancing Akt activity. As described earlier, *S*-nitrosylation of FLIP inhibits its ubiquitination and subsequent proteasomal degradation, thereby, stabilizing the protein and sustaining its anti-apoptotic activity. Although there is no direct evidence for the role of *S*-nitrosylation in HeLa cell motility, indirect evidence suggests its involvement. For example, inhibition of iNOS and NO production was reported to suppress HeLa cell migration and invasion as well as xenograft tumor growth [65, 66].

S-Nitrosylation and MMP-9

There has been a strong correlation between matrix metalloproteinases (MMPs) and ECM degradation and cancer cell invasion [67]. MMP-9 is a key proteinase that efficiently degrades native collagen type IV and V, fibronectin, entactin, and elastin. Its expression is elevated in various solid malignancies, including breast, bladder, prostate and ovarian cancer [68]. *S*-nitrosylation of MMP-9 was facilitated by its colocalization with iNOS at the leading edge of migrating trophoblasts, where NO production occurred. *S*-nitrosylation of MMP-9 resulted in its activation and increased trophoblast migration and invasion [69]. It is conceivable that the promoting effect of NO on tumor cell motility may be mediated, in part, through *S*-nitrosylation of MMP-9.

S-Nitrosylation and Angiogenesis

Angiogenesis, the physical process of new blood vessel formation, is an essential step for tumor growth and metastasis. Angiogenesis involves endothelial cell migration and vascular permeability which are subjected to NO regulation [70, 71]. In this

process, NO is synthesized through eNOS upon stimulation with angiogenic factors such as the vascular endothelial growth factor (VEGF) [72]. While *S*-nitrosylation of eNOS itself suppresses its enzymatic activity and, thus, NO production, *S*-nitrosylation of many other target proteins in the close proximity of eNOS and in the microenvironment enriched with NO enhance angiogenesis as further discussed below.

SDF-1α and S-Nitrosylation of MKP7

Stromal cell-derived factor-1α (SDF-1α), also called CXCL12, is one of the most potent pro-angiogenic CXC chemokines that plays a role in angiogenesis. In aortic endothelial cells, SDF-1α stimulated cell migration through eNOS activation [73]. Increased NO production by eNOS led to *S*-nitrosylation of MAP kinase phosphatase 7 (MKP7) and subsequent suppression of its activity. Under a basal condition, JNK3 is inactivated by MKP7. *S*-nitrosylation of MKP7 causes sustained JNK3 activation and ultimately endothelial cell migration and angiogenesis.

S-nitrosylation and β-catenin

An increase in vascular permeability is one of the early events during angiogenesis and a key characteristic of the newly formed vasculature in tumors. The vascular permeability is controlled by the adherens junction complex consisting of β-catenin and VE-cadherin. In aortic endothelial cells, such complex is regulated by *S*-nitrosylation of β-catenin, which facilitates its dissociation from VE-cadherin and reorganization of the adherens junction [74]. Together with its tyrosine phosphorylation by Src, *S*-nitrosylation of β-catenin promotes the disruption of adherens junction and increases endothelial permeability.

Conclusion

The effect of NO on tumor biology is broad, spanning from tumor initiation of cellular transformation to tumor progression of the metastatic cascade. Protein *S*-nitrosylation is a PTM process that has gained increasing prominence rivaling other known PTMs such as phosphorylation and ubiquitination. *S*-nitrosylation controls the function and activity of many cancer-associated proteins, thus, its dysregulation could lead to carcinogenesis and metastasis. Currently, numerous efforts have been made to develop novel anticancer therapeutics based on *S*-nitrosylation [75]. In this article, we review the role of protein *S*-nitrosylation in anoikis resistance, cell migration and invasion, and angiogenesis, which are key determinants of cancer metastasis. While most studies have indicated the positive regulatory role of

protein *S*-nitrosylation in cancer progression and metastasis, the suppressive role of this post-translational process has also been reported similarly to the observed dichotomous effects of NO. As we move forward, it will be essential to identify the key determining factors of these effects and answer some unresolved questions such as what tumor-associated proteins are involved, what are their mechanisms of action, how localization of NOS contributes to the protein *S*-nitrosylation, and how differential amounts of NO regulate the *S*-nitrosylation process. The past decade has provided exciting new discoveries on the diverse role of protein *S*-nitrosylation in cancer biology, but obviously we have only just started.

Acknowledgements This work was supported by the National Institutes of Health Grants R01-HL095579 and R01-ES022968.

Conflicts of Interest No potential conflicts of interest were disclosed.

References

1. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature. 1987;327:524–6.
2. Knowles RG. Nitric oxide synthases. Biochem Soc Trans. 1996;24:875–8.
3. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S. Nitric oxide synthase activity in human breast cancer. Br J Cancer. 1995;72:41–4.
4. Puhukka A, Kinnula V, Napankangas U, Saily M, Koistinen P, Paakko P, Soini Y. High expression of nitric oxide synthases is a favorable prognostic sign in non-small cell lung carcinoma. APMIS. 2003;111:1137–46.
5. Bakshi A, Nag TC, Wadhwa S, Mahapatra AK, Sarkar C. The expression of nitric oxide synthases in human brain tumours and peritumoral areas. J Neuro Sci. 1998;155:196–203.
6. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumour progression. Nat Rev Cancer. 2006;6:521–534.
7. Tse GMK, Wong FC, Tsang AKH, Lee CS, Lui PCW, Lo AWI, Law BKB, Scolyer RA, Putti TC. Stromal nitric oxide synthase (NOS) expression correlates with the grade of mammary phallodes tumour. J Clin Pathol. 2005;58:600–604.
8. Mocellin S, Bronte V, Nitti D. Nitric oxide a double edged sword in cancer biology: searching for therapeutic opportunities. Med Res Rev. 2007;27:317–352.
9. Heigold S, Sers C, Bechtel W, Ivanovas B, Schafer R, Bauer G. Nitric oxide mediates apoptosis induction selective in transformed fibroblasts compared to nontransformed fibroblasts. Carcinogenesis. 2002;23:929–41.
10. Ridnour LA, Thomas DD, Donzell D, Espey MG, Roberts DD, Wink DA, Isenberg JS. The biphasic nature of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol. 2001;2:149–56.
11. Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolocci N, Ambs S, Colton CA, Harris CC, Roberts DD, Wink DA. The chemical biology of nitric oxide: impications in cellular signaling. Free Radic Biol Med. 2008;45:18–31.
12. Iyer AKV, Azad N, Wang L, Rojanasakul Y. S-nitrosylation—how cancer cells say no to cell death. In: Bonavida B (ed.). Nitric Oxide (NO) and cancer, cancer drug delivery and development. New York: Springer Science + Business Media, LLC;2010.
13. Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. Protein S-Nitrosylation: purview and parameters. Nat Rev Mol Cell Biol. 2005;6:150–66.

14. Stamler JS, Lamas S, Fang FC. Nitrosylation. the prototypic redox-based signaling mechanism. Cell. 2001;78:931–6.
15. Lane P, Hao G, Gross SS. S-nitrosylation is emerging as a specific and fundamental post-translational protein modification: head-to-head comparison with O-Phosphorylation. Sci STKE. 2001;86:RE1.
16. Azad N, Iyer AKV, Wang L, Lu Y, Medan D, Castranova V, Rojansakul Y. Nitric oxide-mediated Bcl-2 stabilization potentiates malignant transformation of human lung epithelial cells. Am J Repair Cell Mol Biol. 2010;42:578–85.
17. Medan D, Luanpitpong S, Azad N, Wang L, Jiang BH, Davis ME, Barnett JB, Guo L, Rojanasakul Y. Multifunctional role of Bcl-2 in malignant transformation and tumorigenesis of Cr(VI)-transformed lung cells. PLoS ONE. 2012;7:e37045.
18. Chaffer CL, Weinberg R. A perspective on cancer cell metastasis. Science. 2011;331:1559–64.
19. Fidler I. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer. 2003;3:453–8.
20. Talmadge JE, Fidler IJ. AACR centennial series: the biology of cancer metastasis: historical perspective. Cancer Res. 2010;70:5649–69.
21. Stenner-Liewen F, Reed JC. Apoptosis and cancer: basic mechanisms and therapeutic opportunities in the postgenomic era. Cancer Res. 2003;63:263–8.
22. Frisch SM, Screaton RA. Anoikis mechanisms. Curr Opin Cell Biol. 2002;14:563–8.
23. Simpson CD, Anyiwe K, Schimmer AD. Anoikis resistance and tumor metastasis. Cancer Lett. 2008;272:177–85.
24. Sakamoto S, Kyprianou N. Targeting anoikis resistance in prostate cancer metastasis. Mol Aspects Med. 2010;31:205–14.
25. Khwaja A, Rodriguez-Viciana P, Wennstrom S., Warne PH, Downward1 J. Matrix adhesion and ras transformation both activate a phosphoinoistide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J. 1997;16:2783–93.
26. Numajiri N, Takasawa K, Nishiya T, Tanaka H, Ohno K, Hayakawa W, Asada M, Matsuda H, Azumi K, Kamata H, Nakamura T, Hara H, Minami M, Lipton SA, Uehara T. On–Off system for PI3-Kinase–Akt signaling through S-Nitrosylation of Phosphatase with sequence. Proc Natl Acad Sci USA. 2011;108:10349–54.
27. Hollander MC, Blumenthal GM, Dennis PA. PTEN loss in the continuum of common cancers, rare syndromes and mouse models. Nat Rev Cancer. 2011;11:289–301.
28. Vitolo MI, Weiss MB, Szmacinski M, Tahir K, Waldman T, Park BH, Martin SS, Weber DJ, Bachman KE. Deletion of PTEN promotes tumorigenic signaling, resistance to anoikis, and altered response to chemotherapeutic agents in human mammary epithelial cells. Cancer Res. 2009;69:2875–83.
29. Kwak YD, Ma T, Diao S, Zhang X, Chen Y, Hsu J, Lipton SA, Masliash E, Xu H, Liao FF. NO signaling and S-Nitrosylation regulate PTEN inhibition in neurodegeneration. Mol Neurodegener. 2010;5:49.
30. Guicciardi ME, Gores GJ. Life and death by death receptors. FASEB J. 2009;23:1625–37.
31. Tait SWG, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol. 2010;11:621–32.
32. Kim YN, Koo KH, Sung JY, Yun UJ, Kim H. Anoikis resistance: an essential prerequisite for tumor metastasis. Int J Cell Biol. 2012. doi:10.1155/2012/306879.
33. Mawji IA, Simpson CD, Hurren R, Gronda M, Williams MA, Filmus J, Jonkman J, Da Costa RS, Wilson BC, Thomas MP, Reed JC, Glinsky GV, Schimmer AD. Critical role for fas-associated death domain-like Interleukin-1-converting enzyme-like inhibitory protein in anoikis resistance and distant tumor formation. J Natl Cancer Inst. 2007;99:811–822.
34. Dingsheng L, Jie F, Weishan C. Bcl-2 and Caspase-8 related anoikis resistance in human osteosarcoma MG-63 cells. Cell Biol Int. 2008;32:1199–206.
35. Joseph MG, Melinda MM, Tawnya LB, Subbulakshmi V, Richard JB. ERK/BCL-2 pathway in the resistance of pancreatic cancer to anoikis. J Surg Res. 2009;152:18–25.

36. Chanvorachote P, Nimmannit U, Wang L, Stehlik C, Lu B, Azad N, Rojanasakul Y. Nitric oxide negatively regulates fas CD95-induced apoptosis through inhibition of ubiquitin-proteasome-mediated degradation of FLICE-inhibitory protein. J Biol Chem. 2005;280:42044–50.
37. Chanvorachote P, Nimmannit U, Stehlik C, Wang L, Jiang BH, Ongpipatanakul B, Rojanasakul Y. Nitric oxide regulates cell sensitivity to cisplatin-induced apoptosis through S-Nitrosylation and inhibition of Bcl-2 ubiquitination. Cancer Res. 2006;66:6353–60.
38. Azad N, Vallyathan V, Wang L, Tantishaiyakul V, Stehlik C, Leonard SS, Rojanasakul Y. S-nitrosylation of Bcl-2 inhibits its ubiquitin-proteasomal degradation. A novel antiapoptotic mechanism that suppresses apoptosis. J Biol Chem. 2006;281:34124–34.
39. Talbott SJ, Luanpitpong S, Stehlik C, Azad N, Iyer AK, Wang L, Rojansakul Y. S-Nitrosylation of FLICE-inhibitory protein determines its interaction with RIP1 and activation of NF-kB. Cell Cycle. 2014;13:1948–57.
40. Chunhacha P, Chanvorachote P. Roles of Caveolin-1 on anoikis resistance in non small cell lung cancer. Int J Physiol Pathophysiol Pharmacol. 2012;4:149–55.
41. Herzog M, Storch CH, Gut P, Kotlyar D, Fullekrug J, Ehehalt R, Haefeli WE, Weiss J. Knockdown of Caveolin-1 decreases activity of breast cancer resistance protein (BCRP/ABCG2) and increases chemotherapeutic sensitivity. Naunyn-Schmied Arch Pharmacol. 2011;383:1–11.
42. Yang G, Truong L, Timme TL, Ren C, Wheeler TM, Park SH, Nasu Y, Bangma CH, Kattan MW, Scardino PT, Thompson TC. Elevated expression of caveolin is associated with prostate and breast cancer. Clin Cancer Res. 1998;4:1873–80.
43. Ho CC, Huang PH, Huang HY, Chen YH, Yang PC, Hsu SM. Up-regulated Caveolin-1 accentuates the metastasis capability of lung adenocarcinoma by inducing filopodia formation. Am J Pathol. 2002;161:1647–56.
44. Patlolla JM, Swamy MV, Raju J, Rao CV. Overexpression of Caveolin-1 in experimental colon adenocarcinomas and human colon cancer cell lines. Oncol Rep. 2004;115:719–24.
45. Ravid D, Maor S, Werner H, Liscovitch M. Caveolin-1 inhibits anoikis and promotes survival signaling in cancer cells. Adv Enzyme Regul. 2006;46:163–75.
46. Ravid D, Moar S, Werner H, Liscovitch M. Caveolin-1 inhibits cell detachment-induced P53 activation and anoikis by upregulation of insulin-like growth factor-1 receptor and signaling. Oncogene. 2005;17:1338–47.
47. Li L, Ren CH, Tahir SA, Ren C, Thompson TC. Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. Mol Cell Biol. 2003;23:9389–404.
48. Chunhacha P, Pongrakananon V, Rojanasakul Y, Chanvorachote P. Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells. Am J Physiol Cell Physiol. 2012;302:C1284–92.
49. Chanvorachote P, Nimmannit U, Lu Y, Talbott S, Jiang BH, Rojanasakul Y. Nitric oxide regulates lung carcinoma cell anoikis through inhibition of ubiquitin-proteasomal degradation of caveolin-1. J Biol Chem. 2009;284:28476–84.
50. Bravo-Cordero JJ, Hodgson L, Condeelis J. Directed cell invasion and migration during metastasis. Curr Opin Cell Biol. 2012;24:277–83.
51. Luanpitpong S, Talbott S, Rojanasakul Y, Nimmannit U, Pongrakhananon V, Wang L, Chanvorachote P. Regulation of lung cancer cell migration and invasion by reactive oxygen species and caveolin-1. J Biol Chem. 2010;285:38832–40.
52. Sanuphan A, Chunhacha P, Pongrakhananon V, Chanvorachote P. Long-Term nitric oxide exposure enhances lung cancer cell migration. BioMed Res Int. 2013;2013:186972.
53. Irby RB, Yeatman TJ. Role of Src expression and activation in human cancer. Oncogene. 2000;19:5636–42.
54. Rahman MA, Senga T, Ito S, Hyodo T, Hasegawa H, Hamaguchi M. S-Nitrosylation at Cysteine 498 of c-Src tyrosine kinase regulates nitric oxide-mediated cell invasion. J Biol Chem. 2010;285:3806–14.
55. Hoadley KA, Weigman VJ, Fan C, Sawyer LR, He X, Troester MA, Sartor CI, Rieger-House T, Bernard PS, Carey LA, Perou CM. EGFR Associated expression profiles vary with breast tumor subtype. BMC Genomics. 2007;8:258.

56. Glynn SA, Boersma BJ, Dorsey TH, Yi M, Yfantis HG, Ridnour LA, Martin DN, Switzer CH, Hudson RS, Wink DA, Lee DH, Stephens RM, Ambs S. Increased NOS2 predicts poor survival in estrogen receptor-negative breast cancer patients. J Clin Invest. 2010;120:3843–54.
57. Switzer CH, Glynn SA, Cheng RYS, Ridnour LA, Green JE, Ambs S, Wink DA. S-Nitrosylation of EGFR and src activates an oncogenic signaling network in human basal-like breast cancer. Mol Cancer Res. 2012;10:1203–15.
58. Stites EC, Ravichandran KS. A systems perspective of ras signaling in cancer. Clin Cancer Res. 2009;15:1510–1513.
59. Campbell PM, Der CJ. Oncogenic ras and its role in tumor cell invasion and metastasis. Sem Cancer Biol. 2004;14:105–14.
60. Raines K, Bonini MG, Campbell SL. Nitric oxide cell signaling: S-Nitrosation of Ras superfamily GTPases. Cardiovasc Res. 2007;75:229–39.
61. Lim KH, Ancrile BB, Kashatus DF, Counter CM. Tumor maintenance is mediated by eNOS. Nature. 2008;452:646–9.
62. Raines K, Cao GL, Lee EK, Rosen GM, Shapiro P. Neuronal nitric oxide synthase-induced S-Nitrosylation of H-Ras inhibits calcium ionophore-mediated extracellular-signal-regulated kinase activity. Biochem J. 2006;397:329–36.
63. Basu Roy UK Henkhaus RS Loupakis F Cremolini C Gerner EW Ignatenko NA. Caveolin-1 is a novel regulator of K-RAS-dependent migration in colon carcinogenesis. Int J Cancer. 2013;133:43–58.
64. Shim E, Lee YS, Kim HY, Jeoung D. Down-regulation of c-FLIP increases reactive oxygen species, induces phosphorylation of serine/threonine kinase akt, and impairs motility of cancer cells. Biotechnol Lett. 2007;29:141–7.
65. Li M, Wang L, Liu H, Su B, Liu B, Lin W, Li Z, Chang L. Curcumin inhibits hela cell invasion and migration by decreasing inducible nitric oxide synthase. Nan Fang Yi Ke Da Xue Xue Bao (J South Med Univ). 2013;33:1752–6.
66. Dong J, Cheng M, Sun H. Function of inducible nitric oxide synthase in the regulation of cervical cancer cell proliferation and the expression of vascular endothelial growth factor. Mol Med Rep. 2014;9:583–9.
67. Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev. 2006;25:9–34.
68. Roy R, Yang J, Moses MA. Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer. J Clin Oncol. 2009;27:5287–97.
69. Harris LK, McCormick J, Cartwright JE, Whitley GStJ, Dash PR. S-Nitrosylation of proteins at the leading edge of migrating trophoblasts by inducible nitric oxide synthase promotes trophoblast invasion. Exp Cell Res. 2008;314:1765–76.
70. Lamalice L, Le Boeuf F, Hout J. Endothelial cell migration during angiogenesis. Circ Res. 2007;100:782–94.
71. Dvorak HF. Vascular permeability factor, vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J Clin Oncol. 2002;20:4368–80.
72. Lima B, Forrester MT, Hess DT, Stamler JS. S-Nitrosylation in cardiovascular signaling. Circ Res 2010;106:633–46.
73. Pi X, Wu Y, Ferguson III JE, Portbury AL, Patterson C. SDF-1a stimulates JNK3 activity via eNOS-dependent nitrosylation of MKP7 to enhance endothelial migration. Proc Natl Acad Sci USA. 2009;106:5675–80.
74. Thibeault S, Rautureau Y, Oubaha M, Faubert D, Wilkes BC, Delisle C, Gratton JP. S-Nitrosylation of b-catenin by eNOS-derived no promotes VEGF-induced endothelial cell permeability. Mol Cell. 2010;39:468–76.
75. Wang Z. Protein S-nitrosylation and cancer. Cancer Lett. 2012;320:123–29.

Part III
Modulation of Anti-tumor Immune Responses by NO

Chapter 9
Nitric Oxide, Immunity and Cancer: From Pathogenesis to Therapy

Hermes J. Garbán

In loving Memory of Professor Hermes Augusto Garbán Mendoza (August 28, 1936–October 08, 2013)

Abstract Defining the specific role of nitric oxide (NO) in the regulation of the immune response against cancer is not a simple task. Despite of being extensively studied, NO, reactive nitrogen species (RNS) and reactive oxygen species (ROS) still maintain their reputation of "double-edge-swords". However, by examining key issues related to their sources, concentrations and chemical nature and, their locations and neighboring molecules that potentially will be reacting with them, we will have a more precise interpretation of the functional aspects of NO and related RNS in the context of the immune response to tumor cells and pathogenesis of cancer. Variations in the local cellular concentration of the same reactive intermediates induce different outcomes of the immune response. NO and related reactive species trigger defined signal transduction pathways in cancer, and immune-related cells in a concentration-dependent manner. NO bioavailability and NO-dependent responses are strictly functions of the reactivity of ROS with NO-forming RNS. In this chapter, we will examine the basic biology of NO and related species in the context of the immune response to cancer in both their potential role in the pathogenesis of malignancies and also in the control and modulation of the immune response against tumor cells. We will discuss the potential use of NO and related species in the induction of specific anti-cancer activity by the immune system and the modulation of resistance or tolerogenic factors derived from the protective mechanism acquired by the tumor cells in order to evade the anti-tumor immune response.

Keywords Immunosensitization · Immune response · Apoptosis · Immunomodulation · Reactive oxygen species (ROS) · Reactive nitrogen species (RNS) · Immunotherapy · Cancer therapy · Tumor immunobiology · Cancer pathogenesis

H. J. Garbán (✉)
NantBioscience, Inc. | NantWorks, LLC. California NanoSystems Institute (CnSI) at the University of California, Los Angeles (UCLA). Jonsson Comprehensive Cancer Center at UCLA. Department of Medicine. Division of Dermatology. David Geffen School of Medicine at UCLA, NantBioscience, Inc. | NantWorks, LLC, 570 Westwood Plaza. Room 4523., Los Angeles, CA 90095, USA
e-mail: hgarban@gmail.com; hgarban@ucla.edu

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_9

Abbreviations

AG	Arginase
Ca^{2+}	Calcium
CaM	Calmodulin
CD#	Cluster of differentiation #
cGMP	Cyclic guanosine monophosphate
CTL	Cytotoxic T lymphocytes
CTLA4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cells
eNOS	Endothelial nos
FOXP3	Forkhead box P3
H_2O_2	Hydrogen peroxide
IFN-γ	Interferon gamma
IKB	Inhibitors kappa B
IKK	IκB kinase
IL-β	Interleukin 1 beta
iNOS	Inducible nos
LPS	Lipopolysaccharide
MDSC	Myeloid derived suppressor cells
NF-κB	Nuclear factor kappa
nNOS	Neuronal NOS
NO	Nitric oxide
NOHA	N hydroxyarginine
NOS	Nitric oxide synthase
O_2^-	Superoxide
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$,	Peroxynitrite
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SLE	Systemic lupus erythematosus
SNO	S-nitrosylation
SOD	Superoxide dismutase
TAA	Tumor associated antigens
TNF	Tumor necrosis factor
TNF-α	TNF Qlpha
YY1	Yin-yang 1

Introduction

Direct implications of nitric oxide (NO) or related species in the regulation of the immune response against malignancies have been addressed from various angles. However, when we refer to NO we are not addressing a single type of molecule,

we are referring to a milieu of reactive molecules termed reactive nitrogen species (RNS) with different chemical and biochemical properties and significant diverse biological functions.

In order to understand the role of these RNS in the induction and regulation of the immune system against malignancies (cancer), it is always useful to consider their sources (e.g., endogenous or exogenous), their concentrations and chemical nature and, their locations and neighboring molecules that potentially will be reacting with them. Nevertheless, the role of RNS such as NO in cancer is not limited to the elimination or control of cancerous cells via activation or modulation of the immune system (directly or indirectly), NO may contribute with the pathogenesis of cancer as well.

NO has recently joined the clinical arena of cancer therapy. There is an increasing amount of preclinical data supporting the specific role of NO in the sensitization of resistant cancerous cells to radio-, chemo-, and immunotherapy. In addition, novel targeted immunotherapeutic alternatives have been developed based on nitroergic modifications of proteins in order to increase the antigenic determinant domains and revealing new immunological targets.

NO can also act in the modulation of the immune system by the enhancement of tumor-specific immune response and the sensitization of resistant tumor cells to immune-related effector mechanisms by regulating the expression of immune response-related genes including those bellowing to the tumor necrosis factor (TNF) receptor family and, inflammatory cytokines and chemokines.

Despite its importance, the specific role of NO signaling in immunity and cancer has remained elusive and many controversial data found in the literature contributes to the difficulty in understanding the specific role of NO against cancer. A broad spectrum of activities has been assigned to either the physiology or the patho-physiology of NO in tumor cells.

Approximately half of the scientific literature will support the general role of NO on the pathogenesis of cancer and the other half will support the role of NO and related species as anti-cancer molecules. This functional dichotomy of NO in cancer could be settled by examining these studies under the criteria abovementioned: sources, concentration and chemical nature and, location of neighboring molecules to react. Understanding this functional landscape of NO and related species, immunity and cancer will contribute to the better design of preventive means and more specific therapeutic alternatives in oncology.

Herein, we will examine the basic biology of NO and related species in the context of the immune response to cancer, in both their potential role in the pathogenesis of malignancies and also in the control and modulation of the immune response against cancer. We will discuss the potential use of NO and related species in the induction of specific anti-cancer activity by the immune system and the modulation of resistance or tolerogenic factors derived from the protective mechanisms acquired by the tumor cells in order to evade the anti-tumor immune response.

Nitric Oxide: Basic Concepts

Nitric oxide is a diatomic molecule that plays important roles as the smallest pleiotropic signaling messenger in mammalian cells [1]. The free radical, $NO^{\bullet}$, is an uncharged molecule containing an unpaired electron, enabling it to undergo several reactions functioning either as a weak oxidant or as an anti-oxidant. $NO^{\bullet}$ is able to react with other inorganic molecules (e.g., oxygen, superoxide or transition metals), nucleic acids (e.g., pyrimidine bases), prosthetic groups (e.g., heme) or with proteins leading to S-nitrosylation of thiol groups, nitration of tyrosine residues or disruption of metal−sulfide clusters such as zinc-finger domains or iron−sulfide complexes [2]. NO can function as an anti-oxidant against reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) by diffusing and concentrating into the hydrophobic core of lipids [3]. In addition, NO can react with O_2^- to form peroxynitrite ($ONOO^-$), a highly oxidizing and nitrating reactive nitrogen species (RNS) responsible for mediating protein oxidation reactions under physiological conditions [4]. Noteworthy, one mechanism of NO-related reactivity is through the addition of an NO group to the thiol side chain of cysteine residues within proteins and peptides, termed S-nitrosylation, which plays a significant role in the ubiquitous influence of NO on cellular signal transduction [5].

NO is biologically synthesized by nitric oxide synthases (NOS). NOS catalyze the oxidation of L-arginine resulting in the formation of NO and L-citrulline. NO is produced by three different NOS, two of which are generally constitutively expressed, primarily in neurons (nNOS or Type I) and endothelial cells (eNOS or Type III), respectively [6–8]. An inducible isoform (iNOS or Type II) can be upregulated considerably in immune cells and many other tissues [9, 10]. It has been shown that IFN-γ alone or in combination with TNF-α, interleukin 1β (IL-1β) and bacterial lipopolysaccharide (LPS) can induce the expression of iNOS in a wide variety of tissue organs and in some tumor cell lines [11–13]. The inducible type of nitric oxide synthase (iNOS) is considered to be a central protein in the regulation of the immune response against tumor cells [14, 15].

Nitric Oxide and Immunity

Nitric oxide is an important component of the immune system. Early studies have shown that a substance that was released by macrophages and exhibited a wide range of pathogen toxicity and antitumor activity also required arginine for its production (Hibbs and coworkers) [16, 17]. These data supported an earlier observation that plasma levels of nitrite and nitrates increased upon infection, suggesting an increase in endogenous production of NO [18]. Furthermore, a pivotal connection between NO and the immune response was the observation that IL-2-mediated immune activation increased NO levels in patients and promoted tumor eradication in mice [19, 20]. Moreover, significant evidence that macrophages made nitrite and nitrate, as well as nitrosamines, was reported by a number of groups [21–23]. Studies by Stuehr and Nathan [24] have shown that NO generated by macrophages

could kill leukemia cells. In addition, it has been demonstrated the formation of iron-NO complexes within activated macrophages [25]. Although some of these studies are referring to the direct toxicity of NO on infectious pathogens or their cellular components, the large majority of these studies have demonstrated an active NO-related anti-tumor immune response.

The Ca^{2+}/CaM-independent inducible isoform iNOS is found in various cell types including macrophages, dendritic cells, fibroblasts, chondrocytes, osteoclasts, astrocytes, epithelial cells, and a variety of cancer cells. iNOS is generally associated with the immune system and is stimulated and upregulated via induction by cytokines and/or microbial agents such as LPS and is responsible for generating large amounts of NO sustained over long periods of time for the host defense against pathogens [26].

NO produced by iNOS within the cell can range from 10 nM to μM amounts for several days [27]. This generation of high levels of NO can control various NO-modulated effects within a tissue, each with potentially different functions. Therefore, induction of iNOS is not only characterized by the generation of NO in high local amounts, it can also generate a wide range of NO for variable periods of time [28]. iNOS provides a unique flexible response to a variety of immunological challenges.

An additional level of immune regulation by iNOS is its capacity to generate products other than NO. These include N-hydroxyarginine (NOHA) and O_2^-. The generation of NOHA by iNOS has been shown to inhibit arginase (AG) activity, affecting the pathways that mediate cell growth (ornithine to polyamines) or tissue matrices (ornithine to proline) [29]. This diversity of NOS activities can produce different temporal and concentration profiles of NO as well as other products to facilitate and broaden the functional versatility of these enzymes during the immune response [30].

Regulation of Immunological Apoptosis-related Genes: The NF-κB Case

The most relevant transcription factor participating in the regulation of genes involved in apoptosis and the immune response is the nuclear factor kappa B (NF-κB) promoting the expression of anti-apoptotic genes and regulating pro-inflammatory cytokine expression [31–34].

NF-κB transcription factors are assembled through the dimerization of five subunits: RelA (p65), c-Rel, RelB, p50/NF-κB1 and p52/NF-κB2 [35]. In resting –unstimulated– state, most NF-κB dimmers are sequestered in the cytoplasm by binding to specific inhibitors IκBs. Cell stimulation activates the IκB kinase (IKK) complex. Activated IKK phosphorylates NF-κB-bound IκB proteins and targets them for polyubiquitination and rapid proteasome-mediated degradation [36]. Freed NF-κB dimers translocate to the nucleus where they control the transcriptional activation of several target genes in concert with other transcription factors [37–39].

For many of the immune pathways that are regulated by NO and ROS, NF-κB is critical in orchestrating the innate immune response outcomes [40, 41]. NF-κB is an oxidative stress-responsive transcription factor activated by reactive oxygen species (e.g., H_2O_2, $O^{\bullet}{}_2^-$, etc.) generated as part of the signaling cascade triggered by many molecules such as TNF-α [42, 43]. ROS have been implicated in the signaling pathways initiated by TNF-α. Stimulation of mammalian cells with TNF-α triggers the generation of various ROS [44, 45]. Moreover, the use of antioxidants resulted in the inhibition of various TNF-α-related effects, such as the activation of transcription factors, gene expression, and cytotoxicity, and exogenous ROS mimic TNF-α biological activity [46]. In biological systems the most important ROS generated upon TNF-α stimulation are the result of enzymatic partial reduction of oxygen yielding $O^{\bullet}{}_2^-$, which is immediately disproportionated by superoxide dismutase (SOD) to H_2O_2 and O_2 or rapidly reacts with NO generating $ONOO^-$ [47–49].

It has been shown that NO sensitizes malignant cells to TNF-α-mediated apoptosis through the specific disruption of the TNF-α-induced generation of H_2O_2 and the subsequent inhibition of the NF-κB-dependent expression of anti-apoptotic genes [50].

In addition, NF-κB can be regulated by NO or related molecules *via* inhibition of its activation. It was originally suggested that NO stabilized the NF-κB inhibitor, IκBα, by preventing its degradation from NF-κB. NO also increased the mRNA expression of IκBα, but not NF-κB subunits, p65 or p50, suggesting specific transcriptional induction of IκBα by NO [51]. Also, NF-κB can be inhibited directly by NO through S-nitrosylation (SNO) of the p50 subunit. This SNO modification of NF-κB has been shown to prevent binding to its target DNA site [52, 53].

NO can act directly or indirectly on the transcriptional machinery, orchestrating the expression of apoptosis/survival genes related to the immune response against cancer, either by affecting the signaling molecules that will activate or repress transcription factors or by directly modifying key transcription factors and their DNA binding activity. It can be also cGMP dependent or independent following the general principles of "small concentrations" of NO, in a tight cellular environment NO will tend to favor a cGMP-dependent mechanism of regulation, whereas "high concentrations" of NO will trigger a cGMP-independent set of actions.

Deregulation of the expression of genes involved in apoptosis and immune response has been shown to be a critical aspect in determining the development and progression of numerous cancer types. Therefore, understanding the molecular mechanism involved in the control of apoptosis-related gene expression might facilitate the development of targeted anti-tumor therapies.

The dynamic coordination of genetic factors plays a major role in the regulation of apoptosis-related gene expression associated to the immune system under physiological or pathophysiological conditions. Uncontrolled activation of several transcription factors regulating the expression of genes involved in either pro-apoptotic or anti-apoptotic pathways have been identified as key players in the acquisition of the resistant phenotype of tumor cells. Among these transcription factors, we have examined the specific role of NO on the activity of the NF-κB as one of the most important regulators of anti-apoptotic gene expression and immune response.

Nevertheless, there are other important factors such as yin-yang 1 (YY1) as a novel regulator (transcriptional repressor) of pro-apoptotic receptors and immune regulator, p53 as a key modulator of cell cycle and pro-apoptosis pathways and FOXP3 as a novel tolerogenic and apoptosis-resistance regulator in tumor cells and immune related cells. Thus, specific targeting of these genetic factors by NO or related species regulating the tumor cell sensitivity to apoptosis represents a plausible therapeutic alternative that can be used alone or in combination with already established anti-cancer immunotherapy [54].

Nitric Oxide: Pathogenesis of Cancer

The specific roles of NO in the immune responses and immunotherapy do not escape from controversy. As we have stated in previous sections, there are confounding data that can mislead the possible role of NO in the control of the immune response against cancer. On one hand, we have the role of NO inducing suppression of the immune system by increasing the killing of tumor reactive T cells, activating suppressive mechanisms or inducing the proliferation of T regulatory cells [55–57].

NO is also involved in immunosuppression by regulating circulating immune cells. For example, myeloid-derived suppressor cells (MDSCs) can be activated by NO-mediated increases in cGMP, which in turn, facilitates their binding to Cytotoxic T lymphocytes (CTLs) and reduces T cell proliferation [58]. When cell-to-cell contacts are formed, the expression of AG and iNOS are required to induce apoptosis [59]. Increased iNOS activity is also found in mature dendritic cells (DCs), where NO is associated with suppression of T cell proliferation. Furthermore, when activated by IFN-γ, TNF-α, or IL-1α/β, MDSCs produce chemokines and iNOS, which lead to the immunosuppression of those T cells in the vicinity of the MDSCs [60]. The resulting increase in chemokines and iNOS leads to the attenuation of T cell responsiveness. In general, T cell responses are decreased by NO.

Nitric Oxide, RNS, ROS and Anti-Cancer Therapy

Oxidative stress, a major component of the immune response, is associated with infection, inflammation, aging, etc. Clinically, a milieu of conditions is associated with oxidative damage including chronic inflammatory and autoimmune diseases, cancer, and age-related disorders [61–65]. As mentioned above, oxidative stress is mediated in its majority by reactive oxygen species (ROS) and reactive nitrogen species (RNS) among others. ROS are oxygen-based molecules possessing high chemical reactivity. These include biologically-produced free radicals (superoxide and hydroxyl radical, NO, etc) and non-radical species such as hydrogen peroxide and peroxynitrite [66].

Free radicals are reactive chemical species containing one or more unpaired electrons occupying an outer orbital. They can arise either by the univalent pathway of oxygen reduction or as a consequence of enzymatic/non-enzymatic reactions. The superoxide anion radical O_2^- is formed by the one electron reduction of O_2. The two electron reduction product of O_2 in the fully protonated form is H_2O_2 while the three electron reduction product of O_2 is the hydroxyl radical ($OH^\bullet$). A number of enzymatic and non-enzymatic reactions reduces oxygen to the more reactive superoxide radical. Though hydrogen peroxide is not a free radical by itself, it can lead to the formation of the more dangerous hydroxyl radical via the Fenton type reaction [67].

Exposure of proteins to ROS and RNS alters their composite amino acids and structure thereby generating neo-antigens (a neo-antigen being typically defined as a previously unrecognized host-derived protein which becomes immunogenic usually due to new physical or genetic modifications). However, the oxidative damage to biomolecules is rarely specific and is dependent on the concentration of the protein, its cellular location with respect to cellular oxidant generating systems and the rate of modified protein clearance [66, 68].

While the direct role of free radicals in causing oxidative damage at the molecular level has been known for decades, the extent to which oxidative damage alters tissue/organ function is still under intense research. In immunology, oxidative damage has been implicated in several autoimmune diseases, including systemic lupus erythematosus (SLE) where aberrant immune responses against neo-antigens suggest impairment of immune tolerance mechanisms (Reviewed in [66]). Factors that induce the formation of neo-antigens include inflammation, infection, drugs, ROS, and environmental factors.

Initial results indicate that the adaptive immune response is indeed enhanced by oxidative processes. With regards to humoral immunity, co-administration of oxidized carbohydrates with antigen increases the secretion of antigen-specific immunoglobulins. Parallel studies of T cell-dependent immune responses demonstrate similar increases in responsiveness when using the Schiff base-forming agent tucaresol during immunization [69]. Furthermore, endogenous NO generation by cytokine induction in immune-related cells and exogenous NO (provided locally by NO-releasing compounds) have been demonstrated to be essential for the priming of the immune response (T cell priming) against specific antigens and some tumor associated antigens (TAAs) [57, 70].

From Autoimmunity to Cancer Therapy

Autoimmune disorders display a spectrum of severities and durations. On one end, improvements in treatment options have allowed patients to enjoy qualities and durations of life nearly identical to those observed in healthy individuals for some forms of autoimmunity. On the other end of the spectrum, certain autoimmune disorders are devastatingly aggressive, incurring intense periods of tissue destruction,

pain, and the shortening of life expectancy to as little as 6 months post diagnosis. Research conducted over the past few decades has focused on identifying many of the environmental and genetic risk factors associated with autoimmunity. The identification of the T cell surface protein cytotoxic T lymphocyte antigen 4 (or CTLA-4) is one of the most interesting discoveries in this field. CTLA-4 serves to inhibit T cell immune responses and competes with the activator protein CD28 for the same ligands, CD80 and CD86 [71]. More recently, blockade of CTLA-4 in cancer patients using monoclonal antibodies has emerged as one of the last lines of therapy against chemotherapy-resistant tumors. The anti-cancer activity of CTLA-4 blockade is believed to arise from subsequent immunological recognition and response against previously "masked" cancer neo-antigens, illustrating the potential of neo-antigen-revealing immunotherapy in combating cancer [72, 73].

Final Remarks, Conclusions

Although extensively studied, the roles of NO and related species in the immunological outcome of cancer still remains as a debatable issue. In order to understand and sort the most realistic interpretation of the data and previous studies, we have to consider the broad spectrum of activities that have been assigned to either the physiology or the pathophysiology of NO in tumor cells (for a review, see [74]. First, we have to consider the amount and sources of NO, RNS and ROS generated. Low-output of NO has been correlated with increased blood flow and new blood vessels (angiogenesis) feeding the tumor area [75]. In addition, the generation of NO by tumor cells may inhibit the activation and proliferation or increase apoptosis of surrounding lymphocytes that can account for the immune suppression observed that accompanies tumor growth. Furthermore, high intratumoral-output of NO could inhibit the activation of caspases and therefore antagonizes the pro-apoptotic signals [76, 77]. However, the opposite effect also has been observed in many other systems whereby the generation of high-output of NO, either by iNOS induction or by the use of NO donors, inhibits tumor growth, metastasis and sensitize to immunotherapy [11, 16, 50, 78, 79]. Therefore, the final outcome of NO-mediated signaling will be determined by many factors including the local concentration and sources of NO in the tissue, and the presence of reactive molecules that might redirect the redox status in the cell with the potential of synergize with other anticancer therapeutic modalities and the development of innovative NO-based therapies.

Acknowledgements This study was partially supported by a private development grant given by California Capital Equity to H.J.G. (CCE-20131672) and an institutional support from the Division of Dermatology and the Department of Medicine of the University of California, Los Angeles (UCLA).

Conflict of Interest Dr. Hermes J. Garbán is currently the Head of Therapeutic Antibody Discovery and fully employed at NantBioscience, Inc. an affiliate of NantWorks, LLC.

References

1. Nathan C. Nitric oxide as a secretory product of mammalian cells. Faseb J. 1992;6(12): 3051–64.
2. Bogdan C. Nitric oxide and the regulation of gene expression. Trends Cell Biol. 2001;11(2):66–75.
3. Benz D, Cadet P, Mantione K, Zhu W, Stefano G. Tonal nitric oxide and health–a free radical and a scavenger of free radicals. Med Sci Monit. 2002;8(1):RA1–4.
4. Tuteja N, Chandra M, Tuteja R, Misra MK. Nitric oxide as a unique bioactive signaling messenger in physiology and pathophysiology. J Biomed Biotechnol. 2004;2004(4):227–37.
5. Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. Protein S-nitrosylation: purview and parameters. Nat Rev Mol Cell Biol. 2005;6(2):150–66.
6. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J. 1994;298(Pt 2): 249–58.
7. Mayer B, Hemmens B. Biosynthesis and action of nitric oxide in mammalian cells [published erratum appears in Trends Biochem Sci 1998 Feb;23(2):87]. Trends Biochem Sci. 1997;22(12):477–81.
8. Nathan C, Xie QW. Regulation of biosynthesis of nitric oxide. J Biol Chem. 1994;269(19):13725–8.
9. Schmidt HH, Walter U. NO at work. Cell. 1994;78(6):919–25.
10. Xie Q, Nathan C. The high-output nitric oxide pathway: role and regulation. J Leukoc Biol. 1994;56(5):576–82.
11. Garban HJ, Bonavida B. Nitric oxide sensitizes ovarian tumor cells to Fas-induced apoptosis. Gynecol Oncol. 1999;73(2):257–64.
12. Geller DA, Nussler AK, Di Silvio M, Lowenstein CJ, Shapiro RA, Wang SC, Simmons RL, Billiar TR. Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. Proc Natl Acad Sci U S A. 1993;90(2):522–6.
13. Sherman PA, Laubach VE, Reep BR, Wood ER. Purification and cDNA sequence of an inducible nitric oxide synthase from a human tumor cell line. Biochemistry. 1993;32(43):11600–5.
14. Hokari A, Zeniya M, Esumi H. Cloning and functional expression of human inducible nitric oxide synthase (NOS) cDNA from a glioblastoma cell line A-172. J Biochem (Tokyo). 1994;116(3):575–81.
15. Langrehr JM, Hoffman RA, Lancaster JR Jr, Simmons RL. Nitric oxide–a new endogenous immunomodulator. Transplantation. 1993;55(6):1205–12.
16. Hibbs JB, Jr, Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Science. 1987;235(4787):473–6.
17. Hibbs JB, Jr, Taintor RR, Vavrin Z, Rachlin EM. Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem Biophys Res Commun. 1988;157(1):87–94.
18. Green LC, Ruiz de Luzuriaga K, Wagner DA, R and W, Istfan N, Young VR, Tannenbaum SR. Nitrate biosynthesis in man. Proc Natl Acad Sci U S A. 1981;78(12):7764–8.
19. Hibbs JB, Jr, Westenfelder C, Taintor R, Vavrin Z, Kablitz C, Baranowski RL, Ward JH, Menlove RL, McMurry MP, Kushner JP, et al. Evidence for cytokine-inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy. J Clin Invest. 1992;89(3):867–77.
20. Yim CY, McGregor JR, Kwon OD, Bastian NR, Rees M, Mori M, Hibbs JB, Jr, Samlowski WE. Nitric oxide synthesis contributes to IL-2-induced antitumor responses against intraperitoneal Meth A tumor. J Immunol. 1995;155(9):4382–90.
21. Stuehr DJ, Marletta MA. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc Natl Acad Sci U S A. 1985;82(22):7738–42.
22. Marletta MA. Mammalian synthesis of nitrite, nitrate, nitric oxide, and N-nitrosating agents. Chem Res Toxicol. 1988;1(5):249–57.
23. Kosaka H, Wishnok JS, Miwa M, Leaf CD, Tannenbaum SR. Nitrosation by stimulated macrophages. Inhibitors, enhancers and substrates. Carcinogenesis. 1989;10(3):563–6.

24. Stuehr DJ, Nathan CF. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med. 1989;169(5):1543–55.
25. Lancaster JR Jr, Hibbs JB Jr. EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. Proc Natl Acad Sci U S A. 1990;87(3):1223–7.
26. Blaise GA, Gauvin D, Gangal M, Authier S. Nitric oxide, cell signaling and cell death. Toxicology. 2005;208(2):177–92.
27. Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolocci N, Ambs S, Colton CA, Harris CC, Roberts DD, Wink DA. The chemical biology of nitric oxide: implications in cellular signaling. Free Radic Biol Med. 2008;45(1):18–31.
28. Espey MG, Miranda KM, Pluta RM, Wink DA. Nitrosative capacity of macrophages is dependent on nitric-oxide synthase induction signals. J Biol Chem. 2000;275(15):11341–7.
29. Boucher JL, Custot J, Vadon S, Delaforge M, Lepoivre M, Tenu JP, Yapo A, Mansuy D. N omega-hydroxyl-L-arginine, an intermediate in the L-arginine to nitric oxide pathway, is a strong inhibitor of liver and macrophage arginase. Biochem Biophys Res Commun. 1994;203(3):1614–21.
30. Wink DA, Hines HB, Cheng RY, Switzer CH, Flores-Santana W, Vitek MP, Ridnour LA, Colton CA. Nitric oxide and redox mechanisms in the immune response. J Leukoc Biol. 2011;89(6):873–91.
31. Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell. 1996;87(3):565–76.
32. Beg AA, Baltimore D. An Essential Role for NF-kB in Preventing TNF-alpha-Induc Cell Death. Science. 1996;274(1):782–4.
33. Van Antwerp DJ Martin SJ Kafri T Green DR Verma IM. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science. 1996;274(5288):787–9.
34. Wang CY, Mayo MW, Baldwin AS Jr. TNF—and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB [see comments]. Science. 1996;274(5288):784–7.
35. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. Cell. 2002;109:S81–96.
36. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol. 2000;18:621–63.
37. Werner SL, Barken D, Hoffmann A. Stimulus specificity of gene expression programs determined by temporal control of IKK activity. Science. 2005;309(5742):1857–61.
38. Park JM, Greten FR, Wong A, Westrick RJ, Arthur JS, Otsu K, Hoffmann A, Montminy M, Karin M. Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis–CREB and NF-kappaB as key regulators. Immunity. 2005;23(3):319–29.
39. Covert MW, Leung TH, Gaston JE, Baltimore D. Achieving stability of lipopolysaccharide-induc NF-kappaB activation. Science. 2005;309(5742):1854–7.
40. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol. 1994;12:141–79.
41. Gerondakis S, Fulford TS, Messina NL, Grumont RJ. NF-kappaB control of T cell development. Nat Immunol. 2014;15(1):15–25.
42. Hong YH, Peng HB, La Fata V, Liao JK. Hydrogen peroxide-mediated transcriptional induction of macrophage colony-stimulating factor by TGF-beta1. J Immunol. 1997;159(5):2418–23.
43. Schreck R, Albermann K, Baeuerle PA. Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). Free Radic Res Commun. 1992;17(4):221–37.
44. Garcia-Ruiz C, Colell A, Mari M, Morales A, Fernandez-Checa JC. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. J Biol Chem. 1997;272(17):11369–77.
45. Hennet T, Richter C, Peterhans E. Tumour necrosis factor-alpha induces superoxide anion generation in mitochondria of L929 cells. Biochem J. 1993;289(Pt 2):587–92.
46. Suzuki YJ, Forman HJ, Sevanian A. Oxidants as stimulators of signal transduction. Free Radic Biol Med. 1997;22(1–2):269–85.

47. Huie RE, Padmaja S. The reaction of no with superoxide. Free Radic Res Commun. 1993;18(4):195–9.
48. Fukuto JM, Wink DA. Nitric oxide (NO): formation and biological roles in mammalian systems. Met Ions Biol Syst. 1999;36:547–95.
49. Szabo C, Ohshima H. DNA damage induced by peroxynitrite: subsequent biological effects. Nitric Oxide. 1997;1(5):373–85.
50. Garban HJ, Bonavida B. Nitric oxide disrupts H2O2-dependent activation of nuclear factor kappa B. Role in sensitization of human tumor cells to tumor necrosis factor-alpha -induced cytotoxicity. J Biol Chem. 2001;276(12):8918–23.
51. Peng HB, Libby P, Liao JK. Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. J Biol Chem. 1995;270(23):14214–9.
52. DelaTorre A, Schroeder RA, Kuo PC. Alteration of NF-kappa B p50 DNA binding kinetics by S-nitrosylation. Biochem Biophys Res Commun. 1997;238(3):703–6.
53. Matthews JR, Botting CH, Panico M, Morris HR, Hay RT. Inhibition of NF-kappaB DNA binding by nitric oxide. Nucleic Acids Res. 1996;24(12):2236–42.
54. Olson SY, Garban HJ. Regulation of apoptosis-related genes by nitric oxide in cancer. Nitric Oxide. 2008;19(2):170–6.
55. Brito C, Naviliat M, Tiscornia AC, Vuillier F, Gualco G, Dighiero G, Radi R, Cayota AM. Peroxynitrite inhibits T lymphocyte activation and proliferation by promoting impairment of tyrosine phosphorylation and peroxynitrite-driven apoptotic death. J Immunol. 1999;162(6):3356–66.
56. Chen H, Li M, Campbell RA, Burkhardt K, Zhu D, Li SG, Lee HJ, Wang C, Zeng Z, Gordon MS, Bonavida B, Berenson JR. Interference with nuclear factor kappa B and c-Jun NH(2)-terminal kinase signaling by TRAF6C small interfering RNA inhibits myeloma cell proliferation and enhances apoptosis. Oncogene. 2006;25:6520–7.
57. Niedbala W, Wei XQ, Piedrafita D, Xu D, Liew FY. Effects of nitric oxide on the induction and differentiation of Th1 cells. Eur J Immunol. 1999;29(8):2498–505.
58. Serafini P, Meckel K, Kelso M, Noonan K, Califano J, Koch W, Dolcetti L, Bronte V, Borrello I. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med. 2006;203(12):2691–702.
59. Talmadge JE. Pathways mediating the expansion and immunosuppressive activity of myeloid-derived suppressor cells and their relevance to cancer therapy. Clin Cancer Res. 2007;13(18 Pt 1):5243–8.
60. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell. 2008;2(2):141–50.
61. Csiszar A, Toth J, Peti-Peterdi J, Ungvari Z. The aging kidney: role of endothelial oxidative stress and inflammation. Acta Physiol Hung. 2007;94(1–2):107–15.
62. Federico A, Morgillo F, Tuccillo C, Ciardiello F, Loguercio C. Chronic inflammation and oxidative stress in human carcinogenesis. Int J Cancer. 2007;121(11):2381–6.
63. Hald A, Lotharius J. Oxidative stress and inflammation in Parkinson's disease: is there a causal link? Exp Neurol. 2005;193(2):279–90.
64. Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. Free Radic Biol Med. 2007;43(4):477–503.
65. Weiskopf D, Schwanninger A, Weinberger B, Almanzar G, Parson W, Buus S, Lindner H, Grubeck-Loebenstein B. Oxidative stress can alter the antigenicity of immunodominant peptides. J Leukoc Biol. 2010;87(1):165–72.
66. Kurien BT, Scofield RH. Autoimmunity and oxidatively modified autoantigens. Autoimmun Rev. 2008;7(7):567–73.
67. Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, Shah AM. NADPH oxidases in cardiovascular health and disease. Antioxid Redox Signal. 2006;8(5–6):691–728.
68. Griffiths HR. Is the generation of neo-antigenic determinants by free radicals central to the development of autoimmune rheumatoid disease? Autoimmun Rev. 2008;7(7):544–9.

69. Charo J, Lindencrona JA, Carlson LM, Hinkula J, Kiessling R. Protective efficacy of a DNA influenza virus vaccine is markedly increased by the coadministration of a Schiff base-forming drug. J Virol. 2004;78(20):11321–6.
70. Koncz A, Pasztoi M, Mazan M, Fazakas F, Buzas E, Falus A, Nagy G. Nitric oxide mediates T cell cytokine production and signal transduction in histidine decarboxylase knockout mice. J Immunol. 2007;179(10):6613–9.
71. Chambers CA, Kuhns MS, Egen JG, Allison JP. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. Annu Rev Immunol. 2001;19:565–94.
72. Naidoo J, Page DB, Wolchok JD. Immune modulation for cancer therapy. Br J Cancer. 2014;111:2214–9.
73. Wolchok JD, Hodi FS, Weber JS, Allison JP, Urba WJ, Robert C, O'Day SJ, Hoos A, Humphrey R, Berman DM, Lonberg N, Korman AJ. Development of ipilimumab: a novel immunotherapeutic approach for the treatment of advanced melanoma. Ann N Y Acad Sci. 2013;1291:1–13.
74. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev. 1991;43(2):109–42.
75. Jenkins DC, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S. Roles of nitric oxide in tumor growth. Proc Natl Acad Sci U S A. 1995;92(10):4392–6.
76. Liu L, Stamler JS. NO: an inhibitor of cell death. Cell Death Differ. 1999;6(10):937–42.
77. Liu H, Sidiropoulos P, Song G, Pagliari LJ, Birrer MJ, Stein B, Anrather J, Pope RM. TNF-alpha gene expression in macrophages: regulation by NF-kappa B is independent of c-Jun or C/EBP beta. J Immunol. 2000;164(8):4277–85.
78. Shi Y, Lee JS, Galvin KM. Everything you have ever wanted to know about Yin Yang 1. Biochem Biophys Acta. 1997;1332(2):F49–66.
79. Garban HJ, Bonavida B. Nitric oxide inhibits the transcription repressor Yin-Yang 1 binding activity at the silencer region of the Fas promoter: a pivotal role for nitric oxide in the up-regulation of Fas gene expression in human tumor cells. J Immunol. 2001;167(1):75–81.

Chapter 10
Regulation of Anti-Tumor Immune Responses

Peter Siesjö

Abstract Nitric oxide has a dual role in both regulation of immune homeostatsis and in responses to pathogens. It is therefore not surprising that a similar duality exists in the response against neoplastic cells. At the same time as NO can exert cytotoxicity against tumor cells it can also inhibit immune reactivity against these cells. The following chapter will recapitulate the basal roles of NO in immune homestasis and immune reactivity against pathogens followed by a more detailed review of the role of NO in immune reactivity against tumors. In experimental therapy against tumors, the positive effects of NO can thus be manipulated by administration of NO donors or by inducing NO secretion from innate immune cells. On the other hand, inhibition of NO release by NOS inhibitors or by inhibition of molecules upstream of NOS induction can boost adaptive immune responses mainly by T cells.

Keywords Cytotoxicity · Dendritic cell · Inducible nitric oxide symthase · Macrophage · Myeloid derived suppressor cells · Nitric oxide · Tumor · T cell

Abbreviations

APC	antigen presenting cell
CCL-2	chemokine, also MCP-1 (monocyte chemotactic protein)
CNS	central nervous system
COX	cyclooxygenase
DC	dendritic cell
eNOS	endothelial nitric oxide synthase
FAS-L	FAS-ligand; death receptor ligand
IFNγ	Interferon gamma
GM-CSF	granulocyte-macrphage colony stimulating factor
G-MDSC	granulocytic-myeloid derived suppressor cells
HIF1α	hypoxia inducible factor1-alfa
IL-1β	interleukin-1beta
IL-2	interleukin-2

P. Siesjö (✉)
Section of Neurosurgery, Department of Clinical Sciences, Lund University, Lund, Sweden
e-mail: peter.siesjo@med.lu.se

Department of Neurosurgery, Skane University Hospital, 22185 Lund, Sweden

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_10

IL-6	interleukin-6
IL-10	interleukin-10
IL-12	interleukin-12
IL-18	interleukin-18
IDO	indoleamine 2, 3-dioxygenase
iNOS	inducible endothelial nitric oxide synthase
LEC	lymphatic endothelial cells
L-NAME	L-NG-nitroarginine methyl ester
L-NIL	N6- (1- iminoethyl)- L- lysine, dihydrochloride
L-NMMA	*N*-Monomethyl-L-arginine, monoacetate
NSAID	non steroidal anti-inflammatory drug
LPS	lipopolysaccarhide
M1	type 1 macrophage
MEG	mercapto ethyl guandinidine
MDSC	myeloid derived suppressor cells
Mo	macrophage
Mo-MDSC	monocytic-myeloid derived suppressor cells
MSC	mesenchymal stromal cells
NF-κβ	Nuclear factor kappa beta
NK	natural killer
PD-L1	programmed death receptor-1ligand; also B7-H1
PGE_2	prostaglandin E_2
ROS	reactive oxygen species
RNS	reactive nitrogen species
STAT3	signal transducer and activator of transcription 3
TAA	tumor associated antigens
TAM	tumor associated macrophages
T cell	T lymphocyte
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th1	type 1 T helper lymphocyte
Th 17	type 17 T helper lymphocyte
TNFα	tumor necrosis factor-alpha
T reg	regulatory T lymphocyte
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	vascular endothelia l growth factor

Introduction

Nitric oxide is a promiscuous molecule regarding its effects on the immune system, as it can both execute and inhibit immune functions. Therefore, it is not unexpected that similar features are encountered concerning its role in anti-tumor immunity and immunotherapy against cancer.

Immunotherapy has since long been investigated for its potential use in cancer treatment. Both in human and experimental animal neoplastic pathology spontaneous responses to tumor-associated antigens (TAAs) are a common phenomenon (for review see Coulie et al [1]). However, although many human tumors contain a varying compartment of infiltrating immune cells, these do not, in the majority of cases, seem to kill tumor cells effectively. Consequentially, the successful cure of experimental tumors after immune intervention did not initially translate into effective clinical immunotherapy. Nevertheless, recent advances in adoptive immunotherapy, dendritic cell based immunotherapy and breaching immune suppression have changed the picture [2, 3]. The previous limited clinical efficacy can partially be explained by immune evasion of tumor cells, e.g. down-regulation of processing or presentation of tumor antigens, shedding of antigens and similar mechanisms. In addition to these, failure of immunotherapy is also due to immunosuppressive mechanisms, triggered by both tumor angiogenesis and hypoxia, tumor cells per se, tumor-associated stromal cells and tumor infiltrating immune cells. These suppressive mechanisms also encompass the action of NOS with the ensuing release of NO and, in certain cases the formation of peroxynitrate that can exert suppression by both direct and indirect mechanisms. However, accumulated results show that not all the effects mediated by NO will down-regulate immune reactivity against tumors. NO is also a potent immune effector molecule and can induce tumor cell eradication by several mechanisms. The mechanisms governing the intricate balance between the two Janus faces of NO in anti-tumor immune responses are still not fully understood. Tentatively manipulation of NO release in tumor tissue could result in anti-tumor immune effects both by boosting direct effector functions but also by dampening immune suppression.

The Dual Role of NO in the Physiological Regulation of the Immune System

In order to understand how NO can be used to manipulate anti-tumor immune responses, its role in basal immune function will briefly be recapitulated. Due to the fact that the adaptive anti-tumor responses discussed in this chapter mainly are T-cell based, B cell responses will be omitted.

NO plays an important and diverse role during the regulation of general immune responses [4]. This diversity is partly dependent on the levels of NO secreted but also

on cellular targets and timing of release. Generally, low levels are required for initiation of immune responses while high levels of NO induce immune suppression [5].

Since the sentinel finding that IFNγsecretion from activated T cells could induce indirect cytotoxicity by release of reactive species from macrophages [6, 7], (later identified as NO [8]), an increasing amount of data on the role of NO in the immune and inflammatory systems have accumulated. NO has been shown to influence different immune functions both during innate and adaptive immune responses, including T cell activation and proliferation, cytokine production, APC expansion and maturation, central and peripheral tolerance, T cell differentiation, as well as T cell apoptosis (for review see [4]).

Effects of NO in Immune Homeostasis

NO has been implicated in the regulation of both central and peripheral tolerance. In central tolerance, APCs in the thymus, including DCs and MOs, were shown to trigger the selection process both to self- and allo-antigens by releasing NO [9]. During peripheral tolerance, on the other hand, regulatory T cells have been shown to induce NO production from APCs, to control other pathogenic T cells, and NO has also been implicated in the direct induction of CD+CD25+Foxp3 regulatory T cells via p53, IL-2 and CD40 release [10, 11]. Recently, a fundamental role of NO in immune regulation was reported by the specific expression of NO after IFNα release from activated T cells in lymph node stromal cells, specifically in the fibroblastic reticular cells (FRCs) and the lymphatic endothelial cells (LECs) [12].

Regulation of T Cell Activation by NO

NO production is initiated by upregulation of iNOS in myeloid cells during or shortly after the antigen encounter by T cells with subsequent IFNγproduction and, initially, this seems to boost T cell activation [5, 13]. Later work showed that also eNOS is induced in T cells shortly after antigen binding, and is involved in the positive regulation of TCR signaling by activating N-Ras [14, 15]. The T cell inhibitory effect exerted by histamine has also been explained by reduced NO production due to histamine release confirming the central role of NO in T cell priming [16]. Once initiated, the T cell response is suppressed or regulated by increasing amounts of NO and was demonstrated in DCs exposed to apoptotic cells [17], anti-CD28-induced immune tolerance [18] and T cell suppression by mesenchymal stromal cells (MSC) [19].

Initially, NO was thought to specifically boost Th1 responses but was also shown to inhibit Th17 cells and ameliorate experimental autoimmune disease. This specificity was extended in a recent study showing that T regs induced by NO preferen-

tially suppressed Th17 cells while natural T reg cells preferentially suppressed Th1 cells [20], but whether this is an exception to general conditions is not clear. NO has also been implicated in the regulation of T cell memory and survival by attenuating memory responses [21].

The Immune Suppressive Role of NO

Immune suppression executed by the release of NO can be categorized as direct and indirect depending on whether NO by itself acts on the effector mechanisms or whether it does so by indirect means. The former can originate in tissue resident macrophages, dendritic cells, immature myeloid cells as myeloid derived suppressor cells (MDSC) and MSC. The latter have been shown to execute their suppressive action by NO in rat and mice but a recent report claimed that human MSC rather orchestrate their suppressive action through IDO [22]. Additionally, rat MDSC were shown to be dichotomous in their suppressive function whereby NO inhibits T cell proliferation while PGE_2 inhibits cytokine production [23]. Indirect action of NO can emanate from various mechanisms as the induction of suppressive T reg cells, tolerogenic DC and mature and immature myeloid cells. Immune suppression by NO has been documented in various non-neoplastic circumstances as prolongation of graft survival in experimental transplantations with NO-secreting MDSC [24] prevailing immune suppression in the liver [25] and immune dysfunction in trauma [26].

In addition to its direct inhibitory effects, NO can also react with other oxidants such as super oxide, and forms additional RNS such as peroxynitrite. Peroxynitrite was shown to inhibit proliferation of T lymphocytes in a dose dependent manner via nitration of tyrosine residues, thereby, blocking the activation-induced protein phosphorylation and by inducing apoptosis in T cells [27].

The Role of NO in the (Pathological) Regulation of Anti-Tumor Responses

Potentiation of Immune Derived Cytotoxicity Against Tumor Cells

Several different mechanisms of NO-mediated or -executed cytotoxicity against tumor cells have been proposed. In response to IL-2, iNOS was induced in rat NK cells and was suggested to be responsible for their cytotoxic functions [28]. Furthermore, it has been shown that the effector function of NK cells during AK-5 tumor rejection was mediated by overproduction of NO, and that NK cells induced tumor cell lysis through NO-mediated cytotoxicity [29].

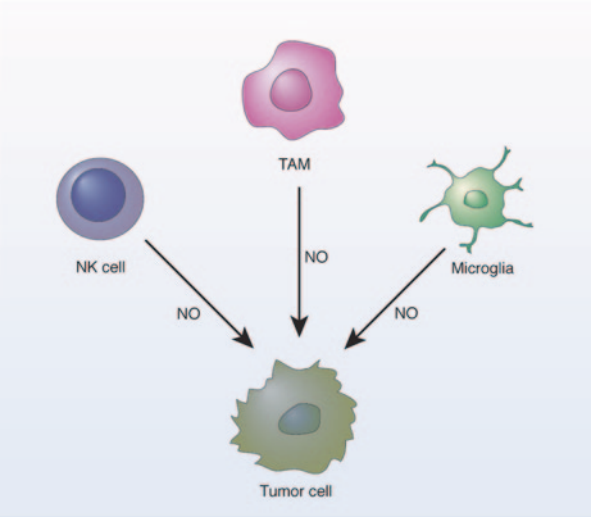


Fig. 10.1 NO can exert cytotoxicity against neoplastic cells by the direct release of NO from the innate immune cells and NK cells, tumor-associated macrophages and organ resident macrophages as microglia

Tumor-associated macrophages (TAM) can, after exposure to proinflammatory cytokines, be reverted to classically activated type 1 macrophages (M1) producing high levels of NO, by targeting the NF-κ signaling pathway [30]. Intracranial administration of LPS and IFNγ significantly prolonged the survival of B16F10 murine melanoma bearing animals, which seemed to be dependent on NO generation, because the survival was decreased using the NOS inhibitor L-NAME. These data show that NO by itself can inhibit tumor progression [31].

Mechanistically NO has been shown to potentiate apoptotic cell death by synergy with members of the TNF family as TRAIL, FAS-L and TNFα even in primary resistant tumor cells as reviewed by Jeannin et al and Bonavida et al [32, 33]. In a model of ocular melanoma NO was shown to be compulsory for T cell function but no explanation for this was presented [34]. IL-2/CD40 therapy of experimental tumors was also shown to depend on iNOS induction in intratumoral tumor associated macrophages (TAM) [35]. Also immune escape and immune suppression during hypoxia induced by HIF1α was counteracted by NO released by iNOS activity in experimental tumors but as for the former two examples the effector mechanisms were mainly innate eg by NK cells [36, 37]. Also microglia mediated tumor cell death requires NO and additional molecules as Cathepsin B [38].

In conclusion, NO can exert direct cytotoxicity from NK cells, TAM and microglia but will then also suppress T cell function. See also Fig. 10.1

NO-Mediated Immune Suppression in Tumor Bearing Hosts

Dendritic Cells

In several non-CNS experimental tumors, the induction of tolerogenic DC was induced by all-trans retinoic acid from hepatic stellate cells through upregulation of both iNOS and arginase-1 [25].

Myeloid Derived Suppressor Cells

In addition to mature DCs and MO, immature myeloid cells, often referred to as myeloid-derived suppressor cells (MDSC), can effectuate suppression of T cells during tumor progression via NO-mediated pathways [39–41]. Phenotypically, MDSC are divided into granulocytic (G-MDSC) and monocytic (Mo-MDSC). G-MDSC, in mice defined as $CD11b^{+}Gr1^{+}$cells, derived from tumor-bearing animals were initially shown to inhibit T cell activation induced through CD3/CD28 co-stimulation via peroxynitrite- dependent mechanisms [42] Initial reports indicated that IFN-γ induces NO in the myeloid cells as $CD11b^{+}Gr1^{+}$MDSCs isolated from iNOS null mice did not inhibit T cell proliferation [43]. However, later work implied that only G-MDSC use peroxynitrite induced by eNOS and gp91 as effector inhibitory mechanisms while Mo-MDSC use NO from iNOS but both mechanisms were dependent on IFNγ [44]. MDSC derived inhibition of lymphocyte proliferation and migration has been linked to various mechanisms as lack of T cell protein tyrosine phosphatase (TC-PTP) [45], CCL-2 increase [40] and downregulation of Notch-1 and 2 in T cells [46].

In MDSC, the iNOS activity and the recruitment of MDSC have been shown to depend on several mechanisms and as expression of Ovarian cancer G-protein-coupled-receptor-1 (OGR1) [47], transmembrane TNF-α [48], FK506 binding protein 51 (FKBP51) [49] and HIF1α [50]. Also, overexpression of human cytomegalovirus (HCMV) or UL 28 from HCMV was shown to induce immune suppression through up-regulation of STAT-3 and eNOS [51]. See also Fig. 10.2.

Mechanisms of NO-Mediated Immune Suppression

Mechanistically, NO blocks signaling through the IL-2 receptor expressed by T lymphocytes by impeding phosphorylation of the intracellular-signaling proteins STAT5, AKT and ERK [52]. Co-stimulatory related ligands have also been shown to participate in the NO-mediated suppression as blockade of B7-H1 (PD-L1) on MOs [53].

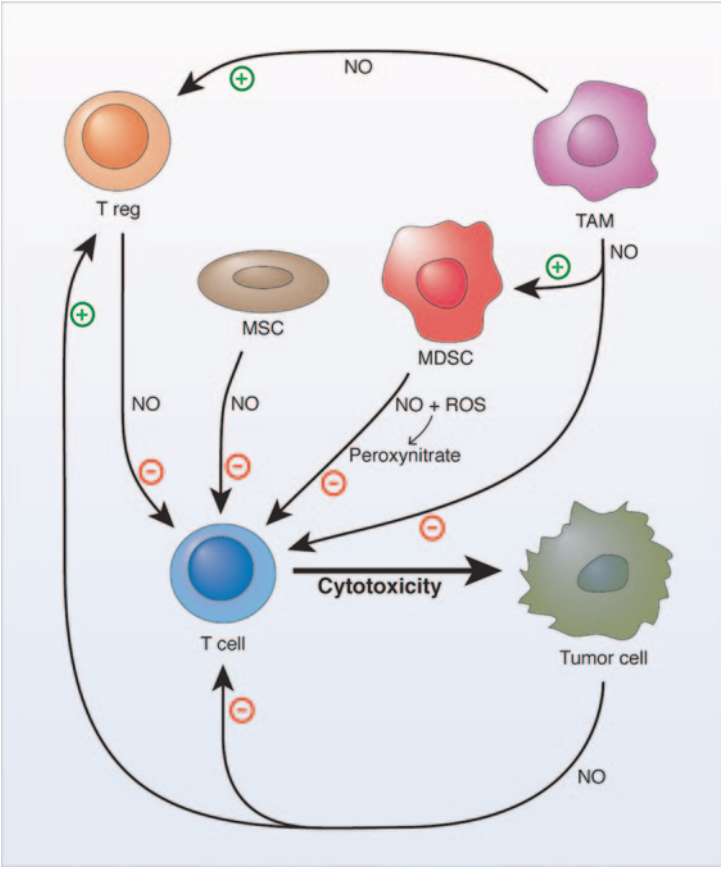


Fig. 10.2 NO can inhibit anti-tumor adaptive T cell responses by the induction of suppressive immune cells and as tolerogenic dendritic cells (*tDC*), myeloid derived suppressor cells (*MDSC*) and regulatory T cells (*T reg*) but also stromal cells as mesenchymal stromal cells (*MSC*). The source of NO is mainly local and systemic myeloid cells but also stromal cells as MSC and tumor cells per see can secrete NO. Note that MDSC are both generated by NO and use NO to suppress T cells

iNOS and subsequent NO secretion also could be induced by immunotherapy with BCG vaccination [54]. In a rat glioma model, iNOS expression was only observed in tumors from animals immunized with tumor cells transfected with IFN-γ, but not in animals immunized with wild type tumor cells [55]. These results also confirm that only tumor infiltrating NK or NK-like cells or T lymphocytes can secrete sufficient amounts of IFN-γ for the induction of iNOS in tumor infiltrating myeloid cells [56]. However, other tumor-derived molecules as TNFα, MCP-1 (CCL2) can induce iNOS in microglia that suppress T lymphocyte responses [57]. Tumor derived pericytes and their progeny mesenchymal stromal cells express iNOS constitutively and are potent immune suppressors but it is not clear whether tumor-derived factors other than IFN-γ can enhance NO production from these

cells [58]. In a recent study, another immune suppressive mechanism was proposed whereby nitrosylation of MCP-1 (CCL-2) blocked T cell influx into tumor tissue and this could be reversed by blocking MCP-1 modification [59]. Although NO can act as an effector molecule in killing of tumor cells higher doses will exert immune suppression as demonstrated in a model of intratumoral IL-12 delivery [60].

Boosting Anti-Tumor Immunity by Modulation of NO-Mediated Immunosuppression

The use of NO-donors, which when released intracellularily can inhibit iNOS, has been reported to be successful in different malignancies. NO-releasing aspirin was able to improve the effects of a GM-CSF-based cancer vaccine in a murine colon cancer model, through inhibition of iNOS and other suppressive enzymes [61].

NO release can also protect against immune suppression by other mechanisms as in the B16 melanoma mouse model, where DCs treated *ex vivo* with NO-donors became resistant to tumor-induced apoptosis, due to prevention of activation of the down-stream pro-apoptotic events including pro- and antiapoptotoc proteins Bcl-2, Bax and caspase-9 [62].

However, the major observed effects of NO during anti-tumor immunotherapy tend to be suppressive. In our earlier studies we showed that the systemic immunosuppression induced by large tumor burdens in tumor-bearing hosts was mediated by NO production [63, 64].

Immunotherapy that directly or indirectly will lead to increased levels of activated T cells or NK cells can lead to immune suppression due to an increased release of NO. Koblish et al demonstrated that an IL-12-based experimental immunotherapy was unsuccessful due to an NO-mediated down-regulation of the immune response but this could be reversed by nonspecific inhibitors of NOS [43].

Down regulation of T lymphocyte responses induced by administration of polyclonal stimulation also involves NO release and we could show that the selective iNOS inibitor L-NIL was superior in reversing this than the nonspecific inhibitor L-NAME [65]. In this report, the *in vivo* combination of L-NIL and immunotherapy with IFN-γ-secreting tumor cells gave a slight prolongation of survival. These results were markedly improved when an inhibitor of both iNOS and COX, mercaptoethylguanidine (MEG), was used in the same experimental model and 40% of rats bearing intracerebral tumors could be cured [65]. These effects were strictly dependent on the timing by which the iNOS inhibitor was administered in relation to the time of immunization, pointing out the important feature of modulating NO during anti-tumor immune responses. This study also demonstrated that systemic levels of NO were actually increased during immunotherapy above the levels of tumor bearers underscoring the fact that immunotherapy per se can induce further immunosuppression, in this case by release of NO.

The reports of successful immunotherapy combined with inhibition of NO have in common that the immunotherapies have directly or indirectly been linked to

release of IFN-γ, the major trigger of NO release. This is also the case of the combined immunotherapy with IL-12 and IL-18-secreting tumor cells in an intrahepatic rat colon cancer model. Due to the abundance of NO producing, myeloid cells in the liver immunotherapy per se did not have any effect but the combination of L-NAME and low doses of an anti-angiogenic compound, combretastatin, significantly prolonged survival [66].

Thus, to obtain a functional immunotherapy we need to understand the specific suppressive mechanisms presented in a particular tumor type. However, in our experience, inhibition of NOS or iNOS during immunizations could abrogate the effects and underscore the importance of crucial levels of NO for T cell priming.

By combining inhibitors of iNOS and COX-2 by separate drugs, we have preliminary shown that there is a synergistic effect of the compounds and that iNOS inhibition boosts memory responses. Following treatment with the iNOS inhibitor L-NIL and/or COX-2 inhibitor parecoxib, in combination with an IFNγ based antitumor immunotherapy, animals receiving the iNOS inhibitor survived more often after rechallenge with tumor cells [67].

Crosstalk in the Immune Suppressive Networks

Suppression of immune effector mechanisms against tumors encompass several enzymes, transcription factors and effector molecules apart from NO and as STAT-3, NF-κB, IDO, ROS, VEGF, PGE_2, TGFβ, IL-13, T reg and IL-10 constituting an immune suppressive network (for review see [68]). Experimental conditions as the tumor model, the therapeutic intervention and the inhibitors used may influence the outcome leading to contradictory results. It is also evident that several of the effectors of immune suppression can act simultaneously in a hierarchal fashion. In many reports, the induction of NOS and secretion of NO is indeed upstream of other suppressive effectors [69–71]. However, several reports imply that PGE_2, a key suppressive agent, acts upstream of NO and inhibitors of COX-2 can thus also block NO release [72, 73]. In humans, MDSC from patients with renal carcinoma, ROS was shown to act upstream of iNOS [74].

Clinical Studies

The combination of immunotherapy with NO-modulating approaches has not yet been explored in patients. However, there are a couple of *ex vivo* studies indicating the involvement of NO in the tumor-induced immunosuppression. In prostate organ cultures, treatment with the specific iNOS inhibitor L-NMMA, was able to restore the suppressed functions of tumor infiltrating T lymphocytes [39]. Induction of apoptosis was obtained after administration of NO-donors on human colon carcinoma cells *in vitro* [75]. NO-NSAIDs such as NO-aspirin, NO-ibuprofen and

NO-sulindac have been shown to induce apoptosis in human colon cancer cells *in vitro* [76]. Although extensively assayed in experimental systems, no clinically available NO inhibitors of NOS and no NO-NSAIDS exist which have blocked the translational development of therapy. However, the use of clinically available COX-2 inhibitors might also inhibit the release of NO and indirectly give proof of concept. It is still also an open question whether the immune suppressive effect of NO in experimental systems is effective in human cancer.

Concluding Remarks and Future Directions

NO has accordingly dual roles and functions in both immune homeostatis and in neoplasia, see Table 10.1. The main obstacle for manipulating NO in clinical tumor therapy is the absence of clinically approved donors and inhibitors. Nevertheless, once these are available, immunotherapeutic approaches should be designed to achieve a positive net effect on NO regulation.

Table 10.1 Summary of NO effects in immune homeostais and tumors

Cell of origin	Target cell	Mechanism and net effect	Ref
Effects of NO in immune homeostasis			
FRC, LEC, APC	T cell	Immune homeostasis, central and peripheral tolerance	[1, 2, 9–11]
cAPC APC	T cell	Low NO from iNOS boosts T cell activation	[5,13]
T lymphocyte	T cell	NO from eNOS boosts T cell activation	[14, 15]
APC, MSC, DC	T cell	High NO from iNOS suppresses T cells	[17–19]
Myeloid cells	T reg, DC, MDSC	NO induces indirect T cell suppression	[24–26]
Potentiation of immune derived cytotoxicity against tumor cells			
NK cell	Tumors, pathogens	Direct tumor cell and pathogen killing by NO	[28, 29, 36, 37]
TAM, microglia	Tumors, pathogens	Direct tumor cell and pathogen killing by NO	[30, 31, 35, 38]
NO mediated immune suppression in tumor bearing hosts			
DC	T cell	T cell suppression in experimental tumors	[25]
MDSC	T cell	NO implicated in both generation and function	[39–41, 42–44]
Myeloid cells	T cell	Systemic myeloid cells inhibit T cells by NO	[63–65]

It is important to consider the fact that the manipulation of the immune system using different immunotherapeutic approaches might itself induce tolerance and/or immunosuppression. Although NO-donors have shown increased induction of apoptosis *in vitro* it is still unclear whether they also inhibit iNOS activity, The induction of cytotoxicity by NO *in vivo* could also boost T cell responses by partially degrading tumor cells thus facilitating antigen presentation by APC. In conclusion, the combination of immunotherapy with NO-modulating approaches has to be specifically tailored, considering the tumor type, timing of immune intervention and also the suppressive network prevailing in the specific tumor.

Acknowledgements The author thanks Edward Visse for illustrations in Figs. 10.1 and 10.2.

No Conflict Statement No potential conflicts of interest were disclosed

References

1. Coulie PG, Van den Eynde BJ, van der Bruggen P, Boon T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. Nat Rev Cancer. 2014;14:135–46.
2. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nat Rev Cancer. 2012;12:265–77.
3. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. Nat Rev Immunol. 2012;12:269–81.
4. Bogdan C. Nitric oxide and the immune response. Nat Immunol. 2001;2:907–16.
5. Niedbala W, Wei XQ, Campbell C, Thomson D, Komai-Koma M, Liew FY. Nitric oxide preferentially induces type 1 T cell differentiation by selectively up-regulating IL-12 receptor beta 2 expression via cGMP. Proc Natl Acad Sci U S A. 2002;99:16186–91.
6. Nathan CF, Murray HW, Wiebe ME, Rubin BY. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J Exp Med. 1983;158:670–89.
7. Nathan CF, Prendergast TJ, Wiebe ME, Stanley ER, Platzer E, Remold HG, Welte K, Rubin BY, Murray HW. Activation of human macrophages. Comparison of other cytokines with interferon-gamma. J Exp Med. 1984;160:600–5.
8. Stuehr DJ, Gross SS, Sakuma I, Levi R, Nathan CF. Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. J Exp Med. 1989;169:1011–20.
9. Aiello S, Noris M, Piccinini G, Tomasoni S, Casiraghi F, Bonazzola S, Mister M, Sayegh MH, Remuzzi G. Thymic dendritic cells express inducible nitric oxide synthase and generate nitric oxide in response to self- and alloantigens. J Immunol. 2000;164:4649–58.
10. Chen C, Lee WH, Zhong L, Liu CP. Regulatory T cells can mediate their function through the stimulation of APCs to produce immunosuppressive nitric oxide. J Immunol. 2006;176:3449–60.
11. Niedbala W, Cai B, Liu H, Pitman N, Chang L, Liew FY. Nitric oxide induces CD4+ CD25+ Foxp3 regulatory T cells from CD4+ CD25 T cells via p53, IL-2, and OX40. Proc Natl Acad Sci U S A. 2007;104:15478–83.
12. Lukacs-Kornek V, Malhotra D, Fletcher AL, Acton SE, Elpek KG, Tayalia P, Collier AR, Turley SJ. Regulated release of nitric oxide by nonhematopoietic stroma controls expansion of the activated T cell pool in lymph nodes. Nat Immunol. 2011;12:1096–104.
13. Huang FP, Niedbala W, Wei XQ, Xu D, Feng GJ, Robinson JH, Lam C, Liew FY. Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. Eur J Immunol. 1998;28:4062–70.

14. Ibiza S, Pérez-Rodríguez A, Ortega A, Martínez-Ruiz A, Barreiro O, García-Domínguez CA, Víctor VM, Esplugues JV, Rojas JM, Sánchez-Madrid F, Serrador JM. Endothelial nitric oxide synthase regulates N-Ras activation on the Golgi complex of antigen-stimulat T cells. Proc Natl Acad Sci U S A. 2008;105:10507–12.
15. Ibiza S, Víctor VM, Boscá I, Ortega A, Urzainqui A, O'Connor JE, Sánchez-Madrid F, Esplugues JV, Serrador JM. Endothelial nitric oxide synthase regulates T cell receptor signaling at the immunological synapse. Immunity. 2006;24:753–65.
16. Koncz A, Pasztoi M, Mazan M, Fazakas F, Buzas E, Falus A, Nagy G. Nitric oxide mediates T cell cytokine production and signal transduction in histidine decarboxylase knockout mice. J Immunol. 2007;179:6613–9.
17. Dugast AS, Haudebourg T, Coulon F, Heslan M, Haspot F, Poirier N, Vuillefroy de Silly R, Usal C, Smit H, Martinet B, Thebault P, Renaudin K, Vanhove B. Myeloid-derived suppressor cells accumulate in kidney allograft tolerance and specifically suppress effector T cell expansion. J Immunol. 2008;180:7898–906.
18. Ren G, Su J, Zhao X, Zhang L, Zhang J, Roberts AI, Zhang H, Das G, Shi Y. Apoptotic cells induce immunosuppression through dendritic cells: critical roles of IFN-gamma and nitric oxide. J Immunol. 2008;181:3277–84.
19. Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, Muroi K, Ozawa K. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. Blood. 2007;109:228–34.
20. Niedbala W, Besnard AG, Jiang HR, Alves-Filho JC, Fukada SY, Nascimento D, Mitani A, Pushparaj P, Alqahtani MH, Liew FY. Nitric oxide-induced regulatory T cells inhibit Th17 but not Th1 cell differentiation and function. J Immunol. 2013;191:164–70.
21. Vig M, Srivastava S, Kandpal U, Sade H, Lewis V, Sarin A, George A, Bal V, Durdik JM, Rath S. Inducible nitric oxide synthase in T cells regulates T cell death and immune memory. J Clin Investig. 2004;113:1734–42.
22. Ling W, Zhang J, Yuan Z, Ren G, Zhang L, Chen X, Rabson AB, Roberts AI, Wang Y, Shi Y. Mesenchymal stem cells use IDO to regulate immunity in tumor microenvironment. Cancer Res. 2014;74:1576–87.
23. Zinöcker S, Vaage JT. Rat mesenchymal stromal cells inhibit T cell proliferation but not cytokine production through inducible nitric oxide synthase. Front Immunol. 2012;3:62.
24. Arakawa Y, Qin J, Chou HS, Bhatt S, Wang L, Stuehr D, Ghosh A, Fung JJ, Lu L, Qian S. Cotransplantation with myeloid-derived suppressor cells protects cell transplants: a crucial role of inducible nitric oxide synthase. Transplantation. 2014;97:740–7.
25. Bhatt S, Qin J, Bennett C, Qian S, Fung JJ, Hamilton TA, Lu L. All-trans retinoic acid induces arginase-1 and inducible nitric oxide synthase-producing dendritic cells with T cell inhibitory function. J Immunol. 2014;192:5098–108.
26. Darwiche SS, Pfeifer R, Menzel C, Ruan X, Hoffman M, Cai C, Chanthaphavong RS, Loughran P, Pitt BR, Hoffman R, Pape HC, Billiar TR. Inducible nitric oxide synthase contributes to immune dysfunction following trauma. Shock. 2012;38:499–507.
27. Brito C, Naviliat M, Tiscornia AC, Vuillier F, Gualco G, Dighiero G, Radi R, Cayota AM. Peroxynitrite inhibits T lymphocyte activation and proliferation by promoting impairment of tyrosine phosphorylation and peroxynitrite-driven apoptotic death. J Immunol. 1999;162:3356–66.
28. Cifone MG, D'Alò S, Parroni R, Millimaggi D, Biordi L, Martinotti S, Santoni A. Interleukin-2-activated rat natural killer cells express inducible nitric oxide synthase that contributes to cytotoxic function and interferon-gamma production. Blood. 1999;93:3876–84.
29. Jyothi MD, Khar A. Induction of nitric oxide production by natural killer cells: its role in tumor cell death. Nitric Oxide. 1999;3:409–18.
30. Hagemann T, Lawrence T, McNeish I, Charles KA, Kulbe H, Thompson RG, Robinson SC, Balkwill FR. "Re-educating" tumor-associated macrophages by targeting NF-{kappa}B. J Exp Med. 2008;205:1261–8.
31. Samdani AF, Kuchner EB, Rhines L, Adamson DC, Lawson C, Tyler B, Brem H, Dawson VL, Dawson TM. Astroglia induce cytotoxic effects on brain tumors via a nitric oxide-dependent pathway both in vitro and in vivo. Neurosurgery. 2004;54:1231–8.

32. Jeannin JF, Leon L, Cortier M, Sassi N, Paul C, Bettaieb A. Nitric oxide-induced resistance or sensitization to death in tumor cells. Nitric Oxide. 2008;19:158–63.
33. Bonavida B, Baritaki S, Huerta-Yepez S, Vega MI, Chatterjee D, Yeung K. Novel therapeutic applications of nitric oxide donors in cancer: roles in chemo- and immunosensitization to apoptosis and inhibition of metastases. Nitric Oxide. 2008;19:152–7.
34. Vicetti Miguel RD, Cherpes TL, Watson LJ, McKenna KC. CTL induction of tumoricidal nitric oxide production by intratumoral macrophages is critical for tumor elimination. J Immunol. 2010;185:6706–18.
35. Weiss JM, Ridnour LA, Back T, Hussain SP, He P, Maciag AE, Keefer LK, Murphy WJ, Harris CC, Wink DA, Wiltrout RH. Macrophage-dependent nitric oxide expression regulates tumor cell detachment and metastasis after IL-2/anti-CD40 immunotherapy. J Exp Med. 2010;207:2455–67.
36. Barsoum IB, Hamilton TK, Li X, Cotechini T, Miles EA, Siemens DR, Graham CH. Hypoxia induces escape from innate immunity in cancer cells via increased expression of ADAM10: role of nitric oxide. Cancer Res. 2011;71:7433–41.
37. Siemens DR, Hu N, Sheikhi AK, Chung E, Frederiksen LJ, Pross H, Graham CH. Hypoxia increases tumor cell shedding of MHC class I chain-related molecule: role of nitric oxide. Cancer Res. 2008;68:4746–53.
38. Hwang SY, Yoo BC, Jung JW, Oh ES, Hwang JS, Shin JA, Kim SY, Cha SH, Han IO. Induction of glioma apoptosis by microglia-secreted molecules: the role of nitric oxide and cathepsin B. Biochim Biophysic Acta. 2009;1793:1656–68.
39. Bronte V, Kasic T, Gri G, Gallana K, Borsellino G, Marigo I, Battistini L, Iafrate M, Prayer-Galetti T, Pagano F, Viola A. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. J Exp Med. 2005;201:1257–68.
40. Hosoi A, Matsushita H, Shimizu K, Fujii S, Ueha S, Abe J, Kurachi M, Maekawa R, Matsushima K, Kakimi K. Adoptive cytotoxic T lymphocyte therapy triggers a counter-regulatory immunosuppressive mechanism via recruitment of myeloid-derived suppressor cells. Int J Cancer. 2014;134:1810–22.
41. Serafini P, Meckel K, Kelso M, Noonan K, Califano J, Koch W, Dolcetti L, Bronte V, Borrello I. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med. 2006;203:2691–702.
42. Kusmartsev SA, Li Y, Chen SH. Gr-1+ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation. J Immunol. 2000;165:779–85.
43. Koblish HK, Hunter CA, Wysocka M, Trinchieri G, Lee WM. Immune suppression by recombinant interleukin (rIL)-12 involves interferon gamma induction of nitric oxide synthase 2 (iNOS) activity: inhibitors of NO generation reveal the extent of rIL-12 vaccine adjuvant effect. J Exp Med. 1998;188:1603–10.
44. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, De Baetselier P, Van Ginderachter JA. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T-cell suppressive activity. Blood. 2008;111:4233–44.
45. Dupuis M, De Jesús Ibarra-Sánchez M, Tremblay ML, Duplay P. Gr-1+ myeloid cells lacking T cell protein tyrosine phosphatase inhibit lymphocyte proliferation by an IFN-gamma- and nitric oxide-dependent mechanism. J Immunol. 2003;171:726–32.
46. Sierra RA, Thevenot P, Raber P, Cui Y, Parsons C, Ochoa AC, Trillo-Tinoco J, Del Valle L, Rodriguez PC. Rescue of Notch 1 signaling in antigen-specific CD8+ T cells overcomes tumor-induced T cell suppression and enhances immunotherapy in cancer. Cancer Immunol Res. 2014;2:800–11.
47. Yan L, Singh LS, Zhang L, Xu Y. Role of OGR1 in myeloid-derived cells in prostate cancer. Oncogene. 2014;33:157–164.
48. Hu X, Li B, Li X, Zhao X, Wan L, Lin G, Yu M, Wang J, Jiang X, Feng W, Qin Z, Yin B, Li Z. Transmembrane TNF-a promotes suppressive activities of myeloid-derived suppressor cells via TNFR2. J Immunol. 2014;192:1320–31.

49. Kim YS, Kim YJ, Lee JM, Kim EK, Park YJ, Choe SK, Ko HJ, Kang CY. Functional changes in myeloid-derived suppressor cells (MDSCs) during tumor growth: FKBP51 contributes to the regulation of the immunosuppressive function of MDSCs. J Immunol. 2012;188:4226–34.
50. Corzo CA, Condamine T, Lu L, Cotter MJ, Youn JI, Cheng P, Cho HI, Celis E, Quiceno DG, Padhya T, McCaffrey TV, McCaffrey JC, Gabrilovich DI. HIF1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. J Exp Med. 2010;207:2439–53.
51. Soroceanu L, Matlaf L, Bezrookove V, Harkins L, Martinez R, Greene M, Soteropoulos P, Cobbs CS. Human cytomegalovirus US28 found in glioblastoma promotes an invasive and angiogenic phenotype. Cancer Res. 2011;71:6643–53.
52. Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, Zanovello P, Segal DM. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J Immunol. 2002;168:689–95.
53. Yamazaki T, Akiba H, Koyanagi A, Azuma M, Yagita H, Okumura K. Blockade of B7-H1 on macrophages suppresses CD4+ T cell proliferation by augmenting IFN-gamma-induced nitric oxide production. J Immunol. 2005;175:1586–92.
54. Martino A, Badell E, Abadie V, Balloy V, Chignard M, Mistou MY, Combadière B, Combadière C, Winter N. *Mycobacterium bovis* bacillus Calmette-Guérin vaccination mobilizes innate myeloid-derived suppressor cells restraining *in vivo* T cell priming via IL-1R-dependent nitric oxide production. J Immunol. 2010;184:2038–47.
55. Johansson AC, Hegardt P, Janelidze S, Visse E, Widegren B, Siesjö P. Enhanced expression of iNOS intratumorally and at the immunization site after immunization with IFNgamma-secreting rat glioma cells. J Neuroimmunol. 2002;123:135–43.
56. van der Veen RC, Dietlin TA, Hofman FM. Tissue expression of inducible nitric oxide synthase requires IFN-gamma production by infiltrating splenic T cells: more evidence for immunosuppression by nitric oxide. J Neuroimmunol. 2003;145:86–90.
57. Shen SC, Wu MS, Lin HY, Yang LY, Chen YH, Chen YC. Reactive oxygen species-dependent nitric oxide production in reciprocal interactions of glioma and microglial cells. J Cell Physiol. 2014;229:2015–26.
58. Ochs K, Sahm F, Opitz CA, Lanz TV, Oezen I, Couraud PO, von Deimling A, Wick W, Platten M. Immature mesenchymal stem cell-like pericytes as mediators of immunosuppression in human malignant glioma. J Neuroimmunol. 2013;265(1–2):106–16.
59. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, De Palma A, Mauri P, Monegal A, Rescigno M, Savino B, Colombo P, Jonjic N, Pecanic S, Lazzarato L, Fruttero R, Gasco A, Bronte V, Viola A. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. J Exp Med. 2011;208:1949–62.
60. Egilmez NK, Harden JL, Virtuoso LP, Schwendener RA, Kilinc MO. Nitric oxide short-circuits interleukin-12-mediated tumor regression. Cancer Immunol Immunother. 2011;60:839–45.
61. De Santo C, Serafini P, Marigo I, Dolcetti L, Bolla M, Del Soldato P, Melani C, Guiducci C, Colombo MP, Iezzi M, Musiani P, Zanovello P, Bronte V. Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. Proc Natl Acad Sci U S A. 2005;102:4185–90.
62. Perrotta C, Falcone S, Capobianco A, Camporeale A, Sciorati C, De Palma C, Pisconti A, Rovere-Querini P, Bellone M, Manfredi AA, Clementi E. Nitric oxide confers therapeutic activity to dendritic cells in a mouse model of melanoma. Cancer Res. 2004;64:3767–71.
63. Hegardt P, Widegren B, Li L, Sjögren B, Kjellman C, Sur I, Sjögren HO. Nitric oxide synthase inhibitor and IL-18 enhance the anti-tumor immune response of rats carrying an intrahepatic colon carcinoma. Cancer Immunol Immunother. 2001;50:491–501.
64. Hegardt P, Widegren B, Sjögren HO. Nitric-oxide-dependent systemic immunosuppression in animals with progressively growing malignant gliomas. Cell Immunol. 2000;200:116–27.
65. Badn W, Visse E, Darabi A, Smith KE, Salford LG, Siesjö P. Postimmunization with IFN-gamma-secreting glioma cells combined with the inducible nitric oxide synthase inhibitor mercaptoethylguanidine prolongs survival of rats with intracerebral tumors. J Immunol. 2007;179:4231–8.

66. Badn W, Kalliomäki S, Widegren B, Sjögren HO. Low-dose combretastatin A4 phosphate enhances the immune response of tumor hosts to experimental colon carcinoma. Clin Cancer Res Off J Am Assoc Cancer Res. 2006;12:4714–9.
67. Eberstål S, Badn W, Fritzell S, Esbjörnsson M, Darabi A, Visse E, Siesjö P. Inhibition of cyclooxygenase-2 enhances immunotherapy against experimental brain tumors. Cancer Immunol Immunother. 2012;61:1191–9.
68. Grimm EA, Sikora AG, Ekmekcioglu S. Molecular pathways: inflammation-associated nitric-oxide production as a cancer-supporting redox mechanism and a potential therapeutic target. Clin Cancer Res Off J Am Assoc Cancer Res. 2013;19:5557–63.
69. Jayaraman P, Parikh F, Lopez-Rivera E, Hailemichael Y, Clark A, Ma G, Cannan D, Ramacher M, Kato M, Overwijk WW, Chen SH, Umansky VY, Sikora AG. Tumor-expressed inducible nitric oxide synthase controls induction of functional myeloid-derived suppressor cells through modulation of vascular endothelial growth factor release. J Immunol. 2012;188:5365–76.
70. Jia W, Jackson-Cook C, Graf MR. Tumor-infiltrating, myeloid-derived suppressor cells inhibit T cell activity by nitric oxide production in an intracranial rat glioma+ vaccination model. J Neuroimmunol. 2010;223:20–30.
71. Samy AS, Igwe OJ. Regulation of IL-1b-induced cyclooxygenase-2 expression by interactions of Ab peptide, apolipoprotein E and nitric oxide in human neuroglioma. J Mol Neurosci. 2012;47:533–45.
72. Obermajer N, Wong JL, Edwards RP, Odunsi K, Moysich K, Kalinski P. PGE(2)-driven induction and maintenance of cancer-associated myeloid-derived suppressor cells. Immunolo Invest. 2012;41:635–57.
73. Veltman JD, Lambers ME, van Nimwegen M, Hendriks RW, Hoogsteden HC, Aerts JG, Hegmans JP. COX-2 inhibition improves immunotherapy and is associated with decreased numbers of myeloid-derived suppressor cells in mesothelioma. Celecoxib influences MDSC function. BMC Cancer. 2010;10:464.
74. Kusmartsev S, Su Z, Heiser A, Dannull J, Eruslanov E, Kübler H, Yancey D, Dahm P, Vieweg J. Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. Clin Cancer Res Off J Am Assoc Cancer Res. 2008;14:8270–8.
75. Millet A, Bettaieb A, Renaud F, Prevotat L, Hammann A, Solary E, Mignotte B, Jeannin JF. Influence of the nitric oxide donor glyceryl trinitrate on apoptotic pathways in human colon cancer cells. Gastroenterology. 2002;123:235–46.
76. Williams JL, Borgo S, Hasan I, Castillo E, Traganos F, Rigas B. Nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NSAIDs) alter the kinetics of human colon cancer cell lines more effectively than traditional NSAIDs: implications for colon cancer chemoprevention. Cancer Res. 2001;61:3285–9.

Chapter 11
Nitric Oxide: Immune Modulation of Tumor Growth

Naveena B. Janakiram and Chinthalapally V. Rao

Abstract Nitric oxide (NO) plays a pivotal role in the physiology of diverse tissues including cells of the immune system. It is well established that the levels of nitric oxide must be regulated carefully to maintain homeostasis. Dysregulation or overproduction of nitric oxide has been implicated in the pathogenesis of many disorders including atherosclerosis, neurodegenerative diseases, autoimmune diseases, and cancer. Tumor-associated generation of NO, predominately via inducible nitric oxide synthase (iNOS), can be produced by the immune-system (dendritic cells, NK cells, mast cells, monocytes, macrophages, Kupffer cells) as well as by other cells involved in tumor growth. Depending upon the levels of NO generated, the potential exists for it to behave like a "double-edged" biological sword. In tumorigenesis assays, both protective and toxic effects of NO generated from immune cells frequently are seen in parallel. Thus, there is no simple, uniform picture of the function of NO in the immune modulation of tumor growth. The striking inter- and intracellular signaling between tumor cells and immune system cells makes it extremely difficult to predict the effect of NOS inhibitors and NO donors. This complexity has delayed evaluation of NO regulatory drugs as frontline therapies for cancer.

Keywords Inflammation · INOS · Nitric Oxide

C. V. Rao (✉) · N. B. Janakiram
Center for Cancer Prevention and Cancer Drug Development, Department of Medicine, Hematology Oncology Section, PCS Cancer Center, University of Oklahoma Health Sciences Center, 975 NE 10th Street, BRC 1203, OUHSC, Oklahoma City, OK 73104, USA
e-mail: cv-rao@ouhsc.edu

N. B. Janakiram (✉)
e-mail: naveena-janakiram@ ouhsc.edu

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_11

Abbreviations

NO	Nitric oxide
iNOS (NOS-II)	Inducible nitric oxide synthase
nNOS (NOS-I)	Neuronal nitric oxide synthase
eNOS (NOS-II)	Endothelial nitric oxide synthase
NK	Natural killer cells
DC	Dendritic cells
T reg	T regulatory cells
IL4	Interleukin 4
LPS	Lipopolysaccharide
IFN-γ	Interferon-γ
IL-10	Interleukin 10
TGF-β	Transforming growth factor-β
Bid	BH3 interacting domain
DnIKK2	Kinase-defective dominant negative form of IKK2
MCP-1	Monocyte chemo attractant protein 1
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
PNT	Peroxynitrite radical
PGE_2	Prostaglandin E_2
TNF-α	Tumor necrosis factor-α
COX-1	Cyclo-oxygenase-1
COX-2	Cyclooxygenase-2
NF-kB	Nuclear factor–κB
VEGF	Vascular endothelial growth factor
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
NSAID	NOn-steroidal anti-inflammatory drug
NO-NSAID	NO-releasing NSAID
L-NAME	*L*-nitro arginine methyl ester
L-NMA	N-monomethyl-L-arginine
Apc	Adenomatouspolyposis coli
CRC	Colorectal cancer
IL8	Interleukin 8
TILs	Tumor-infiltrating lymphocytes
TcR	T cell receptor
CTLs	Cytotoxic T lymphocytes
MDSCs	Myeloid-derived suppressor cells
AOM	Azoxymethane
Se-PBIT	Selenium [*S,S'*-1,4-phenylene*bis*(1,2-ethanediyl) *bis*-isothiourea]
GI	Gastrointestinal
MIP	Macrophage inflammatory protein
DMH	Dimethyl hydrazine
MDFs	Mucin depleted foci

DSS Dextran sulfate sodium
PGF2α Prostaglandin F2α
TxB2 Thromboxane B2

Introduction

Nitric Oxide (NO) is a free radical gas that is generated physiologically by organisms varying from bacteria to mammals. It acts as a signaling molecule in several biological processes. It is synthesized during transformation of the substrate L-arginine to L-citrulline by nitric oxide synthase (NOS) isoforms. The NOS family consists of three isoforms, neuronal NOS (nNOS; NOS-I), inducible NOS (iNOS; NOS-II), and endothelial NOS (eNOS; NOS-III). nNOS and eNOS are both constitutive forms of NOS. NO functions as both an intracellular and an extracellular messenger signaling molecule to regulate the vascular system, neurological functions, and inflammatory immune responses. It has been well established that NO and other reactive nitrogen species (RNS) and reactive oxygen species (ROS) play critical roles in immune responses, particularly in the "killing" of bacteria and other infectious parasites.

Nitric Oxide, Immune Response, and Tumor Growth

Several human cancers are associated with chronic viral, bacterial and parasitic infections and NO formation is elevated in these infections [1]. NO production that is excessive in concentration and/or duration can damage DNA, leading to gene mutations and cancer [2]. Most of the cells comprising a tumor mass (particularly infiltrating immune cells) have been shown to generate NO [3]. Expression of iNOS is reported to be high in various cancers, including esophageal and colon cancers and cancers of the cervix, breast, lung, and head and neck [4]. A significant number of reports suggests a positive role for NO in tumor cell proliferation; however, recently a few reports also suggest that NO may inhibit cell cycle progression and lead to suppression of cell proliferation. These opposing effects of NO suggest that NO has biphasic roles with the effect in a given situation depending on its concentration. Thus, it is critical to determine the functions of NO as an inhibitor and as a enhancer of tumor cell proliferation.

Several reports suggest a tumoricidal role for NO *in vivo*. Chronic inhibition of NO synthesis with N-monomethyl-L-arginine (L-NMA) resulted in increased tumor growth and delayed immune recognition in mice, implicating endogenous NO in the impaired ability of tumor cells to proliferate [5]. Moreover, the daily intraperitoneal administration of L-NMA prevented the tumoricidal activity in mice suggesting NO accounts for the tumoricidal activity [6]. As a result of these studies, NO releasing anti-inflammatory agents with minimal side effects are being tested

preclinically for beneficial antitumor effects [4, 7, 8]. However, cancer growth can be stimulated as well as inhibited by the immune system. The ability of the immune system to inhibit or stimulate tumor growth may relate to the intra-tumor macrophage arginine metabolism. It has been suggested that arginine metabolism in the tumor bed yielding citrulline and NO favors tumor rejection, whereas production of ornithine and urea could promote tumor growth [9]. These observations are consistent with the demonstrated tumor inhibitory effects of difluromethyl ornithine in the suppression of polyamines and tumor growth [10, 11].

Cytokine-Nitric Oxide Interactions

When macrophages are activated with interferon-γ (IFN-γ) and a low dose of lipopolysaccharide (LPS), they produce significant amounts of NO and express high levels of iNOS [12]. This production and expression can be reversed in a dose-dependent manner by interleukin 4 (IL-4). Interleukin 10 (IL-10) and transforming growth factor-β (TGF-β) also can inhibit NO synthesis. In contrast, IFNγ- and tumor necrosis factor-α (TNF-α) transmit a series of signals leading to the expression of iNOS and the synthesis of NO [13]. Advanced neoplasia has long been associated with defective capacity to mount responses to inflammatory stimuli. A small amount of NO may enhance the production of chemokines, such as macrophage inflammatory protein-2 (MIP-2) and monocyte chemo attractant protein 1 (MCP-1), whereas a large amount of NO may suppress these chemokines (Fig. 11.1). When cells such as neutrophils are activated with IFNγ- or other stimulants, they produce a large amount of NO via iNOS, and this concentration can be toxic to target immune cells [14] (Fig. 11.1). Cytokines leaking from advanced tumors can regulate NO levels,

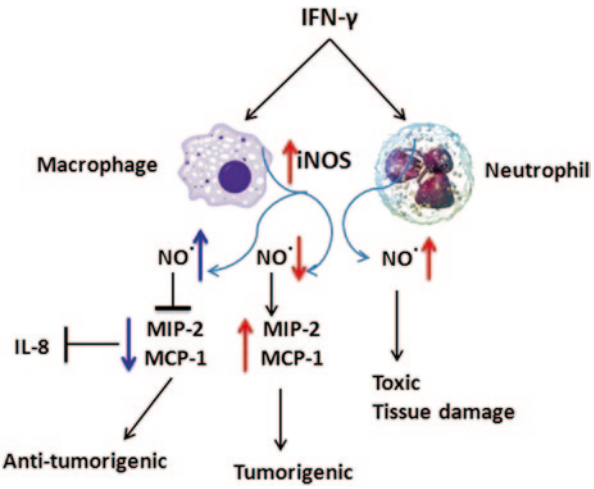


Fig. 11.1 NO and cytokine interactions: NO is synthesized when iNOS is stimulated by IFNγ- and other stimulants. NO in high levels inhibit MIP-2 and MCP-1 leading to anti-tumorigenic effects. However, NO in low levels increases MIP-2 and MCP-1 leading to tumorigenic effects. High NO levels induced by IFNγ- in neutrophils leads to effector functions towards tissue damage

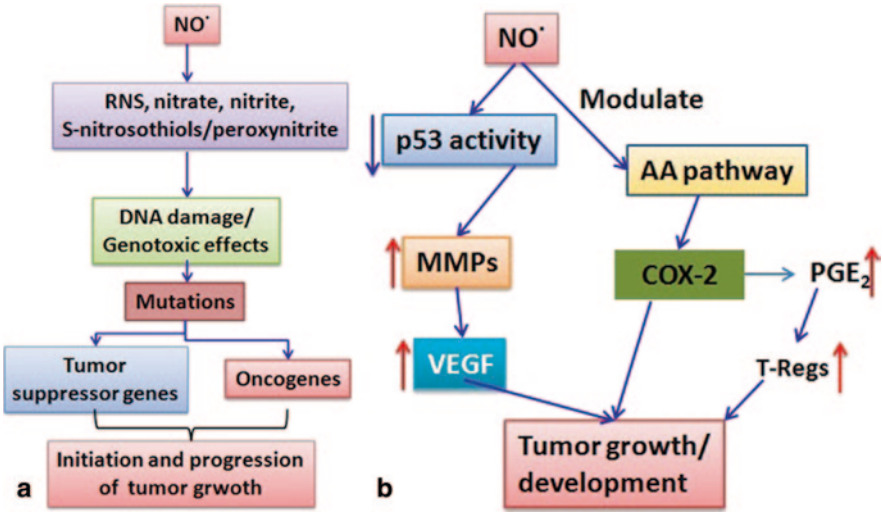


Fig. 11.2 **a.** Chronic NO synthesis leads to the production of reactive nitrogen species, nitrate, nitrite, S-nitrosothiols or peroxynitrites and causes DNA damage or genotoxic effects. This leads to the formation of mutations in tumor suppressor genes and oncogenes causing initiation and progression of tumor growth. **b.** Nitric oxide produced in low levels inactivates TP53 activity and leads to increase in MMPs and VEGF leading to Increase in angiogenesis and causing tumor development. It can also stimulate the arachidonic acid (*AA*) pathway leading to increase in COX-2 and proinflammatory eicosanoids and increase of T-Regulatory cells helping in tumor development

which, in turn, play a role in systemic defects in inflammation and immunity associated with neoplasia [15, 16]. Thus, a balance between chemotactic and inhibitory cytokines may infiltrate tissues, including neoplasms, and the immuno-regulatory effect of NO appears to be determined by the NO level.

Role of NO in Inflammation and Cancer Progression

The role of NO in cancer is controversial. It is reported to promote tumorigenesis and also to exert anti-tumorigenic activities. Various effects have been observed on events that occur during cancer formation, such as apoptosis, angiogenesis, cell cycle, invasion, and metastases. Since NO is a gas, it is highly diffusible in the vasculature, and since it is a highly reactive free radical, it forms various metabolites, usually by reacting with molecular oxygen and heavy metals, to generate different biological effects of NO. The RNS metabolites formed, such as nitrate, nitrite, S-nitrosothiols or peroxynitrite, can contribute to DNA damage and genotoxic effects [17, 18]) (Fig. 11.2). RNS can have direct effects by modifying DNA or indirect effects by inhibiting DNA repair mechanism to cause formation of mutations in tumor suppressor genes and in oncogenes initiating or driving tumor progression [19] (Fig. 11.2). NO is reported to repress p53 activity, influencing apoptotic pathways

[20] (Fig. 11.2b). It is involved in neo-vascular growth in tumors through effects on vascular endothelial growth factor (VEGF), and in tumor invasion by up-regulating matrix metalloproteinase's [21] (Fig. 11.2b). It also can regulate the arachidonic acid pathway, influencing prostaglandins synthesis to contribute to overall tumor cell proliferation, growth and invasion (Fig. 11.2b).

The roles of NO during initiation and maintainance of inflammatory bowel disease (IBD) are well documented. A high concentration of citrulline, which is a co-product of NO synthase resulting from iNOS activity, was observed in ulcerative colitis (UC) biopsy samples as compared with normal histology samples. Incubation of UC biopsy samples with the iNOS inhibitor L-NMA caused a decrease in citrulline, suggesting that NOS plays a vital role in causing UC [22]. Kolios *et al.* reported similar findings that colonic mucosa biopsies from both UC and Crohns disease patients had an eight-fold increase in iNOS activity compared with normal control colon mucosa [23]. The clinicopathological features displayed in these IBD tissues may relate to NO-induced vasodilation, activation of neutrophils, formation of peroxynitrite (PNT) radical and direct toxicity. These data show a definite link between increase in NO due to iNOS and pathological features associated with these chronic inflammatory disorders.

Clinically, and in preclinical animal models, NO production and iNOS expression often are detected at high levels in colon cancer [24, 25]. A recent report suggests that tyrosine nitration, along with iNOS expression is an indicator of colon cancer development and progression [26]. Thus, it is often assumed that continuous formation of NO in a tissue through iNOS is an important step in carcinogenesis leading to neoplastic transformation. Many studies have reported that NO produced by iNOS can help in initiating tumorigenesis or promoting tumor growth and metastases. Recently, it was reported that iNOS expression is correlated with tumor growth and poor prognosis in patients with colon cancer [20, 27]. A combined transgenic mouse having both the adenomatous polyposis coli (Apc) mutation and iNOS-/- condition has reduced formation of polyps in both large and small intestines compared with mice having the Apc mutation alone, suggesting a positive role for iNOS in colon cancer generation [28]. iNOS-/- mice also displayed less gastric carcinogenesis when infected with Helicobacter pylori [29]. Recently, it was reported that iNOS regulates Wnt/β-catenin signaling and increases cell proliferation and tumor growth in human colon cancer cells [30]. These reports suggest that targeting iNOS for prevention or treatment of colorectal cancer (CRC) is valid. Use of iNOS inhibitors alone or in combination with cyclo-oxygenase-2 (COX-2) inhibitors has shown significant inhibitory effects on colon carcinogenesis, reiterating the positive role of iNOS in CRC [31–34]. On the other hand, increasing NO signaling was proposed as an effective strategy in inhibiting CRC [35, 36]. In a xenograft study, Xu *et al*. showed that increasing NO signaling by delivering iNOS-expressing cells in the peritumoral region resulted in inhibition of tumors [37]. Seril *et al*. observed NO difference in iNOS+/+ and iNOS−/− mice when dextran sodium sulfate (DSS) was injected to study ulcerative colitis development, thus suggesting that iNOS is not involved in UC-associated cancer [38]. Most of the preclinical studies are in agreement with a decrease in NO signaling having beneficial anti-tumorigenic effects.

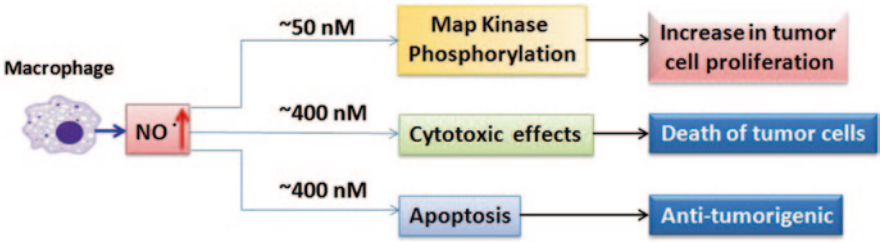


Fig. 11.3 Nitric oxide produced in accumulated macrophages within tumor cells leads to different outcomes at varied concentrations. NO in low levels leads to activation of Map Kinases in which increase tumor cell proliferation. However, NO in high levels causes increase in apoptosis via increase in p53 and p21, or causes cytotoixc effects on tumor cells and displaying anti-tumorigenic effects

However, a thorough analysis is needed to fill in gaps in our understanding of NO regulation in order to target aberrant NO production in tumors without disturbing the normal physiological functions of NO.

Dose-Dependent Effects of NO in Cancer Progression

NO is expressed highly in macrophages in and around the tumor tissue and also in tumor epithelial cells [39]. Depending on the concentrations present in these macrophages (from < 50–> 300 nm), NO behaves as a tumor promoter or inhibitor. Steady state and sustained lower concentrations of NO (< 50 nm) usually are associated with activating or increasing phosphorylation of kinases involving in extracellular signaling, which may enhance proliferation and anti-apoptotic activity and favor tumor growth (Fig. 11.3). Intermediate concentrations of NO are associated with stabilization of hypoxia-inducing factor-1α (HIF-1α). High concentrations such as ~400 nm NO in macrophages lead to induction of cytotoxicity against tumor cells and surrounding tissue. NO or peroxynitrite induce cell death by apoptosis or necrosis. NO was reported to induce the release of cytochrome c from the mitochondrial membrane without involvement of either caspase 8 or BH3-interacting domain (Bid) [40]. NO is also reported to induce apoptosis with an increase in p53 and p21 [41] (Fig. 11.3). p53 has binding sites in the promoters of iNOS and eNOS, which are used to regulate the synthesis of NO and apoptosis. Other cytotoxic effects of NO on tumor cells are through DNA damage [42]. Hence, at high levels, NO leads to both apoptotic and anti-tumorigenic functions in tumors.

Potential Positive and Negative Regulation of Immune Responses

Since NO is a highly reactive, gaseous free radical that diffuses freely across cell membranes, it is unique in possessing both paracrine and autocrine signaling functions in biological systems [43, 44]. It can undergo nitration and nitrosation, nitrosylation, and oxidation reactions and interact with other free radicals such as oxygen and hydrogen to modify metabolites in various cell types and to alter many biological signaling pathways [45]. The signaling mechanisms through which NO regulate immune cells are extremely complex. Both NO and its derivatives regulate immune cell responses by altering the structures of regulatory molecules. Of the three NOS isoforms, all of which are involved in immune responses [45–48], iNOS produces high levels of NO and is present in macrophages, dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), endothelial cells, natural killer (NK) cells, neuronal cells, neutrophils and tumor cells [49–55]. There is growing evidence that NO is versatile in mediating-effects on viral infections, the pathology of human bacterial infections and inflammatory disorders.

NO induced by iNOS plays a role in leukocyte recruitments and cell adhesion under inflammatory conditions. Experiments performed using flow chambers with NO supplied exogenously from NO donors suggested that NO inhibits adhesion of the leukocytes [56–58]. NO, similarly, may inhibit B and T cells; but the exact mechanisms for how NO exerts these functions are needed in order to design treatments. Acting as an intracellular messenger, NO can modulate MCP1 and inhibit interleukin 8 (IL8), and play an important role in chemokine signaling pathways [58, 59] (Fig. 11.1). It is well known, now, that NO is implicated in generation and functions of T-regulatory cells (Tregs). Tregs inhibit T cell-specific antigen-driven proliferation by producing IFN-γ and by direct interaction with antigen presenting cells. The IFN-γ acts by inducing iNOS in macrophages [57, 60] (Fig. 11.1). We and others have observed increased expression of iNOS and Tregs in colon tumors [61]. Immature dnIkk2 DCs loaded with antigens stimulate naïve mouse T cells to generate dnIkk2-Tregs, which express FOXp3 and iNOS (Fig. 11.4a). These dnIkk2-Tregs are reported to inhibit naïve and pre-activated T cell responses *in vitro* [62]. A recent report described the presence of a new Treg population, NO-Tregs, which are induced by NO, and regulated by T cell receptor (TcR) activation [63]. These NO-Tregs are reported to have a Th2 phenotype devoid of FOXp3 and to suppress CD4+CD25- effector T cells (Fig. 11.4b). The role of NO in the generation of NO-Tregs is demonstrated with the use of the NOS inhibitor L-NMMA. The results suggest that NO plays an important role in maintaining chronic inflammatory responses. We have reported an increased number of Tregs in colon tumors, and adoptive transfer of Tregs led to an increase in colon tumors [61]. These results suggest that suppression of iNOS and decreasing of Tregs during colon tumor development may be necessary to inhibit colon cancer development.

NO is reported to cause apoptosis of T cells by nitration of tyrosines in proteins to form peroxynitrite (PNT), leading to blockade of Janus Kinase 3 phosphorylation

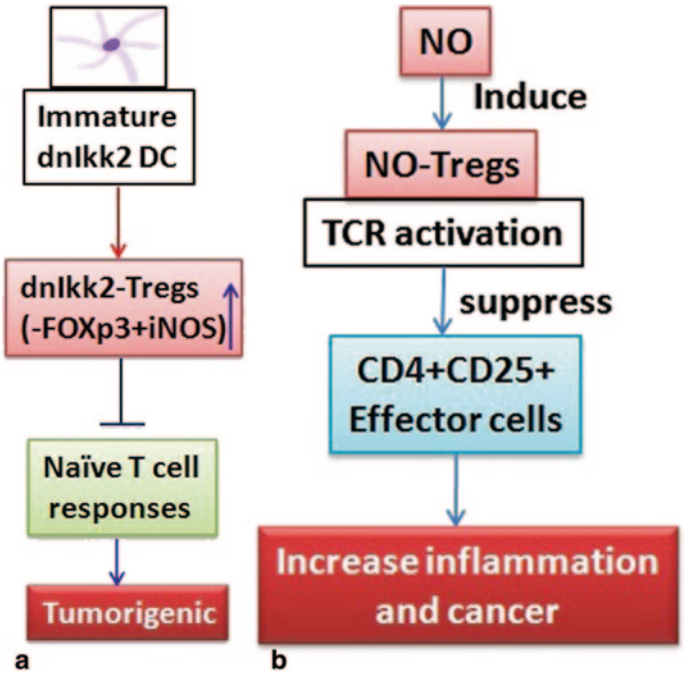


Fig. 11.4 **a.** Immature dendritic cells upon antigen stimulation produce dnIkk2-Tregs, which are FOXp3 negative and iNOS positive. These can inhibit naïve T cell responses causing tumorigenesis. **b.** Nitric oxide induces the formation of NO-Tregs by activation of TCR receptors which suppress effector cells and causing inflammation and cancer

[64] and increased expression of major histocompatibility complex class II (MHCII) proteins [65]. Nitrotyrosine is used as a marker for identifying peroxynitrite activity in cells and tissues [66]. PNT can cause oxidative damage by inducing nitration in tyrosine, cysteine, methionine, and tryptophan [67]. The damage is seen in cell membrane phospholipids, nucleic acids, and proteins. Nitrotyrosines often are identified in high quantities in thymic extracts and in thymic apoptotic cells, indicating a role for nitrotyrosines in thymic apoptosis *in vivo* [68]. The enzymes arginase 1 and NOS2 require the substrate L-arginine for production of NO [69]. High concentrations of PNT have been reported in many cancers, including colon cancer, and its presence in tumor tissues is associated with poor prognosis [25, 70–75].

In a tumor microenvironment, nitration in tumor-infiltrating lymphocytes (TILs) to generate high concentrations of PNT can force TILs to become nonresponsive to various nonspecific stimuli from the host's immune cells, leading to failure to eradicate the tumor [76]. PNT can cause nitration of the TCRs and, thereby, inhibits binding of CD8+T cells to peptide-MHC complexes, causing T cell tolerance against the tumor [77]. The recognition by CD8+and CD4+T cells of MHC class I- and MHC class II-restricted peptides is altered by nitration of a single tyrosine [78, 79]. Nitration of cytotoxic T lymphocytes (CTLs) led to loss of their activity against the

specific peptides as compared with that of non-nitrated CTLs [80]. These and other reports suggest that increased PNT in tumor-infiltrating MDSCs, and to a lesser extent in macrophages, does not affect the expression of MHC class I on the tumor cells; rather it leads to nitration of peptides causing decreased efficiency of their binding to MHC class I [81]. Hence, post-translational modifications of peptide-MHC I complexes on tumor cells induced by PNT need to be inhibited—alone or in combination, with antigens or vaccines designed to elicit tumor-specific immune responses—to enable or the enhance function of the CTLs to eliminate tumor cells.

The chemokine CCL2, a chemoattractant for NK cells, activated T cells and myeloid cells, is reported to be nitrated by PNT production induced by intra-tumoral cells, causing decreased T cell-migration to the tumor site [82, 83]. However, nitration of CCL2 did not alter the infiltration of myeloid cells into the tumor site. Therefore, fewer infiltrated T cells as compared with myeloid cells usually are observed in tumors.

Other tumor-promoting effects of NO include antimicrobial, antiviral, immunostimulatory and cyto-protective functions. NO exerts its antimicrobial functions in several ways including by inhibiting DNA repair and synthesis, causing DNA mutations, causing post-translational alteration of proteins and inhibiting enzymatic functions [57]. Also, during infection, it protects the tissue by inhibiting or terminating immune responses by causing apoptosis of activated T cells [84]. In activated macrophages, NO exhibits antimicrobial activity during pathogenic bacterial infections [85, 86]. Iniesta *et al.* further confirmed this effect by the use of the NOS inhibitor NMMA in infected mice; NMMA administration led to an increase in infection, suggesting a positive role of NO during microbial infections [86]. In the skin, NO generated by commensal bacteria has antimicrobial effects to limit external infections [87]. NO generated from iNOS also is implicated in causing tumor cell death. The IFNγ- and TNF-induced iNOS in CTL cells leads to killing of tumor cells [88]. The production of NO in the vasculature helps to prevent adhesion and release of oxidants by activated neutrophils [89].

Since NO has dual pro-inflammatory and anti-inflammatory roles, its replacement or augmentation with NO-releasing drugs has proved to be an important step in treatment of inflammatory disorders and cancers. NO-releasing non-steroidal inflammatory drugs (NO-NSAIDs) have been developed for the treatment of various inflammatory disorders and for treating cancers. NSAIDs and aspirin inhibit cyclooxygenase (COX) enzymes and are well known drugs prescribed for anti-inflammatory, antipyretic, analgesic and anti-thrombotic effects. The gastrointestinal toxicity of NSAIDs, that inhibit COX-1, led to the discovery of specific COX-2 inhibitors. Although these drugs are effective and tolerable, questions remain about the safety of these new COX-2 inhibitors, which couldn't replace aspirin.

NO and prostaglandin products of COX enzymes play a vital role in maintaining mucosal integrity. NO is reported to protect the mucosa by maintaining the blood flow, scavenging free radicals and functioning in mucus secretion. Hence, inhibition of COX by NSAIDs leads to gastrointestinal toxicity. Due to the overlapping functions of NO and COX, efforts were made to develop compounds that can counter the negative effects of COX inhibition and protect mucosal integrity by releasing

NO. The NO-NSAIDs developed proved to be effective anti-inflammatory and anti-tumorigenic agents and these compounds have been found to be safe and effective compared with traditional NSAIDs.

Nitrate-ester compounds, including 2-acetoxybenzoate 2-(2-nitroxy-methyl)-phenyl ester (NCX-4016) and 2-acetoxybenzoate 2-(2-nitroxy)-butyl ester (NCX-4215), are NO-releasing aspirins. NCX-4016 has been tested in many experimental models for its anti-inflammatory and anti-thrombotic effects [90]. NCX-4016 has been reported to inhibit caspases 1 and 3 [91, 92], T lymphocyte activation [89], cytokine release [92], apoptosis [92], and IL-1Bβ converting enzyme [93]. We tested NCX-4016 and NO-indomethicin (NCX-530) in azoxymethane (AOM)-induced colon cancer in rats at doses of 1500 or 3000 ppm and 0, 40, or 80 ppm, respectively. Both doses of NO-indomethacin and the high dose of NO-aspirin significantly suppressed both tumor incidence ($P<0.01$) and multiplicity ($P<0.001$). Inhibition of colonic tumors by these NO-NSAIDs correlated with inhibition of total COX activity, including COX-2 activity, and formation of prostaglandin E2 (PGE2), prostaglandin F2α (PGF2α), 6-keto-PGF1α, and thromboxane B2 (TxB2) from arachidonic acid. These drugs also suppressed nitric oxide synthase 2 (NOS-II) activity and expression of β-catenin [24].

Other animal colon cancer studies (Apc min study, xenograft study) have shown similar results for NCX-4016. In a study of Apc min study, three weeks of treatment with NCX-4016 caused 55% inhibition of tumors [94]. NCX-4016 (which caused 85% inhibition) was more effective in reducing aberrant crypt foci in AOM-induced rats than was aspirin (which caused 64% inhibition) [95]. The improved efficacy of NCX-4016 may be due to inhibition of MAPK, Wnt, NFKB and NOS signaling pathways. Although preclinical data were very promising, a recent phase I clinical trial with this agent was ended abruptly because of its DNA damaging effect in *in vitro*. Thus, further work is necessary to develop NO-NSAIDs with improved inhibition of tumor growth and better safety profiles, in addition to maintaining mucosal integrity. A recently reported NO-NSAID, Ethyl 2-((2,3-bis(nitrooxy)propyl) disulfanyl)benzoate (GT-094), a NO chimera containing NSAID and NO moieties and also a disulfide pharmacophore, induced apoptosis and inhibited proliferation of the colon cancer cell lines RKO and SW480 [96]. This agent suppressed transcription factors sp1, sp3 and sp4, which are highly expressed in colon cancer [96]. This inhibition was due to effects on the ROS-miR-27a:ZBTB10-Sp transcription factor pathway [96]. GT-094 has passed several milestones in preclinical testing but still needs to be analyzed further to satisfy other requirements before being considered for clinical trials.

Conclusions

NO is a versatile molecule with diverse biological functions having positive and negative effects on the immune system. It acts as both an intracellular and intercellular signaling molecule that plays a vital role in shaping immune responses against various pathogenic conditions and in sustaining homeostasis. Although it has been

more than 20 years since the identification of such diverse roles of NO, until recently attempts at developing safe approaches to modulate NO in cancer treatments progressed very slowly. Since NO has a role both in inflammatory and anti-inflammatory effects, it can switch from a regulator to a destroyer. Over-production of NO is reported to induce inflammatory diseases and tumor cell progression leading to cancer. Very little data are available on the role of NO in colon cancer progression via its effect on immune cell functions. Therefore, a brief overview is written in this chapter on the roles of NO and the immune system specifically in colon cancer initiation and progression. Since NO regulates multiple functions that can impact cancers, it is important to dissect the relative importance and concentration-dependence of these effects in specific situations. Improved understanding of these effects will help in the development of safer and more effective drugs and treatment strategies for colon and other cancers.

Acknowledgment The authors thank Dr. Julie Sando for her critical reading of the chapter. The works cited were supported by in part by grant NIH-NCI R01 CA109247 and the Kerley-Cade Chair Endowment.

References

1. Oshima H, Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutation Res. 1994;305:253–64.
2. Liu RH, Hotchkiss JH. Potential genotoxicity of chronically elevated nitric oxide: a review. Mut Res. 1995;339:73–89.
3. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumor progression. Nat Rev Cancer. 2006;6:521–34.
4. Janakiram NB, Rao CV. iNOS-selective inhibitors for cancer prevention: promise and progress. Future Med Chem. 2012;4:2193–204.
5. Yim CY, Bastian NR, Smith JC, Hibbs Jr JB, Samlowski WE. Macrophage nitric oxide synthesis delays progression of ultraviolet light-induced murine skin cancers. Cancer Res. 1993;53:5507–11.
6. Farias-Eisner R, Sherman MP, Aeberhard E, Chaudhuri G. Nitric oxide is an important mediator for tumoricidal activity in vivo. Proc Natl Acad Sci U S A. 1994;91:9407–11.
7. Rao CV, Reddy BS, Steele VE, Wang CX, Liu X, Ouyang N, Patlolla JM, Simi B, Kopelovich L, Rigas B. Nitric oxide-releasing aspirin and indomethacin are potent inhibitors against colon cancer in azoxymethane-treated rats: effects on molecular targets. Mol Cancer Ther. 2006;5:1530–8.
8. Rigas B, Kashfi K. Nitric-oxide-donating NSAIDs as agents for cancer prevention. Trends Mol Med. 2004;10:324–30.
9. Mills CD, Shearer J, Evans R, Caldwell MD. Macrophage arginine metabolism and the inhibition or stimulation of cancer. J Immunol. 1992;149(8):2709–14.
10. Gerner EW, Meyskens FL. Polyamines and cancer: old molecules, new understanding. Nat Rev Cancer. 2004;4:781–92.
11. Mohammed A, Janakiram NB, Brewer M, Vedala, Steele VE, Rao CV. Multitargeted low-dose GLAD combination chemoprevention: a novel and promising approach to combat colon carcinogenesis. Neoplasia. 2013;15:481–90.
12. Liew FY. Interactions between cytokines and nitric oxide. Adv Neuroimmunol. 1995;5:201–9.
13. Otsuka Y, Nagano K, et al. Inhibition of neutrophil migration by tumor necrosis factor. Ex vivo and in vivo studies in comparison with in vitro effect. J Immunol. 1990;145:2639–43.

14. Kobayashi Y. The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. J Leukoc Biol. 2010;88:1157–62.
15. Mazzoni A, Bronte V, Visintin A, et al. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J Immunol. 2002; 168:689–95.
16. Lejeune P, Lagadec P, Onier N, Pinard D, Ohshima H, Jeannin JF. Nitric oxide involvement intumor-induced immunosuppression. J Immunol.1994;152:5077–83.
17. Ohshima H, Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutat Res. 1994;305:253–64.
18. Ohshima H, Gilibert I, Bianchini F. Induction of DNA strand breakage and base oxidation by nitroxyl anion through hydroxyl radical production. Free Radic Biol Med. 1999;(9–10):1305–13.
19. Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol. 2001;2:149–56.
20. Ambs S, Mofolusara O, Ogunfusika MO, Merriam WG, Bennett WP, Billiard TR, Harris CC. Up-regulation of inducible nitric oxide synthase expression in cancer-prone p53 knockout mice. Proc Natl Acad Sci U S A. 1998;95:8823–8.
21. Babykutty S, Suboj P, Srinivas P, Nair AS, Chandramohan K, Gopala S. Insidious role of nitric oxide in migration/invasion of colon cancer cells by upregulating MMP-2/9 via activation of cGMP-PKG-ERK signaling pathways. Clin Exp Metastasis. 2012;29:471–92.
22. Boughton-Smith NK. Pathological and therapeutic implications for nitric oxide in inflammatory bowel disease. J R Soc Med. 1994;87:312–4.
23. Kolios G, Valatas V, Ward S. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. Immunology. 2004;113:427–37.
24. Rao CV, Reddy BS, Steele VE, Wang C-X, Liu X, Ouyang N, Patlolla JMR, Simi B, Kopelovich L, Rigas B. Nitric oxide–releasing aspirin and indomethacin are potent inhibitors against colon cancer in azoxymethane-treated rats: effects on molecular targets. Mol Cancer Ther. 2006;5:1530–8.
25. Jenkins DC, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S. Roles of nitric oxide in tumor growth. Proc Natl Acad Sci U S A. 1995;92:4392–6.
26. Gochman E1, Mahajna J, Shenzer P, Dahan A, Blatt A, Elyakim R, Reznick AZ. The expression of iNOS and nitrotyrosine in colitis and colon cancer in humans. Acta Histochem. 2012;114:827–35.
27. Ambs S, Bennett WP, Merriam WG, Ogunfusika MO, Oser SM, Harrington AM, Shields PG, Felley-Bosco E, Hussain SP, Harris CC. Relationship between p53 mutations and inducible nitric oxide synthase expression in human colorectal cancer. J Natl Cancer Inst. 1999;91:86–8.
28. Ahn B, Ohshima H. Suppression of intestinal polyposis in Apc(Min/+) mice by inhibiting nitric oxide production. Cancer Res. 2001;61:8357–60.
29. Erdman SE, Rao VP, Poutahidis T, Rogers AB, Taylor CL, Jackson EA, Ge Z, Lee CW, Schauer DB, Wogan GN, Tannenbaum SR, Fox JG. Nitric oxide and TNF-alpha trigger colonic inflammation and carcinogenesis in Helicobacter hepaticus-infected, Rag2-deficient mice. Proc Natl Acad Sci U S A. 2009;106:1027–32.
30. Du Q, Zhang X, Liu Q, Zhang X, Bartels CE, Geller DA. Nitric oxide production upregulates Wnt/b-catenin signaling by inhibiting Dickkopf-1. Cancer Res. 2013;73:6526–37.
31. Janakiram NB, Swamy MV, Patlolla JM, Guruswamy S, Rao CV. A selective iNOS inhibitor N6- iminoethyl-lysine tetrazoleamide (NILT), suppress invasive colonic cancers and improves preventive efficacy of low-dose COX-2 inhibitor, celecoxib in F344 rats. 101st AACR Annual Meeting 2010 held at Washington DC. April 17–21. Volume 70; Abstract 954; 2010.
32. Rao CV, Kawamori T, Hamid R, Reddy BS. Chemoprevention of colonic aberrant crypt foci by an inducible nitric oxide synthase- selective inhibitor. Carcinogenesis. 1999;20:641–4.
33. Rao CV, Indranie C, Simi B, Manning PT, Connor JR, Reddy BS. Chemopreventive properties of a selective inducible nitric oxide synthase inhibitor in colon carcinogenesis, adminis-

tered alone or in combination with celecoxib, a selective cyclooxygenase-2 inhibitor. Cancer Res. 2002;62:165–70.
34. Thomsen, LL, Scott JMJ, Topley P, Knowles RG, Keerie AJ, Frend AJ. Selective inhibition of inducible nitric oxide synthase inhibits tumor growth in vivo: studies with 1400W, a novel inhibitor. Cancer Res. 1997;57:3300–4.
35. Scott D, Hull MA, Cartwright EJ, Lam W, Tisbury A, Poulsom R, et al. Lack of inducible nitric oxide synthase promotes intestinal tumorigenesis in the ApcMin/+ mouse. Gastroenterology. 2001;121: 889–99.
36. Hussain SP, Trivers GE, Hofseth LJ, He P, Shaikh I, Mechanic LE, Doja S, Jiang W, Subleski J, Shorts L, Haines D, Laubach VE, Wiltrout RH, Djurickovic D, Harris CC. Nitric oxide, a mediator of inflammation, suppresses tumorigenesis. Cancer Res. 2004;64:6849–53.
37. Xu W, Liu L, Charles IG. Microencapsulated iNOS-expressing cells cause tumor suppression in mice. FASEB J. 2002;16:213–5.
38. Seril DN, Liao J, Yang G-Y. Colorectal carcinoma development in inducible nitric oxide synthase-deficient mice with dextran sulfate sodium-induced ulcerative colitis. Mol Carcinog. 2007;46:341–53.
39. Mosser DM, Zhang X. Activation of murine macrophages. Curr Protoc Immunol. 2008. doi:10.1002/0471142735.im1402s83.
40. Brookes PS, Salinas EP, Darley-Usmar K, Eiserich JP, Freeman BA, Darley-Usmar VM. Concentration-dependent effects of nitric oxide on mitochondrial permeability transition and cytochrome c release. J Biol Chem. 2000;275:20474–9.
41. Kolb JP. Mechanisms involved in the pro- and anti-apoptotic role of NO in human leukemia. Leukemia. 2000;14:1685–94.
42. Cai H, Dikalov S, Griendling KK, Harrison DG. Detection of reactive oxygen species and nitric oxide in vascular cells and tissues: comparison of sensitivity and specificity. Methods Mol Med. 2007;139:293–311.
43. Ignarro IJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci U S A. 1987;84:9265–9.
44. Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature. 1987;327:524–6.
45. Lancaster JR Jr, Xie K. Tumors face NO problems? Cancer Res. 2006;66:6459–62.
46. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. Nat Rev Cancer. 2003;3:276–85.
47. Lamas S, Lowenstein CJ, Michel T. Nitric oxide signaling comes of age:20 years and thriving. Cardiovasc Res. 2007;75:207–9.
48. Hofseth LJ, Hussain SP, Wogan GN, Harris CC. Nitric oxide in cancer and chemoprevention. Free Radic Biol Med. 2003;34:955–68.
49. Aiello S, Noris M, Piccinini G, Tomasoni S, Casiraghi F, Bonazzola S, Mister M, Sayegh MH, Remuzzi G. Thymic dendritic cells express inducible nitric oxide synthase and generate nitric oxide in response to self- and alloantigens. J Immunol. 2000;164:4649–58.
50. Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. Crit Rev Oncol Hematol. 2008;66:1–9.
51. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol. 2005;5:641–54.
52. Cifone MG, Ulisse S, Santoni A. Natural killer cells and nitric oxide. Int Immunopharmacol. 2001;1:1513–24.
53. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol. 2009;182:4499–506.
54. Rotondo R, Bertolotto M, Barisione G, Astigiano S, Mandruzzato S, Ottonello L, Dallegri F, Bronte V, Ferrini S, Barbieri O. Exocytosis of azurophil and arginase 1-containing granules by activated polymorphonuclear neutrophils is required to inhibit T lymphocyte proliferation. J Leukoc Biol. 2011;89: 721–7.

55. Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest. 2007;117:1155–66.
56. Grisham M, Granger D, Lefer D. Modulation of leukocyte-endothelial interactions by reactive metabolites of oxygen and nitrogen: relevance to ischemic heart disease. Free Radic Res. 1998;25:404–33.
57. Bogdan C. Nitric oxide and the immune response. Nat Immunol. 2001;2:907–15.
58. Bogdan C. The function of nitric oxide in the immune system. In: Mayer B, editor. Handbook of experimental pharmacology. Nitric oxide. Heidelberg: Springer; 2000. pp. 443–92.
59. Cheria R, Ganu R. Stromal cell-derived factor 1a—induced chemotaxis in T cells is mediated by nitric oxide signalling pathways. J Immunol. 2001;166:3067–74.
60. Atochina O, Daly-Engel T, Piskorska D, McGuire E, Harn DA. A schistosome-expressed immunomodulatory glycoconjugate expands peritoneal Gr1 + macrophages that suppress naive CD4 + T cell proliferation via an IFN-γ and nitric oxide-dependent mechanism. J Immunol. 2001;167:4293–302.
61. Janakiram NB, Mohammed A, Bryant T, Brewer M, Biddick L, Lightfoot S, Lang ML, Rao CV. Adoptive transfer of regulatory T cells promotes intestinal tumorigenesis and is associated with decreased NK cells and IL-22 binding protein. Mol Carcinogenesis. 2014. doi:10.1002/mc.22168.
62. Aiello S, Cassis P, Cassis L, Tomasoni S, Benigni A, Pezzotta A, Cavinato RA, Cugini D, Azzollini N, Mister M, Longaretti L, Thomson AW, Remuzzi G, Noris M. DnIKK2-transfected dendritic cells induce a novel population of inducible nitric oxide synthase-expressing CD4+ CD25—cells with tolerogenic properties. Transplantation. 2007;83:474–84.
63. Niedbala W, Cai B, Liu H, Pitman N, Chang L, Liew FY. Nitric oxide induces CD4+ CD25+ Foxp3 regulatory T cells from CD4+ CD25–T cells via p53, IL-2, and OX40. Proc Natl Acad Sci U S A. 2007;104:15478–83.
64. Brito C, Naviliat M, Tiscornia AC, Vuillier F, Gualco G, Dighiero G, Radi R, Cayota AM. Peroxy nitrite inhibits T lymphocyte activation and proliferation by promoting impairment of tyrosine phosphorylation and peroxynitrite-driven apoptotic death. J Immunol. 1999;162:3356–66.
65. Michel T, Feron O. Nitricoxidesynthases:which,where,how,andwhy? J Clin Invest. 1997;100:2146–52.
66. Haqqani AS, Kelly JF, Birnboim HC. Selective nitration of histone tyrosine residues in vivo in mutatect tumors. J Biol Chem. 2002;277: 3614–21.
67. Ekmekcioglu S, Ellerhorst J, Smid CM, Prieto VG, Munsell M, Buzaid AC, Grimm EA. Inducible nitric oxide synthase and nitrotyrosine in human metastatic melanoma tumors correlate with poor survival. Clin Cancer Res. 2000;6:4768–75.
68. Moulian N, Truffault F, Gaudry-Talarmain YM, Serraf A, Berrih-Aknin S. Invivo and invitro apoptosis of human thymocytes are associated with nitrotyrosine formation. Blood. 2001; 97:3521–30.
69. Bronte V, Serafini P, De Santo C, Marigo I, Tosello V, Mazzoni A, Segal DM, Staib C, Lowel M, Sutter G, Colombo MP, Zanovello P. IL-4-induced arginase 1 suppresses alloreactive T cells in tumorbearing mice. J Immunol. 2003;170:270–8.
70. Vickers SM, MacMillan-Crow LA, Green M, Ellis C, Thompson JA. Association of increased immunostaining for inducible nitric oxide synthase and nitrotyrosinewith fibroblast growth factor transformation in pancreatic cancer. Arch Surg. 1999;134:245–51.
71. Cobbs CS, Whisenhunt TR, Wesemann DR, Harkins LE, Van Meir EG, Samanta M. Inactivation of wild-type p53 protein function by reactive oxygen and nitrogen species in malignant glioma cells. Cancer Res. 2003;63:8670–3.
72. Kinnula VL, Torkkeli T, Kristo P, Sormunen R, Soini Y, Pääkkö P, Ollikainen T, Kahlos K, Hirvonen A, Knuutila S. Ultrastructural and chromosomal studies on manganese superoxide dismutase in malignant mesothelioma. Am J Respir Cell Mol Biol. 2004;31:147–53.
73. Nakamura Y, Yasuoka H, Tsujimoto M, Yoshidome K, Nakahara M, Nakao K, Nakamura M, Kakudo K. Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis. Clin Cancer Res. 2006;12:1201–7.

74. Ekmekcioglu S, Ellerhorst JA, Prieto VG, Johnson MM, Broemeling LD, Grimm EA. Tumor iNOS predicts poor survival for stage III melanoma patients. Int J Cancer. 2006;119:861–6.
75. Szaleczky E1, Prónai L, Nakazawa H, Tulassay Z. Evidence of in vivo peroxynitrite formation in patients with colorectal carcinoma, higher plasma nitrate/nitrite levels, and lower protection against oxygen free radicals. J Clin Gastroenterol. 2000;30:47–51.
76. Bronte V, Casic T, Gri G, Gallana K, Borsellino G, Marrigo I, Battistini L, Iafrate M, Prayer-Galetti T, Pagano F, Viola A. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. J Exp Med. 2005;201:1257–68.
77. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, Herber DL, Schneck J. Gabrilovich D Altered recognition of antigen is a mechanism of CD8þT cell tolerance in cancer. Nat Med. 2007;13:828–35.
78. Birnboim HC, Lemay AM, Lam DK, Goldstein R, Webb JR. Cutting associated marker 3-nitrotyrosine evade central tolerance and elicit a robust cell-mediated immune response. J Immunol. 2003;171:528–32.
79. Hardy LL, Wick DA, Webb JR. Conversion of tyrosine to the inflammation-associated analog 30-nitrotyrosine at either TCR- or MHC contact positions can profoundly affect recognition of the MHC class I-restricted epitope of lymphocytic choriomeningitis virus glycoprotein 33 by CD8 T cells. J Immunol. 2008;180:5956–62.
80. Nagaraj S, Schrum AG, Cho HI, Celis E, Gabrilovich DI. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. J Immunol. 2010;184:3106–16.
81. Lu T, Ramakrishnan R, Altiok S, Youn JI, Cheng P, Celis E, et al. Tumor infiltrating myeloid cells induce tumor cell resistance to cytotoxic T cells in mice. J Clin Invest. 2011;121:4015–29.
82. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, De Palma A, Mauri P, Monegal A, Rescigno M, Savino B, Colombo P, Jonjic N, Pecanic S, Lazzarato L, Fruttero R, Gasco A, Bronte V, Viola A. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. J Exp Med. 2011;208:1949–62.
83. Allavena P, Bianchi G, Zhou D, van Damme J, Jilek P, Sozzani S, Montavani A. Induction of natural killer cell migration by monocyte chemotactic protein-1, -2 and -3. Eur J Immunol. 1994;24:3233–6.
84. Dalton D, Haynes L, Chu C, Swain S, Wittmer S. Interferon-g eliminates responding CD4 T cells during mycobacterial infection by inducing apoptosis of activated CD4 T cells. J Exp Med. 2000;192:117–22.
85. Kroncke K, Fehsel K, Kolb-Bachofen V. Inducible nitric oxide synthase in human disease. Clin Exp Immunol. 1998;113:147–56.
86. Iniesta V, Gomez-Nieto LC, Corraliza I. Theinhibition of arginase by N(omega)-hydroxy-L-arginine controls the growth of Leishmania inside macrophages. J Exp Med. 2001;193:777–84.
87. Weller R, Pattullo S, Smith L, Golden M, Ormerod A, Benjamin N. Nitric oxide Is generat on the skin surface by reduction of sweat nitrate. J Investig Dermatol. 1996;107:327–31.
88. Abramson SB, Attur M, Amin AR, Clancy R. Nitric oxide and inflammatory mediators in the perpetuation of osteoarthritis. Curr Rheumatol Rep. 2001;3:535–41.
89. Kwak J, Han MK, Choi KS, Park IH, Park SY, Sohn MH, Kim UH, McGregor JR, Samlowski WE, Yim CY. Cytokines secreted by lymphokine-activated killer cells induce endogenous nitric oxide synthesis and apoptosis in DLD-a colon cancer cells. Cellular Immunology. 2000;203:84–94.
90. Del Soldato P, Sorrentino R, Pinto A. NO-aspirins, a class of new-inflammatory and antithrombotic agents. Trends Pharmacol Sci. 1999;20:319–23.
91. Fiorucci S, Antonelli E, Santucci L, Morelli O, Miglietti M, Federici B, Mannucci R, Del Soldato P, Morelli A. Gastrointestinal safety of nitric oxide derived aspirin is related to inhibition if ICE-like cysteine proteases in rats. Gastroenterology. 1999;116:1089–106.
92. Fiorucci S, Santucci L, Antonelli E, Distrutti E, Del Sero G, Morelli O, Romani L, Federici B, Del Soldato P, Morelli A. NO aspirin protects from T cell-mediated liver injury by inhibiting caspase dependent processing of Th1-like cytokines. Gastroenterology. 2000;118:404–21.

93. Fiorucci S, Santucci L, Cirino G, Mencarelli A, Familiari L, Soldato PD, Morelli A. IL-1Bb converting enzyme is a target for nitric oxide-releasing aspirin: new insights in the antiinflammatory mechanism of nitric oxide-releasing nonsteroidal antiinflammatory drugs. J Immunol. 2000;165:5245–54.
94. Williams JL, Kashfi K, Ouyang N, del Soldato P, Kopelovich L, Rigas B. NO-donating aspirin inhibits intestinal carcinogenesis in Min (APC(Min/+) mice. Biochem Biophys Res Commun. 2004;313:784–8.
95. Bak AW, McKnight W, Li P, Del Soldato P, Calignano A, Cirino G, Wallace JL. Cyclooxygenase-independent chemoprevention with an aspirin derivative in a rat model of colonic adenocarcinoma. Life Sci. 1998;62:L367–73.
96. Pathi SS, Jutooru I, Chadalapaka G, Sreevalsan S, Anand S, Thatcher GRJ, Safe S. GT-094, a NO-NSAID, inhibits colon cancer cell growth by activation of a reactive oxygen species-MicroRNA-27a: ZBTB10-specificity protein pathway. Mol Cancer Res. 2011;9:195–205.

Part IV
Therapeutics and Overcoming Resistance

Chapter 12
Pivotal Role of Nitric Oxide in Chemo and Immuno Sensitization of Resistant Tumor Cells to Apoptosis

Benjamin Bonavida

Abstract Nitric oxide (NO) has been the subject of many reports with respect to its role in cancer. These various reports were not consistent and often contradictory. On one hand, the levels of iNOS correlated with both the progression and carcinogenesis of certain tumors, whereas, on the other hand, the levels of iNOS or treatment with NO donors correlated with tumor regression. These contradictory findings were interpreted as due to the relative levels of generated NO, such as lower levels were pro-tumorigenic whereas high levels were anti-tumorigenic. Initial findings by us and others demonstrated that the induction of iNOS in resistant tumors or the treatment with NO donors resulted in the reversal of resistance and the tumor cells were chemo and immuno sensitized to cytotoxic stimuli. Consequently, we have extended our investigations to examine the biochemical and molecular underlying mechanisms responsible for NO-mediated chemo-immunosensitizing activities to apoptosis by cytotoxic agents. In our investigations, we have established, in several tumor cell lines, the highly dysregulated NF-κB/Snail/YY1/RKIP/PTEN/PI3K-AKT loop that regulates both drug/immune resistance and EMT/metastasis. This dysregulated loop was established by the demonstration that most tumor cells exhibit constitutively hyper-activated NF-κB and PI3K-AKT pathways that regulate cell viability, proliferation and anti-apoptotic pathways through a large number of downstream target gene products. Independent analyses revealed that the transcription factor Yin Yang 1 (YY1), downstream of NF-κB, was a repressor of the Fas and DR5 death receptors expression on many tumor cells and correlated with the tumor cells' resistance to corresponding FasL and TRAIL death ligands, respectively, on the surface of cytotoxic lymphocytes. In addition, the overexpression of YY1 in tumor cells regulates positively the transcription repressor factor Snail

B. Bonavida (✉)
Department of Microbiology, Immunology & Molecular Genetics, David Geffen School of Medicine, Jonsson Comprehensive Cancer Center, University of California at Los Angeles, 90095 Los Angeles, CA, USA
e-mail: bbonavida@mednet.ucla.edu

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_12

that regulates both EMT and resistance. The recent findings demonstrated that the metastasis-suppressor gene product, Raf kinase inhibitor protein, RKIP, which is minimally expressed in many cancers, was involved in the reversal of drug/immune resistance when it is overexpressed. Overexpression of RKIP was also reported to inhibit both the Raf/MEK/ERK and NF-κB pathways and, thus, also inhibited YY1 and Snail downstream of NF-κB. Snail-mediated inhibition of RKIP and PTEN expressions resulted in maintaining the activation of the PI3K-AKT survival pathway and its crosstalk with the NF-κB pathway. Overexpression of RKIP resulted in the upregulation of PTEN and inhibition of PI3K-AKT and NF-κB and resulting in the downregulation of YY1 and Snail and sensitization to chemo-immune-mediated apoptosis.

Altogether, the above findings established the tightly dysregulated NF-κB/Snail/YY1/RKIP/PTEN/PI3K-AKT loop that regulates cell survival, cell proliferation, EMT/metastasis, and resistance to apoptotic stimuli by cytotoxic agents. This dysregulated loop was shown to be significantly altered by NO due to the findings that treatment with NO donors resulted in the S-nitrosylation of p50/p65 chains of NF-κB and S-nitrosylation of both YY1 and Snail and, thus, inhibiting their activities and resulting in the upregulation of RKIP and PTEN. These gene products, in turn, also inhibit NF-κB and PI3K, respectively, and potentiate the NO-mediated inhibitory activity. These effects result in the inhibition of cell viability, proliferation, EMT/metastasis, and sensitization to apoptosis by cytotoxic immune-chemo drugs. The findings in vitro of the sensitizing activities of NO donors were corroborated in part *in vivo* in mice bearing tumor xenografts.

In this chapter, we briefly review our findings and those of others of the anti-tumor chemo and immune sensitizing activities of NO donors through their interference, in part, of the highly dysregulated NF-κB/Snail/YY1/RKIP/PTEN/PI3K-AKT resistance and metastatic loop. We suggest that NO donors are a new class of therapeutic agents that can exert simultaneously multiple anti-tumor effects at the level of cell viability, cell proliferation, induction of EMT, and chemosensitizing activities. The development and clinical application of various NO donors in combination with various therapeutics in the treatment of resistant and metastatic tumors are highly warranted, provided they are subtoxic to normal tissues.

Keywords Akt · Apoptosis · Chemotherapy · Immunotherapy · NF-κB · Nitric oxide · Resistance · RKIP · Snail · YY1

Abbreviations

CRC	Colorectal cancer
CTL	Cytotoxic T lymphocyte
DETANONOate	Diethylenetriamine NONOate
DR4	Death receptor 4
DR5	Death receptor 5
EMT	epithelial mesenchymal transition
FasL	Fas ligand

iNOS	Inducible nitric oxide synthase
NK	Natural killer
NPI-0052	Proteasome inhibitor
PDT	Photodynamic therapy
Rituximab	Chimeric anti-CD20 mAb
RKIP	Raf kinase inhibitor protein
TRAIL	TNF-related apoptosis-inducing ligand
YY1	Yin-Yang 1

This paper has not been published nor is it under consideration to be published by any other journal.

Introduction

General

The free radical NO chemical exerts pleiotropic activities. It is involved in cell signaling [1, 2] and cell regulation [3]. It is also involved in mediating cytotoxicity by blood cells [4, 5]. The various activities mediated by NO depend on concentrations; cytotoxicity by NO requires high levels of NO [6] and the regulation of platelets secretions, for example, requires low levels of NO [7]. The levels are, in part, regulated by various NOS isoforms present in the cells and their activation. These include endothelial (eNOs), a calcium-dependent form, which functions outside the nervous system, neuronal (nNOs), a calcium-dependent form involved in cell signaling of the CNS and inducible (iNOs), involved in immunological responses and the generation of high levels of NO [8]. The NO products of nNOs and eNOs act in a cGMP-dependent manner [9, 10] whereas the NO produced by iNOS is cGMP-independent [11]. NO has been shown to play a role in the regulation of immune responses [12–14].

Inducible NOS (iNOS) exerts many biological properties including its role in immune response mediated by CTL and macrophages [15]. It also exerts a negative feedback mechanism by inhibition of NF-κB activity, which transcribes iNOS [16]. The concentration of NO released by iNOS is very high (0.1–4 μM) depending on the stimulus [17].

NO and Cancer

The role of NO in cancer was first reported by Shinki et al. [18], whereby, murine activated macrophages synthesized nitrite and nitrate and mediated cytotoxicity against bacteria and tumor cells [19, 20] and subsequently, many reviews were published on the role of NO in cancer [21–23]. High levels of NO exert anti-neoplastic activ-

ity whereas low levels are pro-tumorigenic. Sustained NO production leads to the activation of caspases and apoptosis whereas low doses have opposing effects [24].

Evidence in the literature suggested that low levels of NO are pro-tumorigenic and enhance cell survival, induce proliferation, and protect cells from apoptosis; however, at higher levels, NO induces cytotoxic activities and inhibits tumor progression [25]. In addition, NO has also been reported to exert pro- and anti-metastatic activities [25, 26].

High levels of NO manifest their activities against tumor cells by various mechanisms including (1) apoptotic stimuli by (a) upregulation of p53 [27] (2) proteasomal degradation of anti-apoptotic molecules [28] (3) release of Smac/DIABLO [29] (4) cytochrome C release and increased mitochondrial permeability [30] and (5) formation of peroxinitrite $ONOO^-$ that leads to increased p53 [21] (b) cell cycle arrest [31] (c) cell death by necrosis [32] (d) inhibition of angiogenesis [33] and (e) cytotoxicity [2]. Other mechanisms *in vivo* have been reviewed [9].

NO-Mediated Sensitization of Resistant Cancer Cells to Drug-Induced Apoptosis

General

Many patients who have initially responded to cancer treatment therapies experience relapses and recurrences and no longer respond to further treatments. The development of resistance is accompanied by cross-resistance to a variety of unrelated apoptotic stimuli. The underlying mechanism of resistance through anti-apoptotic mechanisms is the result of constitutively hyperactivated survival pathways that regulate the apoptotic pathways. One predominant hyperactivated pathway is the NF-κB [34]. Hence, NF-κB regulates the expression of a number of genes involved in tumoregenesis, including anti-apoptotic genes *CIPs, survivin, TRAF, cFLIP, Bcl-2, Bcl-$_{xl}$, Mcl1*, etc. and genes encoding adhesion molecules and invasion, examples include *COX-2, MMP-9 and VEGF*, genes encoding chemokines and inflammatory cytokines and cell cycle-regulating genes such as *cyclin-D1* [35]. FDA approved therapeutics that inhibit NF-κB are exemplified by bortezomib for the treatment of multiple myelomas [36, 37]. Since NO has been reported to inhibit NF-κB activity by nitrosylation of p50 [38], we have used NO donors as potential agents for the reversal of resistance.

Several classes of NO donors have been reported in cancer and include organic nitrates, metal NO cyclases, sodium nitroprusside (SNP), S-nitrothiols, syndonimines, diazeniumdiolates (NONOates) and NO drug hybrids. The NO donors belonging to the class diazeniumdiolates have been used and shown to be potent chemo-immunosensitizing agents in various cancer cells [39].

Chemosensitization of Drug-Resistant Cancer Cells by NO

Nagai et al. [40] investigated the sensitization by NO to pemetrexed (PEM), a multitargeted antifolate and an antineoplastic agent, in resistant tumor cells and resulted in the induction of cytotoxicity. PEM is a novel antifolate, antineoplastic agent for NSCLC or malignant pleural mesothelioma. The NO donor NOC-18 and GTN combination therapy with PEM reduced tumor growth *in vivo* compared with PEM alone.

Ye et al. [41] used cationic liposome-mediated iNOs gene transfection and low dose CDDP on human lung adenocarcinoma *in vitro* and *in vivo*. The combination treatment resulted in the inhibition of cell proliferation, invasion, migration and apoptosis. The intratumoral administration of the liposome improved the low dose CDDP-mediated inhibition of tumor growth. In addition, intravenous administration inhibited lung metastasis.

Patients with metastatic colon cancer (mCRC) do not respond to conventional therapies and experience a short survival span. Clearly, mCRC exhibits intrinsic and acquired resistance to a variety of cytotoxic therapeutics. These patients need urgently novel therapeutic approaches to alter the fate of the disease. The cross-resistance exhibited by mCRC is exemplified in CRC resistance to CDDP but also resistance to a variety of unrelated drugs [42]. Analysis of agents that can overcome resistance to mCRC was examined by established tumor cell lines derived from patients. The SW480 colon cancer cell line was established from a primary, stage II legion while the SW620 was obtained from the same patient a year later from a metastatic lymph node (stage III). SW620 is resistant to CDDP [43–45]. In the study by Huerta et al. [46], the NO donor DETANONOate was used as a chemosensitizing agent based on previous findings of its sensitizing activity [47, 48]. In addition, the molecular mechanism of chemosensitization was examined. The findings demonstrated that treatment of SW620 with DETA sensitized the tumor cells to CDDP apoptosis. Previous findings analyzing gene products that regulate apoptosis and that are differentially expressed in the primary SW480 and the metastatic SW620 were reported [49]. In these studies, SW620 cells showed a markedly reduced level of AIF compared to its expression in SW480 and, thus, its reduced level may have had a role in the inhibition of CDDP-mediated activation of caspases 3 and 9 and apoptosis. AIF is present in the cytosol as a 67 kDa protein and then imported in the mitochondria by a chaperon protein which is then therefore processed to a 62 kDa protein [50]. Apoptotic signaling resulted in the cleavage of the 62 kDa into a 57 kDa for their release into the cytosol and interaction with cylophillin. A prior to its translocation into the nucleus and acquisition of DNase activity [49, 51].

The role of AIF expression in resistance was examined. It was found that treatment of SW620 with DETA resulted in the upregulation of AIF expression, and in combination with CDDP, led to significant apoptosis. The direct role of AIF in sensitization was corroborated in cells transfected with siRNA AIF, which reversed DETA-mediated sensitivity to CDDP. Hence, sensitization was found to occur via the mitochondrial type II apoptotic pathway. *In vivo*, mice bearing SW620 tumor

xenografts and treated with DETA and CDDP showed tumor regression and tumor tissue examined *ex vivo* demonstrated upregulation of AIF. In addition, tissues from patients with colorectal cancer and examined by immunocytochemistry demonstrated downregulation of AIF [49].

Immunosensitization of Immune-Resistant Tumor Cells by NO

In addition to chemotherapy and radiotherapy, immunotherapy has been recently introduced in the treatment of various cancers through the generation of antitumor monoclonal antibodies and also various means to generate antitumor CTLs.

CTLs mediate their cytotoxic effect by both necrosis (perforin and granzyme) and apoptosis (Fas-L, TNF-α and TRAIL) [52]. The apoptotic signaling by membrane-bound ligands and their interactions with corresponding receptors leads to cell death provided a target cell is sensitive. However, many tumors are resistant to killing by CTL and by their ligands. While the use of Fas-L and TNF-α has been considered as therapy, they are however, toxic. In contrast, TRAIL is not toxic and TRAIL receptors, agonist antibodies (anti-DR-4 and anti-DR-5) have been used in the clinic [53, 54].

1. Sensitization to TRAIL

TRAIL is a type II transmembrane protein of the TNF-α family and forms homotrimers that bind three receptor molecules [55]. Four homologous human TRAIL receptors (DR-4, DR-5, DCR-1 and DCR-2) and a fifth soluble receptor osteoprotegerin (OPG) have been identified [56–58]. DR-4 and DR-5 contain a conserved death domain (DD) motif and signal the cells for apoptosis. Although many tumors are sensitive to TRAIL, the majority is resistant. Resistance to TRAIL may be governed, in part, by overexpression of anti-apoptotic proteins such as Bcl-2 family proteins [59], cFLIPShort (cFLIPS), [60] or inhibitors of apoptosis (IAPs) [61]. These anti-apoptotic gene products are part of the constitutively activated NF-κB survival pathway in cancer [62]. Overcoming resistance to TRAIL apoptosis may be accomplished by the use of sensitizing agents that modulate or inhibit the expression of anti-apoptotic gene products [48, 63, 64].

Sodium nitroprusside (SNP) sensitizes human gastric cells to TRAIL apoptosis. Using gastric cell lines, TRAIL-induced apoptosis and cell cycle arrest were mediated by NO and implicated both the intrinsic and extrinsic pathways of apoptosis. SNP sensitization to TRAIL was found to be via activation of caspases 8 and 9. Synergy was also achieved [65].

2. Sensitization of tumor cells to TNF-α apoptosis by NO

NF-κB activity has been implicated in the regulation of tumor cell resistance to apoptotic stimuli [66, 67]. TNF-α signals the cells via two receptors, TNF-R1 and TNF-R2, and exerts distinct signaling pathways, one of which triggers the apoptotic pathway and the other the inflammatory response. Alone, TNF-α triggers the gen-

eration of various ROS [68]. The use of exogenous ROS mimics the biological activity of TNF-α. IFN-γ treatment sensitized Fas-resistant tumor cells to Fas-ligand apoptosis via the induction of iNOS and NO.

NF-κB is an oxidative stress response transcription factor that responds to small concentrations of exogenous hydrogen peroxide (H2O2) or to reactive oxygen species triggered by many agents including TNF-α [69]. In the present study, it was reported that IFN-γ sensitizes tumor cells to TNF-α cytotoxicity. Further, NO disrupted the hydrogen peroxide-dependent activity of NF-κB and resulted in the inhibition of anti-apoptotic gene products and, therefore, sensitized cells to TNF-α apoptosis [70].

Sensitization to TNF-α by IFN-γ was inhibited by the specific NOS inhibitor L-NMA and was mimicked by the NO-donor SNAP. Overall, sensitization of tumor cells by NO to TNF-α apoptosis was the result of the specific disruption of TNF-α-induced generation of H_2O_2 and its subsequent inhibition of NF-κB-dependent expression of anti-apoptotic gene products. This finding was a novel mechanism of NO-mediated sensitization to apoptosis [70].

3. Sensitization to FasL by NO

We have reported that interferon-gamma treatment sensitized FASL resistant tumor cells to FasL-induced apoptosis. Sensitization was mediated by iNOS induction and exogenous NO (DETA) mimicked interferon-gamma-induced sensitization [47]. Sensitization was paralleled with upregulation of Fas expression. The mechanism by which NO modulated the expression of Fas was examined and we found that NO inhibits the repressor activity of YY1 acting at the Fas promoter. The human *Fas* gene promoter consists of three major regions within the ~2000 bp5'-flanky region. There was a silencer region between nucleotides position −1781 and −1007 and a strong enhancerregion between −1007 and −425 in the hiuman *Fas* gene. The response of NO was due to its activity on the silencer region and deletion of the silencer region resulted in enhancing Fas expression. YY1 has been reported to act as a transcription repressor in the human *interferon-gamma* gene [71]. NO inhibits YY1 DNA-binding activity [47, 72].

Leon-Bollote et al. [73] reported another mechanism by which NO sensitizes tumor cells to FasL apoptosis. Tumor cells were treated with glyceryl-trinitrate and demonstrated that Fas was S-nitrosykated at residues 199 and 304 in the cytoplasmic and tail regions of Fas. and recruited to lipid rafts. Mutation of Fas at residue 304 inhibited migration to lipid rafts and sensitization to FasL apoptosis. However, mutation at residue 199 had no effect.

Sensitization to Radiotherapy by NO

The radiosensitizing effect of DETA in the radioresistant HT29 CRC was examined. This line is positive for c-myc, K-Ras, H-Ras, N-Ras, Myb and Fos oncogene. The HT29 cells have mutations in both alleles of p53 [74] and is also iNOS-negative [75]. HT29 cells are resistant to apoptosis by irradiation as the levels of Bcl-2 and

survivin are elevated. Treatment with DETA and irradiation sensitizes cells to apoptosis with cleavage of PARP-1, elevation of p21, p27 and Bax, and a decrease of Bcl-2 [76]. The in vitro data were corroborated in vivo in SCID mice bearing a tumor xenograft. The irradiated treated mice received 2.0GYIR directly to the tumor over a period of five days. While the tumor reduction of irradiated treated mice resulted in 37% tumor growth reduction, treatment with the combination of irradiation and DETA resulted in a more significant tumor regression (70.1%) [76].

NO-Mediated Induction of Resistance

Godoy et al. [77] reported that patients with melanoma showed that then cancers have high levels of iNOs and nitrotyrosine (derived from NO). The cells used expressed constitutively iNOs and generated nM levels of NO intracellularly. Inhibition of NO synthesis or inhibition of NO sensitizes cells to CDDP apoptosis. In addition, inhibition of s-nitrosylation increased CDDP cytotoxicity. They showed that S-nitrosylation of caspase 3 and proxy hydrolase (and enzyme responsible for targeting HIF-1α) were inhibited by NO and resulted in resistance.

The VP-16 interaction with NO generates nitrozo VP-16. Nitric oxide products of VP-16 diminish cleavage of DNA-dependent topoisomerase 2 and inhibited cytotoxicity. Thus, detoxification of VP-16 reduces its cytotoxic effect [78].

Long exposures to NO (7–14 days) rendered lung cancer cells resistant to drugs (CDDP, doxorubicin, VP-16) in a tumor-dependent fashion. The mechanism is due to adaptive responses of the cell and increased survival by overexpression of caveolin-1 and Bcl-2 and AKT. These were responsible for the drugs tested and resistance was reversible by removing NO [79].

NO and related molecules can exert contrasting effects, that is, pro and anti-apoptotic effects, depending on the type of cells and the stimuli [80–82]. For instance, NO inhibits apoptosis through S-nitrosylation of caspases [83]. However, our findings are compatible with those of others regarding sensitization to apoptosis [30, 84]. Long-lasting production of NO acts as a modulator of pro-apoptotic effects by activating caspases whereas low and physiological concentrations of NO prevent cells from undergoing apoptosis [85].

NO-Mediated Alteration of the Dysregulated NF-κB/Snail/RKIP/YY1 Loop

In several cancers, the apoptotic signaling pathways are modified to protect the tumor cells from lethal damage. These dysregulated pathways participate in the pathogenesis and progression of tumors. They include (a) cell accumulation centered by circumventing cell turnover mechanisms and thus creating the proper environment for genetic instability and oncogenic activation, (b) acquisition of resistance to immune attack, (c) development of mechanism of resistance to chemotherapeutic

drugs and radiation, (d) tumor cell survival under hypoxic conditions and (e) promoting metastasis [86–89].

The constitutively activated NF-κB survival/apoptotic pathway regulates downstream the transcription and expression of the metastasis inducer Snail and the DR5 YY1 repressor in a loop that also regulates the repression of RKIP by the repressor Snail. NO interferes in this loop and modifies the expression and the activities of all the factors by inhibiting NF-κB, Snail, and YY1 and upregulating RKIP. Such modifications by NO result in the inhibition of metastasis [90] and reversal of resistance by chemo-immunosensitizing agents.

NO modifies the NF-κB/Snail/RKIP loop and inhibits EMT. Each gene product is involved, since it is inter-regulated in the loop. The direct role of Snail inhibition by DETA was corroborated by treatment with siRNA Snail and the cells mimicked DETA-mediated inhibition of EMT. Further, overexpression of the stable form of Snail induced the EMT phenotype in an EMT-negative cell line. These findings were in agreement with other reports [91, 92]. The direct role of RKIP induction by DETA and its inhibition of EMT was demonstrated by ectopic expression of RKIP and such cells mimicked DETA treatment and inhibited the EMT phenotype along with inhibition of both NF-κB and Snail. In contrast, silencing RKIP by siRNA induced EMT in an EMT-negative cell line [90, 93]. The induction of RKIP by DETA is supported by findings showing that treatment of normal keratinocytes with the NO donor SNAP resulted in elevated levels of RKIP mRNA and protein and inhibition of proliferation [94].

Establishment of the NF-κB/Snail/YY1/RKIP loop and its role in resistance [90]

Most cancer cells express constitutively hyperactivated survival pathways, such as the NF-κB pathway [95]. The NF-κB regulates many genes, including both pro- and anti-apoptotic proteins. Among the anti-apoptotic proteins that regulate resistance are Bcl-$_{xl}$, XIAP and YY1 [96, 97]. In addition, NF-κB regulates the transcription of Snail [98]. NF-B also regulates the transcription of YY1. Hence, it is clear that a loop consisting of NF-κB, Snail, and YY1 coexists. In addition, Yeung et al. [99, 100] reported that RKIP inhibits both the Raf/MEK/ERK and NF-κB pathways. Further, Snail represses RKIP. Hence, the loop is further modified to consist of the NF-κB/Snail/YY1/RKIP, and tumor cells overexpress NF-κB, Snail and YY1 whereas underexpress RKIP. Below, a brief description of the role of each gene product of the loop in the regulation of resistance is provided.

Role of NO-Mediated Inhibition of NF-κB in Chemo-Immunosensitization

One predominantly constitutively activated survival/anti-apoptotic pathway in cancer is the NF-κB pathway. It plays several roles in tumoregenesis such as proliferation, resistance and metastasis [101]. The findings that NO inhibits NF-κB activity by S-nitrosylation of p50 [38], we hypothesized that NO donors may be considered a therapeutic strategy to reverse resistance.

We and others have shown that NO induced by DETANONOate inhibits NF-κB activity in tumor cell lines by various mechanisms, such as inhibition of the NF-κB promoter activity, and the NF-κB DNA-binding activity by EMSA [48, 102]. Various NO donors showed inhibition of the activation status of NF-κB and reversal of tumor cell lines resistance [103–105]. Treatment with NO nitrosylates the p50 subunit of NF-κB [48]. Park and Wei [106] also reported that NO release by sodium nitroprusside (SNP) inhibits activation of the c-myc promoter in mouse embryonic carcinoma cells.

Treatment of tumor cell lines with DETA inhibited the constitutively hyperactivated NF-κB activity through inhibition of its DNA-binding activity and S-nitrosylation of p50 and p65. The direct role of NO-mediated inhibition of NF-κB in sensitization was corroborated by the use of the specific NF-κB inhibitor DHMEQ, which mimicked DETA in sensitizing the tumor cells to CDDP and TRAIL apoptotosis. The sensitization correlated with the inhibition of NF-κB target anti-apoptotic gene products such as XIAP, Bcl-$_{xl}$ and survivin [48, 107].

Role of NO-Mediated Inhibition of YY1 in Chemo-Immunosensitization

Treatment of prostate carcinoma cells with DETA sensitized the cells to CDDP apoptosis. Sensitization was due, in part, to the inhibition of YY1 and Bcl-$_{xl}$. The direct role of YY1 inhibition in sensitization was corroborated by the use of siRNA YY1, which mimicked DETA [108]. The expression of YY1 in prostate cancer cells was reported to be elevated [109]. In addition, the overexpression of Bcl-$_{xl}$ has been shown to be in 100 % of prostate carcinoma and has been associated with advanced disease, poor prognosis and shortened survival [110]. Both YY1 and Bcl-$_{xl}$ are regulated by NF-κB [102]. Treatment with DETA inhibited NF-κB activity through S-nitrosylation of both p65 and p50 [48, 111]. Treatment of tumor cells with YY1 siRNA mimicked DETA and resulted in the inhibition of YY1 and Bcl-$_{xl}$ and sensitization to CDDP apoptosis. Treatment with Bcl-$_{xl}$ siRNA resulted in the inhibition of both Bcl-$_{xl}$ and YY1 and sensitization to CDDP. The BH3 mimetic Gossypol inhibits Bcl-2, Bcl-$_{xl}$ and Mcl-1 expressions and synergizes with docetaxel *in vitro* and *in vivo* and inhibits PC3 tumor cell growth [112]. The findings by DETA in the desensitization of prostate cancer cells to CDDP was validated in tumor xenografts in mice. Treatment with a single agent had no effect, but treatment with the combination resulted in significant reduction of tumor growth. Tumor tissue examination demonstrated inhibition of YY1 and Bcl-$_{xl}$. Patient-derived prostate cancer TMA showed co-expression of YY1 and Bcl-$_{xl}$, and YY1 expression levels correlated with tumor grade [108].

Reports in the literature demonstrated that treatment of TRAIL-resistant tumor cells with certain chemotherapeutic drugs sensitizes cells to TRAIL apoptosis with the concomitant upregulation of DR-5 and/or DR-4 [113–115]. However, the molecular mechanism was not reported. Thus, we have reported that the treatment of

TRAIL-resistant tumor cells with DETA sensitizes cells to TRAIL-apoptosis [48]. Sensitization resulted from the inhibition of the DR-5 transcription factor YY1 and upregulation of DR-5. The relationship between YY1 inhibition and DR-5 upregulation suggested the possibility that YY1 may be a repressor of DR-5 transcription. These were investigated and validated by Huerta-Yepez et al. [107]. The findings revealed that the inhibition of YY1 by DETA was in large part responsible for sensitization of tumor cells to TRAIL apoptosis. This finding was corroborated by the use of YY1 siRNA, which mimicked DETA and these cells were sensitized to TRAIL apoptosis. In addition, we demonstrated that YY1 is a direct repressor of DR-5 transcription as assessed by ChIP analysis and mutations of the DR-5 promoter where the putative binding of YY1 was taking place. The *in vitro* findings were validated in a murine model bearing a human tumor xenograft and treated with DETA. Tumor tissues analyzed *ex vivo* demonstrated inhibition of YY1 and upregulation of DR-5.

Overexpression of DR-5 in TRAIL-resistant cells restored sensitivity [116]. The direct role of DR-5 upregulation and sensitization, however, is not yet clear. The finding with DETA corroborated reports by Chawla-Sarkar et al. [117], who reported that the NO donor nitrosylcobalim sensitized tumor cells to TRAIL apoptosis.

Tumor cells treated with DETA inhibited YY1 expression and activity as a result of inhibiting NF-κB. The YY1 DNA-binding activity was abolished by DETA and was due, partially, to S-nitrosylation of YY1 [118]. The direct role of YY1 in sensitization was shown by treatment with siRNA YY1, which mimicked DETA, and resulted in sensitization to CDDP and TRAIL apoptosis [107, 119, 120]. The inhibition of YY1 and sensitization to TRAIL by DETA resulted in the upregulation of DR5, since YY1 is a repressor of DR5 transcription. Also, DETA treatment resulted in the upregulation of DR5 as a result of YY1 inhibition [107].

Role of NO-Mediated Inhibition of Snail in Chemo-Immunosensitization [126]

Treatment of prostate cancer cell lines with DETA inhibited NF-κB and, in turn, inhibited downstream Snail, resulting in the derepression and induction of RKIP, and this resulted in both the inhibition of EMT and chemoimmunosensitizing activities.

Snail is a member of the Snail superfamily zinc-finger transcription factors. It plays a role in tumor progression and invasion and triggers EMT [121]. Snail transcription is regulated by both NF-κB and YY1 [98, 122]. Snail acts as a repressor of both E-cadherin and RKIP [123, 124].

Treatment of tumor cells expressing Snail with an NO donor resulted in the inhibition of Snail mRNA and protein expressions. In addition, treatment with DETA resulted in the S-nitrosylation of Snail and inhibition of its DNA-binding activity. Inhibition of Snail by DETA sensitizes tumor cells to both CDDP and TRAIL apoptosis. The direct role of Snail inhibition in sensitization was corroborated by the use of siRNA Snail, which mimicked DETANO-treated cells. Treatment with siRNA Snail resulted in the upregulation of RKIP and the inhibition of both NF-κB and YY1 [125, 126].

Role of NO-Mediated Induction of RKIP in Chemo-Immunosensization

The Raf-kinase inhibitor protein, RKIP, was cloned by Yeung et al. [99, 100] and reported its mediated inhibition of the Raf/MEK/ERK pathway. RKIP blocks Raf1-induced phosphorylation of MEK via its direct interaction with Raf1 kinase and consequently, inhibition of ERK activity. Yeung et al. [100] reported that RKIP also inhibits the NF-κB pathway via its interaction with the NF-κB-inducing kinase (NIK) and TGF-β-activated kinase1 (TAK1). Inhibition of these pathways resulted in the inhibition of many anti-apoptotic targeted gene products [101, 127]. Inhibition of these pathways and targeted gene products should render the cells more sensitive to apoptotic stimuli.

Chatterjee et al. [128] reported that induction of RKIP-sensitized resistant cells to Camptothecin (CPT-1)-induced apoptosis. We have also reported that rituximab sensitized tumor cells to cytotoxic drugs via inhibition of NF-κB activity and concomitantly to the induction of RKIP [127, 129]. We also reported that the overexpression of RKIP sensitized tumor cells to TRAIL apoptosis and upregulation of DR5 [120].

Based on the above findings, we have postulated that NO-mediated inhibition of NF-κB and chemosensitization may also result in the induction of RKIP. Accordingly, treatment of tumor cells with DETA induced RKIP expression, both transcriptionally and post-transcriptionally. The direct role of RKIP in sensitization was shown by treatment of these tumor cells with shRNA RKIP and reversal of tumor cell sensitivity to TRAIL [120]. The mechanism by which NO induced RKIP expression may have been the result of NO-mediated inhibition of Snail, a repressor of RKIP [124]. Snail belongs to the Snail superfamily of zinc finger transcription factors and plays an important role in the induction of EMT [130]. Further, Snail is transcriptionally regulated by NF-κB as well as by GSK-3β-mediated phosphorylation of Snail and its cytoplasmic localization and proteasome degradation [131, 132].

Treatment of tumor cells will DETA resulted in significant induction of RKIP both transcriptionally and post-transcriptionally. The direct role of RKIP in sensitization was shown in cells transfected with shRNA RKIP which inhibited sensitization to apoptosis [119]. The mechanism by which RKIP was induced by NO is the result of NO-mediated inhibition of NF-κB target gene product, the RKIP suppressor Snail [124].

Yeung et al. [100] reported that RKIP inhibits NF-κB activity through its interaction with upstream kinases TAK1, NIK1 and IKK. RKIP physically interacts with and blocks TAK1 and NIK — MEKK1 or NAK1. RKIP interacts with the α and β subunits of IKK and inhibits the phosphorylation of IkBα, thus inhibiting NF-κB. Treatment of tumor cell lines with DETA potentiated the expression of RKIP similar to findings in which treatment of tumor cells with chemotherapeutic drugs also induced the upregulation of RKIP [128]. Snail is the only transcription factor that has been reported to repress RKIP transcription in prostate and breast cancer cells [124]. Hence, NO-mediated upregulation of RKIP may be the result of NO-medi-

ated inhibition of Snail and derepression of RKIP transcription. We have reported that the overexpression of the stable form of Snail, Snail-6 A, in prostate tumor cells resulted in the inhibition of RKIP expression. We also showed that treatment with DETA resulted in the S-nitrosylation of Snail [125]. Thus, inhibition of Snail by NO results in the induction of RKIP.

NO and PDT

Photodynamic therapy (PDT) is a therapeutic strategy to treat various solid tumors. PDT consists of three elements working together, namely, a photosensitizer, light and oxygen. Under conditions of culture, cell growth arrest and cell death are observed following PDT treatment through the generation of oxygen species (ROS) and/or singlet oxygen ($^1O_2$). It has been reported that NO is detected in tumor tissues, and its level and persistence result in either the progression or regression of the tumor [31, 133, 134]. These contradictory effects by NO result from the findings that high levels of NO are cytotoxic whereas low levels are cytoprotective [26]. The NO level in the tumor microenvironment influences the response of the tumor to PDT [135, 136]. PDT regulates the NO level, as it is able to induce NO in tumors through the induction of iNOS [137]. Also, tumor infiltrated macrophages are, in part, the source of PDT-induced iNOS [138–140]. It was reasoned that the antitumor efficacy of PDT may be augmented by the addition of NO drugs, such as NO donors. A model system consisting of the murine B78-H1 amelanotic tumor cell line was used and examined both *in vitro* and *in vivo* in C57BL/6 mice. Under suboptimal conditions of PDT, which normally results in tumor recurrence, treatment with PDT and DETA resulted in significant inhibition of cell proliferation *in vitro* and inhibition of tumor growth *in vivo* [141]. The mechanism by which the combination was effective was examined. Previous findings demonstrated that the NO donor DETA inhibited EMT and sensitized tumor cells to chemo and immunodrugs [90]. This was due to interference by DETA in the dysregulated pro-survival NF-κB/Snail/YY1/RKIP loop. NO inhibited the expression and activity of NF-κB and downstream targets Snail and YY1, while it upregulated RKIP expression through the inhibition of the RKIP repressor Snail. While low dose of PDT elicited low levels of NO and activated the survival loop above, the addition of NO donors increased the levels of NO and modified the loop as reported above. Indeed, the combination of PDT and DETA inhibited NF-κB, Snail and YY1 and upregulated RKIP expression.

In Vivo Application of NO Donors in Tumor Regression in Pre-Clinical Animal Models

The diazeniumdiolates JS-K generates NO on enzymatic activation by glutathione and glutathione-S-transferase (GST). Weyerbrock et al. [142] used U87 gliomiac cells treated with JS-K. They found inhibition of proliferation and apoptosis *in vi-*

tro. The growth of U87 zenograft in mice was significantly inhibited. The response to JS-K correlated with the expression of GST, mRNA and protein, and amount of NO released.

Huerta et al. [76] investigated the effect of NO donors in colorectal carcinoma (CRC). The median survival time of patients with metastatic CRC is dismal, and the mortality rate is high [143]. The activation of proto-oncogenes and the loss of tumor suppressor genes appear to be present in CRC. The chemoresistance of the metastatic CRC cell line SW620 and the radioresistant HT29 CRC cell line were used as models. Treatment with the NO donor DETA resulted in both chemo and radiosensitizing activities of the cell lines [76].

In breast cancer cells, treatment with DETA inhibited cyclin D1 and resulted in cell cycle arrest [144]. Treatment with DETA of NO-synthase deficient breast cancer cells resulted in apoptotic cell death [145]. The combination of DETA and farnesyltransferase inhibitors potentiated apoptosis in breast cancer cells with minimal toxicity in breast tumor cells [146]. Matthews et al. [147] also reported the reversal of resistance of breast cancer cells to 5FU and doxorubicin by DETA. It was also reported enhanced CDDP-mediated cytotoxicity by DETA in chimeric hamster V79 lung fibroblasts and head and neck cancer cells [148, 149]. In a model of *in vivo* melanoma tumor xenografts treated intratumorally with DETA and intraperitoneally with CDDP resulted in significant inhibition of tumor growth [150]. In the reported study by Huerta et al. [46], they showed that the sensitizing activities by DETA to CDDP apoptosis implicated the role of AIF.

Snail and RKIP exhibit opposing effects in the regulation of resistance [125]. We have reported that NO inhibits the EMT phenotype in several human cancer cell lines. The inhibition was due, in large part, to the inhibition of NF-κB and downstream inhibition of the metastatic inducer Snail, which led to induction of the metastatic suppressor RKIP. The *in vitro* findings were validated *in vivo* in mice bearing human tumor xenografts [126].

Concluding Remarks

We have reported of the presence of a dysregulated loop in cancer cells that regulates drug immune resistance and EMT. Treatment of tumor cells with the NO donor DETA modified the gene products of the loop in such a way that the cells became more sensitive to killing by cytotoxic agents and also resulted in the inhibition of EMT [48, 102, 107, 125, 129].

During the last decade, investigations on the role of NO in cancer have resulted in several controversies and, not until recently, it became clear that both the pro- and anti-tumorogenic properties of NO are fundamentally sound. Hence, the controversies were resolved, in large part, by the findings that the levels of NO dictate the outcome in cancer cells. Clearly, these findings supported the role of high levels of NO as anti-tumorogenic and introduced the potential therapeutic roles of NO in the pathogenesis, progression and response to cytotoxic drugs in cancer. This review,

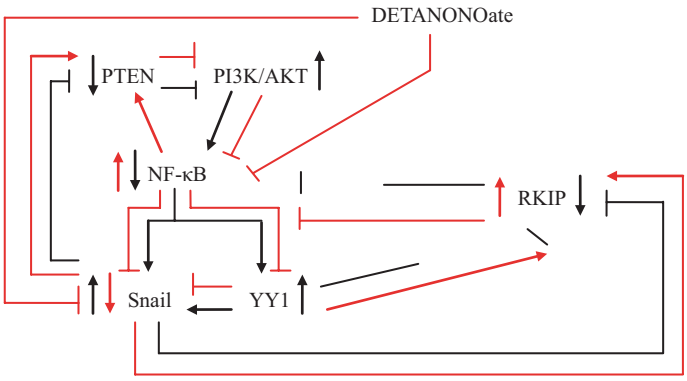


Fig. 12.1 Schematic diagram of the anti-cancer therapeutic activity of NO donors. *Black lines* represents the dysregulated NFκ-B/Snail/YY1/PI3K/RKIP/PTEN loop in cancer cells. The constitutively activated NFκ-B in cancer regulates downstream its target gene products, Snail and YY1. Overexpression of YY1 regulates also Snail transcription and expression. Overexpression of Snail, the metastasis inducer, represses the metastasis suppressor gene product RKIP and also inhibits e-cadherin metastasis. The PI3K/AKT pathway is hyperactivated due, in part, to the repression of PTEN and also contributes to the activation of NFκ-B. Hence, the above disregulated loop, thus, regulates cell proliferation, cell death by apoptosis and EMT/metastasis. *Red lines* treatment of cancer cells with NO donors, such as DETANONOate, results in the inhibition of NFκ-B, in part, via S-nitrosylation of p50 and p65 and resulting in the inhibition downstream of Snail and YY1. In addition, treatment with DETANONOate S-nitrosylates Snail and inhibits its DNA-binding activity. The inhibition of Snail will result in the derepression of PTEN and RKIP and resulting in their upregulation and, consequently, inhibition of the PI3K/AKT and NFκ-B pathways, respectively. Overall, treatment with NO donors results in the inhibition of cell proliferation, sensitization to apoptosis by chemo-immunotherapeutic drugs, and inhibition of EMT and metastasis

and others in this volume, report the NO-mediated effects in the inhibition of tumor cell survival, cell growth and proliferation, EMT and metastasis. In addition, the important role of NO in mediating chemo-immuno-radiosensitizing activities led to the reversal of resistance to apoptotic cytotoxic drugs. Tumor cells have been shown to express a highly dysregulated NF-κB/Snail/YY1/RKIP/PTEN/Akt circuitry that controls cell survival, metastasis and resistance to cytotoxic drugs. Treatment of tumor cells with NO donors has been shown to interfere with this circuitry and reverses its role and, thereby, results in the inhibition of tumor cell growth and their response to apoptotic stimuli. Thus, clearly, NO donors exert anti-tumor activities which were shown both *in vitro* and *in vivo* tumor model systems. (See Fig. 12.1)

These above findings suggested strongly the adoption of NO donors in the clinic and this was supported by the few clinical trials that showed a beneficial effect of the use of NO donors, either alone or in combination with chemotherapy. Clearly, the findings that NO donors can also prevent EMT and metastasis as well as reverse resistance when used in combination with drugs should be exploited due to the pleiotropic activities that NO mediates in tumor cells. There have been several newly generated classes of NO donors as single molecules or coupled with other molecules, including tumor-targeted moieties. These latter compounds, clearly, may be more effective and can be used at lower doses in combination and less toxic to

normal tissues. Some examples are found in this volume. It is also noteworthy that NO conjugated with porphyrin have been investigated in PDT against cancer cell lines, a potential novel application in cancer therapy (see Rapozzi in this volume). In addition, the finding that NO donors can sensitize tumor cells to immunotherapy suggests that the *in vivo* administration of NO donors may sensitize immune resistant tumor cells to host natural/immune cytotoxic lymphocytes or sensitize tumor cells following passive cellular immunotherapy and/or *in vivo* blocking of immune suppressor checkpoints on cytotoxic lymphocytes such as the administration of anti-PD1/PDL1 antibodies. The versatile nature of NO donors, including the FDA approved nitroglycerin, in cancer therapy is now clearly warranted for additional clinical studies to validate its anti-tumor activities.

Acknowledgments The author acknowledges the various students, fellows and collaborators whose published reports were the subject of this review. They are Drs. S Baritaki, J Berenson, D Chatterjee, H Chen, H Garban, F Hongo, S Huerta-Yepez, H Liu, M Vega and K Yeung. The author also acknowledges the Jonsson Comprehensive Cancer Center at UCLA for its assistance. The following funding agencies that supported the various publications referred to in this review (1999-present, in alphabetical order) are listed below: the Bodossaki Foundation, the Boiron Research Foundation, the Department of Defense (DOD)/US Army, DAMD. 17-02-1-0023, the Department of Surgery at the University of Texas Southwestern (Shannon Funds), the Fogarty Fellowships (D43DW0001 13–14), the National Council of Science and Technology, NCI RO1CA107023-02 S1, NIH COBRE Grant Number P20RR17695, PRIN 2009 and FVG 2009 (Italy), Terry Fox Funds Grant HIM/2007/061, UC MEXUS, the UCLA AIDS Institute, the UCLA Gene Therapy Program, the UCLA SPORE in Prostate Cancer, Veterans' Affairs VISN17 and the Vollmer Foundation. The author also acknowledges the assistance of Samantha Kaufhold, Daphne Liang, Kathy Nguyen and Anna Shvartsur for their assistance in the preparation of this manuscript.

No Conflict Statement "No potential conflicts of interest were disclosed."

References

1. Hughes MN. Chemistry of nitric oxide and related species. In: Robert KP, editor. Methods in enzymology. Waltham: Academic. 2008. pp. 3–19.
2. Kroncke KD, Fehsel K, Kolb-Bachofen V. Nitric oxide: cytotoxicity versus cytoprotection—how, why, when and where? Nitric Oxide. 1997;1:107–20.
3. Osorio J, Recchia F. The role of nitric oxide in metabolism regulation: from basic sciences to the clinical setting. Intensive Care Med. 2000;26:1395–8.
4. Albina J, Reichner J. Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. Cancer Metastasis Rev. 1998;17:39–53.
5. Umansky V, Shirrmacher V. Nitric oxide-induced apoptosis in tumor cells. Adv Cancer Res. 2001;82:107–131.
6. Leiro J, Iglesias R, Paramá A, Sanmartin ML, Ubeira FM. Respiratory burst responses of rat macrophages to microsporidian spores. Exp Parasitol. 2001;98:1–9.
7. Gkaliagkousi E, Ferro A. Nitric oxide signalling in the regulation of cardiovascular and platelet function. Front Biosci. 2011;16:1873–97.
8. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. Cardiovasc Res. 1999;43:521–31
9. Huerta S1, Chilka S, Bonavida B. Nitric oxide donors: novel cancer therapeutics (review). Int J Oncol. 2008;33:909–27.

10. Derbyshire ER, Marletta MA. Structure and regulation of soluble guanylate cyclase. Ann Rev Biochem. 2012;81:533–59.
11. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Ann Rev Immunol. 1997;15:323–50.
12. Farias-Eisner R, Sherman MP, Aeberhard E, Chaudhuri G. Nitric oxide is an important mediator for tumoricidal activity in vivo. Proc Natl Acad Sci USA. 1994;91:9407–11.
13. Binder C, Schulz M, Hiddemann W, Oellerich M. Induction of inducible nitric oxide synthase is an essential part of tumor necrosis factor-alpha-induced apoptosis. Lab Invest. 1999;79:1703–12.
14. Wink D, Hines H, Cheng R, Switzer C, Flores-Santana W, Vitek M, Ridnour L, Colton C. Nitric oxide and the redox mechanisms in the immune response. J Leukocyte Biol. 2011;89:873–91.
15. Brennan PA, Downie IP, Langdon JD, Zaki GA. Emerging role of nitric oxide in cancer. Br J Oral Maxillofac Surg. 1999;37:370–3.
16. Chang K, Lee SJ, Cheong I, et al. Nitric oxide suppresses inducible nitric oxide synthase expression by inhibiting posttranslational modification of IkappaB. Exp Mol Med. 2004;36:311–24.
17. Lechner M, Lirk P, Rieder J. Inducible nitric oxide synthase (iNOS) in tumor biology: the two sides of the same coin. Semin Cancer Biol. 2005;15:277–289.
18. Magee PN, Barnes JM. The production of malignant primary hepatic tumors in the rat by feeding dimethyl-nitrosamine. Br J Cancer. 1956;10:114–22.
19. Hibbs JB Jr, Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Science. 1987;235:473–6.
20. Hibbs JB Jr, Taintor RR, Vavrin Z, Rachlin EM. Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem Biophys Res Commun. 1988;157:87–94.
21. Bonavida B, Khineche S, Huerta-Yepez S, Garban H. Therapeutic potential of nitric oxide in cancer. Drug Resist Updat. 2006;9:157–73.
22. Bonavida B, Baritaki S, Huerta-Yepez S, Vega MI, Jazirehi AR, Berenson J. Nitric oxide donors are a new class of anti-cancer therapeutics for the reversal of resistance and inhibition of metastasis. In: Bonavida, B, editor. Nitric Oxide Cancer Cancer Drug Discov Dev. Springer. 2010; PP. 459–78.
23. Alexandrova R, Mileva M, Zvetkova E. Nitric oxide and cancer (minireview). Exp Pathol Parasitol. 2001;4:13–8.
24. Brune H. Surface diffusion: shifting strings. Nat Mater. 2003;2:778–9.
25. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumor progression. Nat Rev Cancer. 2006;6:521–34.
26. Wink DA, Vodovotz Y, Cook JA, Krishna MC, Kim S, Coffin D, et al. The role of nitric oxide chemistry in cancer treatment. Biochemistry (Mosc). 1998;63:802–9.
27. Forrester K, Ambs S, Lupold SE, et al. Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53. Proc Natl Acad Sci USA. 1996;93:2442–7.
28. Glockzin S, von KA, Scheffner M, Brune B. Activation of the cell death program by nitric oxide involves inhibition of the proteasome. J Biol Chem. 1999;274:19581–6.
29. Li CQ, Robles AI, Hanigan CL, Hofseth LJ, Trudel LJ, Harris CC, Wogan GN. Apoptotic signaling pathways induced by nitric oxide in human lymphoblastoid cells expressing wild-type or mutant p53. Cancer Res. 2004;64:3022–9.
30. Boyd CS, Cadenas E. Nitric oxide and cell signaling pathways in mitochondrial-dependent apoptosis. Biol Chem. 2002;383:411–23.
31. Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol. 2001;2:149–156.
32. Schaer DA, Hirschhorn-Cymerman D, Wolchok JD. Targeting tumor-necrosis factor receptor pathways for tumor immunotherapy. J Immunother Cancer. 2014;2:7.
33. Pipili-Synetos E, Papageorgiou A, Sakkoula E, Sotiropoulou G, Fotsis T, Karakiulakis G, Maragoudakis ME. Inhibition of angiogenesis, tumour growth and metastasis by the NO-

releasing vasodilators, isosorbide mononitrate and dinitrate. Br J Pharmacol. 1995;116:1829–34.
34. Kumar A, Takada Y, Boriek AM, Aggarwal BB. Nuclear factor-kappaB: its role in health and disease. J Mol Med (Berl). 2004;82:434–48.
35. Aggarwal BB. Nuclear factor-kappaB: the enemy within. Cancer Cell. 2004;6:203–8.
36. Adams J. Preclinical and clinical evaluation of proteasome inhibitor PS-341 for the treatment of cancer. Curr Opin Chem Biol. 2002;6:493–500.
37. Adams J. The proteasome a suitable antineoplastic target. Nat Rev Cancer. 2004;4:349–60.
38. Marshall HE, Stamler JS. Inhibition of NF-kappa B by S-nitrosylation. Biochemistry. 2001;40:1688–93.
39. Huerta S, Chilka S, Bonavida B. Nitric oxide donors: novel cancer therapeutics. Int J Oncol. 2008;33:909–27.
40. Nagai H, Yasuda H, Hatachi Y, Xue D, Sasaki T, Yamaya M, Sakamori Y, Togashi Y, Masago K, Ito I, Kim YH, Mio T, Mishima M. Nitric oxide (NO) enhances pemetrexed cytotoxicity via NO-cGMP signaling in lung adenocarcinoma cells in vitro and in vivo. Int J Oncol. 2012;41:24–30.
41. Ye S, Yang W, Wang Y, Ou W, Ma Q, Yu C, Ren J, Zhong G, Shi H, Yuan Z, Zhu W. Cationic liposome-mediated nitric oxide synthase gene therapy enhances the antitumor effects of cisplatin in lung cancer. Int J Mol Med. 2013;31:33–42.
42. Jamieson ER, Lippard SJ. Structure, recognition, and processing of cisplatin-DNA adducts Chem Rev. 1999;99:2467–2498.
43. Hewitt RE, McMarlin A, Kleiner D, Wersto R, Martin P, Tsokos M, Stamp GW, Stetler-Stevenson WG. Validation of a model of colon cancer progression. J Pathol. 2000;192:446–454.
44. Huerta S, Harris DM, Jazirehi A, Bonavida B, Elashoff D, Livingston EH, Heber D. Gene expression profile of metastatic colon cancer cells resistant to cisplatin-induced apoptosis Int J Oncol. 2003;22:663–670.
45. Huerta S, Goulet EJ, Huerta-Yepez S, Livingston EH. Screening and detection of apoptosis. J Surg Res. 2007;139:143–156.
46. Huerta S, Baay-Guzman G, Gonzalez-Bonilla CR, Livingston EH, Huerta-Yepez S, Bonavida B. In vitro and in vivo sensitization of SW620 metastatic colon cancer cells to CDDP-induced apoptosis by the nitric oxide donor DETANONOate: involvement of AIF. Nitric Oxide. 2009;20:182–94.
47. Garbán HJ, Bonavida B. Nitric oxide sensitizes ovarian tumor cells to Fas-induced apoptosis. Gynecol Oncol. 1999;73:257–64.
48. Huerta-Yepez S, Vega M, Jazirehi A, Garban H, Hongo F, Cheng G, Bonavida B. Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-kappa B and inhibition of Bcl-xl expression. Oncogene. 2004;23:4993–5003.
49. Huerta S, Heinzerling JH, Nguiano-Hernandez YM, Huerta-Yepez S, Lin J, Chen D, Bonavida B, Livingston EH. Modification of gene products involved in resistance to apoptosis in metastatic colon cancer cells: roles of Fas, Apaf-1, NFkappaB, IAPs, Smac/DIABLO, and AIF. J Surg Res. 2007;142:184–94.
50. Modjtahedi N, Giordanetto F, Madeo F, Madeo F, Kroemer G. Apoptosis-inducing factor: vital and lethal. Trends Cell Biol. 2006;16:264–72.
51. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature. 1999;397:441–6.
52. Shresta S, Pham CT, Thomas DA, Graubert TA, Ley TJ. How do cytotoxic lymphocytes kill their targets?. Curr Opin. Immunol. 1998;10:581–7.
53. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med. 1999;5:157–63.

54. Buchsbaum DJ, Forero-Torres A, LoBuglion AF. TRAIL-receptor antibodies as a potential cancer treatment. Future Oncol. 2007;3:405–9.
55. Hymowitz SG, Christinger HW, Fuh G, Ultsch M, O'Connell M, Kelley RF, Ashkenazi A, de Vos AM. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. Mol Cell. 1999;4:563–71.
56. Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P, Ashkenazi A. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science. 1997;277:818–21.
57. MacFarlane M, Ahmad M, Srinivasula SM, Fernandes-Alnemri T, Cohen GM, Alnemri ES. Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. J Biol Chem. 1997;272:25417–20.
58. Pan G, Ni J, Wei YF, Yu GL, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science. 1997;277:815–818.
59. Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. Annu Rev Immunol. 1998;16:395–419.
60. Tschopp J, Irmler M, Thome M. Inhibition of fas death signals by FLIPS. Curr Opin Immunol. 1998;10:552–8.
61. Deveraux QL, Reed JC. IAP family proteins: suppressors of apoptosis. Genes Dev. 1999;13:239–252.
62. Lee H, Dadgostar H, Cheng Q, Shu J, Cheng G. NF-kB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. Proc Natl Acad Sci USA. 1999;96:9136–9141.
63. Jazirehi AR, Ng CP, Gan XH, Schiller G, Bonavida B. Adriamycin sensitizes the adriamycin-resistant 8226/Dox40 human multiple myeloma cells to Apo2L/tumor necrosis factor-related apoptosis-inducing ligand-mediated (TRAIL) apoptosis. Clin Cancer Res. 2001;7:3874–83.
64. Tillman DM, Izeradjene K, Szucs KS, Douglas L, Houghton JA. Rottlerin sensitizes colon carcinoma cells to tumor necrosis factor-relat apoptosis-inducing ligand-induced apoptosis via uncoupling of the mitochondria independent of protein kinase C. Cancer Res. 2003;63:5118–25.
65. Yang L, Lan C, Fang Y, Zhang Y, Wang J, Guo J, Wan S, Yang S, Wang R, Fang D. Sodium nitroprusside (SNP) sensitizes human gastric cancer cells to TRAIL-induced apoptosis. Int Immunopharmacol. 2013;17:383–9.
66. Wang CY, Mayo MW, Baldwin AS Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science. 1996;274:784–7.
67. Wang CY, Cusack JC Jr, Liu R, Baldwin AS Jr. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. Nat Med. 1999;5:412–7.
68. Garcia-Ruiz C, Colell A, Mari M, Morales A, Fernandez-Checa JC. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. J Biol Chem. 1997;272:11369–77.
69. Hong YH, Peng HB, La Fata V, Liao JK. Hydrogen peroxide-mediated transcriptional induction of macrophage colony-stimulating factor by TGF-beta1. J Immunol. 1997;159:2418–23.
70. Garbán HJ, Bonavida B. Nitric oxide disrupts H2O2-dependent activation of nuclear factor kappa B. Role in sensitization of human tumor cells to tumor necrosis factor-alpha-induced cytotoxicity. J Biol Chem. 2001;276:8918–23.
71. Ye J, Ghosh P, Cippitelli M, Subleski J, Hardy KJ, Ortaldo JR, Young HA. Characterization of a silencer regulatory element in the human interferon-g promoter. J Biol Chem. 1994;269:25728.
72. Garbán HJ, Bonavida B. Nitric oxide inhibits the transcription repressor Yin-Yang 1 binding activity at the silencer region of the Fas promoter: a pivotal role for nitric oxide in the up-regulation of Fas gene expression in human tumor cells. J Immunol. 2001;167:75–81.
73. Leon-Bollotte L, Subramaniam S, Cauvard O, Plenchette-Colas, S, Paul C, Godard C, Martinez-Ruiz A, Legembre P, Jeannin JF, Bettaieb A. S-nitrosylation of the death receptor fas promotes fas ligand-mediated apoptosis in cancer cells. Gastroenterology. 2011;140:2009–18.

74. Shibata D, Peinado MA, Ionov Y, Malkhosyan S, Perucho M. Genomic instability in repeated sequences is an early somatic event in colorectal tumorigenesis that persists after transformation. Nat Genet. 1994;6:273–81.
75. Siegert A, Rosenberg C, Schmitt WD, Denkert C, Hauptmann S. Nitric oxide of human colorectal adenocarcinoma cell lines promotes tumour cell invasion. Br J Cancer. 2002;86:1310–5.
76. Huerta S, Gao X, Bonavida B. DETANONO is a potent chemo/radio-sensitizing agen in colon and colorectal cancers as assessed in in vitro and in vivo established tumor xenografts. Forum Immunopathol Dis Ther. 2010;1:281–95.
77. Godoy LC, Anderson CT, Chowdhury R, Trudel LJ, Wogan GN. Endogeneously produced nitric oxide mitigates sensitivity of melanoma cells to cisplatin. Proc Natl Acad Sci USA. 2012;109:20373–8.
78. Sinha BK, Bhattacharjee S, Chatterjee S, Jiang J, Motten AG, Kumar A, Espey MG, Mason RP. Role of nitric oxide in the chemistry and anticancer activity of etoposide (VP-16,213). Chem Res Toxicol. 2013;18:379–87.
79. Wongvaranon P, Pongrakhananon V, Chunhacha P, Chanvorachote P. Acquired resistance to chemotherapy in lung cancer cells mediated by prolonged nitric oxide exposure. Anticancer Res. 2013;33:5433–44.
80. Brune B, von Knethen A, Sandau KB. Nitric oxide and its role in apoptosis. Eur J Pharmacol. 1998;351:261–72.
81. Brune B, von Knethen A, Sandau KB. Nitric oxide (NO): an effector of apoptosis. Cell Death Differ. 1999;6:969–75.
82. Brune B, Cantoni O. Nitric oxide-mediated redox reactions in pathology, biochemistry and medicine. Cell Death Differ. 2000;7:1018–20.
83. Liu L, Stamler JS. NO: an inhibitor of cell death. Cell Death Differ. 1999;6:937–42.
84. Moncada S, Erusalimsky JD. Does nitric oxide modulate mitochondrial energy generation and apoptosis?. Nat Rev Mol Cell Biol. 2002;3:214–20.
85. Brune B. Nitric oxide: NO apoptosis or turning it ON? Cell Death Differ. 2003;10:864–9.
86. Raguz S, Yague E. Resistance to chemotherapy: new treatments and novel insights into an old problem. Br J Cancer. 2008;99:387–91.
87. Reed JC. Dysregulation of apoptosis in cancer. J Clin Oncol. 1999;17:2941–53.
88. Tan DS, Gerlinger M, Teh BT, Swanton C. Anti-cancer drug resistance. Understanding the mechanisms through the use of integrative genomics and functional RNA interference. Eur J Cancer. 2010;46:2143–4.
89. DeClerck K, Elble RC. The role of hypoxia and acidosis in promoting metastasis and resistance to chemotherapy. Front Biosci. 2010;15:213–25.
90. Bonavida B, Baritaki S. Dual role of NO donors in the reversal of tumor cell resistance and EMT: downregulation of the NF-kB/Snail/YY1/RKIP circuitry. Nitric Oxide. 2011;24:1–7.
91. Yang MH, Chang SY, Chiou SH, Liu CJ, Chi CW, Chen PM, et al. Overexpression of NBS1 induces epithelial-mesenchymal transition and co-expression of NBS1 and Snail predicts metastasis of head and neck cancer. Oncogene. 2007;26:1459–67.
92. Olmeda D, Jorda M, Peinado H, Fabra A, Cano A. Snail silencing effectively suppresses tumor growth and invasiveness. Oncogene. 2007; 26:1862–74.
93. Bonavida B, Baritaki S. Inhibition of epithelial-to-mesenchymal transition (EMT) in cancer by nitric oxide: pivotal roles of nitrosylation of NF-kB, YY1 and Snail. Forum Immunopathol Dis Ther. 2012;3:125–33.
94. Tsao DA, Yu HS, Chang HR. Nitric oxide enhances expression of raf kinase inhibitor protein in keratinocytes. Exp Dermatol. 2009;18:571–3.
95. Lin Y, Bai L, Chen W, Xu S. The NF-kappaB activation pathways, emerging molecular targets for cancer prevention and therapy. Expert Opin Ther Targets. 2010;14:45–55.
96. Wang H, Hertlein E, Bakkar N, Sun H, Acharyya S, Wang J, Carathers M, Davuluri R, Guttridge DC. NF-kappaB regulation of YY1 inhibits skeletal myogenesis through transcriptional silencing of myofibrillar genes. Mol Cell Biol. 2007;27:4374–87.
97. Kleinberg L, Davidson B. Cell survival and apoptosis-related molecules in cancer cells in effusions: a comprehensive review. Diagn Cytopathol. 2009;37:613–24.

98. Julien S, Puig I, Caretti E, Bonaventure J, Nelles L, van Roy F, Dargemont C, de Herreros AG, Bellacosa A, Larue L. Activation of NF-kappaB by Akt upregulates Snail expression and induces epithelium mesenchyme transition. Oncogene. 2007;26:7445–56.
99. Yeung K, Seitz T, Li S, Janosch P, McFerran B, Kaiser C, Fee F, Katsanakis KD, Rose DW, Mischak H, Sedivy JM, Kolch W. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. Nature. 1999;401:173–7.
100. Yeung KC, Rose DW, Dhillon AS, Yaros D, Gustafsson M, Chatterjee D, McFerran B, Wyche J, Kolch W, Sedivy JM. Raf kinase inhibitor protein interacts with NF-kappaB-inducing kinase and TAK1 and inhibits NF-kappaB activation. Mol Cell Biol. 2001;21:7207–17.
101. Kumar A, Takada Y, Boriek AM, Aggarwal BB. Nuclear factor-kappaB its role in health and disease. J Mol Med. 2004;82:434–48.
102. Vega MI, Jazirehi AR, Huerta-Yepez S, Bonavida B. Rituximab-induced inhibition of YY1 and Bcl-xL expression in Ramos non-Hodgkin's lymphoma cell line via inhibition of NF-kappa B activity: role of YY1 and Bcl-xL in Fas resistance and chemoresistance, respectively. J Immunol. 2005;175:2174–83.
103. Santos-Silva MC, Sampaio de Freitas M, Assreuy J. Killing of lymphoblastic leukemia cells by nitric oxide and taxol: involvement of NF-kappaB activity. Cancer Lett. 2001;173:53–61.
104. Santos-Silva MC, Freitas MS, Assreuy J. Involvement of NF-kappaB and glutathione in cytotoxic effects of nitric oxide and taxol on human leukemia cells. Leuk Res. 2006;30:145–152.
105. Sun Y, Rigas B. The thioredoxin system mediates redox-induced cell death in human colon cancer cells: implications for the mechanism of action of anticancer agents. Cancer Res. 2008;68:8269–7.
106. Park SW, Wei LN. Regulation of c-myc gene by nitric oxide via inactivating NF-kappa B complex in P19 mouse embryonal carcinoma cells. J Biol Chem. 2003;278:29776–82.
107. Huerta-Yepez S, Vega M, Escoto-Chavez SE, Murdock B, Sakai T, Baritaki S, Bonavida B. Nitric oxide sensitizes tumor cells to TRAIL-induced apoptosis via inhibition of the DR5 transcription repressor Yin Yang 1. Nitric Oxide. 2009;20:39–52.
108. Huerta-Yepez S, Baritaki S, Baay-Guzman G, Hernandez-Luna MA, Hernandez-Cueto A, Vega MI, Bonavida B. Contribution of either YY1 or BclXL-induced inhibition by the NO-donor DETANONOate in the reversal of drug resistance, both in vitro and in vivo. YY1 and BclXL are overexpressed in prostate cancer. Nitric Oxide. 2013;29:17–24.
109. Seligson D, Horvath S, Huerta-Yepez S, Hanna S, Garban H, Roberts A, et al. Expression of transcription factor Yin Yang 1 in prostate cancer. Int J Oncol. 2005;27:131–41.
110. Sun A, Tang J, Hong Y, Song J, Terranova PF, Thrasher JB, et al. Androgen receptor-dependent regulation of Bcl-xL expression: implication in prostate cancer progression. Prostate. 2008;68:453–61.
111. Marshall HE, Hess DT, Stamler JS. *S* -nitrosylation: physiological regulation of NF-kappaB. Proc Natl Acad Sci USA. 2004;101:8841–2.
112. Lebedeva I, Raffo A, Rando R, Ojwang J, Cossum P, Stein CA. Chemosensitization of bladder carcinoma cells by bcl-xL antisense oligonucleotides. J Urol. 2001;166:461–9.
113. Shigeno M, Nakao K, Ichikawa T, Suzuki K, Kawakami A, Abiru S, Miyazoe S, Nakagawa Y, Ishikawa H, Hamasaki K, Nakata K, Ishii N, Eguchi K. Interferon-alpha sensitizes human hepatoma cells to TRAIL-induced apoptosis through DR5 upregulation and NF-kappa B inactivation. Oncogene. 2003;22:1653–62.
114. LaVallee TM, Zhan XH, Johnson MS, Herbstritt CJ, Swartz G, Williams MS, Hembrough WA, Green SJ, Pribluda VS. 2-methoxyestradiol up-regulate death receptor 5 and induces apoptosis through activation of the extrinsic pathway. Cancer Res. 2003;63:468–75.
115. Johnston JB, Kabore AF, Strutinsky J, Hu X, Paul JT, Kropp DM, Kuschak B, Begleiter A, Gibson SB. Role of the TRAIL/APO2-L death receptors in chlorambucil- and fludarabine-induced apoptosis in chronic lymphocytic leukemia. Oncogene. 2003;22:8356–69.
116. Fulda S, Wick W, Weller M, Debatin KM. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. Nat Med. 2002;8:808–15.

117. Chawla-Sarkar M, Bauer JA, Lupica JA, Morrison BH, Tang Z, Oates RK, Almasan A, Di-Donato JA, Borden EC, Lindner DJ. Suppression of NF-kappa B survival signaling by nitrosylcobalamin sensitizes neoplasms to the anti-tumor effects of Apo2L/TRAIL. J Biol Chem. 2003;278:39461–9.
118. Hongo F, Garban H, Huerta-Yepez S, Vega M, Jazirehi AR, Mizutani Y, Miki T, Bonavida B. Inhibition of the transcription factor Yin Yang 1 activity by S-nitrosation. Biochem Biophys Res Commun. 2005;336:692–701.
119. Baritaki S, Katsman A, Chatterjee D, Yeung KC, Spandidos DA, Bonavida B. Regulation of tumor cell sensitivity to TRAIL-induced apoptosis by the metastatic suppressor Raf kinase inhibitor protein via Yin Yang 1 inhibition and death receptor 5 up-regulation. J Immunol. 2007;179:5441–53.
120. Baritaki S, Suzuki E, Umezawa K, Spandidos DA, Berenson J, Daniels TR, Penichet ML, Jazirehi AR, Palladino M, Bonavida B. Inhibition of Yin Yang 1-dependent repressor activity of DR5 transcription and expression by the novel proteasome inhibitor NPI-0052 contributes to its TRAIL-enhanced apoptosis in cancer cells. J Immunol. 2008;180:6199–210.
121. Nieto MA. The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol. 2002;3:155–66.
122. Palmer MB, Majumder P, Cooper JC, Yoon H, Wade PA, Boss JM. Yin yang 1 regulates the expression of snail through a distal enhancer. Mol Cancer Res. 2009;7:221–9.
123. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, Del Barrio MG, Portillo F, Nieto MA. The transcription factor snail controls epithelial–mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol. 2000;2:76–83.
124. Beach S, Tang H, Park S, Dhillon AS, Keller ET, Kolch W, Yeung KC. Snail is a repressor of RKIP transcription in metastatic prostate cancer cells. Oncogene. 2008;27:2243–8.
125. Baritaki S, Yeung K, Palladino M, Berenson J, Bonavida B. Pivotal roles of snail inhibition and RKIP induction by the proteasome inhibitor NPI-0052 in tumor cell chemoimmunosensitization. Cancer Res. 2009;69:8376–85.
126. Baritaki S, Huerta-Yepez S, Sahakyan A, Karagiannides I, Bakirtzi K, Jazirehi AR, Bonavida B. Mechanisms of nitric oxide-mediated inhibition of EMT in cancer: inhibition of the metastasis-inducer Snail and induction of the metastasis-suppressor RKIP. Cell Cycle. 2010;9:4931–40.
127. Jazirehi AR, Bonavida B. Cellular and molecular signal transduction pathways modulated by rituximab (rituxan, anti-CD20 mAb) in non-Hodgkin's lymphoma implications in chemosensitization and therapeutic intervention. Oncogene. 2005;24:2121–43.
128. Chatterjee D, Bai Y, Wang Z, Beach S, Mott S, Roy R, Braastad C, Sun Y, Mukhopadhyay A, Aggarwal BB, Darnowski J, Pantazis P, Wyche J, Fu Z, Kitagwa Y, Keller ET, Sedivy JM, Yeung KC. RKIP sensitizes prostate and breast cancer cells to drug-induced apoptosis. J Biol Chem. 2004;279:17515–23.
129. Jazirehi AR, Vega MI, Chatterjee D, Goodglick L, Bonavida B. Inhibition of the Raf-MEK1/2-ERK1/2 signaling pathway, Bcl-xL down-regulation, and chemosensitization of non-Hodgkin's lymphoma B cells by Rituximab. Cancer Res. 2004;64:7117–26.
130. Thiery JP. Epithelial–mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002;2:442–54.
131. BarberÃ MJ, Puig I, Domínguez D, Julien-Grille S, Guaita-Esteruelas S, Peiró S, Baulida J, Francí C, Dedhar S, Larue L, García de Herreros A. Regulation of Snail transcription during epithelial to mesenchymal transition of tumor cells. Oncogene. 2004;23:7345–54.
132. Ko H, Kim HS, Kim NH, Lee SH, Kim KH, Hong SH, Yook JI. Nuclear localization signals of the E-cadherin transcriptional repressor Snail. Cells Tissues Organs. 2007;185:66–72.
133. Hirst D, Robson T. Targeting nitric oxide for cancer therapy. J Pharm Pharmacol. 2007;59:3–13.
134. Mocellin S, Bronte V, Nitti D. Nitric oxide, a double-edged sword in cancer biology: searching for therapeutic opportunities. Med Res Rev. 2007;27:317–52.
135. Korbelik M, Parkins CS, Shibuya H, Cecic I, Stratford MR, Chaplin DJ. Nitric oxide production by tumour tissue: impact on the response to photodynamic therapy. Br J Cancer. 2000;82:1835–43.

136. Reeves KJ, Reed MWR, Brown NJ. Is nitric oxide important in photodynamic therapy?. J Photochem Photobiol. 2009;95:141–7.
137. Gupta S, Ahmad N, Mukhtar H. Involvment of nitric oxide during phthalocyanine (Pc4) photodynamic therapy-mediated apoptosis. Cancer Res. 1998;58:1785–8.
138. McCall TB, Boughton-Smith NK, Palmer RM, Whittle BJ, Mocada S. Synthesis of nitric oxide from l-arginine by neutrophils. Release and interaction with superoxide anion. Biochem J. 1989;261:293–6.
139. Mehta J, Lawson DL, Nicolini FA, Ross MH, Player DW. Effects of activated polymorphonuclear leukocytes on vascular smooth muscle tone. Am J Physiol. 1991;261:327–34.
140. Evans TJ, Buttery LDK, Carpenter A, Springall DR, Polak JM, Cohen J. Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. Proc Natl Acad Sci USA. 1996;93:9553–8.
141. Rapozzi V, Della Pietra E, Zorzet S, Zacchigna M, Bonavida B, Xodo LE. Nitric oxide-mediated activity in anti-cancer photodynamic therapy. Nitric Oxide. 2013;30:26–35.
142. Weyerbrock A, Osterberg N, Psarras N, Baumer B, Kogias E, Werres A, Bette S, Saavedra JE, Keefer LK, Papazoglou A. JS-K, a glutathione S-transferase-activated nitric oxide donor with antineoplastic activity in malignant gliomas. Neurosurgery. 2012;70:497–510.
143. O'Connell JB, Maggard MA, Ko CY. Colon cancer survival rates with the new American joint committee on cancer sixth edition staging. J Natl Cancer Inst. 2004;96:1420–5.
144. Pervin S, Singh R, Chaudhuri G. Nitric oxide-induced cytostasis and cell cycle arrest of a human breast cancer cell line (MDA-MB-231): potential role of cyclin D1. Proc Natl Acad Sci USA. 2001;98:3583–8.
145. Muir CP, Adams MA, Graham CH. Nitric oxide attenuates resistance to doxorubicin in three-dimensional aggregates of human breast carcinoma cells. Breast Cancer Res Treat. 2006;96:169–76.
146. Pervin S, Singh R, Gau CL, Edamatsu H, Tamanoi F, Chaudhuri G. Potentiation of nitric oxide-induced apoptosis of MDA-MB-468 cells by farnesyltransferase inhibitor: implications in breast cancer. Cancer Res. 2001;61:4701–6.
147. Matthews NE, Adams MA, Maxwell LR, Gofton TE, Graham CH. Nitric oxide-mediated regulation of chemosensitivity in cancer cells. J Natl Cancer Inst. 2001;93:1879–85.
148. Wink DA, Cook JA, Christodoulou D, Krishna MC, Pacelli R, Kim S, DeGraff W, Gamson J, Vodovotz Y, Russo A, Mitchell JB. Nitric oxide and some nitric oxide donor compounds enhance the cytotoxicity of cisplatin. Nitric Oxide. 1997;1:88–94.
149. Azizzadeh B, Yip HT, Blackwell KE, Horvath S, Calcaterra TC, Buga GM, Ignarro LJ, Wang MB. Nitric oxide improves cisplatin cytotoxicity in head and neck squamous cell carcinoma. Laryngoscope. 2001;111:1896–900.
150. Perrotta C, Bizzozero L, Falcone S, Rovere-Querini P, Prinetti A, Schuchman EH, Sonnino S, Manfredi AA, Clementi E. Nitric oxide boosts chemoimmunotherapy via inhibition of acid sphingomyelinase in a mouse model of melanoma. Cancer Res. 2007;67:7559–64.

Chapter 13
Emerging Role of NO-Mediated Therapeutics

Cian M. McCrudden and Helen O. McCarthy

Abstract The free radical nitric oxide (NO) is fundamental in the neoplastic environment. At low concentrations, the gasotransmitter manifests a pathological phenotype characterized by uncontrolled proliferation, increased invasion and metastasis, stimulation of angiogenesis and inhibited apoptosis. Paradoxically, at superphysiological concentrations, a less aggressive phenotype exists, where tumor cells are less likely to metastasize, angiogenesis is inhibited, and apoptotic machinery operates appropriately. This dichotomy of response to NO has created a divergence in the field, with some researchers set on interfering with NO signaling in cancer cells, and others endeavoring to boost it. The purpose of this chapter is to examine the activity of NO in oncology, and to highlight the recent advances in NO-mediated therapeutics. We have focused on emerging strategies that act either by promotion of or interference with NO signaling, including genetic therapies, and have largely limited topics discussed to discoveries from the past 18 months. Attention is paid to agents that have been or are being assessed at clinical trial, and the chapter concludes with a cautionary note on the appropriate use of NO-mediated therapies in oncology.

Keywords Cancer · Donor · Gene therapy · Hypoxia · Nitric oxide

Abbreviations

CMV	Cytomegalovirus
DMBA	7,12-dimethylbenz[a]anthracene
DT-D	NAD(P)H:(quinone-acceptor) oxidoreductase
EGF(R)	Epidermal growth factor (receptor)
ELIP	Echogenic liposomes
ER	Estrogen receptor

H. O. McCarthy (✉) · C. M. McCrudden
Experimental Therapeutics Research Group, School of Pharmacy, Queen's University Belfast, Lisburn Road, Belfast BT9 7BL, UK
e-mail: h.mccarthy@qub.ac.uk

C. M. McCrudden
e-mail: c.mccrudden@qub.ac.uk

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_13

FOLFIRI	Folinic acid, Fluorouracil, Irinotecan
GST	Glutathione S-transferase
HNO	Nitroxyl
HUVEC	Human umbilical vein endothelial cell
IR	Ionizing radiation
L-NAME	L-NG-Nitroarginine methyl ester
MLF2	Myeloid leukemia factor 2
MMP	Matrix metalloproteinase
NO(S)	Nitric oxide (synthase)
NONOates	Diazeniumdiolates
NSAID	Non-steroidal anti-inflammatory drug
NSCLC	Non-small cell lung cancer
P-gp	P-glycoprotein
PARP	Poly(ADP)ribose polymerase
Pb	Pheophorbide
PLGA	Poly(lactic-co-glycolic acid)
PZP	Polyethylene glycol-conjugated zinc protoporphyrin IX
ROS/RNS	Reactive oxygen/nitrogen species
RPL39	Ribosomal protein L39
TIMP	Tissue inhibitor of metalloproteinase
TPP	Triphenylphosphonium
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

NO Donors

DEA/NO	Diethylamine/NO
DETA/NO	Diethylenetriamine/NO
GLYN	Glycidyl nitrate
GSNO	S-nitrosoglutathione
GTN	Glyceryl trinitrate
INDQ/NO	Indolequinone-diazeniumdiolate/NO
IPA/NO	Isopropylamine/NO
JS-K	(*O*2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1- yl] diazen-1-ium-1,2-diolate)
MNBI	2-mercapto-5-nitro benzimidazole
NOSH	Nitric oxide/hydrogen sulfide donor
PABA/NO	*p*-aminobenzoic acid/NO
RRx-001	1-bromoacetyl-3,3-dinitroazetidine (ABDNAZ)
SIN-1	3-morpholinosydnonimine
SNAP	S-nitroso- N-acetylpenicillamine
SNO(-HSA)	*S*-nitrosothiol (-human serum albumin)
SNP	Sodium nitroprusside

Introduction—NO as a Therapeutic

Produced by the actions of three nitric oxide synthase (NOS) enzymes, the free radical nitric oxide (NO) primarily acts by activation of soluble guanylate cyclase, and functions in maintenance of the normal physiological condition and in the regulation of pathophysiological processes. In transformed tissues, concentrations of the free radical that normally elicit normal physiological activities instead provoke pathophysiological manifestations, including stimulating uncontrolled proliferation, activation of matrix metalloproteinases (MMPs) and inactivation of tissue inhibitors of metalloproteinases (TIMPs), and promotion of angiogenesis, invasion and metastasis [1]. The dichotomous relationship between NO and the tumor, where low concentrations of the radical provoke tumor progression, but high concentrations are tumoricidal, is paradoxical, and represents an inversion of hormesis.

The functionality of NO is dependent on at least one of a range of possible fates of the radical, but generally involves post-translational modification of proteins, that occur by formation of S-nitrosothiols, nitrosylation of metal complexes and metalloproteins or other biological targets [2]. NO can react with inorganic molecules (i.e. oxygen, superoxide or transition metals), structures in DNA, prosthetic groups (eg heme) or proteins, and can elicit beneficial or detrimental responses dependent on the concentration of the radical and the environment of the reaction [3]. NO has also been implicated as being central to the Warburg effect in ovarian cancer cells, ensuring maintenance of a high metabolic rate that is characteristic of cancer cells [4].

Figure 13.1 highlights some of the functions of NO in cancer, and the concentration-dependence on which the function is determined.

NO Donor Classes

A range of NO donating agents have been developed and characterized in terms of their NO release kinetics and therapeutic applications. For relatively recent, comprehensive introductions to NO donors, see reviews by Huerta and colleagues [1] and Miller and Megson [5]. The major classes of NO donor drugs are the organic nitrates, metal-NO complexes (eg sodium nitroprusside (SNP)), *S*-nitrosothiols, sydnonimines, diazeniumdiolates (NONOates), and NO-drug hybrids [1].

The most commonly used NO donor agents are the organic nitrates, which include glyceryl trinitrate (GTN—nitroglycerin). GTN is among the oldest recognized NO donors, and has long been used for its vasodilatory properties for the treatment of hypertension and other cardiovascular complaints. More recently, the donor compound has been trialed for its ability to impact tumor vasculature by increasing tumor perfusion and improving tumor oxygenation [6]. GTN applied topically onto murine sarcoma S-180 tumor xenografts increased tumor perfusion, measured by macromolecular drug (Evans blue/albumin) accumulation, without altering peripheral local blood flow. GTN treatment (0.1 mg/tumor in Vaseline) improved delivery of the

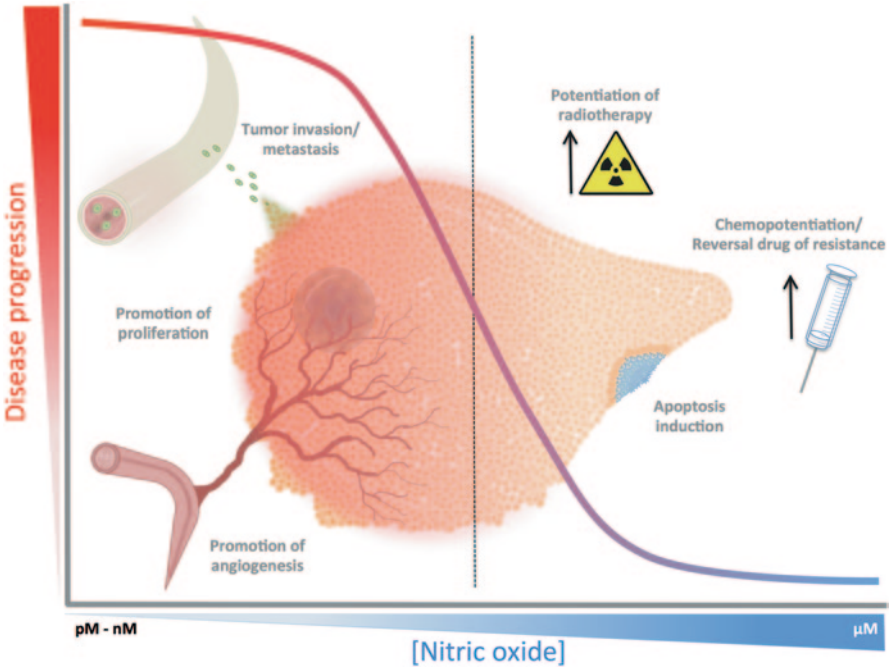


Fig. 13.1 Low concentrations of NO increase cancer progression by promoting angiogenesis and metastasis and by stimulating cancer cell progression. On the other hand, higher concentrations of NO inhibit cancer progression by sensitizing tumors to radiotherapy or chemotherapy, reversing resistance to chemotherapy, or induction of apoptosis, as well as hampering the metastatic and angiogenic cascades

heme oxygenase-1 inhibitor, polyethylene glycol-conjugated zinc protoporphyrin IX (PZP) two-fold, and potentiated the tumor growth delay properties of PZP in S-180 xenografts and 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced tumors, as well as augmenting aclarubicin's potency in C38 colon adenocarcinoma xenografts [7]. In two cohorts of patients with previously untreated stage IIIB/IV non-small-cell lung cancer, transdermal GTN (25 mg/patch, one patch per day for five consecutive days from three days pre-chemotherapy) improved response to combination vinorelbine/cisplatin treatment, with median time to progression increasing in one trial (327 v 185 days) [8], and improved overall response rate in the second (35.3 vs. 18.8%) [9]. Safe combination of the above triumvirate of therapeutics with radiotherapy was demonstrated in a Phase II trial; the absence of toxicity attributable to GTN justifies implementation of a randomized trial according to the authors [10]. The impact of NO produced by GTN was demonstrated in patients with operable lung adenocarcinoma; expression of HIF-1α, VEGF and P-gp was lower in tumor sections of lung adenocarcinoma patients who received transdermal GTN for 72 h before surgery than in sections from patients not administered the NO donor [11].

A transdermal patch delivering GTN improved prostate specific antigen (PSA) level doubling time in men with increasing PSA levels post-surgery/radiotherapy

for prostate cancer up to 24 months post commencement of treatment. The authors used the data as justification for a further trial, as increased PSA doubling time should concomitantly increase the time before commencement of hormone therapy [12]. The same lead investigator, Dr. D. Robert Siemens, is the responsible party for a trial to delineate the impact of transdermal GTN on expression of biomarkers in men with recurrent prostate cancer (ClinicalTrials.gov identifier: NCT01704274). A trial to determine the therapeutic value of GTN in non-small cell lung cancer (NSCLC) is recruiting participants in the MAASTRO clinic in the Netherlands (NCT01210378).

The therapeutic potential of SNP has been demonstrated in a range of cancer models, and exerted anti-cancer effects by suppression of invasion, HIF-1α a interference, and radiosensitization, among other mechanisms [1]. The therapeutic interest in SNP therapy has waned recently, possibly as a result of toxicity of the molecule (SNP generates cyanide as a by-product). A recent report showed that SNP sensitized SGC-7901, AGS, MKN45 and MKN28 gastric cancer cell lines to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis [13]. Although promising results were forthcoming from this study, effects were not investigated *in vivo*.

Diazeniumdiolates (NONOates) consist of a diolate group [N(O−)N=O] bound to a nucleophile adduct, such as a primary or secondary amine or polyamine, via a nitrogen atom. Release of NO from NONOates follows first order kinetics, making the degree of NO release predictable. As recently reviewed by Bonavida and Baritaki, DETA NONOate interferes with the oncologic state by reversing or preventing drug resistance, and inhibiting epithelial-mesenchymal transition, resultant of interference with the NF-κB/Snail/YY1/RKIP cascade [14].

Owing to its potency against a range of cancer cell lines, and apparent specificity for tumor cells, JS-K (*O*2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl] diazen-1-ium-1,2-diolate) was accepted onto the National Cancer Institute's Rapid Access to Interventional Development (RAID) program, ensuring its progression as a potential therapeutic [5]. Its status as a front-runner in drug development has led to a great deal of research into this promising molecule. A study revealed that JS-K treatment down-regulates the expression of and signaling produced by the androgen receptor in 22-RV-1 prostate cancer cells *in vitro*, and interferes with Wnt signaling in a concentration-dependent manner. The donor is also cytotoxic in the same concentration range [15]. A more recent study suggested that the aryl ring in the structure of JS-K may have been responsible for toxicity of the molecule in SFFV-MEL murine erythroleukemia cells; both JS-K and CNDB, it's diazeniumdiolate-lacking relative (incapable of donating NO), induced caspase-dependent apoptosis [16]. It is possible that arylation is partly responsible for the effects of JS-K in other models.

Of the *S*-Nitrosothiols, S-nitroso- N-acetylpenicillamine (SNAP) and S-nitrosoglutathione (GSNO) have progressed farthest in the field, with their therapeutic potential in cancer conditions being assessed for almost 20 years [17]. As well as therapeutic benefit, SNAP treatment has also resulted in development of a carcinogenic phenotype, upregulating iNOS and COX-2 expression in head and neck cancer cells. GSNO is similarly dichotomous, either promoting or inhibiting the cancer phenotype [1].

The most extensively studied sydnonimine is 3-morpholinosydnonimine (SIN-1), a molecule that donates NO, and in the presence of oxygen, peroxynitrite generation results from superoxide also released [1], although it is more studied for its peroxynitrite-donating properties than purely NO, and for that reason is beyond the scope of this chapter.

New Therapeutics

NO Donors

Glycidyl nitrate (GLYN) is a novel energetic dinitroazetidine NO-releasing compound developed by ATK Aerospace Systems that instigated NO release from the M21 melanoma cell line and the SCC VII murine squamous cell carcinoma cell line *in vitro*. *In vivo*, GLYN sensitized SCC VII xenografts to cisplatin and gamma irradiation, and increased tumor blood flow in SCC VII tumors [18]. GLYN was well tolerated at the effective dose of up to 150 mg/kg in mice, and its improvement of tumor blood flow could make it particularly beneficial in tumors with significant hypoxic regions.

RRx-001 (1-bromoacetyl-3,3-dinitroazetidine—ABDNAZ), another ATK Aerospace Systems product generated for its energy release potential, is a NO-liberating drug that had a cytotoxicity profile similar to that of cisplatin in a panel of 12 human cancer cell lines, and cytotoxicity was enhanced by hypoxia [19]. γ-H2AX expression was increased by RRx-001, and apoptosis was induced. Moreover, RRx-001 was as potent as cisplatin in SCC VII xenograft-bearing mice, and appeared to lack the widespread toxicity associated with cisplatin. RRx-001 also sensitized SCC VII to fractionated radiotherapy [19]. Case studies of the potential benefit of RRx-001 in two advanced colorectal adenocarcinoma patients for whom treatment options had been exhausted revealed the potential that RRx-001 holds; RRx-001 re-sensitized Patient 1 to FOLFIRI (Folinic acid, Fluorouracil, Irinotecan) therapy after resistance developed previously, and carcinoembryonic antigen (CEA) levels remained relatively stable while the patient was receiving RRx-001, but increased dramatically when RRx-001 was withdrawn. In Patient 2, an increased response to FOLFIRI was observed following RRx-001. Furthermore the growth of the patient's target lesion's longest diameter was slowed during RRx-001 treatment and accelerated after withdrawal of RRx-001 [20]. RRx-001 is being assessed clinically in solid tumors for which no suitable treatment options exist (NCT01359982).

NO Conjugates

A NO-releasing doxorubicin conjugate, NitDox has emerged that reverses doxorubicin resistance in human colon cancer cells [21]. Complexation of the drug conjugates into liposomal formulations further boosts cytotoxicity and NO-releasing

potency in parental- and doxorubicin-resistant HT29 colon cancer cells. Moreover, liposomal NitDox was more potent than Caelyx, a liposomal doxorubicin approved for carcinomas of the ovary and breast *in vitro* [22]. Nitration of P-glycoprotein (P-gp) (NO reverses doxorubicin resistance in human colon cancer cells by inhibiting the drug efflux by nitration of enzymes such as P-gp [23]) was evident in all NitDox-treated doxorubicin-resistant MCF-7 cells, and absent in cells that received doxorubicin or Caelyx. Impressively, the liposomal NitDox formulations appear to lack the cardiotoxic properties that limit doxorubicin's use [22].

A previously developed macromolecular NO delivery vehicle, poly-NO conjugated human serum albumin (poly-SNO-HSA) [24] that was capable of evoking NO accumulation in tumors, was further improved by dimerization and PEGylation. A recent report by Ishima and colleagues detailed the addition of poly(ethylene) glycol to poly-SNO-HSA for enhancement of stability *in vitro* and *in vivo*. Dimerization of the poly-SNO-HSA boosted by an order of magnitude the anti-cancer potential of poly-SNO-HSA in Colon-26 xenograft-bearing mice [25], leading the authors to conclude that the PEG-Poly-SNO-HSA dimer bears all the hallmarks of a clinically-relevant drug candidate.

Conjugation of two actives into a bifunctional prodrug is a strategy that has produced impressive results. The poly(ADP)ribose polymerase (PARP) inhibitor olaparib, itself undergoing clinical trials for a number of malignancies, was conjugated to JS-K, producing a prodrug, that upon exposure to glutathione S-transferase P1 (GSTP1), an enzyme frequently overexpressed in cancers, is metabolized into two active drugs that concomitantly induce DNA damage (through NO), and inhibit the repair of this induced damage (olaparib). Olaparib's PARP-1 inhibitory function was marginally weakened by JS-K conjugation, while activation of the pro-drug (ie NO liberation) returned olaparib activity to normal levels, and the prodrug was more potent an anti-proliferative agent than olaparib alone in a range of NCSLC cell lines, and induced DNA damage more potently than olaparib in H1703 cells [26]. The NO-donating PARP-1 inhibitor also slowed A549 xenograft growth *in vivo* more potently than the parent molecule. In combination with Bortezomib, a proteasome inhibitor that has been studied extensively in NCSLC, colony-forming ability of A549 cells was all but abolished, indicating a possible therapeutic combination between the two agents [26]. Given the upsurge in PARP inhibitor development in the last decade, and the huge therapeutic potential of these molecules [27], conjugation of a NO donor to olaparib is an exciting development.

A prodrug (termed '21') of O^2-(2,4-dinitrophenyl)diazeniumdiolate and oleanolic acid derivatized with a GSTπ-cleavable bond (GSTπ is commonly overexpressed in cancer) was more stable in rat blood plasma than standard NO donors JS-K and PABA/NO. Intraperitoneally-delivered '21' was more potent than 5-fluorouracil in a mouse model of hepatoma, in that it achieved equivalent tumor growth delay at a lower concentration (38.3 μmol/kg '21' v 153.8 μmol/kg 5-fluorouracil), and induced apoptosis of HepG2 cells *in vitro*. Inhibition of GSTπ or scavenging of NO abrogated the effects of '21' on apoptosis-associated proteins including pJNK, p38, BCL-2 and Bax [28]. In the current environment when personalized medication is a driving force behind product development, targeting of proteins that are aberrantly expressed in neoplastic conditions to facilitate NO delivery is likely to continue its upwards trend.

NO-NSAIDs

Supplementation of the anti-cancer potential of non-steroidal anti-inflammatory drugs (NSAID) with NO donating agents has been widely reported previously [1]. Nortcliffe and colleagues armored the NSAID sulindac with various NO donors (nitrate esters, furoxans and sydnonimines), publishing the anti-cancer properties of 56 such products. Compounds 1k and 1n (both furoxan–sulindac analogues, with hexyl and cis-butenyl linkers respectively) were most impressive in terms of cytotoxicity against PC-3 prostate cancer cells *in vitro*, and NO release in glutathione buffer [29]. NO-sulindac also exhibited anti-melanoma potential, preventing UVB-induced tumorigenesis in hairless mice [30]. A library of 24 1,2,4-triazole/oxime hybrids similarly produced a lead compound that inhibited growth of A498 renal cancer cell line by 50 %, and had an improved gastric response compared to the NO donor-lacking NSAID [31].

NBS-242 (flurbiprofen benzyl nitrate) is another emerging NO-NSAID that has been tested for its cancer targeting ability. NBS-242 inhibited the viability (inhibiting proliferation and inducing apoptosis) of A-341 epidermoid carcinoma cell line five times more potently than benzyl nitrate and 15 times more potently than flurbiprofen itself. This interference with cell kinetics was associated with increased activation of caspase-3 [32]. Further testing of the potential of NBS-242 in *in vivo* models of human cancer are required before the potential of this NO-NSAID will be known.

Two novel NONO-NSAIDs, IPA/NO-aspirin and DEA/NO-aspirin appreciably inhibited the proliferation of A549 lung adenocarcinoma cells, while the NSAID or NO/nitroxl (HNO) donors alone had limited/no impact on viability. The conjugation of the NSAID to IPA/DEA also ameliorated gastric toxicity that is a common side effect of NSAID therapy, as measured in a stomach ulceration assay in Sprague Dawley rats [33]. Both conjugates also maintained their NSAID functionality. This paper was the first report of a HNO-donating NSAID. As toxicity was limited to cancer cells (non-transformed HUVEC cells were not susceptible to drug-elicited cytotoxicity), it is likely that further investigation of the therapeutic applications of these or related compounds can be expected.

Recently, NO-NSAIDs have been produced to also contain donating compounds for another gasotransmitter, hydrogen sulfide (H_2S). H_2S is receiving attention for anti-cancer potential of its own, and a push for H_2S-donating agents for therapeutic applications has led to the development and characterization of a plethora of compounds [34] Two such compounds that contain NO- and H_2S-donating motifs, NOSH-naproxen (AVT-219) and NOSH-sulindac (AVT-18 A) were potent tumor cell growth inhibitors (HT-29 colon cancer, Jurkat leukemia, MCF-7 breast cancer, and BxPC-3 pancreatic cancer), with potencies ranging from 2500 to 34,000-fold more potent than parent NSAIDs, and both provoked NO and H_2S plasma accumulation *in vivo* [35]. Both compounds retained the anti-inflammatory activity of their parent compounds, as assessed in a carrageenan-induced rat paw edema model. In this model, drugs were given by oral gavage one hour prior to carrageenan, and paw volume was measured at four hours post injection. Longer-term animal studies to determine normal tissue toxicity are of course required.

Nanocarriers of NO

The therapeutic activity of NO donors can be improved by rational addition of functional groups. Gold nanoparticles have received attention recently for their anti-cancer functionality [36]. Functionalization of gold nanoparticles with the light-sensitive NO-releasing 2-mercapto-5-nitro benzimidazole (MNBI) afforded the particles toxicity to HeLa cervical cancer cells when irradiated. MNBI was only moderately toxic to the same cells [37]. Follow up studies to determine *in vivo* performance of these particles is anticipated.

An elegant strategy for the targeted controlled release of NO was devised by Xu and colleagues, namely a carbon dot-based nanoplatform system functionalized with a mitochondrion targeting agent (triphenylphosphonium (TPP)) and NO donor, SNO. HeLa cells treated with the nanoplatform, termed Cdot-TPP-SNO, released NO in the absence or presence of white light irradiation. Cdot-TPP avoided endosomal entrapment, and localized to the mitochondria, where NO release dose-dependently increased the apoptotic fraction; targeting NO release to the mitochondria more efficiently provoked apoptosis, supporting the use of this targeting strategy [38]. To date, only *in vitro* results on this technology have been forthcoming. As the targeting strategy targets an organelle present in almost every cell in the body, it may be that further functionalization of the therapy is required to limit NO release to the target tissues.

MDA-MB-231 and MDA-MB-468 breast cancer cells were highly susceptible to NO generated by a novel NO-loaded echogenic liposomes (ELIP) that comprised four phospholipids and cholesterol hydrochloride. The ELIP itself was without impact on viability, but when loaded with NO, reduced viability of MDA-MB-468 to $<20\,\%$ of control, and almost completely abolished viability of MDA-MB-231 cells [39]. Incorporation of NO donor JS-K into micelles composed of P123 Pluronic resulted in improved delivery of the donor to the nuclei of HL-60 myeloid leukaemia cells [40]. NO donating compounds ruthenium tetraamine (*trans*-$[Ru(NO)(NH_3)_4(py)]^{3+}$ (py = pyridine)) and tetraazamacrocyclic nitrosyl compounds (*trans*-$[Ru(NO)Cl(cyclam)]^{2+}$ (cyclam = 1,4,8,11-tetraazacyclotetradecane)) were complexed into nanoscale particles for enhanced delivery and NO-releasing ability. Poly(lactic-*co*-glycolic) acid (PLGA) complexation slowed the release of NO from particles, facilitating, as the authors suggest, controlled release of NO from the complexes [41].

Highly efficient release of NO from GSNO-loaded polymeric nanoparticles was demonstrated by Duong et al. Particles 40–50 nm in diameter harboring GSNO provoked NO release in neuroblastoma cells (BE(2)-C) cells, and co-delivery of nanoparticle with cisplatin evoked synergistic cytotoxic activity [42]. In an attempt to boost toxicity, Fraix and colleagues engineered a polymeric nanoplatform capable of the co-delivery of NO and singlet oxygen ($^1O_2$), another cytotoxic gaseous transmitter. Nanoparticles (~60 nm diameter) co-encapsulating the photosensitive donors of the two transmitters localized in the cytoplasm of melanoma cells. In the absence of photo-activation, the nanoparticles were ineffectual, while nanoparticles harboring both donors, when photo-activated, obliterated melanoma cell viability [43]. As photoactivtion is required, such a therapy may only be suitable in superficial

tumors or in tumors in luminal organs. Another compound designed to donate both NO and $^1O_2$, $[Ru(phthalocyanine)(pz)_2\{Ru(bpy)_2NO\}_2]^{6+}$, was cytotoxic against B16F10 mouse melanoma cells *in vitro* [44]. As with many of these emerging nanotechnologies, until a full *in vivo* toxicological evaluation is performed, the most promising candidates are difficult to identify. Nevertheless, a Pubmed search for the term 'nanomedicine' a decade ago would have returned ten results from that year (2004); a similar search in 2013 returned more than 160 times that number. Given the explosion in nanomedical research, it is unsurprising that NO-delivering nanoscale devices are being produced.

Tumor Targeting

The short half-life of NO requires that location and time of generation of the radical is critical, in order to maximize its therapeutic index. In a strategy to ensure appropriate delivery and release of NO, the HNO and NO donor IPA/NO, a diazeniumdiolate anion that hydrolyses to a mixture of NO and HNO, was conjugated to a β-galactosidase substrate, tetraacetylated galactosyl bromide. Rate of IPA/NO release was proportional to the concentration of β-galactosidase used, indicating that enzymatic activation of the prodrug is a suitable strategy for controlled release. Although no cancer applications were investigated, it is likely that alternative groups could be conjugated to the donor agent to exploit tumors' differential enzyme expression patterns [45]. One such NO-donating prodrug, 1-(2-methylpiperidin-1-yl) diazen-1-ium-1,2-diolate, was designed to rely on activation by nitroreductase which is highly expressed in malignancies. DLD-1 adenocarcinoma and HeLa cervical cancer cells were cytotoxically sensitive to the prodrug, and the NO-induced toxicity was abolished in the absence of nitroreductase [46].

Blockade of the activation of the EGF receptor is a successful strategy in therapy of cancers of various origins. Coupling a phenylsulfonylfuroxan NO donor to an EGFR T790M-targeting anilinopyrimidine produced a compound with remarkable properties; compound 10h was almost as potent as gefitinib in gefitinib-sensitive HCC827 NCSLC cells, and retained this potency in gefitinib-resistant H1975 lung adenocarcinoma cells. Unsurprisingly, gefitinib itself did not impact on NO release from H1975 and scavenging of NO using hemoglobin revealed that NO generation was partly responsible for cytotoxicity. Compound 10h also impaired EGFR signaling in a similar manner as WZ4002, a third generation EGFR inhibitor [47]. These tantalizing *in vitro* studies should be followed by investigations in mouse models to delineate the therapeutic potential of such exciting molecules.

INDQ/NO is a NO-donating prodrug that was functionalized to be activatable by the activity of NAD(P)H:quinone oxidoreductase (DT-diaphorase; DT-D—elevated in non-small cell lung carcinoma, colorectal carcinoma, liver cancers and breast carcinomas [48]), an enzyme highly expressed in numerous cancers. In physiological buffer, INDQ/NO was completely reliant on the presence of DT-D for NO liberation which resulted in impaired viability of cancer cells irrespective of the organ of origin and provoked congregation of γ-H2AX foci in HeLa cells [49].

NO-Mediated Effects of Other Drugs

Calycopterin, a flavonoid from *Dracocephalum kotschyi* induced apoptosis in HepG2 hepatoblastoma cells, and boosted NO and ROS levels 90- and three-fold respectively. Scavenging of ROS protected cells from calycopterin-induced apoptosis. The responsibility of NO was curiously not assessed [50].

Luteolin, a bioflavonoid, was capable of preventing azoxymethane-induced colon carcinogenesis; the success of the strategy was deemed to be down to the bioflavanoid's inhibition of iNOS and COX-2 signalling [51]. Similar inhibition of iNOS and COX-2 was evoked by Garcinol, a polyiso- prenylated benzophenone derivative in ICR mice. Tumor initiation (by combined delivery of dextran sulfate sodium [DSS] and azoxymethane [AOM]) produced tumors in 100 % of control mice, but in only 44 % of mice that received garcinol at 500 ppm in their diet. Garcinol inhibited DSS-evoked iNOS and COX-2 activation, and further, also inhibited VEGF activation by DSS/AOM combination treatment [52].

Classically prescribed for control of blood cholesterol levels, the statins are now also receiving attention for anti-cancer properties. Simvastatin and fluvastatin negatively impacted cell viability of MCF-7 [53], MDA-MB-231 and BT-549 [54] breast cancer cells, but were without potency in non-transformed MCF-10 A cells. Fluvastatin provoked NO accumulation in MDA-MB-231 cells, and greatly enhanced iNOS expression in both cell lines. This was in turn abrogated by NOS inhibition, implicating the statin-induced increase in iNOS expression as being responsible for the NO generation. The authors concluded that NO generation was at least partly responsible for cytotoxity of the statins in triple negative breast cancer cell lines [54]. The cytotoxic properties of calprotectin, an abundant heterodimeric cytosolic protein of neutrophils, were found to be due in part to the generation of ROS and NO in LNCaP prostate cancer cells [55].

Combination of the anti-diabetes drug metformin with photodynamic therapy also inhibited iNOS and NO generation, with consequent COX-2 and MMP-2 activity in Wistar rats bearing Walker 256 carcinosarcoma [56]. The photosensitizer pheophorbide *a* (Pb *a*), when irradiated, manifested NO accumulation (increasing iNOS expression) in B78-H1 murine amelanotic melanoma cells, as did DETA/NO; combination of the two resulted in additive NO accumulation, with consequent enhancement of cytotoxicity and inhibition of cellular invasive properties. Clonogenic survival *in vitro* and xenograft growth *in vivo* were both similarly maximally inhibited when both agents were used in combination [57]. Rapozzi and colleagues, the authors of this second study, conclude by revealing that investigations on a drug conjugate of a photosensitizer and an NO donor are ongoing within their laboratories. As they are studying a model of melanoma, photodynamic therapy is therapeutically viable, and we look forward with anticipation to further reports on the therapy.

Tumor immunotherapy is a cancer therapeutic strategy that is limited by tumor cell resistance to infiltrating effector T cells. Klug and colleagues recently demonstrated low dose irradiation-mediated infiltration of RT5 tumors by activated T cells. Tumor antigen-specific $CD8^{+}$T cell transfer coupled with 2 Gy irradiation

significantly boosted survival of RT5 tumor-bearing mice. Abolition of the macrophage complement of mice using clodronate in turn abolished the anti-tumor ability of T cell/2 Gy therapy. The authors noted increased iNOS expression in irradiated RT5 tumor-bearing mice and in irradiated peritoneal MAC-1-positive macrophages of RT5 mice and tumor-infiltrating macrophages from RT5 mice, and emphasized the therapeutic importance of iNOS expression/NO generation by the abrogation of tumor infiltration when NOS was inhibited using 1400 W [58].

iNOS Gene Therapy

It is perhaps curious that elevated iNOS levels have been shown to predict a poorer prognosis in cancer patients, as it seems reasonable to assume that higher iNOS expression would be coupled with higher NO levels, and that this would improve patient prognosis as elevated NO levels inhibit cancers. Notwithstanding this, it has also been shown convincingly that high iNOS expression correlates with earlier mortality. iNOS expression has consistently been shown to be associated with progression of breast carcinomas. In a cohort of 151 breast cancer patients, higher iNOS expression was significantly ($P<0.001$) correlated with malignancy [59]. In a separate cohort there was a correlation between those patients with iNOS expression and increasing grades of *in situ* breast carcinoma, indicating that iNOS may play a role in carcinogenesis [60]. Although conversely, iNOS expression was negatively correlated with lesion grade in a cohort of invasive ductal breast carcinomas [61], indicating a possible role in the prevention of metastasis. A cohort of breast cancer patients was stratified based on their expression of the estrogen receptor and iNOS expression was assessed; high iNOS expression was associated with significantly poorer survival in ER-negative patients, while ER-positive patient survival was not impacted by iNOS expression [62].

Neuronal NOS and endothelial NOS are primarily responsible for production of NO for maintenance of physiological processes, while the activity of inducible NOS is controlled by cytokine levels, so that, when required, appropriate levels of NO can be generated to affect an appropriate response. As elevated NO levels are desirable to elicit tumoricidal effects, some researchers have attempted to harness the cancer cell's own transcriptional and translational machinery to boost NO production [63].

DU145 androgen-independent prostate cancer cells were sensitive to apoptotic cell death on transfection with an iNOS-encoding plasmid [64]. ZR-75-1 breast cancer cells were exquisitely sensitive to iNOS gene therapy delivered using a bioinspired nanovector that contained discreet motifs to facilitate DNA condensation, ZR-75-1-specific targeting, endosomal disruption and nuclear localization. Transfection of ZR-75-1 with the nanoparticulate therapy at the optimal vector:DNA ratio (termed N:P ratio) obliterated clonogenic survival of the cells [65].

Cationic liposome-mediated delivery of pVAX-iNOS gene therapy was cytotoxic in A549 lung carcinoma cells, cell death occurring apoptotically. Combination

iNOS gene therapy with low dose cisplatin produced additive benefit, and inhibited the invasion/migration ability of the cells. *In vivo*, the combination therapy was an effective tumor growth inhibitor in an intradermal A549 xenograft model, and also in an experimental A549 metastasis model, where cells were delivered via the tail vein. Amelioration of MMP-2 signalling and enhancement of p53 expression are at least partly responsible for the success of the combination gene-/chemotherapy approach [66].

Recombinant adenoviral technology was used to introduce iNOS expression into bladder carcinoma T24 cells. Infection of T24 cells with the adenoviral product increased iNOS expression, with consequent boosting of p53 at the message and protein levels. NO production was massively boosted by infection, and numbers of apoptotic cells was almost trebled by infection [67].

Refer to Table 13.1 for a synopsis of some of the strategies that have been used to deliver iNOS to transformed cells for therapeutic applications, and a summary of findings.

NO Interference

Due to the dichotomy of the NO relationship with cancer, an appetite exists for abrogating NO's activities in cancer. Barakat et al. recently reported a suite of 22 zwitterionic adduct compounds for the scavenging of NO, two of which were considerably more potent scavengers than their positive control, ascorbic acid [82] The authors concluded that the antioxidant products could have therapeutic uses in conditions such as diabetes, strokes, cardiovascular complications associated with chronic inflammatory disease and heart failure.

Ma et al. synthesized 52 compounds analogous to 5-Nitropyrimidine-2,4-dione and analyzed them for interference with NO signaling. A single compound was identified that potently inhibited LPS-induced release of NO from RAW264.7 and iNOS activity, while lacking cytotoxicity [83]. The analogue effectively abrogated carrageenan-induced paw edema formation *in vivo*; it is unclear whether the anti-cancer potential of the agent will be assessed.

An array of pyrazolopyrimidine derivatives was synthesized and assayed for their inhibition of iNOS and COX-1 and −2. Despite lacking direct cytotoxicity against SK-MEL malignant melanoma, KB epidermal carcinoma, BT-549 breast ductal carcinoma and SK-OV-3 ovarian cancer cells *in vitro*, the compounds could prove beneficial in oncological applications [84].

siRNA targeting of ribosomal protein L39 (RPL39) and myeloid leukemia factor 2 (MLF2) (two proteins that promote breast cancer stem cell self-renewal) significantly abrogated MDA-MB-231 xenograft development. Proteomic analysis revealed that iNOS expression and signaling was inhibited by knockdown of RPL39 and MLF2. Migratory ability of SUM149 and BT549 breast cancer cells was boosted by overexpression of both RPL39 and MLF2, and this was reversed by NOS inhibition using L-NAME, strengthening the proposal that interference with the two targets has anti-cancer potential, and that this potential is dependent on iNOS [85].

Table 13.1 Summary of iNOS gene therapy strategies for cancer targeting

Delivery method	Targeting strategy	Disease model	Major outcomes
pAd adenovial vector	NA	T24 (human bladder carcinoma)	Increased apoptosis of T24 cells infected with pAD-iNOS construct [67]
Cationic liposomes	NA	A549 (NSCLC)	Increased cell sensitivity to cisplatin *in vitro*, and produced additive anti-tumor effects *in vivo* in a subcutaneous xenograft model and a model of experimental metastasis [66]
Bio-inspired recombinant vector	ZR-75-1 targeting motif	ZR-75-1 (human breast cancer)	Abolition of clonogenic survival of ZR-75-1 cells. Specificity of vector for ZR-75-1 over MDA-MB-231 and MCF-10 A breast cells [65]
Cationic liposomes	Human osteocalcin (hOC)/prostate specific membrane antigen (PSMA) promoter	PC-3, DU145 and LNCaP prostate cancer; HT29 colon cancer; MCF-7 breast cancer	PSMA-driven iNOS expression reduced clonogenicity of prostate cancer cells only; hOC-driven iNOS expression reduced clonogenicity in PC-3, DU145, HT29 and MCF-7, but not in LNCaP. PSMA- and hOC-driven iNOS expression delayed PC-3 xenograft growth *in vivo* [68]
Rat mesenchymal stem cell (MSC)-based delivery	NA, although MSCs migrated to the tumor site on intravenous administration	RIF-1 nurine fibrosarcoma	MSCs delivering iNOS reduced RIF-1 proliferative capacity by 40% (MSCs lacking iNOS were without effect) and inhibited clonogenicity; systemic delivery of MSC-iNOS significantly slowed xenograft growth [69]
Cationic liposome	NA	RIF-1, PC-3, DU145, HT29 and HCT116 (colon carcinoma)	RIF-1 were sensitized to cisplatin *in vitro* and *in vivo* in intradermal xenografts. Cisplatin sensitization varied with cell type. Unlike all other cells tested, HCT116 did not release NO following transfection [70]
Cationic liposome	Radiation-inducible pE9 (CArG) promoter	RIF-1	pE9/iNOS sensitized normoxic and hypoxic (0.1% O_2) RIF-1 to ionizing radiation, reducing clonogenicity *in vitro* and delaying tumor growth *in vivo*. Targeted therapy (pE9) was more potent than non-targeted (CMV-driven iNOS) [71, 72]
Cationic liposome	Hypoxia-inducible WAF-1promoter	RIF-1 and HT29	Intratumoral WAF-1 iNOS delivery delayed tumor growth in RIF-1 and HT-29 xenografts—this was potentiated by combination with fractionated radiotherapy [73]
Adenoviral delivery	NA	MCF-7	Transduction with iNOS increased sensitivity to doxorubicin *in vitro* [74]

Table 13.1 (continued)

Delivery method	Targeting strategy	Disease model	Major outcomes
Adenoviral delivery	NA	HCT-116, SNU-1040 human colon adenocarcinoma	Radiosensitization *in vitro* and improved tumor growth delay when combined with single dose of IR *in vivo*. Tumors from AdiNOS-receiving mice have 2.9-fold more vascularity than control [75, 76]
Retroviral delivery	anti-CEA scFv-envelope chimeric protein to target carcinoembryonic antigen	CEA non-expressing MKN-74 cells and CEA-expressing cells MKN-45, KATO-III (all human gastric carcinoma) and CHO-CEA (CHO variant)	Cytotoxicity that was reliant on target cell expression of CEA. 70 % inhibition of tumor growth *in vivo* in MKN-45 xenografts [77, 78]
Recombinant cells in a semipermeable alginate-poly-L-lysine membrane	Tetracycline-dependent iNOS expression	SK-OV-3 and DLD-1 ovarian cancer	Almost complete retardation of *in vivo* growth when oral doxycycline was delivered concomitantly with intratumoral gene therapy delivery [79]
Retrovirus	NA	SN12PM6 renal carcinoma K-1735 melanoma cells	Cells iNOS-infected prior to orthotopic injection failed to develop tumors and metastasize, unlike control [80] Subcutaneous xenografts of infected cells were slow growing and failed to metastasize, unlike control which grew quickly and produced lung metastases [81]

Two analogues of the teratogenic drug thalidomide inhibited the angiogenic phenotype of HUVEC cells, potentially through inhibition of NO synthesis [86] MHY-794 (4-[thiazolidin-2-yl]benzene-1,2-diol), a tyrosinase inhibitor is also capable of NO scavenging. MHY-794 was as effective a tyrosinase inhibitor as kojic acid, and was as equipotent a scavenger of NO released from sodium nitroprusside as hemoglobin. MHY-794 prevented the development of UVB-induced skin pigmentation in hairless melainin-expressing mice, without directly impacting the viability of B16-F10 melanoma cells, indicating potential of NHY-794 as an inhibitor of melanogenesis [87].

Amino acid structures incorporating furoxan and sydnonimine ring system NO donor motifs were synthesized and evoked NO release in glutathione buffer, and could be incorporated into bioactive peptides for cellular targeting of NO release [88]. An encouraging observation was that the amino acids retained peptide bond-forming ability, so the heterocycles appended to the amino acid side chains shouldn't impact on peptide structure. The authors did not speculate on possible bioactive peptides whose sequences could be supplemented with the novel amino acids, although peptide inhibitors of HIF-1 [89], MMP-2 and −9 [90] and the angiogenic process [91] (among others) are all well known, and could be candidates.

Conclusions

There is a multitude of evidence to suggest that elevated iNOS levels are indicative of poor clinical prognosis. Notwithstanding the paradox that high iNOS expression should result in high NO levels, which are generally associated with favorable outcomes, comparably little research is being made into interference with NO, and far more effort is going into development of new NO generators.

Much of the effort that has gone into iNOS gene therapy has involved delivery of an iNOS-encoding plasmid under the control of the CMV promoter from cytomegalovirus. The CMV promoter ensures exceptional transgene expression, and in *in vitro* settings, evokes impressive results. There are concerns, however, regarding the indiscriminate delivery of a transgene under the control of a promoter that ensures constitutive expression of iNOS; toxicities of such a therapy are likely, the most obvious possibility being hypotension. Limiting transgene expression to the disease is paramount. Strategies to ensure appropriate gene expression include the use of promoters whose activation is dependent on tumor-specific regulators, or targeting delivery of the therapy to the tumor itself. The latter was alluded to above, when iNOS gene therapy was directed at ZR-75-1 breast cancer cells by a targeting motif contained within a bio-inspired recombinant nanovector; the same vector was unable to transfect MDA-MB-231 and MCF-7 breast cancer cells [65].

The former strategy mentioned, use of inducible or tumor-specific activators, has also been undertaken by us for iNOS gene therapy. Using promoters such as human osteocalcin (hOC—activated by regulators such as RUNX2) [92], prostate specific membrane antigen (PSMA) [93] and pE9 (radiation-inducible) [94] we

have delivered iNOS gene therapy, and observed tumor growth delay as impressive as that evoked by CMV-driven gene therapy in a range of prostate cancer models.

One, when considering NO in oncology, must always bear in mind the dichotomy of response of tumors to NO. While the general rule is that increasing the concentration of NO evokes a response that is generally attractive, it is not always the case. Inhibition of iNOS signaling using amminoguanidine slowed xenograft growth and abrogated the metastatic spread of orthotopically-implanted MDA-MB-231 in mice [95]. The role of iNOS in development of drug resistence is emerging, with a recent report implicating an iNOS/GST-/topoisomerase II (TOPO II) pathway as being responsible for development of resistance in NCSLC [96].

The point should be labored, that high concentrations of NO are not universally beneficial in cancer. NO donors spermine/NO and DETA/NO were cytotoxic against OVCAR3 and SK-OV-3 ovarian cancer cells [97]; puzzlingly, both donors inhibited the release of the tumor angiogenesis activator VEGF in OVCAR3, but failed to do so in SK-OV-3 [98]. The same inhibitors abrogated MMP-2 release from both cell lines, although MMP-2 activity was inhibited only in the SK-OV-3 cells [98]. These studies above were performed only *in vitro*; despite the encouraging results from the first study, it is likely that *in vivo* investigations might reveal limitations of the strategy in these cell types. Thorough and systematic proteomic comparison of the cell types may reveal an explanation for the disparity in tumor aggression markers' response to NO donors, and could ultimately provide rationale for personalized medication strategies.

NO was found to be essential for the autophagic survival of MCF-7 breast cancer cells following treatment with 4-hydroxytamoxifen (4OHTAM—the active metabolite of tamoxifen) [99]. Long-term exposure of lung cancer cells to NO increased the resistance of the cells to cisplatin, doxorubicin, and etoposide [100]; similar 7–14 day NO exposure (5 and 10 μM—marginally below the concentration at which cytotoxicity was observed) increased the motility (migration and invasion) of NCI-H460 NSCLC cells, resulting in a more aggressive phenotype [101]. It could be argued that levels of NO marginally below the cytotoxic concentration are sufficient to manifest a disease-progressing phenotype, as opposed to a disease-limiting one.

This chapter, dealing with the use of NO for therapeutic purposes, has alluded to chemo- and radio-sensitization afforded by NO therapy. Incongruously, rather than potentiating the chemotherapy, NO actually reacts with etoposide, forming several products, including etoposide-*o*-quinone; such reactivity abolished the cytotoxic effect of etoposide in HL-60 leukemia cells *in vitro*, and inhibited topo II-dependent DNA cleavage as induced by etoposide [102].

Therapy-limiting effects of aberrant iNOS expression are also known. Upregulation of iNOS by fractionated doses of ionizing radiation in U87 and U373 glioblastoma cells was responsible for cisplatin and taxol resistance and expansion of glioma stem-like cells *in vitro*. siRNA knockdown of iNOS attenuated the expression of radiation-induced stemness regulators Sox2, Notch-2 and β-catenin. In patient-derived glioma X01 cells, IR also evoked NO release, and expansion of a CD133+ (a putative glioma stem cell marker) population that was preventable by siRNA knockdown of iNOS. Stemness regulators were upregulated by IR in patient-derived

cells as they were in cell lines, and this was preventable by iNOS-targeting siRNA as before. Irradiated X01 cells developed resistance to cisplatin, Taxol and IR (10 Gy), and iNOS interference ameliorated this [103].

Apoptosis of breast cancer cells by oxidative stress evoked by photodynamic therapy with the pro-sensitizer 5-aminolevulinic acid was in turn abrogated by upregulation of iNOS with subsequent increase in NO concentration. iNOS upregulation was dependent on NF-κB activation, and reversible by blockade of the PI3K pathway with wortmannin [104]. Pharmacological inhibition of iNOS or scavenging of NO itself equally abrogated the protection from apoptosis afforded by NO [105]. The phenomenon was not limited to the breast model used; the PC-3 prostate cancer cell line responded similarly to photodynamic therapy, increasing iNOS expression and NO production, ultimately protecting the cell from apoptosis [106].

It is without question that NO has a place in the oncology clinic. Nevertheless, it is of paramount importance that the level of NO directed at the tumor is appropriate, as the dichotomous relationship between the gasotransmitter and the tumor makes using NO a high risk strategy. Nevertheless, a NO-delivering agent that is targeted and capable of the controlled release of appropriate levels of NO to evoke a desired response is likely to have applications beyond the oncology clinic [5].

No Conflict Statement No potential conflicts of interest were disclosed.

References

1. Huerta S, Chilka S, Bonavida B. Nitric oxide donors: novel cancer therapeutics (review). Int J Oncol. 2008;33:909–27.
2. Heinrich TA, da Silva RS, Miranda KM, Switzer CH, Wink DA, Fukuto JM. Biological nitric oxide signalling: chemistry and terminology. Br J Pharmacol. 2013;169:1417–29.
3. Singh S, Gupta AK. Nitric oxide: role in tumour biology and iNOS/NO-based anticancer therapies. Cancer Chemother Pharmacol. 2011;67:1211–24.
4. Caneba CA, Yang L, Baddour J, Curtis R, Win J, Hartig S, Marini J, Nagrath D. Nitric oxide is a positive regulator of the Warburg effect in ovarian cancer cells. Cell Death Dis. 2014;5:e1302.
5. Miller MR, Megson IL. Recent developments in nitric oxide donor drugs. Br J Pharmacol. 2007;151:305–21.
6. Yasuda H. Solid tumor physiology and hypoxia-induced chemo/radio-resistance: novel strategy for cancer therapy: nitric oxide donor as a therapeutic enhancer. Nitric Oxide. 2008;19:205–16.
7. Seki T, Fang J, Maeda H. Enhanced delivery of macromolecular antitumor drugs to tumors by nitroglycerin application. Cancer Sci. 2009;100:2426–30.
8. Yasuda H, Yamaya M, Nakayama K, Sasaki T, Ebihara S, Kanda A, Asada M, Inoue D, Suzuki T, Okazaki T, Takahashi H, Yoshida M, Kaneta T, Ishizawa K, Yamanda S, Tomita N, Yamasaki M, Kikuchi A, Kubo H, Sasaki H. Randomized phase II trial comparing nitroglycerin plus vinorelbine and cisplatin with vinorelbine and cisplatin alone in previously untreated stage IIIB/IV non-small-cell lung cancer. J Clin Oncol. 2006;24:688–94.
9. Reinmuth N, Meyer A, Hartwigsen D, Schaeper C, Huebner G, Skock-Lober R, Bier A, Gerecke U, Held CP, Reck M. Randomized, double-blind phase II study to compare nitroglycerin plus oral vinorelbine plus cisplatin with oral vinorelbine plus cisplatin alone in patients with stage IIIB/IV non-small cell lung cancer (NSCLC). Lung Cancer. 2014;83:363–8.

10. Arrieta O, Blake M, de la Mata-Moya MD, Corona F, Turcott J, Orta D, Alexander-Alatorre J, Gallardo-Rincón D. Phase II study. Concurrent chemotherapy and radiotherapy with nitroglycerin in locally advanced non-small cell lung cancer. Radiother Oncol. 2014;111:311–5.
11. Yasuda H, Nakayama K, Watanabe M, Suzuki S, Fuji H, Okinaga S, Kanda A, Zayasu K, Sasaki T, Asada M, Suzuki T, Yoshida M, Yamanda S, Inoue D, Kaneta T, Kondo T, Takai Y, Sasaki H, Yanagihara K, Yamaya M. Nitroglycerin treatment may enhance chemosensitivity to docetaxel and carboplatin in patients with lung adenocarcinoma. Clin Cancer Res. 2006;12:6748–57.
12. Siemens DR, Heaton JP, Adams MA, Kawakami J, Graham CH. Phase II study of nitric oxide donor for men with increasing prostate-specific antigen level after surgery or radiotherapy for prostate cancer. Urology. 2009;74:878–83.
13. Yang L, Lan C, Fang Y, Zhang Y, Wang J, Guo J, Wan S, Yang S, Wang R, Fang D. Sodium nitroprusside (SNP) sensitizes human gastric cancer cells to TRAIL-induced apoptosis. Int Immunopharmacol. 2013;17:383–9.
14. Bonavida B, Baritaki S. Dual role of NO donors in the reversal of tumor cell resistance and EMT: downregulation of the NF-kappaB/Snail/YY1/RKIP circuitry. Nitric Oxide. 2011;24:1–7.
15. Laschak M, Spindler KD, Schrader AJ, Hessenauer A, Streicher W, Schrader M, Cronauer MV. JS-K, a glutathione/glutathione S-transferase-activated nitric oxide releasing prodrug inhibits androgen receptor and WNT-signaling in prostate cancer cells. BMC Cancer. 2012;12:130.
16. Kaczmarek MZ, Holland RJ, Lavanier SA, Troxler JA, Fesenkova VI, Hanson CA, Cmarik JL, Saavra JE, Keefer LK, Ruscetti SK. Mechanism of action for the cytotoxic effects of the nitric oxide prodrug JS-K in murine erythroleukemia cells. Leuk Res. 2014;38:377–82.
17. Wink DA, Cook JA, Christodoulou D, Krishna MC, Pacelli R, Kim S, DeGraff W, Gamson J, Vodovotz Y, Russo A, Mitchell JB. Nitric oxide and some nitric oxide donor compounds enhance the cytotoxicity of cisplatin. Nitric Oxide. 1997;1:88–94.
18. Ning S, Bednarski M, Oronsky B, Scicinski J, Knox SJ. Novel nitric oxide generating compound glycidyl nitrate enhances the therapeutic efficacy of chemotherapy and radiotherapy. Biochem Biophys Res Commun. 2014;447:537–42.
19. Ning S, Bednarski M, Oronsky B, Scicinski J, Saul G, Knox SJ. Dinitroazetidines are a novel class of anticancer agents and hypoxia-activated radiation sensitizers developed from highly energetic materials. Cancer Res. 2012;72:2600–8.
20. Reid T, Dad S, Korn R, Oronsky B, Knox S, Scicinski J. Two case reports of resensitization to previous chemotherapy with the novel hypoxia-activated hypomethylating anticancer agent RRx-001 in metastatic colorectal cancer patients. Case Rep Oncol. 2014;7:79–85.
21. Chegaev K, Riganti C, Lazzarato L, Rolando B, Guglielmo S, Campia I, et al. Nitric oxide donor doxorubicins accumulate into Doxorubicin-resistant human colon cancer cells inducing cytotoxicity. ACS Med Chem Lett. 2011;2:494–7.
22. Pedrini I, Gazzano E, Chegaev K, Rolando B, Marengo A, Kopecka J, Fruttero R, Ghigo D, Arpicco S, Riganti C. Liposomal nitrooxy-doxorubicin: one step over caelyx in drug-resistant human cancer cells. Mol Pharm. 2014;11:3068–79.
23. Riganti C, Miraglia E, Viarisio D, Costamagna C, Pescarmona G, Ghigo D, Bosia A. Nitric oxide reverts the resistance to doxorubicin in human colon cancer cells by inhibiting the drug efflux. Cancer Res. 2005;65:516–25.
24. Katsumi H, Nishikawa M, Yamashita F, Hashida M. Development of polyethylene glycol-conjugated poly-S-nitrosated serum albumin, a novel S-Nitrosothiol for prolonged delivery of nitric oxide in the blood circulation in vivo. J Pharmacol Exp Ther. 2005;314:1117–24.
25. Ishima Y, Fang J, Kragh-Hansen U, Yin H, Liao L, Katayama N, Watanabe H, Kai T, Suenaga A, Maeda H, Otagiri M, Maruyama T. Tuning of poly-S-nitrosated human serum albumin as superior antitumor nanomedicine. J Pharm Sci. 2014;103:2184–8.
26. Maciag AE, Holland RJ, Kim Y, Kumari V, Luthers CE, Sehareen WS, Biswas D, Morris NL, Ji X, Anderson LM, Saavra JE, Keefer LK. Nitric oxide (NO) releasing poly ADP-ribose polymerase 1 (PARP-1) inhibitors targeted to glutathione S-transferase P1-overexpressing cancer cells. J Med Chem. 2014;57:2292–302.

27. Curtin NJ, Szabo C. Therapeutic applications of PARP inhibitors: anticancer therapy and beyond. Mol Aspects Med. 2013;34:1217–56.
28. Fu J, Liu L, Huang Z, Lai Y, Ji H, Peng S, Tian J, Zhang Y. Hybrid molecule from O2-(2,4-dinitrophenyl)diazeniumdiolate and oleanolic acid: a glutathione S-transferase pi-activated nitric oxide prodrug with selective anti-human hepatocellular carcinoma activity and improved stability. J Med Chem. 2013;56:4641–55.
29. Nortcliffe A, Ekstrom AG, Black JR, Ross JA, Habib FK, Botting NP, O'Hagan D. Synthesis and biological evaluation of nitric oxide-donating analogues of sulindac for prostate cancer treatment. Bioorg Med Chem. 2014;22:756–761.
30. Chaudhary SC, Singh T, Kapur P, Weng Z, Arumugam A, Elmets CA, Kopelovich L, Athar M. Nitric oxide-releasing sulindac is a novel skin cancer chemopreventive agent for UVB-induced photocarcinogenesis. Toxicol Appl Pharmacol. 2013;268:249–55.
31. Abdel-Aziz M, Abuo-Rahma G, Beshr EA, Ali TF. New nitric oxide donating 1,2,4-triazole/oxime hybrids: synthesis, investigation of anti-inflammatory, ulceroginic liability and antiproliferative activities. Bioorg Med Chem. 2013;21:3839–49.
32. Nath N, Liu X, Jacobs L, Kashfi K. Flurbiprofen benzyl nitrate (NBS-242) inhibits the growth of A-431 human epidermoid carcinoma cells and targets beta-catenin. Drug Des Devel Ther. 2013;7:389–96.
33. Basudhar D, Bharadwaj G, Cheng RY, Jain S, Shi S, Heinecke JL, Holland RJ, Ridnour LA, Caceres VM, Spadari-Bratfisch RC, Paolocci N, Velázquez-Martínez CA, Wink DA, Miranda KM. Synthesis and chemical and biological comparison of nitroxyl- and nitric oxide-releasing diazeniumdiolate-bas aspirin derivatives. J Med Chem. 2013;56:7804–20.
34. Kashfi K, Olson KR. Biology and therapeutic potential of hydrogen sulfide and hydrogen sulfide-releasing chimeras. Biochem Pharmacol. 2013;85:689–703.
35. Kodela R, Chattopadhyay M, Kashfi K. Synthesis and biological activity of NOSH-naproxen (AVT-219) and NOSH-sulindac (AVT-18 A) as potent anti-inflammatory agents with chemotherapeutic potential. Medchemcomm. 2013;4:10.1039/C3MD00185G.
36. Coulter JA, Jain S, Butterworth KT, Taggart LE, Dickson GR, McMahon SJ, et al. Cell type-dependent uptake, localization, and cytotoxicity of 1.9 nm gold nanoparticles. Int J Nanomedicine. 2012;7:2673–85.
37. Sudhesh P, Tamilarasan K, Arumugam P, Berchmans S. Nitric oxide releasing photoresponsive nanohybrids as excellent therapeutic agent for cervical cancer cell lines. ACS Appl Mater Interfaces. 2013;5:8263–6.
38. Xu J, Zeng F, Wu H, Hu C, Yu C, Wu S. Preparation of a mitochondria-targeted and no-releasing nanoplatform and its enhanced pro-apoptotic effect on cancer cells. Small. 2014;10:3750–60.
39. Lee SY, Rim Y, McPherson DD, Huang SL, Kim H. A novel liposomal nanomedicine for nitric oxide delivery and breast cancer treatment. Biomed Mater Eng. 2014;24:61–7.
40. Kaur I, Terrazas M, Kosak KM, Kern SE, Boucher KM, Shami PJ. Cellular distribution studies of the nitric oxide-generating antineoplastic prodrug O(2) -(2,4-dinitrophenyl)1-((4-ethoxycarbonyl)piperazin-1-yl)diazen-1-ium-1,2-diolate formulated in Pluronic P123 micelles. J Pharm Pharmacol. 2013;65:1329–36.
41. Gomes AJ, Espreafico EM, Tfouni E. trans-[Ru(NO)Cl(cyclam)](PF6)2 and [Ru(NO)(Hedta)] incorporated in PLGA nanoparticles for the delivery of nitric oxide to B16-F10 cells: cytotoxicity and phototoxicity. Mol Pharm. 2013;10:3544–54.
42. Duong HT, Kamarudin ZM, Erlich RB, Li Y, Jones MW, Kavallaris M, Boyer C, Davis TP. Intracellular nitric oxide delivery from stable NO-polymeric nanoparticle carriers. Chem Commun (Camb). 2013;49:4190–2.
43. Fraix A, Kandoth N, Manet I, Cardile V, Graziano AC, Gref R, Sortino S. An engineered nanoplatform for bimodal anticancer phototherapy with dual-color fluorescence detection of sensitizers. Chem Commun (Camb). 2013;49:4459–61.
44. Heinrich TA, Tedesco AC, Fukuto JM, da Silva RS. Production of reactive oxygen and nitrogen species by light irradiation of a nitrosyl phthalocyanine ruthenium complex as a strategy for cancer treatment. Dalton Trans. 2014;43:4021–5.

45. Holland RJ, Paulisch R, Cao Z, Keefer LK, Saavedra JE, Donzelli S. Enzymatic generation of the NO/HNO-releasing IPA/NO anion at controlled rates in physiological media using beta-galactosidase. Nitric Oxide. 2013;35:131–6.
46. Sharma K, Sengupta K, Chakrapani H. Nitroreductase-activated nitric oxide (NO) prodrugs. Bioorg Med Chem Lett. 2013;23:5964–7.
47. Han C, Huang Z, Zheng C, Wan L, Lai Y, Peng S, Ding K, Ji H, Zhang Y. Nitric oxide donating anilinopyrimidines: synthesis and biological evaluation as EGFR inhibitors. Eur J Med Chem. 2013;66:82–90.
48. Danson S, Ward TH, Butler J, Ranson M. DT-diaphorase: a target for new anticancer drugs. Cancer Treat Rev. 2004;30:437–49.
49. Sharma K, Iyer A, Sengupta K, Chakrapani H. INDQ/NO, a bioreductively activated nitric oxide prodrug. Org Lett. 2013;15:2636–9.
50. Esmaeili MA, Farimani MM, Kiaei M. Anticancer effect of calycopterin via PI3K/Akt and MAPK signaling pathways, ROS-mediated pathway and mitochondrial dysfunction in hepatoblastoma cancer (HepG2) cells. Mol Cell Biochem. 2014;397(1-2):17-31.
51. Pandurangan AK, Kumar SA, Dharmalingam P, Ganapasam S. Luteolin, a bioflavonoid inhibits azoxymethane-induced colon carcinogenesis: Involvement of iNOS and COX-2. Pharmacogn Mag. 2014;10:S306–10.
52. Tsai ML, Chiou YS, Chiou LY, Ho CT, Pan MH. Garcinol suppresses inflammation-associated colon carcinogenesis in mice. Mol Nutr Food Res. 2014;58:1820–9.
53. Kotamraju S, Williams CL, Kalyanaraman B. Statin-induced breast cancer cell death: role of inducible nitric oxide and arginase-dependent pathways. Cancer Res. 2007;67:7386–94.
54. Kanugula AK, Gollavilli PN, Vasamsetti SB, Karnewar S, Gopoju R, Ummanni R, Kotamraju S. Statin-induced inhibition of breast cancer proliferation and invasion involves attenuation of iron transport: intermediacy of nitric oxide and antioxidant defence mechanisms. FEBS J. 2014;281:3719–38.
55. Sattari M, Pazhang Y, Imani M. Calprotectin induces cell death in human prostate cancer cell (LNCaP) through survivin protein alteration. Cell Biol Int. 2014. doi: 10.1002/cbin.10328. [Epub ahead of print].
56. Nenu I, Popescu T, Aldea MD, Craciun L, Olteanu D, Tatomir C, Bolfa P, Ion RM, Muresan A, Filip AG. Metformin associated with photodynamic therapy—a novel oncological direction. J Photochem Photobiol B. 2014;138:80–91.
57. Rapozzi V, Della Pietra E, Zorzet S, Zacchigna M, Bonavida B, Xodo LE. Nitric oxide-mediated activity in anti-cancer photodynamic therapy. Nitric Oxide. 2013;30:26–35.
58. Klug F, Prakash H, Huber PE, Seibel T, Bender N, Halama N, Pfirschke C, Voss RH, Timke C, Umansky L, Klapproth K, Schäkel K, Garbi N, Jäger D, Weitz J, Schmitz-Winnenthal H, Hämmerling GJ, Beckhove P. Low-dose irradiation programs macrophage differentiation to an iNOS(+)/M1 phenotype that orchestrates effective T cell immunotherapy. Cancer Cell. 2013;24:589–602.
59. Bulut AS, Erden E, Sak SD, Doruk H, Kursun N, Dincol D. Significance of inducible nitric oxide synthase expression in benign and malignant breast epithelium: an immunohistochemical study of 151 cases. Virchows Arch. 2005;447:24–30.
60. Vakkala M, Kahlos K, Lakari E, Paakko P, Kinnula V, Soini Y. Inducible nitric oxide synthase expression, apoptosis, and angiogenesis in in situ and invasive breast carcinomas. Clin Cancer Res. 2000;6:2408–16.
61. Tschugguel W, Schneeberger C, Unfried G, Czerwenka K, Weninger W, Mildner M, Gruber DM, Sator MO, Waldhör T, Huber JC. Expression of inducible nitric oxide synthase in human breast cancer depends on tumor grade. Breast Cancer Res Treat. 1999;56:145–51.
62. Glynn SA, Boersma BJ, Dorsey TH, Yi M, Yfantis HG, Ridnour LA, Martin DN, Switzer CH, Hudson RS, Wink DA, Lee DH, Stephens RM, Ambs S. Increased NOS2 predicts poor survival in estrogen receptor-negative breast cancer patients. J Clin Invest. 2010;120:3843–54.
63. Bian K, Ghassemi F, Sotolongo A, Siu A, Shauger L, Kots A, Murad F. NOS-2 signaling and cancer therapy. IUBMB Life. 2012;64:676–83.

64. Chen YF, Jiang XZ, He LY, Tang YX, Long Z. Transfection of iNOS suppresses the growth of androgen-independent prostate cancer DU145 cells. Zhonghua Nan Ke Xue. 2012;18: 697–702.
65. McCarthy HO, Zholobenko AV, Wang Y, Canine B, Robson T, Hirst DG, Hatefi A. Evaluation of a multi-functional nanocarrier for targeted breast cancer iNOS gene therapy. Int J Pharm. 2011;405:196–202.
66. Ye S, Yang W, Wang Y, Ou W, Ma Q, Yu C, Ren J, Zhong G, Shi H, Yuan Z, Su X, Zhu W. Cationic liposome-mediated nitric oxide synthase gene therapy enhances the antitumor effects of cisplatin in lung cancer. Int J Mol Med. 2013;31:33–42.
67. Tan J, Zeng Q, Jiang XZ, He LY, Wang JR, Yao K, Wang CH. Apoptosis of bladder transitional cell carcinoma T24 cells induced by adenovirus-mediated inducible nitric oxide synthase gene transfection. Chin J Cancer Res. 2013;25:593–9.
68. Coulter JA, Page NL, Worthington J, Robson T, Hirst DG, McCarthy HO. Transcriptional regulation of inducible nitric oxide synthase gene therapy: targeting early stage and advanced prostate cancer. J Gene Med. 2010;12:755–65.
69. Xiang J, Tang J, Song C, Yang Z, Hirst DG, Zheng QJ, Li G. Mesenchymal stem cells as a gene therapy carrier for treatment of fibrosarcoma. Cytotherapy. 2009;11:516–26.
70. Adams C, McCarthy HO, Coulter JA, Worthington J, Murphy C, Robson T, Hirst DG. Nitric oxide synthase gene therapy enhances the toxicity of cisplatin in cancer cells. J Gene Med. 2009;11:160–68.
71. Coulter JA, McCarthy HO, Worthington J, Robson T, Scott S, Hirst DG. The radiation-inducible pE9 promoter driving inducible nitric oxide synthase radiosensitizes hypoxic tumour cells to radiation. Gene Ther. 2008;15:495–503.
72. Worthington J, Robson T, Scott S, Hirst D. Evaluation of a synthetic CArG promoter for nitric oxide synthase gene therapy of cancer. Gene Ther. 2005;12:1417–23.
73. Worthington J, McCarthy HO, Barrett E, Adams C, Robson T, Hirst DG. Use of the radiation-inducible WAF1 promoter to drive iNOS gene therapy as a novel anti-cancer treatment. J Gene Med. 2004;6:673–80.
74. Evig CB, Kelley EE, Weydert CJ, Chu Y, Buettner GR, Burns CP. Endogenous production and exogenous exposure to nitric oxide augment doxorubicin cytotoxicity for breast cancer cells but not cardiac myoblasts. Nitric Oxide. 2004;10:119–29.
75. Wang Z, Cook T, Alber S, Liu K, Kovesdi I, Watkins SK, Vodovotz Y, Billiar TR, Blumberg D. Adenoviral gene transfer of the human inducible nitric oxide synthase gene enhances the radiation response of human colorectal cancer associated with alterations in tumor vascularity. Cancer Res. 2004;64:1386–95.
76. Chung P, Cook T, Liu K, Vodovotz Y, Zamora R, Finkelstein S, Billiar T, Blumberg D. Overexpression of the human inducible nitric oxide synthase gene enhances radiation-induced apoptosis in colorectal cancer cells via a caspase-dependent mechanism. Nitric Oxide. 2003;8:119–26.
77. Khare PD, Liao S, Hirose Y, Kuroki M, Fujimura S, Yamauchi Y, Miyajima-Uchida H, Kuroki M. Tumor growth suppression by a retroviral vector displaying scFv antibody to CEA and carrying the iNOS gene. Anticancer Res. 2002;22:2443–6.
78. Khare PD, Shao-Xi L, Kuroki M, Hirose Y, Arakawa F, Nakamura K, Tomita Y, Kuroki M. Specifically targeted killing of carcinoembryonic antigen (CEA)-expressing cells by a retroviral vector displaying single-chain variable fragmented antibody to CEA and carrying the gene for inducible nitric oxide synthase. Cancer Res. 2001;61:370–5.
79. Xu W, Liu L, Charles IG. Microencapsulated iNOS-expressing cells cause tumor suppression in mice. FASEB J. 2002;16:213–5.
80. Juang SH, Xie K, Xu L, Shi Q, Wang Y, Yoneda J, Fidler IJ. Suppression of tumorigenicity and metastasis of human renal carcinoma cells by infection with retroviral vectors harboring the murine inducible nitric oxide synthase gene. Hum Gene Ther. 1998;9:845–54.
81. Juang SH, Xie K, Xu L, Wang Y, Yoneda J, Fidler IJ. Use of retroviral vectors encoding murine inducible nitric oxide synthase gene to suppress tumorigenicity and cancer metastasis of murine melanoma. Cancer Biother Radiopharm. 1997;12:167–75.

82. Barakat A, Al-Majid AM, Al-Najjar HJ, Mabkhot YN, Javaid S, Yousuf S, Choudhary MI. Zwitterionic pyrimidinium adducts as antioxidants with therapeutic potential as nitric oxide scavenger. Eur J Med Chem. 2014;84:146–54.
83. Ma L, He L, Lei L, Liang X, Lei K, Zhang R, Yang Z, Chen L. Synthesis and biological evaluation of 5-Nitropyrimidine-2,4-dione analogues as inhibitors of nitric oxide and iNOS activity. Chem Biol Drug Des. 2014. doi:10.1111/cbdd.12386. [Epub ahead of print].
84. Abdelazeem AH, Abdelatef SA, El-Saadi MT, Omar HA, Khan SI, McCurdy CR, El-Moghazy SM. Novel pyrazolopyrimidine derivatives targeting COXs and iNOS enzymes; design, synthesis and biological evaluation as potential anti-inflammatory agents. Eur J Pharm Sci. 2014;62:197–211.
85. Dave B, Granados-Principal S, Zhu R, Benz S, Rabizadeh S, Soon-Shiong P, Yu KD, Shao Z, Li X, Gilcrease M, Lai Z, Chen Y, Huang TH, Shen H, Liu X, Ferrari M, Zhan M, Wong ST, Kumaraswami M, Mittal V, Chen X, Gross SS, Chang JC. Targeting RPL39 and MLF2 reduces tumor initiation and metastasis in breast cancer by inhibiting nitric oxide synthase signaling. Proc Natl Acad Sci USA. 2014;111:8838–43.
86. El-Aarag BY, Kasai T, Zahran MA, Zakhary NI, Shigehiro T, Sekhar SC, Agwa HS, Mizutani A, Murakami H, Kakuta H, Seno M. In vitro anti-proliferative and anti-angiogenic activities of thalidomide dithiocarbamate analogs. Int Immunopharmacol. 2014;21:283–92.
87. Chung KW, Jeong HO, Jang EJ, Choi YJ, Kim DH, Kim SR, Lee KJ, Lee HJ, Chun P, Byun Y, Moon HR, Chung HY. Characterization of a small molecule inhibitor of melanogenesis that inhibits tyrosinase activity and scavenges nitric oxide (NO). Biochim Biophys Acta. 2013;1830:4752–61.
88. Nortcliffe A, Botting NP, O'Hagan D. Novel amino acids: synthesis of furoxan and sydnonimine containing amino acids and peptides as potential nitric oxide releasing motifs. Org Biomol Chem. 2013;11:4657–71.
89. Miranda E, Nordgren IK, Male AL, Lawrence CE, Hoakwie F, Cuda F, Court W, Fox KR, Townsend PA, Packham GK, Eccles SA, Tavassoli A. A cyclic peptide inhibitor of HIF-1 heterodimerization that inhibits hypoxia signaling in cancer cells. J Am Chem Soc. 2013;135:10418–25.
90. Kim SR, Eom TK, Byun HG. Inhibitory effect of the carnosine-gallic acid synthetic peptide on MMP-2 and MMP-9 in human fibrosarcoma HT1080 cells. J Pept Sci. 2014;20:716–24.
91. Valentine A, O'Rourke M, Yakkundi A, Worthington J, Hookham M, Bicknell R, McCarthy HO, McClelland K, McCallum L, Dyer H, McKeen H, Waugh DJ, Roberts J, McGregor J, Cotton G, James I, Harrison T, Hirst DG, Robson T. FKBPL and peptide derivatives: novel biological agents that inhibit angiogenesis by a CD44-dependent mechanism. Clin Cancer Res. 2011;17:1044–56.
92. McCarthy HO, Coulter JA, Worthington J, Robson T, Hirst DG. Human osteocalcin: a strong promoter for nitric oxide synthase gene therapy, with specificity for hormone refractory prostate cancer. J Gene Med. 2007;9:511–20.
93. Coulter JA, Page NL, Worthington J, Robson T, Hirst DG, McCarthy HO. Transcriptional regulation of inducible nitric oxide synthase gene therapy: targeting early stage and advanced prostate cancer. J Gene Med. 2010;12:755–65.
94. Coulter JA, McCarthy HO, Worthington J, Robson T, Scott S, Hirst DG. The radiation-inducible pE9 promoter driving inducible nitric oxide synthase radiosensitizes hypoxic tumour cells to radiation. Gene Ther. 2008;15:495–503.
95. Heinecke JL, Ridnour LA, Cheng RY, Switzer CH, Lizardo MM, Khanna C, Glynn SA, Hussain SP, Young HA, Ambs S, Wink DA. Tumor microenvironment-based feed-forward regulation of NOS2 in breast cancer progression. Proc Natl Acad Sci USA. 2014;111:6323–8.
96. Li Y, Ma C, Shi X, Wen Z, Li D, Sun M, Ding H. Effect of nitric oxide synthase on multiple drug resistance is related to Wnt signaling in non-small cell lung cancer. Oncol Rep. 2014;32:1703–8.
97. Kielbik M, Klink M, Brzezinska M, Szulc I, Sulowska Z. Nitric oxide donors: spermine/NO and diethylenetriamine/NO induce ovarian cancer cell death and affect STAT3 and AKT signaling proteins. Nitric Oxide. 2013;35:93–109.

98. Kielbik M, Szulc I, Brzezinska M, Bednarska K, Przygodzka P, Sulowska Z, Nowak M, Klink M. Nitric oxide donors reduce the invasion ability of ovarian cancer cells in vitro. Anticancer Drugs. 2014;25(10):1141-51.
99. Duan L, Danzer B, Levenson VV, Maki C. Critical roles for Nitric oxide and ERK in the completion of prosurvival autophagy in 4OHTAM-treated estrogen receptor-positive breast cancer cells. Cancer Lett. 2014;353:290–300.
100. Wongvaranon P, Pongrakhananon V, Chunhacha P, Chanvorachote P. Acquired resistance to chemotherapy in lung cancer cells mediated by prolonged nitric oxide exposure. Anticancer Res. 2013;33:5433–44.
101. Sanuphan A, Chunhacha P, Pongrakhananon V, Chanvorachote P. Long-term nitric oxide exposure enhances lung cancer cell migration. Biomed Res Int. 2013;2013:186972.
102. Sinha BK, Bhattacharjee S, Chatterjee S, Jiang J, Motten AG, Kumar A, Espey MG, Mason RP. Role of nitric oxide in the chemistry and anticancer activity of etoposide (VP-16,213). Chem Res Toxicol. 2013;26:379–87.
103. Kim RK, Suh Y, Cui YH, Hwang E, Lim EJ, Yoo KC, Lee GH, Yi JM, Kang SG, Lee SJ. Fractionated radiation-induced nitric oxide promotes expansion of glioma stem-like cells. Cancer Sci. 2013;104:1172–7.
104. Bhowmick R, Girotti AW. Cytoprotective signaling associated with nitric oxide upregulation in tumor cells subjected to photodynamic therapy-like oxidative stress. Free Radic Biol Med. 2013;57:39–48.
105. Bhowmick R, Girotti AW. Cytoprotective induction of nitric oxide synthase in a cellular model of 5-aminolevulinic acid-based photodynamic therapy. Free Radic Biol Med. 2010;48:1296–301.
106. Bhowmick R, Girotti AW. Pro-survival and pro-growth effects of stress-induced nitric oxide in a prostate cancer photodynamic therapy model. Cancer Lett. 2014;343:115–22.

Chapter 14
Photodynamic Therapy and Nitric Oxide

Emilia Della Pietra and Valentina Rapozzi

Abstract Photodynamic therapy (PDT) is a clinically approved, minimally invasive therapeutic treatment that exerts a selectively cytotoxic activity towards cancer cells. This technique involves administration of a photosensitizer followed by irradiation at a wavelength corresponding to its absorbance band. In the presence of oxygen, a cascade of stress oxidative reactions leads to direct tumor cell death, damage to the microvasculature and induction of a local inflammatory reaction. Clinical studies showed that PDT can be curative particularly in early-stage tumors. Moreover, with many cancers becoming resistant to treatment, PDT offers a mechanistically distinct alternative, mitigating chemoresistance but also synergizing with chemotherapy and molecularly targeted therapies. A well-known phrase of Tayyaba Hasan, one of the experts in PDT, stated "with PDT no matter what you do, if you are lucky, there is a prodeath response, simultaneously, there is a prosurvival molecular response, which mitigates the desidered outcome with PDT". These opposing molecular responses represent the challenge for basic science researchers and clinicians to enhance the photodynamic-mediated chemicals. Noteworthy, one of the major effectors that modulate the efficacy of PDT is nitric oxide, whose role will be discussed in this chapter.

Keywords Cancer · Molecular mechanisms · Nitric oxide · NO-PDT Conjugates · Photodynamic therapy

V. Rapozzi (✉) · E. Della Pietra
Department of Medical and Biological Sciences,
University of Udine, P.le Kolbe 4, 33100 Udine, Italy
e-mail: valentina.rapozzi@uniud.it

E. Della Pietra
e-mail: emydellapietra@yahoo.it

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_14

Abbreviations

AIF	Apoptosis-inducing factor
ALA	5-aminolevulinic acid
cGMP	Cyclic guanosine monophosphate
COX-2	Cyclooxygenase:2
CPTIO	2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DETA/NO	Diethylenetriamine NONOate
EMT	Epithelial mesenchymal transition
GSNO	S-nitrosoglutathione
HbO_2	Oxyhemoglobin
HIF-1α	Hypoxia:inducible factor 1α
$HO^{\bullet}$	Hydroxyl radical
H_2O_2	Hydrogen peroxide
HpD	Hematoporphyrin derivative
JNK	c-Jun NH2-terminal kinase
LDL	Low density protein
LED	Light emitting diode
L-NAME	L-NG-Nitroarginine Methyl Ester
MAPK	Mitogen-Activated Protein Kinase
MMP-9	Matrix metallopeptidase 9
MOMP	Mitochondria outer membrane permeabilization
NF-kB	Nuclear Factor-KappaB
NO	Nitric oxide
$^1O_2$	Singlet oxygen
$ONOO^-$	Peroxynitrite anion
Pb*a*	Pheophorbide *a*
PDT	Photodynamic therapy;
PS	Photosensitizer
RIP1	Receptor interacting protein 1
RKIP	Raf kinase inhibitor protein
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SNT	S-nitrosothiol
TNF-α	Tumor necrosis factor-alpha
YY1	Yin Yang 1
VEGF	Vascular endothelial growth factor.

Basic Components of Photodynamic Therapy

Photodynamic therapy (PDT) consists of three essential components—a photosensitizer (PS), light and oxygen [1, 2]. None of these is individually toxic, but altogether they initiate a photochemical reaction that culminates in the generation of a highly reactive product termed singlet oxygen ($^1O_2$) and/or reactive oxygen species

(ROS). Both oxidative products can rapidly cause significant toxicity leading to cell death. The anti-tumor effects of PDT can involve three inter-related mechanisms: direct cytotoxic effects on tumor cells, damage to the tumor vasculature, and induction of an inflammatory reaction that can induce the activation of systemic immunity. The "choice" among these mechanisms depends on the type and dose of the PS used, the dose-light interval, and the total light dose and its fluence rate (i.e. the number of particles that intersect a unit area in a given amount of time, typically measured in Watts per m^2).

The Photosensitizer

Most of the PSs used in cancer therapy possess a structure similar to a tetrapyrrole ring of the protoporphyrin contained in hemoglobin. The ideal photosensitizer would be a pure compound in order to permit quality control analysis with low manufacturing costs and good stability in storage. It should have a high absorption peak between 600 and 800 nm, because in this wavelength range the penetration of light into tissue is very high. In fact, PSs, such as chlorins, bacteriochlorins and phtalocyanines, that present a strong absorbance in the deep red, offer improvements in tumor control (Table 14.1). Moreover, good PSs should have relatively rapid clearance from normal tissues, thereby, minimizing phototoxic side-effects and no dark toxicity [3]. The optimal photosensitizer should not preclude the combined use of others types of treatment such as chemotherapy, surgery or radiation [4–6].

The first PS to be clinically employed for cancer therapy was a water-soluble mixture of phorphyrins called hematoporphyrin derivative (HpD), a purified form

Table 14.1 Properties of various photosensitizers and their applications in cancer

PS	Structure	Wavelenght (nm)	Cancer types
Photofrin	Porphyrin	630	Lung, esophagus, bile duct, bladder, brain, ovarian
ALA	Porphyrin	635	Skin, bladder, brain, esophagus
ALA esters	Porphyrin precursor	635	Skin, bladder
Foscan	Chlorin	652	Head and neck, lung, skin, bile duct
Verteporfin	Chlorin	690	Ophtalmic, pancreatic, skin
HPPH	Chlorin	665	Head and neck, esophagus, lung, skin, breast
Purlytin	Chlorin	660	Skin, breast
Taloporfin	Chlorin	660	Liver, colon, brain
Fotolon	Chlorine	660	Nasopharyngeal, sarcoma, brain
Silicon pthalocyanine	Phtalocyanine	675	Cutaneous T cell lymphoma
Padoporfin	Bacteriochlorin	762	Prostate
Motexafin lutetium	Texaphyrin	732	Breast

of which later it became known as Photofrin [1, 4, 7]. Even if Photofrin is still the most employed PS, the product has some disadvantages such as a long-lasting skin photosensitivity and a relatively low absorbance at 630 nm. There has been a significant work among medicinal chemists to discover second generation PSs that have an absorbance at the longer wavelengths. Thus, over a hundred compounds have been proposed as potentially useful for the treatment of cancer with PDT. The discovery that 5-aminolevulinic acid (ALA) was a biosynthetic precursor of the PS protoporphyrin IX [8] has led to many applications in which ALA or ALA-ester can be topically applied or administered orally. These are considered to be "pro-drugs" and are required to be converted to protoporhyrin to be active photosensitizers.

Normally, the PSs are very selective towards tumors. Many hypotheses have been suggested to justify the tumor-localizing properties in PDT [9]. These include the prevalence of leaky and twisted tumor blood vessels due to the neovascularization and the absence of lymphatic drainage known to enhanced permeability and retention (EPR) [10]. Some of the most successful compounds bind to low density proteins (LDL) suggesting that the overexpression of LDL receptors found on tumor cells could be important [11]. Targeting studies have demonstrated an increase of tumor uptake when the PSs were covalently attached to various molecules that present some affinity for neoplasia or to receptors expressed on specific tumors [12]. The purpose is to count on the ability of the targeting vehicle to control localization factors so that the PS can be chosen based on its photochemical properties. These vehicles include monoclonal antibodies, antibody fragments, peptides, proteins such as transferrin, epidermal growth factor, insulin, LDL, various carbohydrates, somatostatin, folic acid and many others.

Light Sources

A crucial role in PDT is to ascribe to light irradiation. Considering the different light wavelengths, it is known that blue light penetrates least efficiently through the tissue while red and infrared radiations penetrate more deeply. Even if the region between 600 and 1200 nm is called the optical window for tissues, light up to only about 800 nm can generate $^1O_2$, and in fact longer wavelengths have insufficient energy to initiate a photodynamic reaction [13]. There are other phenomena that limit PDT such as (i) the processes of reflection, refraction and scattering, during light propagation, that reduce the beam power and the penetration in the tissue [14–16]; and (ii) the light absorption by tissue chromophores such as water, oxyhemoglobin (HbO_2) and deoxyhemoglobin, melanin and cytochrome [6, 17], that reduce the PS activation.

Choice of the light source should be based on PS absorption (fluorescence excitation and action spectra), disease (location, size of lesions, accessibility, and tissue chartacteristics), cost and size. The fluence rate also affects the PDT response [18].

Different kinds of lamps can be used including halogen, fluorescent, tungsten or xenon lamps (inexpensive); lasers (more expensive) and light emitting diodes (LEDs) that have narrow spectral bandwidths and high fluence rates [19–20]. La-

sers can be coupled into fibers equipped with diffusing tips to treat tumors present in the urinary bladder and the digestive tract. It is also possible to implant a light source in solid organs deep in the body under image guidance. Inflatable balloons, covered on the inside with a strongly scattering material and formed to fit an organ, are also commercially available [21]. The choice of optimal combinations of PSs, light sources, and treatment parameters is crucial for successful PDT [15–22].

Photophysics and Photochemistry

Most of PSs in their energetically stable state (ground state) possess two electrons with opposite spins located in an energetically most favorable molecular orbital. Absorption of light leads to a transfer of one electron to a higher-energy orbital determining the excited state. In this form, the PS is very unstable and releases this surplus energy as fluorescence and/or heat. Otherwise, an excited PS may through an intersystem crossing to change into a more stable triplet state with inverted spin of one electron. The PS in the triplet state can either decay radiationlessly to the ground state or transfer its energy to molecular oxygen (O_2). This step leads to the formation of singlet oxygen ($^1O_2$), and the reaction is known as a Type II process [23]. In another case, the PS may react directly with an organic molecule in the cellular microenvironment, acquiring a hydrogen atom or an electron to form a radical. This reaction is known as a Type I process. Subsequent autoxidation of the reduced PS produces a superoxide anion radical ($O_2^{\bullet-}$). Dismutation or one-electron reduction of $O_2^{\bullet-}$ gives hydrogen peroxide (H_2O_2), which in turn can undergo one-electron reduction to a powerful and virtually indiscriminate oxidant-hydroxyl radical ($HO^{\bullet}$). ROS generation via the Type II process is much simpler than the Type I, Most PSs are believed to operate via the Type II mechanism (Fig. 14.1).

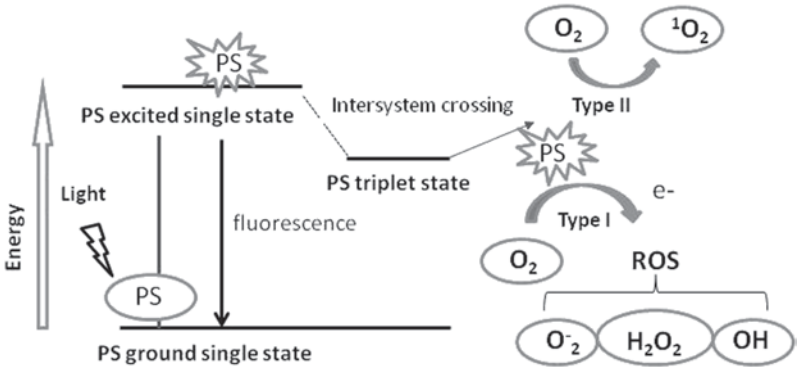


Fig. 14.1 Schematic illustration of photodynamic therapy including the Jablonski diagram. The PS initially absorbs a photon that excites it to the first excited singlet state and this can relax to the more long lived triplet state. This triplet PS can interact with molecular oxygen in two pathways, type I and type II, leading to the formation of reactive oxygen species (ROS) and singlet oxygen respectively [24].

Mechanisms of PDT-Mediated Cytotoxicity

PDT can evoke three main cell death pathways: apoptotic, necrotic, and autophagy-associated cell death determined by the subcellular localization of different PSs [25–26] (Fig. 14.2).

Apoptosis is generally the major cell death modality in PDT. When the PSs are associated to membrane mitochondria, the photodamage determines the release of Bcl-2 [27–29] that causes the mitochondria outer membrane permeabilization (MOMP) and the subsequent release of caspase activators such as cytochrome c and Smac/DIABLO, or other proapoptotic molecules, including the apoptosis- inducing factor (AIF) [30]. If the PSs are located on lysosome membrane, the rupture and leakage of cathepsins from photooxidation [31–32] induce Bid cleavage and MOMP [32]. The phototoxicity can involve other proteases, such as calpains, as well as nonapoptotic pathways [30]. Usually the inhibition or genetic deficiency of caspases can delay phototoxicity or shift the cell death modality toward necrotic cell death [33]. The molecular mechanisms of programmed necrosis are still unclear, but certain events including activation of receptor interacting protein 1 (RIP1) kinase, excessive mitochondrial ROS production, lysosomal damage, and intracellular Ca^{2+}overload have been involved [34–35]. Severe inner mithocondria photodamage or intracellular Ca^{2+} overload could promote mithochondrial permeability transition, an event that may favor the necrotic rather than the apoptotic-mediated phototoxicity [30, 36]. Autophagy is another cell death pathway since it can occur during in vitro tests involving PSs that are localized in the endoplasmatic reticulum

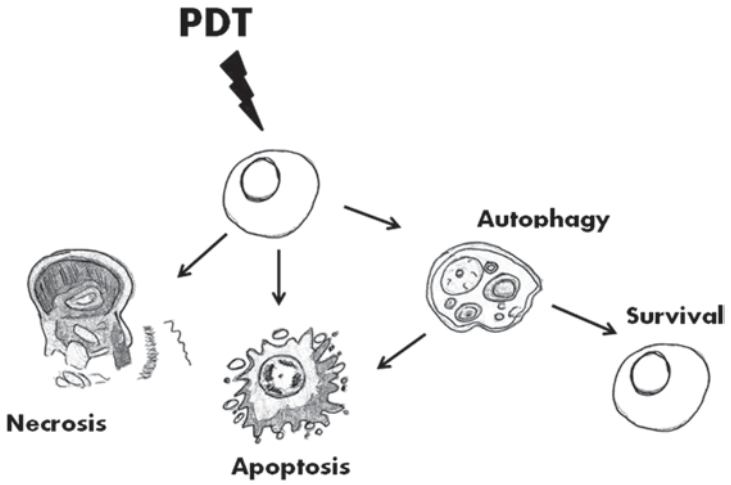


Fig. 14.2 Cell death pathways. Exposure to PDT leads to cellular damage that may result in cell death via different pathways.

(ER). This is a lysosomal pathway for the degradation and recycling of intracellular proteins and organelles. Autophagy can be stimulated by various stress signals including oxidative stress [37]. This process can have both a cytoprotective and a prodeath role after cancer chemotherapies, including those involving ROS as primary damaging agents [37]. Recent studies delineate autophagy as a mechanism to preserve cell viability after PDT [38].

PDT and the Microenvironment

It is important to know that PDT-mediated changes to the tumor microenvironment can modulate treatment responsiveness. The tumor microenvironment is made up of malignant cancer cells and connective tissue as well as a myriad of host cells including endothelial cells, pericytes, and inflammatory leukocytes (macrophages and neutrophils). Leukocytes are recruited into tumors and through the release of a lot of factors, stimulate the endothelium, and indirectly activate tumor vascularization. Also the neutrophil recruitment in tumors can be followed by VEGF and MMP-9 release with associated angiogenesis and invasion, respectively [39–40]. Moreover, tumor-associated macrophages exhibit a phenotype that favors tissue growth, angiogenesis, and tissue remodelling.

All the cellular factors associated with PDT, such as necrosis, apoptosis, and hypoxia, can function as stimuli within the tumor microenvironment. Likewise, PDT-induced hypoxia can lead to the transcriptional activation of VEGF via the HIF-1 pathway [41].

Several laboratories have also shown that PDT can induce the expression and/or activation of additional pro-angiogenic molecules including COX-2 and prostaglandins, TNF, matrix metalloproteinases (MMPs), integrins, IL-6, and IL-8 within the tumor microevironment [41–47]. Preclinical investigations indicate that combining PDT with targeted therapies directed at attenuating the pro-survival actions of the tumor microenvironment can enhance the therapeutic potential of PDT [41–45].

Nitric Oxide and PDT

Nitric oxide (NO) is recognized as a major effector molecule in a diverse array of physiologic and pathologic processes. It is also evident that this radical, produced by many cells in the human body, not only controls important functions in tumor progression, but may have a major influence on the outcome of cancer therapies, in particular those dependent on oxygen and the generation of reactive oxygen species [1, 48–50] such as photodynamic therapy [48, 51–55].

How NO Influences the PDT Antitumor Response?

1. During PDT

The endogenous tumor level of nitric oxide (NO) varies considerably in both human solid tumors [49, 56–59] and murine tumors [48–60]. As described above, the hallmark characteristics in the tumor microenvironment immediately following PDT treatment include tumor vasculature disruption, reduced blood flow, vascular occlusion with subsequent reperfusion injury, in addition to a marked infiltration of inflammatory cells [48]. NO is known to directly influence a number of these biological processes involved in PDT-induced anti-tumor effects. [48, 61]. It has, therefore, been suggested that the intrinsic level of tumor NO may be a determinant in the response to PDT [48, 62].

Both *in vitro* [53] and *in vivo* studies [48, 55] on several tumor models expressing different NO levels reported that a low production of NO makes tumors more sensitive to PDT, in contrast to high NO production. In tumors exhibiting high levels of NO, the vasculature events including vasoconstriction, ischaemia and hypoxia, in addition to the inflammatory reaction induced during PDT, may be reduced [51, 62]. This could result from the following effects of NO: (i) it acts as a potent vasodilator (ii) it prevents platelet aggregation and adhesion to the endothelium (iii) it suppresses the aggregation of accumulated inflammatory neutrophils (iv) it inhibits the expression of leukocyte adhesion molecules and, hence, the adhesion and extravasation of circulating leukocytes and (v) it averts mast cell degranulation [63–65]. On the other hand, elevated NO levels may maintain vessel dilation during PDT treatment, which can limit the decrease in tumor oxygenation and sustain in this way the oxygen-dependent generation of phototoxic damage [51]. Additionally, NO increases the vascular permeability and consequent vascular leakage, which are characteristic occurrences in PDT-treated vasculature (1). The NO-sensitive processes that unfold after termination of photodynamic light treatment include: (i) ischaemia-reperfusion injury, where NO can have a protective role resulting in increased tumor oxygenation (ii) apoptosis of tumor cells which can be stimulated by NO and (iii) development of the immune reaction against the treated tumor, whereby NO has immunoregulatory functions [1, 51].

2. Following PDT

Marked changes in tumor NO levels may be expected to occur after PDT. An increase in the generation of NO attributed to enhanced nitric-oxide synthase (NOS) expression has been observed following PDT using both silicon-phtalocyanine [66] and ALA [67]. Furthermore, the stimulation of cellular signal transduction pathways by PDT-oxidative stress leads to the activation of nuclear transcription factors [1], which may also result in the upregulation of genes encoding NOS. On the other hand, activated inflammatory cells accumulated in PDT-treated tumors may be responsible themselves for the release of NO [68–71].

Cytoprotective Role of NO in PDT

Depending on the concentration of NO presents in the tumor (both endogenous and that induced by PDT), it is important to determine the underlying molecular pathways modulated by NO, with the aim to improve the efficacy of PDT. Once generated, NO combines with other oxidants to form reactive nitrogen species (RNS) which can damage a variety of cellular targets such as DNA and proteins that regulate various intracellular and intercellular signaling events, ultimately leading to apoptosis and mutagenesis [72] (Fig. 14.3).

When high levels of NO are present in a tumor, the superoxide, generated by PDT, can react with an NO unpaired electron and form a peroxynitrite anion ($ONOO^-$) [69, 73–74]. This reaction inactivates the superoxide, hence, the neutrophils are subsequently not activated [75–77], diminishing the damage to the vasculature and surrounding tissue.

Tumors generating low levels of NO are much more sensitive to PDT than those containing high levels of NO, and the administration of NOS inhibitors together with PDT treatment enhances tumor regression [48, 52]. Administration of the NO inhibitors reduces the blood flow in the tumor, and this could explain the increase in PDT efficiency [48].

Several hypotheses have been raised to explain the mechanisms of protection induced by NO in the inhibition of apoptosis [72, 78–79]. NO can inhibit the activa-

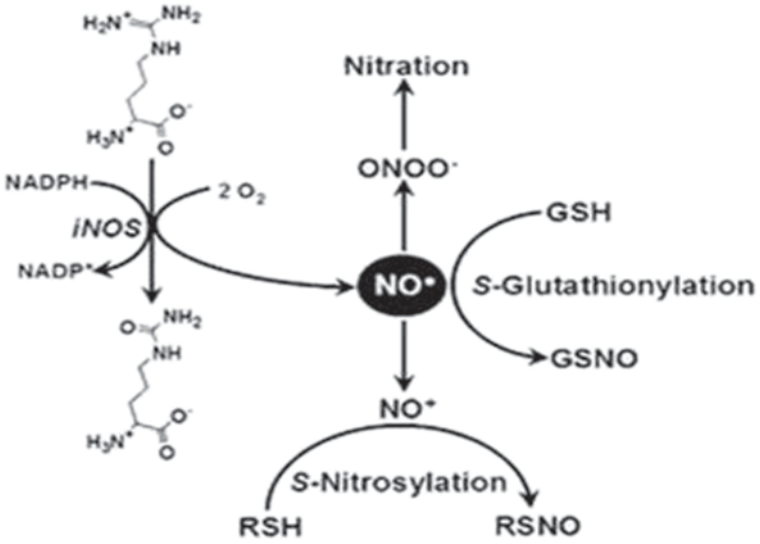


Fig. 14.3 NO chemistry of biological significance. NO is synthesized endogenously from L-arginine, NADPH and oxygen. NO freely diffuses creating concentration gradients across subcellular compartments. Redox or additive reactions with constitutents of cellular microenvironment convert NO to a number of NO_x species, which in turn, dictate the biological effects of NO.

tion of caspases directly by S-nitrosylation [80–81], or through a cyclic guanosine monophosphate (cGMP)-dependent mechanism, by activating protein kinase G (PKG) [82]. More recently, Girotti et al [83–84] demonstrated that the cytoprotective effect of nitric oxide in PDT is due not only to the cGMP involvement but also to the suppression of pro-apoptotic JNK and p38 MAPK activations.

NO Modulates Tumor Cell Death Induced by PDT Through the NF-κB/Snail/YY1/RKIP Loop

The NF-κB/Snail/YY1/RKIP loop is a pivotal molecular circuitry modulated by NO that controls the tumor progression [85]. Several studies have implicated the role of NO in the regulation of tumor cell behavior and have shown that NO either promotes or inhibits tumorigenesis [50, 86–87]. These conflicting findings have been resolved, in part, by the levels of NO used such that low levels promote tumor growth and higher levels inhibit tumor growth. The underlying mechanisms by which NO sensitizes tumor cells to apoptosis were shown to be regulated, in part, by NO-mediated inhibition of NF-κB survival/antiapoptotic pathways and downstream of NF-κB by inhibition of the pro-survival transcriprtion factors Snail and YY1. In addition, it has been shown [85] that NO induces the expression of the metastasis-suppressor/immunosurveillance cancer gene product, Raf-1 kinase inhibitor protein (RKIP). Overexpression of RKIP mimics NO in tumor cell-induced sensitization to apoptosis. The induction of RKIP by NO was the result of the inhibition of the RKIP repressor, Snail, downstream of NF-κB. In the presence of a dysregulated NF-κB/Snail/YY1/RKIP circuitry in tumor cells, the treatment with NO modifies this loop in favor of the inhibition of tumor cell survival and the response to cytotoxic drugs. In addition, the NF-κB/Snail/YY1/RKIP loop consists of gene products that regulate the epithelial mesenchymal transition (EMT) and, thus, tumor metastasis [88] (Fig. 14.4).

Considering that PDT modulates NF-κB activity [89–90] and induces the NO release [66, 83, 91] we have focused our attention on the role of the NF-κB/Snail/YY1/RKIP loop in the tumor response to pheophorbide *a* (a chlorofill derivative, Pb*a*)/PDT. Although, in general, Pb*a*/PDT has been found to be an efficient inducer of cell death by apoptosis and/or necrosis [92–93], however, when the PS is not used at its optimal dose ($< IC_{50}$), it can activate rescuing pathways, leading to tumor survival and recurrence. As the level of NO generated by a low-dose PDT is not sufficient to trigger apoptosis, we investigated how this limited NO release influenced the NF-κB/Snail/YY1/RKIP loop. We have observed that under low-dose Pb*a*/PDT conditions the expression of Snail is increased while the expression of RKIP is decreased: an expression pattern associated to the activation of anti-apoptotic and pro-survival pathways [90].

Moreover, with repeated treatments (8 times) of a low-dose Pb*a*/PDT in prostate cancer cell lines (PC3 and LNCaP), we have found that the NF-κB/YY1/RKIP

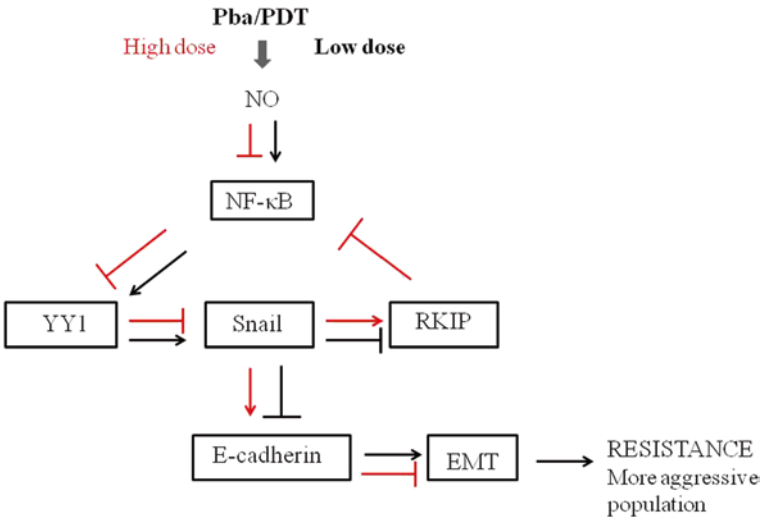


Fig. 14.4 A schematic diagram representing the effect of Pb*a*/PDT on the NF-κB/YY1/Snail/RKIP loop**.** Briefly, a low-dose PDT stimulates NF-κB and the pro-survival genes YY1 and Snail. The up-regulation of Snail results in the downregulation of the metastasis tumor suppressor RKIP. Snail is also correlated to EMT inducing a decrease of E-cadherin expression. Contrasting findings wese observed with a high-dose PDT

circuitry stimulates the EMT. In fact, we have observed a decreased expression of E-cadherin, the main transmembrane adhesion molecule responsible for cell-to-cell interactions and tissue organization in epithelial cells [94–95] and an increase of vimentin, a cytoskeletal component responsible for maintaining cell integrity. As a consequence, loss of E-cadherin expression is considered a crucial event on the disruption of cell-cell adhesion and cytoskeletal architecture and in the acquisition of an invasive phenotype in tumor cells [96]. In particular, in prostate carcinoma, a lower expression of E-cadherin has been associated with more advanced tumor stage and grade [97–98] and higher expression of vimentin is correlated with the invasive capacity [99]. Based on these findings, we have succeeded to isolate a rare cell subpopulation characterized by the CD24+and CD44+phenotypes [100–101]. This subpopulation within the tumor possesses the characteristics of self-renewal capacity, resistance to Pb*a*/PDT in comparison to normal PC3 cells and with tumorigenic ability (unpublished data). All of the above results, yet unpublished, indicate that repeated treatments with a suboptimal dose of PDT determine the presence of a subset cell population with properties of stem cells, that plays a vital role in the initiation, progression and recurrence of cancer [102]. With the administration of L-NAME, a non specific inhibitor of iNOS, in combination with Pb*a*/PDT, we highlighted the role of NO in this tumor progression, through the NK-κB/Snail/YY1/RKIP loop.

Role of Nitric Oxide in Improving the Effectiveness of PDT

NO has been found to be a pivotal factor in the chemosensitization of tumor cells to various chemotherapeutic drugs [72]. Regarding the involvement of NO in PDT, there are some parameters that must be considered in order to improve the efficacy of phototoxic treatment, such as the type of sensitizer (precursor or direct), the type of tumor (in terms of high or low levels of endogenous NO), the interval of time between the administration of the PS and the light exposure.

Many authors have reported that the use of NOS inhibitors (L-NAME, 1400 W) or a nitric oxide scavenger (CPTIO) improves the efficacy of ALA/PDT in tumors with high levels of NO [48, 53, 55, 84], indicating a cytoprotective role for NO. Also in our model, after repeated treatments with low-dose Pb*a*/PDT in PC3, we have observed the same cytoprotective behavior by NO (data unpublished). This effect might be due to trapping lipid-derived radicals generated by one-electron turnover of primary LOOHs [103]. Furthermore, it has been proposed that moderate levels of NO may inhibit caspases by S-nitrosylation [104], induce the downregulation of pro-apoptotic Bax and the upregulation of the the anti-apoptotic Bcl-xL [105] and induce the cytoprotective heme oxygenase-1 [106].

To increase the efficacy of PDT, we have proposed a combined treatment with an NO donor. This treatment is based on the following considerations: (i) the dual role of NO in tumor biology is due to its capacity to promote or inhibit tumor growth dependent on the NO concentration [88] (ii) NO modulates the activity of the NF-κB pathway [107] (iii) Pb*a*/PDT induces the release of cellular NO according to the dose used [90] and (iv) Pb*a*/PDT, in a dose-dependent way, inhibits or stimulates the NF-κB/YY1/Snail/RKIP loop (90) leading to cell growth arrest or cell recurrence.

As a proof of principle, we used in conjunction with Pb*a*, DETANONOate (DETA/NO), a molecule that spontaneously releases in the cytoplasm 2 mol of NO per mole of compound [108]. We found, indeed, that the combination of the PS with an NO donor resulted in a significant modulation of the NF-κB/YY1/Snail/RKIP loop towards the expression of the pro-apoptotic RKIP and the inhibition of anti-apoptotic NF-κB and Snail gene products. The clinical relevance of increasing the RKIP expression by NO correlates with a favorable clinical outcome resulting in tumor regression and in inhibition of metastatic spread [109].

The dual treatment with DETA/NO and mPEG-Pb*a*/PDT [110] has been administered in C57BL/6 mice inoculated s.c. with the B78-H1 murine amelanotic melanoma. The results obtained showed that the use of an NO donor significantly increased the anti-tumor efficacy of PDT. Noteworthy, the group of mice treated with mPEG-Pb*a* and DETA/NO showed a significant delay of tumor growth compared to the untreated group. Furthermore, the Kaplan-Meier survival analyses showed a difference of the median survival times between the mice treated with DETA/NO + mPEG-Pb*a* (59 days) and the mice treated with mPEG-Pb*a*/PDT alone (52.5 days) [91].

The data obtained both *in vitro* and *in vivo* with the combined treatment of an NO donor and PDT [91] are significant and open new horizons for the optimization of photodynamic treatment.

New Therapeutic Strategies with Nitric Oxide and PDT

The effect of the combined treatment DETA/NO + Pb*a*/PDT in an *in vivo* application may be more complex than its effects *in vitro,* due essentially to a systemic effect of the NO donor and especially to its lack of organ or tissue specificity. Therefore, it is exceedingly challenging to selectively deliver NO to a target compartment, to prevent changes of vascular dynamics that result in systemic hypotension [111]. An alternative approach is to deliver NO via the site specific activation of a prodrug, which minimizes adverse drug reactions.

In collaboration with Dr. Greta Varchi, ISOF-CNR, Bologna, Italy, we have synthesized a new compound, named DRPDT2 (Fig. 14.5).

This is a conjugate between Pb*a* (as photosensitizer) and an NO donor that allows a controlled release of NO in the tumor at the time of irradiation of the photosensitizer. The combination between singlet oxygen ($^1O_2$), reactive oxygen species (ROS) and NO should culminate in synergistic cytotoxicity, increasing the efficacy of PDT used alone. Moreover, the linker between the two molecules is intended to increase specificity towards a particular target in prostate carcinoma. Preliminary results have demonstrated that DRPDT2 is a good PS in terms of no toxicity in the dark, easy and cheap synthesis and rapid clearance. It must be irradiated with a white light in order to activate both Pb*a* ($\lambda = 670$ nm) and the release of NO ($\lambda = 400$ nm). The DRPDT2 treatment performed *in vitro* with different lines of prostate cancer cells demonstrated a higher cytotoxic activity then Pb*a* treatment alone. Moreover, DRPDT2 acts through the NF-κB/YY1/Snail/RKIP loop causing an inhibition of NF-κB and, consequently, a strong upregulation of RKIP (unpublished data).

An alternative approach reported in the literature is to deliver NO via PDT: inert PS is activated by irradiation, followed by the decomposition of the excited electronic state to release NO. This technique has been tested with thionitrites, also

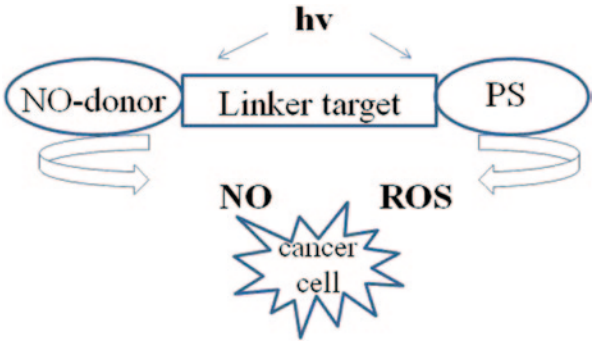


Fig. 14.5 Design of a PDT-NO conjugate. The conjugate is constituted by a an NO donor and a photosensitizer linked together through a linker that allows to increase the selectivity to the tumor cells. The light irradiation causes, at the same time, the release of units of NO and the activation of the PS

known as S-nitrosothiols (SNTs) of glutathione (GSH) forming S-nitrosoglutathione (GSNO) [112–113] and penicilamine [114], as well as photolabile metal-NO complexes [115–116]. Recently, an NO donor has been developed that combines thermal and chemical stabilities to increase the kinetics of NO release during photoactivation [117].

Conclusions and Future Directions

The ability to readily control the kinetics of NO release from these new conjugates, reported above, opens up a range of PDT applications [118]. In particular, regulation of the NO flux has the potential to provide therapies for hypoxia-reperfusion disorders and cardiovascular disease, while a higher exposure levels to these PDT agents could be used to selectively kill malignant cells. The use of these particular PDT-NO conjugates is desirable in cancer therapy to improve classical PDT, taking advantage of nitric oxide by excluding its harmful systemic effects.

Acknowledgements This work has been carried out with the financial support of AIRC (Italian Association for Cancer Research), Project code 10546 (2010-13) and of Rapozzi's Department Research Fund. We also acknowledge Drs. Bonavida, Varchi and Xodo for their collaborative efforts.

No Conflict Statement No potential conflicts of interest were disclosed.

References

1. Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M, Moan J, Peng Q. Photodynamic therapy. J Natl Cancer Inst. 1998;90:889–905.
2. Dolmans DE, Fukumura D, Jain RK. Photodynamic therapy for cancer. Nat Rev Cancer. 2013;3:380–7.
3. Allison RR, Sibata CH. Oncologic photodynamic therapy photosensitizers: a clinical review. Photodiagnosis Photodyn Ther. 2010;7:61–75.
4. Triesscheijn M, Baas P, Schellens JH, Stewart FA. Photodynamic therapy in oncology. Oncologist. 2006;9:1034–44.
5. Plaetzer K, Krammer B, Berlanda J, Berr F, Kiesslich T. Photophysics and photochemistry of photodynamic therapy: fundamental aspects. Lasers Med Sci. 2009;24:259–68.
6. Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, Hahn SM, Hamblin MR, Juzeniene A, Kessel D, Korbelik M, Moan J, Mroz P, Nowis D, Piette J, Wilson BC, Golab J. Photodynamic therapy of cancer: an update. CA Cancer J Clin. 2011;61:250–81.
7. Moan J. Porphyrin photosensitization and phototherapy. Photochem Photobiol. 1986;43:681–90.
8. De Rosa FS Bentley MV. Photodynamic therapy of skin cancers: sensitizers, clinical studies and future directives. Pharm. Res. 2000;17:1447–55.
9. Hamblin MR, Newmann EL. On the mechanism of the tumour-localising effect in photodynamic therapy. J Photochem Photobiol B. 1994;23:3–8.

10. Iyer AK, Greish K, Seki T, Okazaki S, Fang J, Takeshita K, Maeda H. Polymeric micelles of zinc protoporhyrin for tumor targeted delivery based on EPR effect and singlet oxygen generation. J Drug Target. 2007;15:496–506.
11. Kessel D. The role of low-density lipoprotein in the biodistribution of photosensitizing agents. J Photochem Photobiol B. 1992;14:261–2.
12. Sibani SA, McCarron PA, Woolfson AD, Donnelly RF. Photosensitizer delivery for photodynamic therapy. Part 2: systemic carrier platforms. Expert Opin Drug Deliv. 2008;5:1241–54.
13. Juzeniene A, Nielsen KP, Moan J. Biophysical aspects of photodynamic therapy. J Environ Pathol Toxicol Oncol. 2006;25:7–28.
14. Nowis D, Makowski M, Stokłosa T, Legat M, Issat T, Gołab J. Direct tumor damage mechanisms of photodynamic therapy. Acta Biochim Pol. 2005;52:339–52.
15. Wilson BC, Patterson MS The physics, biophysics and technology of photodynamic therapy. Phys Med Biol. 2008;53:R61–R109.
16. Allison RR, Moghissi K. Photodynamic Therapy (PDT): PDT Mechanisms. Clin Endosc 2013;46:24–9.
17. Robertson CA, Evans DH, Abrahamse H. Photodynamic therapy (PDT): a short review on cellular mechanisms and cancer research applications for PDT. J Photochem Photobiol B. 2009;96:1–8.
18. Henderson BW, Busch TM, Snyder JW. Fluence rate as a modulator of PDT mechanisms. Lasers Surg Med. 2006;38:489–93.
19. Juzeniene A, Juzenas P, Ma LW, Iani V, Moan J. Effectiveness of different light sources for 5-aminolevulinic acid photodynamic therapy. Lasers Med Sci. 2004;19:139–49.
20. Szeimies RM, Morton CA, Sidoroff A, Braathen LR. Photodynamic therapy for non-melanoma skin cancer. Acta Derm Venerol. 2005;85:483–90.
21. Beyer W. Systems for light application and dosimetry in photodynamic therapy. J Photochem Photobiol B. 1996;36:153–6.
22. Platzer K, Krammer B, Berlanda J, Berr F, Kiesslich T. Photophysics and photochemistry of photodynamic therapy: fundamental aspects. Lasers Med Sci. 2009;24:259–68.
23. Foote CS. Mecganisms of photosensitized oxidation. There are several different types of photosensitized oxidation which may be important in biological systems. Science. 1968;162:963–70.
24. Dai T, Fuchs BB, Coleman JJ, Prates RA, Astrakas C, St Denis TG, Ribeiro MS, Mylonakis E, Hamblin MR, Tegos GP. Concepts and principles of photodynamic therapy as an alternative antifungal discovery platform. Front Microbiol. 2012;3:1–16.
25. Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: part two-cellular signaling, cell metabolism and modes of cell death. Photodiagnosis Photodyn Ther. 2005;2:1–23.
26. Ortel B, Shea CR, Calzavara-Pinton P. Molecular mechanisms of photodynamic therapy. Front Biosci. 2009;14:4157–72.
27. Kessel D, Castelli M. Evidence that bcl-2 is the target of three photosentizers that induce a rapid apoptotic response. Photochem Photobiol. 2001;74:318–22.
28. Xue LY, Chiu SM, Oleinick NL. Photochemical destruction of the Bcl-2 oncoprotein during photodynamic therapy with the phthalocyanine photosensitizer Pc4. Oncogene. 2001;20:3420–7.
29. Usuda J, Chiu SM, Murphy ES, Lam M, Niemimen AL, Oleinick NL. Domain-dependent photodamage to Bcl-2. A membrane anchorage region is needed to form the target of phthalocyanine photosensitization. J Biol Chem. 2003;278:2021–9.
30. Buytaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. Biochim Biophys Acta. 2007;1776:86–107.
31. Berg K, Moan J. Lysosomes as photochemical targets. Int J Cancer. 1994;59:814–22.
32. Reiners JJ Jr, Caruso JA, Mathieu P, Chelladurai B, Yin XM, Kessel D. Release of cytochrome c and activation of pro-caspase-9 following lysosomal photodamage involves Bid cleavage. Cell Death Differ. 2002;9:934–44.

33. Kessel D. Relocalization of cationic porphyrins during photodynamic therapy. Photochem Photobiol Sci. 2002;1:837–40.
34. Vanlangenakker N, Vanden Berghe T, Krysko DV, Festjens N, Vandenabeele P. Molecular mechanisms and pathophysiology of necrotic cell death. Curr Mol Med. 2008;8:207–20.
35. Zong WX, Thompson CB. Necrotic death as a cell fate. Genes Dev. 2006;20:1–15.
36. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but non apoptotic cell death. Nature. 2005;434:652–8.
37. Dewaele M, Maes H, Agostinis P. ROS-mediated mechanisms of autophagy stimulation and their relevance in cancer therapy. Autophagy. 2010;6:838–54.
38. Reiners JJ Jr, Agostinis P, Berg K, Oleinick NL, Kessel D. Assessing autophagy in the context of photodynamic therapy. Autophagy. 2010;6:7–18.
39. Karin M. Cancer research in flames; tracking inflammation's role in promoting malignancy could lead to better treatments. Scientist. 2005;19:24–5.
40. Benelli R, Morini M, Carrozzino F, Ferrari N, Minghelli S, Santi L, Cassatella M, Noonan DM, Albini A. Neuthrophils as a key cellular target for angiostatin: implications for regulation of angiogenesis and inflammation. FASEB J. 2002;16:267–9.
41. Ferrario A, von Tiehl KF, Rucker N, Schwarz MA, Gill PS, Gomer CJ. Anti-angiogenic treatment enhances photodynamic therapy responsiveness in a mouse mammary carcinoma. Cancer Res. 2000;60:4066–9.
42. Ferrario A, von Tiehl KF, Wong S, Luna M, Gomer CJ. Cyclooxygenase-2 inhibitor treatment enhances photodynamic therapy-mediated tumor response. Cancer Res. 2002;62:3956–61.
43. Ferrario A, Fisher AM, Rucker N, Gomer CJ. Celecoxib and NS-398 enhance photodynamic therapy by increasing in vitro apoptosis and decreasing in vivo inflammatory and angiogenic factors. Cancer Res.2005;65:9473–9.
44. Ferrario A, Chantrain CF, von Tiehl KF, Buckley S, Rucker N, Shalinsky DR, Shimada H, DeClerck YA, Gomer CJ. The matrix metalloproteinase inhibitor Prinomastat enhances photodynamic therapy responsiveness in a mouse tumor model. Cancer Res. 2004;64:2328–32.
45. Ferrario A, Gomer CJ. Avastin enhances photodynamic therapy treatment of Kaposi's sarcoma in a mouse tumor model. J Environ Path Tox Oncol. 2006;25:251–9.
46. Gollnick SO, Evans SS, Baumann H, Owczarczak B, Maier P, Vaughan L, Wang WC, Unger E, Henderson BW. Role of cytokines in photodynamic therapy –induced local and systemic inflammation. Br J Cancer. 2003;88:1772–9.
47. Makowski M, Grzela T, Niderla J, Azarczyk M, Mroz P, Kopee M, Legat M, Strusinska K, Koziak K, Nowis D, Mrowka P, W, Nishikawa N, Uchida K, Yoshikawa K, Noguchi T, Miyaishi O, Shimozato K, Saga S, Matsumoto Y. Cyclooxygenase-2 is a possible target of treatment approach in conjunction with photodynamic therapy for various disorders in skin and oral cavity. Br J Dermatol. 2004;151:472–80.
48. Korbelik M, Parkins CS, Shibuya H, Cecic I, Stratford MR, Chaplin DJ. Nitric oxide production by tumor tissue: impact on the response to photodynamic therapy. Br J Cancer. 2000;82:1835–43.
49. Tozer GM, Everett SA. Nitric oxide in tumor biology and cancer therapy. Part 2: therapeutic implications. Clin Oncol. 1997;9:357–64.
50. Jenkins CD, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S. Roles of nitric oxide in tumor growth. Proc Nat Acad Sci USA. 1995;92:4392–6.
51. Korbelik M, Shibuya H, Cecic I. Relevance of nitric oxide to the response of tumours to photodynamic therapy. SPIE. 1998;3247:98–105.
52. Henderson B, Sitnik-Busch TM, Vaughan LA. Potentiation of photodynamic therapy antitumor activity in mice by nitric oxide synthase inhibition is fluence rate dependent. Photochem Photobiol. 1999;70:64–71.
53. Di Venosa G Perotti C Fukuda H Battle A Casas A. Sensitivity to ALA-PDT of cell lines with different nitric oxide production and resistance to NO cytotoxicity. J Photochem Photobiol B. 2005;80:195–202.

54. Yamamoto F, Ohgari Y, Yamaki N, Kitajima S, Shimokawa O, Matsui H, Taketani S. The role of nitric oxide in delta-aminolevulinic acid (ALA)-induced photosensitivity of cancerous cells. Biochem Biophys Res Commun. 2007;353:541–6.
55. Reeves KJ, Reed MWR, Brown NJ. The role of nitric oxide in the treatment of tumours with aminolevulinic acid-induced photodynamic therapy. J Photochem Photobiol B. 2010;101:224–32.
56. Thomsen LL, Lawton FG, Knowles RG, Beesley JE, Riveros-Moreno V, Moncada S. Nitric oxide synthase activity in human gynecological cancer. Cancer Res. 1994;54:1352–4.
57. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S. Nitric oxide synthase activity in human cancer. Br J Cancer. 1995;72:41–4.
58. Cobbs CS, Brenman JE, Aldape KD, Bredt DS, Israel MA. Expression of nitric oxide synthase in human central nervous system tumors. Cancer Res. 1995;15:727–30.
59. Rosbe KW, Prazma J, Petrusz P, Mims W, Ball SS, Weissler MC. Immunohistochemical characterization of nitric oxide synthase activity in squamous cell carcinoma of the head and neck. Otolaryngol Head Neck Surg. 1995;113:541–9.
60. Parkins CS, Dennis MF, Stratford MR, Hill SA, Chaplin DJ. Ischemia reperfusion injury in tumors: the role of oxygen radicals and nitric oxide. Cancer Res. 1995;55:6026–9.
61. Reeves KJ, Reed MWR, Brown NJ. Is nitric oxide important in photodynamic therapy? J Photochem Photobiol. 2009;95:141–7.
62. Gilissen MJ, van de-Merbel-de Wit, Star WM, Koster JF, Sluiter W. Effect of photodynamic therapy on the endothelium-dependent relaxation of isolated rat aortas. Cancer Res. 1993;53:2548–52.
63. Schmidt HHH, Walker U. NO at work. Cell. 1994;78:919–25.
64. Vanhoutte PM. Endothelium and responsiveness of vascular smooth mucle. J Hypertens. 1987;5:S115–20.
65. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci USA. 1991;88:4651–5.
66. Gupta S, Ahmad N, Mukhtar H. Involvement of nitric oxide during phthalocyanine (Pc4) photodynamic therapy-mediated apoptosis. Cancer Res. 1998;58:1785–8.
67. Dalbasti T, Cagli S, Killnc E, Oktar N, Ozsoz M. Online electrochemical monitoring of nitric oxide during photodynamic therapy. Nitric Oxide. 2002;7:301–5.
68. Cecic I, Korbelik M. Mediators of peripheral blood neutrophilia induced by photodynamic therapy of solid tumors. Cancer Lett. 2002;183:43–51.
69. McCall TB, Boughton-Smith NK, Palmer RM, Whittle BJ, Moncada S. Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. Biochem J. 1989;261:293–6.
70. Mehta JL, Lawson DL, Nicolini FA, Ross MH, Player DW. Effects of activated polymophonuclear leukocytes on vascular smooth muscle tone. Am J Physiol. 1991;261:H327–34.
71. Evans TJ, Buttery LDK, Carpenter A, Springall DR, Polak JM, Cohen J. Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested reeves bacteria. Proc Natl Acad Sci USA. 1996;93:9553–8.
72. Singh S, Gupta AK. Nitric oxide: role in tumour biology and iNOS/NO-based anticancer therapies. Cancer Chemother Pharmacol. 2011;67:1211–24.
73. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxinitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA. 1990;87:1620–4.
74. Beckman JS, Crow JP. Pathological implications of nitric oxide, superoxide and peroxynitrite formation. Biochem Soc Trans. 1993;21:330–4.
75. Korbelik M, Parkins CS, Shibuya H, Cecic I, Stratford MR, Chaplin DJ. Nitric oxide production by tumour tissue: impact on the response to photodynamic therapy. Br J Cancer. 2002;82:1835–43.
76. Gaboury JP, Anderson DC, Kubes P. Molecular mechanisms involved in superoxide-induced leukocyte-endothelial cell interactions in vivo. Am J Physiol. 1994;266:H637–42.

77. Kurose I, Wolf R, Grisham MB, Granger DN. Modulation of ischemia/reperfusion-induced microvascular dysfunction by nitric oxide. Circ Res. 1994;74:376–82.
78. Liu L, Stamler JS. NO: an inhibitor of cell death. Cell Death Diff. 1999;6:937–42.
79. Melino G, Catani MV, Corazzari M, Guerrieri P, Bernassola F. Nitric oxide can inhibit apoptosis or switch it into necrosis. Cell Mol Life Sci. 2000;57:612–22.
80. Rossig L, Fichrlscherer B, Breitschopf K, Haendeler J, Zeither AM, Mulsch A, Dimmeler S. Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. J Biol Chem. 1999;274:6823–6.
81. Dimmeler S, Haendeler J, Nehls M, Zeiher AM. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. J Exp Med. 1997;185:601–7.
82. Gomes ER, Almeida RD, Carvalho AP, Duarte CB. Nitric oxide modulates tumor cell death induced by photodynamic therapy through a cGMP-dependent mechanism. Photochem Photobiol.2002;76:423–30.
83. Bhowmick R, Girotti AW. Cytoprotective signaling associated with nitric oxide upregulation in tumor cells subjected to photodynamic therapy-like oxidative stress. Free Radic Biol Med. 2013;57:39–48.
84. Bhowmick R, Girotti AW. Pro-survival and pro-growth effects of stress-induced nitric oxide in a prostate cancer photodynamic therapy model. Cancer Lett. 2014;343:115–22.
85. Baritaki S, Yeung K, Palladino M, Berenson J, Bonavida B. Pivotal roles of Snail inhibition and RKIP induction by the proteosoma inhibitor NPI-0052 in tumor cell chemosensitization. Cancer Res. 2009;69:8376–85.
86. Wink DA, Mitchell JB. Chemical biology of nitric oxide: insights into the regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Radic Bio Med. 1998;24:434–56.
87. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumor progression. Nat Rev Cancer. 2006;6:521–34.
88. Baritaki S, Huerta-Yepez S, Sahakyan A, Karagiannides I, Bakirtzi K, Jazirehi A, Bonavida B. Mechanisms of nitric oxide-mediated inhibition of EMT in cancer: inhibition of the metastasis-inducer Snail and induction of the metastasis-suppressor RKIP. Cell Cycle. 2010;9:4931–40.
89. Coupienne I, Fettweis G, Rubio N, Agostinis P, Piette J. NF-kappaB inhibition improves the sensitivity of human glioblastoma cells to 5-aminolevulinic acid-based photodynamic therapy. Biochem Pharmacol. 2011;81:606–16.
90. Rapozzi V, Umezawa K, Xodo LE. Role of NF-kB/Snail/RKIP loop in the response of tumor cells to photodynamic therapy. Lasers Surg Med. 2011;43:575–85.
91. Rapozzi V, Della Pietra E, Zorzet S, Zacchigna M, Bonavida B, Xodo LE. Nitric oxide-mediated activity in anti-cancer photodynamic therapy. Nitric Oxide. 2013;30:26–35.
92. Tang PMK, Chan JYM; Au SWN, Kong SK, Tsui SKW, Waye MMY, Mak TC, Fong WP, Fung KP. Pheophorbide *a*, an active compound isolated from *Scutellaria barbata*, possess photodynamic activities by inducing apoptosis in human hepatocellular carcinoma. Cancer Biol Ther. 2006;5:1111–6.
93. Rapozzi V, Miculan M, Xodo LE. Evidence that photoactivated pheophorbide *a* causes in human cancer cells a photodynamic effect involving lipid peroxidation. Cancer Biol Ther. 2009;8:1318–27.
94. Damsky CH, Richa J, Solter D, Knudsen K, Buck CA. Identification and purification of a cell surface glycoprotein mediating intercellular adhesion in embryonic and adult tissue. Cell 1983;34:455–66.
95. Takeichi M. Cadherin cell adhesion receptors as a morphogenic regulator. Science. 1991;251:1451–5.
96. Vleminckx K, Vakaet I Jr, Mareel M, Fiers W, van Roy F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell. 1991:66:107–19.
97. Umbas R, Isaac WB, Bringuier PP, Schaafsma HE, Karhaus HFM, Oosterhof GO, Debruyne FM, Schalken JA. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Res. 1994;54:3929–33.

98. De Marzo AM Knudsen B Chan-Tack K Epstein JI. E-cadherin expression as a marker of tumor aggressiveness in routinely processed radical prostatectomy specimens. Urology. 1999;53:707–13.
99. Lang SH, Hyde C, Reid IN, Hitchcock IS, Hart CA, Bryden AA, Villette JM, Stower MJ, Maitland NJ. Enhanced expression of vimentin in motile prostate cell lines and in poorly differentiated and metastatic prostate carcinoma. Prostate. 2002;52:253–63.
100. Salvatori L, Caporuscio F, Verdina A, Starace G, Crispi S, Nicotra MR, Russo AN, Calogero RA, Morgante E, Natali PG, Russo MA, Petrangeli E. Cell-to-cell signaling influences the fate of prostate cancer stem cells and their potential to generate more aggressive tumors. Plos One. 2012;7:e31467.
101. Whicha MS. Cancer stem cells and metastasis: lethal seeds. Clin Cancer Res. 2006;12:5606–7.
102. Mani SA, Guo W, Laio MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA. The epitheial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133:704–15.
103. Niziolek M, Korytowski W, Girotti AW. Chaion-breaking antioxidant and cytoprotective action of nitric oxide on photodynamically stressed tumor cells. Photochem Photobiol 2003;78:262–70.
104. Li C, Wogan GN. Nitric oxide as a modulator of apoptosis. Cancer Lett. 2005;225:1–15.
105. Bhowmick R, Girotti AW. Cytoprotective induction of nitric oxide synthase in a cellular model of 5-aminolevulinic acid-based photodynamic therapy. Free Radic Biol Med. 2010;48:1296–301.
106. Bhowmick R, Girotti AW. Signaling events in apoptotic photokilling of 5-aminolevulinic acid-treated tumor cells: inibitory effects of nitric oxide. Free Radic Biol Med. 2009;47:731–40.
107. Reynaert NL, Ckless K, Korn SH, Vos N, Guala AS, Wouters EF, van der Vliet A, Janssen-Heininger YM. Nitric oxide represses inhibitory kappaB kinase through S-nitrosylation. Proc Natl Acad Sci USA. 2004;101:8945–50.
108. Davies KM, Wink DA, Saavedra JE, Keefer LK. Chemistry of the diazeniumdiolates. 2. Kinetics and mechanism of dissociation to nitric oxide in aqueous solution. J Am Chem Soc. 2001;208:5473–81.
109. Huerta-Yepez S, Yoon NK, Hernandez-Cueto A, Mah V, Rivera-Pazos CM, Chatterjee D, Vega MI, Maresh EL, Horvath S, Chia D, Bonavida B, Goodglick L. Expression of phosphorylated raf kinase inhibitor protein (pRKIP) is a predictor of lung cancer survival. BMC Cancer. 2011;11:259–67.
110. Rapozzi V, Zacchigna M, Biffi S, Garrovo C, Cateni F, Stebel M, Zorzet S, Bonora GM, Drioli S, Xodo LE. Conjugated PDT drug: photosensitizing activity and tissue distribution of PEGylated pheophorbide *a*. Cancer Biol.Ther. 2010;10:471–82.
111. Shan SQ, Rosner GL, Braun RD, Hahn J, Pearce C, Dewhirst MW. Effects of diethylamine/nitric oxide on blood perfusion and oxygenation in the R3230Ac mammary carcinoma. Br J Cancer. 1997;76:429–37.
112. Sexton DJ, Muruganandam A, McKenney DJ, Mutus B. Visible light photochemical release of nitric oxide from S-nitrosoglutathione: potential photochemotherapeutic applications. Photochem Photobiol. 1994;59:463–7.
113. Zhelyaskov VR, Gee KR, Godwin DW. Control of NO concentration in solutions of nitrosothiol compounds by light. Photochem Photobiol. 1998;67:282–8.
114. Singh RJ, Hogg N, Joseph J, Kalyanaraman B. Photosensitized decomposition of S-nitrosothiols and 2-methyl-2-nitrosopropane possible use for site-directed nitric-oxide production. FEBS Lett. 1995;360:47–51.
115. Carneiro ZA, de Moraes JC, Rodrigues FP, de Lima RG, Curti C, de Rocha ZN, Paulo M, Benhack LM, Tedesco AC, Formiga AL, da Silva RS. Phototoxic activity of a nitrosyl phtalocyanine ruthenium complex- a system capable of producing nitric oxide and singlet oxygen. J Inorg Biochem. 2011;105:1035–43.

116. Ostrowski AD, Deakin SJ, Azhar B, Miller TW, Franco N, Chemey MM, Lee AJ, Burstyn JN, Fukuto JM, Megson IL, Ford PC. Nitric oxide photogeneration from trans-Cr(cyclam) $(ONO)_2^+$ in a reducing environment. Activation of soluble guanylyl cyclase and arterial vasorelaxation. J Med Chem. 2010;53:715–22.
117. Giles NM, Kumari S, Gang BP, Yuen CWW, Billaud EMF, Giles GI. The molecular design of S-bitrosothiols as photodynamic agents for controlled nitric oxide release. Chem Biol Drug Des. 2012;80:471–8.
118. Lancaster JR Jr, Giles GI. Nitrogen monoxide (nitric oxide):bioinorganic chemistry. Encyclopedia of Inorganic and Bioinorganic Chemistry. Wiley. 2011.

Chapter 15
Regulation of Cell Death Signaling by Nitric Oxide in Cancer Cells

Jordi Muntané, Francisco Gallardo-Chamizo, Sheila Pereira, Ángela M. De los Santos, Ángeles Rodríguez-Hernández, Luís M. Marín, Jose M. Álamo and Francisco J. Padillo

Abstract Nitric oxide (NO) is a lipophillic, highly diffusible, and short-lived physiological messenger which regulates a variety of important physiological responses, including vasodilation, respiration, cell migration, immune response and apoptosis. NO is synthesized by three differentially gene-encoded NOS in mammals: neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). NO may exert its cellular action by cGMP-dependent as well as by cGMP-independent pathways that include post-translational modifications in cysteine (S-nitrosylation), tyrosine nitration and S-nitrosoglutathione-derived compounds. NO sensitizes tumor cells to chemotherapeutic compounds. The antitumoral activity of NO may be related to the regulation of the stress response mediated by the hypoxia inducible factor-1 (HIF-1) and p53 that generally lead to growth arrest, apoptosis or adaptation. In addition, the post-translational regulation of cell death receptors modulates the apoptotic pathways.

Keywords Angiogenesis · Cell proliferation · Cell death receptors · cGMP-dependent and independent pathways · HIF-1 · Hypoxia · Nitric oxide synthase · p53 · Tumorogenesis

Abbreviations

BH4	(6R)-5,6,7,8-tetrahydrobiopterin
bZip	Basic Leucine Zipper
CaM	Calmodulin

J. Muntané (✉) · L. M. Marín · J. M. Álamo · F. J. Padillo
Department of General Surgery, Hospital Universitario Virgen del Rocío-Virgen Macarena/IBiS/CSIC/Universidad de Sevilla, Av. Manuel Siurot s/n, 41013 Seville, Spain
e-mail: jmuntane-ibis@us.es

Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHo Ciberehd), Instituto de Salud Carlos III, Madrid, Spain

J. Muntané · F. Gallardo-Chamizo · S. Pereira · Á. M. De los Santos · Á. Rodríguez-Hernández
Instituto de Biomedicina de Sevilla (IBiS), IBiS/Hospital Universitario Virgen del Rocío-Virgen Macarena/Universidad de Sevilla/CSIC, Av. Manuel Siurot s/n, 41013 Seville, Spain

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_15

DR	Death Receptor
ECM	Extracellular Matrix
eNOS (NOS-3)	Endothelial NOS
GTN	Glyceryl Trinitrate
GSH	Reduced Glutathione
GSNO	S-nitrosoglutathione
haeme	Iron Protoporphyrin IX
HIF-1	Hypoxia Inducible Factor-1
iNOS	Inducible NOS
MAPK	Mitogen-Activated Protein Kinases
nNOS (NOS-1)	Neuronal NOS
NO	Nitric Oxide
NOS	Nitric Oxide Synthases
$O^{2\bullet-}$	Anion Superoxide
PI3K	Phosphatidylinositol-3 kinase
PSA	Prostate-Specific Antigen
sGC	Soluble Guanylate Cyclase
TRAIL	Tumor Necrosis Factor-Related Apoptosis Inducing Ligand
TRAIL-R1	TRAIL Receptor 1
TRAIL-R2	TRAIL Receptor 2
TNF-R1	Tumor Necrosis Factor Receptor Type 1

Introduction

Nitric Oxide, Nitric Oxide Synthase and Subcellular Localization

Nitric oxide (NO) is a lipophillic, highly diffusible, and short-lived physiological messenger [1]. NO regulates a variety of important physiological responses, including vasodilation, respiration, cell migration, immune response and apoptosis. NO is synthesized by three diffrentially gene-encoded nitric oxide synthases (NOS) in mammals. The brain proved to be a rich source of NO synthesis and allowed the first NOS (nNOS or NOS-1) to be cloned and purified [2–4]. The *NOS-1* gene has the most complex genomic organization in humans with multiple splice variants being produced [5, 6]. The inducible NOS (iNOS or NOS-2) is readily induced in many tissues by proinflammatory cytokines [7]. The endothelial NOS (eNOS or NOS-3) binds to plasma membranes and is typically associated with caveolin [8]. The full active NOS form requires two NOS monomers associated with two Ca^{2+}-binding protein molecules, calmodulin (CaM). NOS contains relatively tightly-bound cofactors such as (6R)-5,6,7,8-tetrahydrobiopterin (BH4), FAD, FMN and iron protoporphyrin IX (haeme), and catalyzes the reaction of L-arginine, NADPH, and oxygen to NO, L-citrulline and NADP [7]. HB_4 acts as a redox cofactor and

prevents the uncoupling of NOS and the generation of anion superoxide (O_2^-). All NOS isoforms are differentially regulated at the transcriptional, translational and post-translational levels. However, the activity of NOS-1 and NOS-3 is highly dependent upon intracellular Ca^{2+} concentration, whereas NOS-2 forms an active complex with CaM.

There are compartments that allow the full activation of NOS with free access to substrates and cofactors, as well as the presence of activators [9]. Noteworthy, accumulating evidence indicates that NOSs are subject to specific targeting to subcellular compartments (plasma membrane, Golgi, cytosol, nucleus and mitochondria) and that this trafficking is crucial for NO production and specific posttranslational modifications of target proteins [10, 11].

Nitric Oxide Cell Signaling

The biological activity of NO is classified by cGMP-dependent and cGMP-independent pathways, both attributed to physiological and pathological conditions [12–14]. cGMP-dependent protein kinases, cyclic-nucleotide-gated ion channels and cGMP-regulated phosphodiesterases mediate several cellular effects. However, during the last decade cGMP-independent reactions gained considerable interest. A variety of effects are achieved through NO interactions with targets via redox and additive chemistry, that may promote covalent modifications of proteins as well as oxidation events that do not require attachment of the NO group. In fact, NO is the prototypic redox-signaling molecule that is more versatile than O_2^- or H_2O_2 and is clearly better identified with redox-related modifications of intracellular proteins [15].

Nitric Oxide cGMP-Dependent Pathways

Soluble guanylate cyclase (sGC) is a receptor for NO [16], and is intimately involved in many signal transduction pathways, most notably in the cardiovascular system (e.g., in the regulation of vascular tone and platelet function) and in the nervous system (e.g. in neurotransmission and, possibly, long-term potentiation and depression). There are three known targets for cGMP which mediate the transmission of the NO/cGMP pathway signal downstream fromguanylate cyclase: cGMP-dependent protein kinase [17], cGMP-regulated phosphodiesterase [18, 19] and cGMP-gated ion channels [20]. The first of these, cGMP-dependent protein kinase, phosphorylates target proteins in response to an increase in the cGMP concentration. For example, in smooth muscle cells, cGMP-dependent protein kinase phosphorylates the inositol 1,4,5-triphosphate receptor, leading to a decrease in Ca^{2+} concentration and, ultimately, smooth muscle relaxation. The second, cyclic nucleotide phosphodiesterase, catalyzes the hydrolysis of the 3′-phosphodiester bond of cAMP and cGMP to yield AMP and GMP, respectively. The third, cyclic nucleotide-gated

ion channels, are non-specific cation channels found in a variety of tissues. Most notably, these channels are found in the retina and in the olfactory epithelium where they are involved, respectively, in visual phototransduction and in olfaction.

Nitric Oxide cGMP-Independent Pathways

The most prominent and recognized NO reaction with thiol groups of cysteine residues is called S-nitrosylation, which leads to the formation of more stable nitrosothiols [21]. However, other modifications such as disulfide, mixed disulfide formation with reduced glutathione (S-nitrosoglutathione or GSNO), or oxidation towards sulfonic acid are also important, since they are reversible. Higher thiol oxidation states such as sulfinic or sulfonic acids are irreversible modifications with subsequent loss of functional control. Nitrosothiol formation can be the result of a direct reaction with NO or of an oxidative nitrosylation reaction involving the preformation of $ONOO^-$ [22]. The pattern of nitrosylated proteins is specific and is probably dependent of the presence of specific consensus motifs which influence the accessibility of the thiols groups to NO [23]. Different proteins such as NMDA and ryanodine receptors, ras, caspases, glyceraldehyde-3-phosphate dehydrogenase and DNA repair proteins are widely post-translationally modified by nitrosylation [24].

The NO-dependent post-translational modification of key factors exerts transcriptional regulation. The S-nitrosation of specific cysteines in active zinc fingers sequences in SP-1, EGR-1 and glucocorticoid receptors induces Zn^{2+} release, concomitantly with conformational changes, and reduced DNA-binding [25]. The impact of NO on other transcription factors such as NF-κB may affect at different levels such as on the IκB expression and stability, NF-κB activation, nuclear translocation, or cysteine residue modification involving alteration of DNA-binding. The administration of NO donors reduces NF-κB activation and downstream the expression of anti-apoptotic gene products [26], which is relevant for NO-dependent sensitization of chemotherapy-resistant tumor cells [27, 28]. It now seems more certain that reducing conditions are required in the nucleus for NF-κB DNA-binding, whereas oxidizing conditions in the cytoplasm promote NF-κB activation [29]. AP-1 is a transcription factor that belongs to the basic leucine zipper (bZip) family in which a single cysteine residue is present that confers redox sensitivity [30]. NO, mostly by S-nitrosylation [31] and glutathiolation [32] of cysteines, inhibits c-Jun and c-Fos DNA-binding in a reversible manner. p53 also binds to its specific DNA sites in a reducing environment, and mutations of cysteine residues in the p53 core binding-domain (loop-sheet-helix motif linked to a loop-helix motif) prevents its DNA-binding and p53-induced transcription [33]. HIF-1α has a single cysteine in the basic-helix-loop-helix of the carboxyl-terminal trans-activating domain, which participates in protein–protein interactions that activate transcription [34]. Other transcription factors whose binding to DNA are facilitated under reducing conditions include c-Myb, USF, NFI, NF-Y, HLF, PEBP2, GABPa, TTF-1, and Pax-8 [29].

The generation of O_2^- and NO may lead to the production of the harmful molecule $ONOO^-$ [35]. $ONOO^-$ may result in S-nitrosylation and tyrosine nitration of proteins with a concomitant change in their functions [36]. The reaction of $ONOO^-$ with Akt and BH4 altered NO production generated by NOS [37, 38]. Proteins that can be nitrated on tyrosine residues include actin, histone proteins, protein kinase C, prostacyclin synthase, manganese superoxide dismutase, tyrosine hydroxylase, cytochrome P450B1, transcription factor STAT1 and p53 [39]. NO may induce indirectly gene transcription via activation/modulation of signaling pathways such as mitogen-activated protein kinases (MAPK), G-proteins, the Ras pathway, or phosphatidylinositol-3 kinase (PI3K) pathways [40].

Nitric Oxide, Cell Proliferation and Cancer

NO may participate in the induction of genotoxic lesions, as well as promoting angiogenesis, tumor cell growth, and invasion [41]. The effects of NO in tumors seem to depend on the activity and localization of NOS isoforms, concentration and duration of NO exposure, and cellular sensitivity to NO [42].

Importance of the Extracelular Matrix in Tumor Progression

Basic cancer research has mainly focused on mutations in cancer cells that result in either gain-of-function in oncogenes or loss-of-function in tumor-suppressor genes [43]. However, the extracellular matrix (ECM) of tumors and the non-cancerous stromal cells within tumors also have an important impact on tumor progression [44]. NOS-2 is present in fibroblast, inflammatory and endothelial cells; and NOS-3 is present in endothelial cells which has a relevant impact in the cancer-cell growth, differentiation, apoptosis, migration and invasion, and the regulation of angiogenesis and immunity [45].

Participation of NO in Carcinogenesis

Expression of NOSs and production of NO have been detected in several established human tumors [42]. The infectious and non-infectious generation of chronic injury and irritation initiate an inflammatory response [46]. Neoplastic transformation is a key initial step in cancer. Chronic inflammation and continuous exposure to moderate/high concentrations of NO that is produced by NOS-2 are thought to promote neoplastic transformation. A subsequent respiratory burst, an increased uptake of oxygen that leads to the release of free radicals from leukocytes, including activated macrophages, can damage surrounding cells. This process can drive carcinogenesis by altering targets and pathways that are crucial to normal tissue

homeostasis. NO and NO-derived reactive nitrogen species induce oxidative and nitrosative stress, which result in DNA damage (such as nitrosative deamination of nucleic acid bases, transition and/or transversion of nucleic acids, alkylation and DNA strand breakage) and inhibition of DNA repair enzymes (such as alkyltransferases and DNA ligases) through direct or indirect mechanisms [42]. By contrast, there are several studies that show inhibitory effects of NO on tumorigenesis [47, 48]. DNA damage and/or modification that is produced by NO induces the accumulation of wild-type p53 and activation of poly(ADP-ribose) synthase, and results in death of transformed cells [49, 50]. The induction of mutations in cancer-related genes or post-translational modifications of proteins by nitration, nitrosation, phosphorylation, acetylation or polyADP-ribosylation are some key events that can increase the cancer risk. However, NO may also influence the carcinogenesis process by alteration of cell-cycle checkpoints [51], apoptosis [52] and DNA repair [53]. NO donors sensitize tumor cells to chemotherapeutic compounds by nitrosylation of critical thiols in DNA repair enzymes in hepatoma cell line [54]. Other studies have demonstrated increased susceptibility to chemotherapy to cisplatin [55] and melphalan [56] by NO donors in different cell lines. However, the expression level, duration and timing of NO delivery, the microenvironment, the genetic background and the cell type might determine NO sensitivity and the overall effect of NO [42]. These results implied substantial modifications of key biological target(s) including DNA repair proteins and transcription factors that are known to be inhibited by NO.

Hypoxia, Tumorogenesis and NO

Evidence has accumulated showing that up to 50–60 % of locally advanced solid tumors may exhibit hypoxic and/or anoxic tissue areas that are heterogeneously distributed within the tumor mass. Tumor-to-tumor variability in oxygenation is greater than intra-tumor variability [57]. Major pathogenetic mechanisms involved in the development of hypoxia in solid tumors are (a) severe structural and functional abnormalities of tumor microvessels (perfusion- limited O2 delivery) (b) increased resistance to blood flow (c) deterioration of diffusion geometry (diffusion-limited O2 delivery) (d) tumor-associated and/or therapy-induced anemia leading to a reduced O2 transport capacity of the blood (anemic hypoxia) and (e) fewer functional lymph vessels.

Cells exposed to hypoxia respond by arresting their proliferation and subsequent cell death, thus, *in vitro* hypoxia can hinder or even completely inhibit tumor cell proliferation [57]. Additionally, hypoxia can induce programmed cell death (apoptosis) both in normal and in neoplastic cells via HIF-1and p53. All these features occur under anoxia (short hypoxia); however, sustained hypoxia may promote tumor progression via mechanisms enabling cells to overcome nutritive deprivation, to escape from the hostile environment and to favor unrestricted growth [58]. Tumor hypoxia is classically associated with resistance to radiotherapy, but it has also been shown to diminish the efficacy of certain forms of chemotherapy, of photodynamic therapy and immunotherapy [58–60].

NO and Tumor Angiogenesis

NO has been reported to contribute to tumor biology and vascularization with multiple cellular and molecular effects and sometimes with divergent properties. Early experimental studies have shown that the induction of iNOS in tumor cells promotes angiogenesis (by upregulating VEGF expression), which increases microvascular density and tumor progressions [61–65]. A histological examination of tumor specimens has revealed a significant relationship between high angiogeneic activity (i.e. microvessel density or VEGF expression) and iNOS expression in human brain, head and neck, lung, breast, stomach, colon tumors, etc. [66–75]. These findings definitely indicate that cancer-derived NO mediates tumor angiogenesis, invasion and growth. As we described previously, one of the most important transcription factors that activates the expression of O_2-regulated genes is hypoxia-inducible factor 1 (HIF-1) [76, 77].

The administration of nitroglycerin, isosorbide dinitrate and sodium nitroprusside differentially modulates hypoxia-induced gene expression mediated by HIF-1. SNP treatment reduced HIF-1a protein levels and HIF-1 transcriptional activity in Hep3B cells that were untreated or exposed to hypoxia [78]. Similar results were observed in the human embryonic kidney HEK293 cells and HUVECs, suggesting that the phenomenon is not dependent on the cell or tissue type [79]. Nitroglycerin dramatically decreased the rates of immunoreactive cells for HIF-1α protein in cancer tissues in patients with operable lung adenocarcinoma, beside a decrease in plasma levels of VEGF and P-glycoprotein [80]. This suggested that decreases in plasma VEGF levels following nitroglycerin treatment may enhance chemosensitivity to Docetaxel and Carboplatin via a decrease in HIF-1α-dependent pathways such as P-gp in tumor tissues [80]. Moreover, low-dose of glyceryl trinitrate (GTN) has a consistent, inhibitory effect on prostate-specific antigen (PSA) progression in men with recurrent prostate cancer after primary treatment failure, suggesting a non-random drug effect of GTN [81].

NO and Cell Death Receptors in the Liver

Cell death receptors include CD95 (Apo-1), tumor necrosis factor receptor type 1 (TNF-R1), tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptor 1 (TRAIL-R1/Death receptor 4 [DR 4]); TRAIL receptor 2 (TRAIL-R2/DR5/Killer/TRICK2), death receptor 3 (DR3/Apo-3/TRAMP/WSL-1/LARD), and death receptor 6 (DR6). The administration of an NO donor mediates mitochondrial dysfunction and cell death in cells [82, 83]. The intratumoral injection of microencapsulated NOS-2-expressing cells upregulates the CD95/CD95L system and induces cell death in colon- and ovarian-derived tumors [84]. Similarly, the intratumoral injection of NOS-2 over-expressing plasmids significantly reduced tumor growth developed in medullary thyroid implanted tumor cell cells in nude mice [85].

We have recently shown that the administration of an NO donor or NOS-3 overexpression increased the oxidative/nitrosative stress, CD95/CD95L expression and cell death in HepG2 cells, as well as it increased cell death receptors (TNF-R1, CD95/CD95L and TRAIL-R1) expression and reduced tumor cell growth after implantation of the HepG2 cells in a xenograft mouse model [86, 87]. Our study also showed that p53-mediated induction of cell death and increased CD95 expression were induced by NOS-3 overexpression in HepG2 cells [87]. A series of NO-dependent post-translational modifications also modulate CD95 dependent cell death. Interestingly, both exogenous and endogenous NO induce S-nitrosylation of Cys199 and Cys304 of the CD95 receptor, and this correlates with enhanced expression of CD95 at the plasma membrane and its translocation into lipid rafts [88].

The induction of DNA damage by NO increases p53 accumulation and cell death in transformed cells [49, 50]. NO donors sensitize tumor cells to chemotherapeutic compounds by nitrosylation of critical thiols in DNA repair enzymes in a hepatoma cell line [54]. The induction of extrinsic and intrinsic cell death pathways induced by chemotherapy in hepatocellular carcinoma cells involves increased expression of several p53 gene family members [89], which have been additionally related to increased expressions of TNF-R1, CD95 and TRAIL-R1 in a hepatoma cell line [90]. Other studies have also shown that NO donors increased susceptibility to cisplatin [55] and melphalan [56] in different cell lines.

Acknowledgements We thank the Biomedical Research Network Center for Liver and Digestive Diseases (CIBERehd) founded by “Carlos III” Health Institute for its support.

Conflict Statement No potential conflicts of interest were disclosed.

References

1. Lancaster JR Jr. A tutorial on the diffusibility and reactivity of free nitric oxide. Nitric Oxide. 1997;1:18–30.
2. Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature. 1990;347:768–70.
3. Bredt DS, Snyder SH. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc Natl Acad Sci U S A. 1989;86:9030–3.
4. Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc Natl Acad Sci U S A. 1990;87:682–5.
5. Grasemann H, Drazen JM, Deykin A, Israel E, De Sanctis GT, Pillari A, Yandava CH. Simple tandem repeat polymorphisms in the neuronal nitric oxide synthase gene in different ethnic populations. Hum Hered. 1999;49:139–41.
6. Raghuram N, Fortenberry JD, Owens ML, Brown LA. Effects of exogenous nitric oxide and hyperoxia on lung fibroblast viability and DNA fragmentation. Biochem Biophys Res Commun. 1999;262:685–91.
7. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001;357:593–615.
8. Feron O, Belhassen L, Kobzik L, Smith TW, Kelly RA, Michel T. Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. J Biol Chem. 1996;271:22810–4.

9. Boo YC, Kim HJ, Song H, Fulton D, Sessa W, Jo H. Coordinated regulation of endothelial nitric oxide synthase activity by phosphorylation and subcellular localization. Free Radic Biol Med. 2006;41:144–53.
10. Iwakiri Y, Satoh A, Chatterjee S, Toomre DK, Chalouni CM, Fulton D, Groszmann RJ, Shah VH, Sessa WC. Nitric oxide synthase generates nitric oxide locally to regulate compartmentalized protein S-nitrosylation and protein trafficking. Proc Natl Acad Sci U S A. 2006;103:19777–82.
11. Oess S, Icking A, Fulton D, Govers R, Muller-Esterl W. Subcellular targeting and trafficking of nitric oxide synthases. Biochem J. 2006;396:401–9.
12. Stamler JS. Redox signaling: nitrosylation and related target interactions of nitric oxide. Cell. 1994;78:931–6.
13. Schmidt HH, Walter U. NO at work. Cell. 1994;78:919–25.
14. Friebe A, Koesling D. Regulation of nitric oxide-sensitive guanylyl cyclase. Circ Res. 2003;93:96–105.
15. Stamler JS, Hausladen A. Oxidative modifications in nitrosative stress. Nat Struct Biol. 1998;5:247–9.
16. Rall TW, Sutherland EW. Formation of a cyclic adenine ribonucleotide by tissue particles. J Biol Chem. 1958;232:1065–76.
17. Lohmann SM, Vaandrager AB, Smolenski A, Walter U, De Jonge HR. Distinct and specific functions of cGMP-dependent protein kinases. Trends Biochem Sci. 1997;22:307–12.
18. Degerman E, Belfrage P, Manganiello VC. Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). J Biol Chem. 1997;272:6823–6.
19. Houslay MD, Milligan G. Tailoring cAMP-signalling responses through isoform multiplicity. Trends Biochem Sci. 1997;22:217–224.
20. Zagotta WN, Siegelbaum SA. Structure and function of cyclic nucleotide-gated channels. Annu Rev Neurosci. 1996;19:235–63.
21. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nat Cell Biol. 2001;3:193–7.
22. Espey MG, Thomas DD, Miranda KM, Wink DA. Focusing of nitric oxide mediated nitrosation and oxidative nitrosylation as a consequence of reaction with superoxide. Proc Natl Acad Sci U S A. 2002;99:11127–32.
23. Hess DT, Matsumoto A, Nudelman R, Stamler JS. S-nitrosylation: spectrum and specificity. Nat Cell Biol. 2001;3:E46–49.
24. Govers R, Oess S. To NO or not to NO: ‘where?’ is the question. Histol Histopathol. 2004;19:585–605.
25. Kroncke KD, Carlberg C. Inactivation of zinc finger transcription factors provides a mechanism for a gene regulatory role of nitric oxide. FASEB J. 2000;14:166–73.
26. Bonavida B, Baritaki S, Huerta-Yepez S, Vega MI, Chatterjee D, Yeung K. Novel therapeutic applications of nitric oxide donors in cancer: roles in chemo- and immunosensitization to apoptosis and inhibition of metastases. Nitric Oxide. 2008;19:152–7.
27. Huerta-Yepez S, Vega M, Jazirehi A, Garban H, Hongo F, Cheng G, Bonavida B. Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-kappa B and inhibition of Bcl-xl expression. Oncogene. 2004;23:4993–5003.
28. Jazirehi AR, Bonavida B. Cellular and molecular signal transduction pathways modulated by rituximab (rituxan, anti-CD20 mAb) in non-Hodgkin’s lymphoma: implications in chemosensitization and therapeutic intervention. Oncogene. 2005;24:2121–43.
29. Marshall HE, Merchant K, Stamler JS. Nitrosation and oxidation in the regulation of gene expression. FASEB J. 2000;14:1889–900.
30. Abate C, Patel L, Rauscher FJ, 3rd, Curran T. Redox regulation of fos and jun DNA-binding activity in vitro. Science. 1990;249:1157–61.
31. Nikitovic D, Holmgren A, Spyrou G. Inhibition of AP-1 DNA binding by nitric oxide involving conserved cysteine residues in Jun and Fos. Biochem Biophys Res Commun. 1998;242:109–12.

32. Klatt P, Molina EP, Lamas S. Nitric oxide inhibits c-Jun DNA binding by specifically targeted S-glutathionylation. J Biol Chem. 1999;274:15857–64.
33. Rainwater R, Parks D, Anderson ME, Tegtmeyer P, Mann K. Role of cysteine residues in regulation of p53 function. Mol Cell Biol. 1995;15:3892–903.
34. Ema M, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, Poellinger L, Fujii-Kuriyama Y. Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J. 1999;18:1905–14.
35. Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem. 1991;266:4244–50.
36. Hanafy KA, Krumenacker JS, Murad F. NO, nitrotyrosine, and cyclic GMP in signal transduction. Med Sci Monit. 2001;7:801–19.
37. Zou MH, Hou XY, Shi CM, Nagata D, Walsh K, Cohen RA. Modulation by peroxynitrite of Akt- and AMP-activated kinase-dependent Ser1179 phosphorylation of endothelial nitric oxide synthase. J Biol Chem. 2002;277:32552–7.
38. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. J Biol Chem. 2003;278:22546–54.
39. Ischiropoulos H. Biological selectivity and functional aspects of protein tyrosine nitration. Biochem Biophys Res Commun. 2003;305:776–83.
40. Lander HM. An essential role for free radicals and derived species in signal transduction. FASEB J. 1997;11:118–24.
41. Lala PK, Chakraborty C. Role of nitric oxide in carcinogenesis and tumor progression. Lancet Oncol. 2001;2:149–56.
42. Fukumura D, Kashiwagi S, Jain RK. The role of nitric oxide in tumor progression. Nat Rev Cancer. 2006;6:521–34.
43. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57–70.
44. Bissell MJ, Radisky D. Putting tumors in context. Nat Rev Cancer. 2001;1:46–54.
45. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002;2:161–74.
46. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860–7.
47. Hofseth LJ, Hussain SP, Wogan GN, Harris CC. Nitric oxide in cancer and chemoprevention. Free Radic Biol Med. 2003;34:955–68.
48. Crowell JA, Steele VE, Sigman CC, Fay JR. Is inducible nitric oxide synthase a target for chemoprevention? Mol Cancer Ther. 2003;2:815–23.
49. Ambs S, Merriam WG, Ogunfusika MO, Bennett WP, Ishibe N, Hussain SP, Tzeng EE, Geller DA, Billiar TR, Harris CC. p53 and vascular endothelial growth factor regulate tumor growth of NOS2-expressing human carcinoma cells. Nat Med. 1998;4:1371–6.
50. Zhang J, Dawson VL, Dawson TM, Snyder SH. Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. Science. 1994;263:687–9.
51. Pervin S, Singh R, Chaudhuri G. Nitric oxide-induced cytostasis and cell cycle arrest of a human breast cancer cell line (MDA-MB-231): potential role of cyclin D1. Proc Natl Acad Sci U S A. 2001;98:3583–8.
52. Melino G, Bernassola F, Knight RA, Corasaniti MT, Nistico G, Finazzi-Agro A. S-nitrosylation regulates apoptosis. Nature. 1997;388:432–3.
53. Jaiswal M, LaRusso NF, Nishioka N, Nakabeppu Y, Gores GJ. Human Ogg1, a protein involved in the repair of 8-oxoguanine, is inhibited by nitric oxide. Cancer Res. 2001;61:6388–93.
54. Laval F, Wink DA. Inhibition by nitric oxide of the repair protein, O6-methylguanine-DNA-methyltransferase. Carcinogenesis. 1994;15:443–7.
55. Wink DA, Cook JA, Christodoulou D, Krishna MC, Pacelli R, Kim S, DeGraff W, Gamson J, Vodovotz Y, Russo A, Mitchell JB. Nitric oxide and some nitric oxide donor compounds enhance the cytotoxicity of cisplatin. Nitric Oxide. 1997;1:88–94.
56. Cook JA, Krishna MC, Pacelli R, DeGraff W, Liebmann J, Mitchell JB, Russo A, Wink DA. Nitric oxide enhancement of melphalan-induced cytotoxicity. Br J Cancer. 1997;76:325–34.

57. Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev. 2007;26:225–39.
58. Vaupel P, Mayer A, Hockel M. Tumor hypoxia and malignant progression. Methods Enzymol. 2004;381:335–54.
59. Vaupel P. The role of hypoxia-induced factors in tumor progression. Oncologist. 2004;9(Suppl 5):10–7.
60. Vaupel P, Thews O, Hoeckel M. Treatment resistance of solid tumors: role of hypoxia and anemia. Med Oncol. 2001;18:243–59.
61. Jenkins DC, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, Moncada S. Roles of nitric oxide in tumor growth. Proc Natl Acad Sci U S A. 1995;92:4392–6.
62. Chin K, Kurashima Y, Ogura T, Tajiri H, Yoshida S, Esumi H. Induction of vascular endothelial growth factor by nitric oxide in human glioblastoma and hepatocellular carcinoma cells. Oncogene. 1997;15:437–42.
63. Frank S, Stallmeyer B, Kampfer H, Schaffner C, Pfeilschifter J. Differential regulation of vascular endothelial growth factor and its receptor fms-like-tyrosine kinase is mediated by nitric oxide in rat renal mesangial cells. Biochem J. 1999;338(Pt 2):367–74.
64. Jadeski LC, Hum KO, Chakraborty C, Lala PK. Nitric oxide promotes murine mammary tumor growth and metastasis by stimulating tumor cell migration, invasiveness and angiogenesis. Int J Cancer. 2000;86:30–9.
65. Yang HT, Yan Z, Abraham JA, Terjung RL. VEGF(121)- and bFGF-induced increase in collateral blood flow requires normal nitric oxide production. Am J Physiol Heart Circ Physiol. 2001;280:H1097–104.
66. Thomsen LL, Lawton FG, Knowles RG, Beesley JE, Riveros-Moreno V, Moncada S. Nitric oxide synthase activity in human gynecological cancer. Cancer Res. 1994;54:1352–4.
67. Cobbs CS, Brenman JE, Aldape KD, Bredt DS, Israel MA. Expression of nitric oxide synthase in human central nervous system tumors. Cancer Res. 1995;55:727–30.
68. Gallo O, Masini E, Morbidelli L, Franchi A, Fini-Storchi I, Vergari WA, Ziche M. Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. J Natl Cancer Inst. 1998;90:587–96.
69. Kojima M, Morisaki T, Tsukahara Y, Uchiyama A, Matsunari Y, Mibu R, Tanaka M. Nitric oxide synthase expression and nitric oxide production in human colon carcinoma tissue. J Surg Oncol. 1999;70:222–9.
70. Swana HS, Smith SD, Perrotta PL, Saito N, Wheeler MA, Weiss RM. Inducible nitric oxide synthase with transitional cell carcinoma of the bladder. J Urol. 1999;161:630–4.
71. Marrogi AJ, Travis WD, Welsh JA, Khan MA, Rahim H, Tazelaar H, Pairolero P, Trastek V, Jett J, Caporaso NE, Liotta LA, Harris CC. Nitric oxide synthase, cyclooxygenase 2, and vascular endothelial growth factor in the angiogenesis of non-small cell lung carcinoma. Clin Cancer Res. 2000;6:4739–44.
72. Vakkala M, Kahlos K, Lakari E, Paakko P, Kinnula V, Soini Y. Inducible nitric oxide synthase expression, apoptosis, and angiogenesis in in situ and invasive breast carcinomas. Clin Cancer Res. 2000;6:2408–16.
73. Aaltoma SH, Lipponen PK, Kosma VM. Inducible nitric oxide synthase (iNOS) expression and its prognostic value in prostate cancer. Anticancer Res. 2001;21:3101–6.
74. Uotila P, Valve E, Martikainen P, Nevalainen M, Nurmi M, Harkonen P. Increased expression of cyclooxygenase-2 and nitric oxide synthase-2 in human prostate cancer. Urol Res. 2001;29:23–8.
75. Massi D, Franchi A, Sardi I, Magnelli L, Paglierani M, Borgognoni L, Maria Reali U, Santucci M. Inducible nitric oxide synthase expression in benign and malignant cutaneous melanocytic lesions. J Pathol. 2001;194:194–200.
76. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci U S A. 1995;92:5510–4.

77. Hirota K. Hypoxia-inducible factor 1, a master transcription factor of cellular hypoxic gene expression. J Anesth. 2002;16:150–9.
78. Leek RD, Stratford I, Harris AL. The role of hypoxia-inducible factor-1 in three-dimensional tumor growth, apoptosis, and regulation by the insulin-signaling pathway. Cancer Res. 2005;65:4147–52.
79. Takabuchi S, Hirota K, Nishi K, Oda S, Oda T, Shingu K, Takabayashi A, Adachi T, Semenza GL, Fukuda K. The inhibitory effect of sodium nitroprusside on HIF-1 activation is not dependent on nitric oxide-soluble guanylyl cyclase pathway. Biochem Biophys Res Commun. 2004;324:417–23.
80. Yasuda H, Nakayama K, Watanabe M, Suzuki S, Fuji H, Okinaga S, Kanda A, Zayasu K, Sasaki T, Asada M, Suzuki T, Yoshida M, Yamanda S, Inoue D, Kaneta T, Kondo T, Takai Y, Sasaki H, Yanagihara K, Yamaya M. Nitroglycerin treatment may enhance chemosensitivity to docetaxel and carboplatin in patients with lung adenocarcinoma. Clin Cancer Res. 2006;12:6748–57.
81. Siemens DR, Heaton JP, Adams MA, Kawakami J, Graham CH. Phase II study of nitric oxide donor for men with increasing prostate-specific antigen level after surgery or radiotherapy for prostate cancer. Urology. 2009;74:878–83.
82. Kurose I, Miura S, Fukumura D, Yonei Y, Saito H, Tada S, Suematsu M, Tsuchiya M. Nitric oxide mediates Kupffer cell-induced reduction of mitochondrial energization in hepatoma cells: a comparison with oxidative burst. Cancer Res. 1993;53:2676–82.
83. Abou-Elella AM, Siendones E, Padillo J, Montero JL, De la Mata M, Muntane Relat J. Tumor necrosis factor-alpha and nitric oxide mediate apoptosis by D-galactosamine in a primary culture of rat hepatocytes: exacerbation of cell death by cocultured Kupffer cells. Can J Gastroenterol. 2002;16:791–9.
84. Xu W, Liu L, Charles IG. Microencapsulated iNOS-expressing cells cause tumor suppression in mice. FASEB J. 2002;16:213–5.
85. Soler MN, Bobe P, Benihoud K, Lemaire G, Roos BA, Lausson S. Gene therapy of rat medullary thyroid cancer by naked nitric oxide synthase II DNA injection. J Gene Med. 2000;2:344–52.
86. Aguilar-Melero P, Ferrin G, Muntane J. Effects of nitric oxide synthase-3 overexpression on post-translational modifications and cell survival in HepG2 cells. J Proteomics. 2012;75:740–55.
87. Gonzalez R, Ferrin G, Aguilar-Melero P, Ranchal I, Linares CI, Bello RI, De la Mata M, Gogvadze V, Barcena JA, Alamo JM, Orrenius S, Padillo FJ, Zhivotovsky B, Muntane J. Targeting hepatoma using nitric oxide donor strategies. Antioxid Redox Signal. 2013;18:491–506.
88. Leon-Bollotte L, Subramaniam S, Cauvard O, Plenchette-Colas S, Paul C, Godard C, Martinez-Ruiz A, Legembre P, Jeannin JF, Bettaieb A. S-nitrosylation of the death receptor fas promotes fas ligand-mediated apoptosis in cancer cells. Gastroenterology. 2011;140:2009–18, 2018.e1–4.
89. Seitz SJ, Schleithoff ES, Koch A, Schuster A, Teufel A, Staib F, Stremmel W, Melino G, Krammer PH, Schilling T, Muller M. Chemotherapy-induced apoptosis in hepatocellular carcinoma involves the p53 family and is mediated via the extrinsic and the intrinsic pathway. Int J Cancer. 2010;126:2049–66.
90. Gressner O, Schilling T, Lorenz K, Schulze Schleithoff E, Koch A, Schulze-Bergkamen H, Lena AM, Candi E, Terrinoni A, Catani MV, Oren M, Melino G, Krammer PH, Stremmel W, Muller M. TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. EMBO J. 2005;24:2458–71.

Chapter 16
Discovery and Development of RRx-001, a Novel Nitric Oxide and ROS Mediated Epigenetic Modulator

Jan Scicinski, Bryan Oronsky, Shoucheng Ning, Gary R. Fanger, Susan J. Knox and Mark Bednarski

Abstract Methods to find compounds that interact favorably with anti-cancer biological targets typically start from a limited set of structural types and focus on target-based approaches resulting in limited breakthroughs, mostly incremental improvements, and many structurally similar compounds that fall into 'me too' or 'me better' categories. Due to the very low success rate for drug development in oncology, different, 'non-me too' approaches that utilize novel chemotypes together with phenotypic discovery approaches are required to provide truly novel treatments. The defense and aerospace industry offers a huge resource of largely untapped chemical diversity that lends itself to a phenotypic approach to drug discovery. Herein we describe a novel program focused on discovering drug candidates from energetic materials that arose from a unique partnership between a defense contractor specializing in the research and utilization of energetic materials and an academic institution, under the umbrella of a start-up and with an innovative funding and organizational structure. We describe the most advanced compound, RRx-001, the first of a new class of NO-mediated epigenetic anticancer agents that bind hemoglobin and drive RBC-mediated redox reactions under hypoxia.

Keywords Cancer · Chemotherapy · Epigenetic resensitization · Nitric oxide · Oxidation · Radiotherapy

J. Scicinski (✉) · B. Oronsky · G. R. Fanger
EpicentRx, Inc, 800 W El Camino Real, Suite 180, Mountain View, CA 94040, USA
e-mail: jscicinski@epicentrx.com

S. Ning · S. J. Knox
Department of Radiation Oncology, Stanford University Medical Center, Stanford, CA 94305, USA

M. Bednarski
Department of Radiology, Stanford University Medical Center, Stanford, CA 94305, USA

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_16

Abbreviations

ABAZ	N-bromoacetylazetidine
ADNAZ	N-acetyl-3,3-dinitroazetidine
BSO	Buthionine sulphoximine
CDDP	Cisplatin
CEA	carcinoembryonic antigen
CMC	Chemistry, Manufacturing and Controls
CMO	Contract Manufacturing Organization
CPT	Cold Pressor Test
DLT	Dose Limiting Toxicity
DNMT	DNA methyltransferase
FOLFIRI	Chemotherapy regimen for colorectal cancer: Folinic acid/ Fluorouracil/irinotecan
GMP	Good Manufacturing practice
HDAC	Histoine deacetylases
HMNAZ	1-tert-butyl-3- hydroxymethyl-3-nitroazetidine
IP	Intraperitoneal route of administration
IV	Intravenous route of administration
MTase	Methyl transferases
MTD	Maximum Tolerated Dose
NAC	N-acetyl Cysteine
NO	Nitric Oxide
PO	Oral route of administration
QD	Once daily
RBC	Red Blood Cell
RNS	Reactive Nitrogen Species
RONS	Reactive Oxygen and Nitrogen Species
ROS	Reactive Oxygen Species
SCCVII	A syngeneic murine squamous cell carcinoma cell line
TBDNAZ	1-tert-butyl-3,3-dinitroazetidine
TNAZ	Trinitroazetidine

Introduction

Cancer accounts for approximately one-quarter of deaths in the United States and is second only to ischemic heart disease as a cause of mortality [1]. Improving the treatment of cancer, therefore, represents an important therapeutic endeavor. The ability of an anti-cancer agent to confer very high specificity to tumors with low toxicity to normal tissues is one of the holy grails of medical oncology.

Natural products are potential sources of novel pharmacophores that are, on the whole, structurally dissimilar from the mainstream, though in general they have fallen out of favor due to complications and challenges with toxicity and manufacture or isolation. Thus, as a generalization, with few exceptions, most new chemical

entities in cancer are based on established chemical structures with similar pharmacological activities targeting the same or related mechanisms. This kind of imitation arises from traditional target and structure-based drug discovery approaches that are focused on identifying and optimizing compounds with 'on-target' specificity and selectivity profiles that often follow in the footsteps of popular and profitable predecessors like Gleevec.

Methods to find compounds that interact favorably with biological targets typically start from a limited set of structural types, often reflecting the molecular sub-types that are seen in biological systems such as aromatic heterocycles and/or amino acid-like compounds. Regardless of the drug discovery source, e.g. high throughput screening or fragment-based drug discovery, the emerging molecules often end up looking similar, leading to intellectual property and freedom to operate issues [2–4] While research breakthroughs do occur, improvements in treatments tend to be incremental, with many compounds falling into the 'me too' or 'me better' category. Clearly, this copycat strategy has failed to live up to expectations as the current success rate for drug development in oncology is approximately 5%, and more effective alternatives and strategies are required.

By contrast, the defense and aerospace industries offer a huge untapped resource of chemical diversity for the discovery and development of novel drugs for a variety of indications. The relatively easy accessibility of these materials together with their unique structures lend themselves well to phenotypic approaches to the discovery of new drug candidates [5]. Here, we describe the discovery and development of RRx-001, arising from a unique partnership between a defense contractor specializing in the discovery, synthesis, large scale manufacture and utilization of energetic materials and an academic institution, under the umbrella of a start-up and with an innovative funding and organizational structure set up to discover and develop novel medicinal agents using energetic materials as start-points.

Energetic Materials as Medicinal Agents

The subset of chemical compounds known as energetic materials store and release significant quantities of energy in the process of breaking chemical bonds; these characteristics are 'tinker-friendly', either through modification of their chemical scaffolds or through the preparation of mixtures of materials for controllable storage and release of the chemical energy under specific conditions [6, 7]. In addition, many of the materials used in solid propulsion motors and military charges are extensively evaluated for human and environmental toxicity, allowing information on the relative toxicity of constituent functional groups to inform the drug discovery process [8, 9].

Molecular free radicals, which mediate the effects of radiation therapy, are important intermediaries in the decomposition of these compounds. Under the leadership of Stanford University radiation oncologist, Dr. Susan Knox, radiologist Dr. Mark Bednarski, and venture capitalist, Dr. Arnold Oronsky, at InterWest Partners,

a collaborative research program with the defense contractor ATK Thiokol was initiated and a company, RadioRx Inc, was formed to identify potential therapeutic leads from these energetic molecules. This unique collaboration led to the discovery and development of a highly innovative anticancer compound, called RRx-001.

An initial set of commonly used energetic compounds (Table 16.1) was selected in collaboration between Stanford University and ATK Thiokol and sent for initial evaluation at Stanford. Compounds were shipped as solutions in DMSO to minimize transportation hazards. Most of the materials tested *in vitro* showed moderate to good activity against tumor cells in a methyl tetrazolium (MTT) assay.

In a separate assay, a subset of these were discovered to augment the effect of ionizing radiation on cancer cells; within this subset the highly energetic molecule, TNAZ [10], not only demonstrated promising single agent activity, but also radiosensitizing properties *in vivo*, confirming the initial hypothesis (Fig. 16.1). As shown in Fig. 16.1, below, the activity of two 2 mg/kg doses of TNAZ, combined with a single 10 Gy dose of radiation showed favorable anti-tumor properties in the SCCVII syngeneic mouse tumor model.

In spite of its highly promising activity, TNAZ was less than an ideal clinical candidate, owing to its explosive properties, which would have led to potentially difficult CMC and regulatory hurdles. Consequently, lead optimization prioritized not only improved activity, both as a single agent and in combination with radiation, but also the identification of a molecule that, unlike TNAZ, would be safe to manufacture, handle and transport. A series of compounds were prepared [11] based on the dinitroazetidine scaffold (Table 16.2) and evaluated *in vitro*, of which RRx-001 was the most promising.

RRx-001 represents a new class of compounds that was rationally designed by combining two structural components: a dinitroazetidine, derived from TNAZ and an α-bromoacetamide. Mark Bednarski's rationale for combining these two structural elements was twofold: (1) to reduce explosive potential and (2) to increase biological activity while enabling the assembly of antibody and prodrug constructs. The individual contribution of these two functionalities to the overall activity of the molecule was assessed by the synthesis and biological evaluation of close analogs of RRx-001 in which these key functionalities were replaced. To probe the effect of the gem-dinitro group, a molecule containing an unsubstituted azetidine, and lacking the gem-dinitro group, (ABAZ, N-bromoacetylazetidine) was synthesized. The importance of the bromoacetamide moiety was assessed by preparation of N-acetyl dinitroazetidine (ADNAZ) (Fig. 16.2).

The activity of these structural analogues was compared *in vitro* and *in vivo* in the mouse SCCVII syngeneic tumor model against RRx-001. While RRx-001 was found to be the most potent of the analogs, the data supported the conclusion that both the gem-dinitro and bromoacetamide groups are required for optimal activity. The bromoacetamide group likely serves a dual function—depletion of glutathione (and glutathione precursors) leading to increase in ROS and RNS and conjugation to an endogenous carrier molecule, RBCs, by hemoglobin adduction (see below). In addition, due to the presence of this alkylating center, RRx-001 could readily be linked to antibodies and prodrugs, resulting in macromolecules that had promising activity in both tumor cell lines and in animal models.

Table 16.1 Initial screening set of energetic molecules. IC_{50} values are from an MTT assay in HT29 cells

No.	Name	Abbreviation	IC_{50} (mM)
1	4,4'-diamino-3,3'azoxyfurazan	DAAOF	0.011
2	1,3,3-trinitroazetidine	TNAZ	0.022
3	N-Acetyl-3,3-dinitroazetidine	ADNAZ	0.04
4	1-tert-butyl-3,3-dinitroazetidium trifluoroacetate	TBDNAZ	0.041
5	2,4,6,8,10,12-hexanitro-2-12-hexaazatetracyclo-dodecane	CL-20	0.07
6	4,10-dinitro-2,6,8,12-tetraoxa-4,10-diazatetrocyclo [5.5.0.0.sup.5,9,0.sup.3,11]-dodecane	TEX	0.088
7	1,1,1-trimethylolethane trinitrate	TMETN	0.089
8	Glycidyl nitrate	GLYN	0.13
9	N-butyl-2-nitratoethyl-nitramine	BuNENA	0.13
10	Cyclotetramethylene tetranitramine	HMX	0.14
11	Diglycerol tetranitrate	DGTN	0.35
12	3,4'-diaminofurazan	DAF	0.56
13	2,3,5,6-tetrahydroxyl-1,4-diformyl- piperazine	THDFP	0.63
14	Hexahydro-1,3,5-trinitro-1,3,5-triazine	RDX	0.70
15	Triethylene glycol dinitrate	TEGDN	1.33
16	3,3-bis-nitratomethyl oxetane	BNMO	2.2
17	Ammonium Dinitriamide	ADN	4.46
18	Nitrotriazolone	NTO	4.5
19	3-nitratomethyl-3-methyl oxetane	NMMO	4.5
20	Pentaerythrite tetranitrate	PETN	5.8

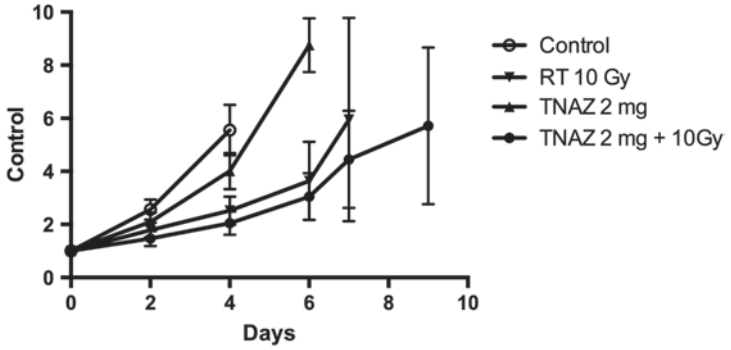
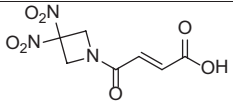
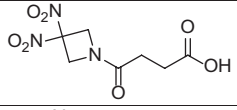
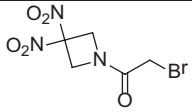
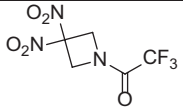
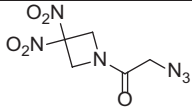
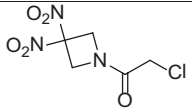
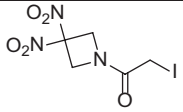


Fig. 16.1 Activity of two 2 mg/kg doses of TNAZ with or without a single 10 Gy dose of radiation against control in the SCCVII syngeneic mouse tumor model

Table 16.2 Activity of close analogs of RRx-001. IC_{50} values are from an MTT assay in HT29 cells

Name	Structure	Abbreviation	IC_{50} (mM)
1-Fumaryl-3,3-dinitroazetidine		FDNAZ	0.029
1-Succinyl-3,3-dinitroazetidine		SDNAZ	0.087
1-Acetylbromo-3,3-dinitroazetidine		ABDNAZ, RRx-001	0.001
1-Trifluoroacetyl-3,3-dinitroazetidine		TFADNAZ	0.092
1-Azidoacetyl-3,3-dinitroazetidine		AzADNAZ	0.07
1-Chloroacetyl-3,3-dinitroazetidine		ClADNAZ	0.011
1-Iodoacetyl-3,3-dinitroazetidine		IADNAZ	0.0014

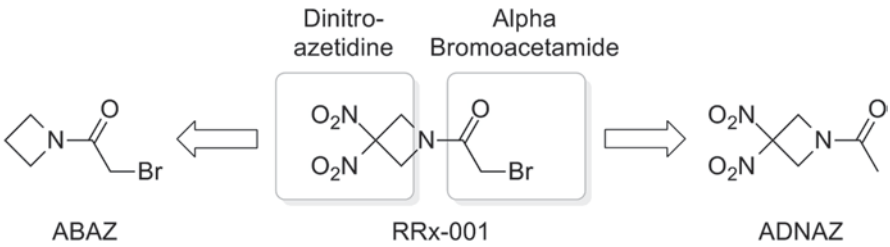


Fig. 16.2 Probing structure activity relationships of RRx-001

Anti-Tumor Activity of RRx-001

RRx-001 was highly active as a single agent *in vitro* against a variety of cell lines (Table 16.3) under conditions of different oxygen concentrations and *in vivo* against multiple tumor types (Figs. 16.3 and 16.4). Moreover, it also synergized with radiation therapy, while acting as a radioprotectant to normal GI epithelium while activity was retained through multiple routes of administration (IV, IP, PO and SC) [12].

Single dose activity of RRx-001 was investigated in a murine SCCVII syngeneic tumor model. Mice bearing SCCVII tumors were treated with a single 15 mg/kg IP injection of RRx-001 or cisplatin (CDDP) 10 mg/kg. The growth inhibitory effects over time were determined by measurement of tumor volumes. At these doses, both drugs exhibited similar activity in reducing tumor growth. In addition, from observations of the animals during the study, RRx-001 treated mice appeared healthier than the cisplatin-dosed mice in terms of their coat condition and lack of substantial weight loss (Fig. 16.3).

The activity of multiple doses of RRx-001 in combination with radiation was determined in the murine syngeneic tumor models. Radiotherapy was administered

Table 16.3 In vitro activity in tumor cell lines as a single agent

Cell line	Cell line type	Mean IC_{50} (μM)	S.D.
SCCVII	SCC	1.8	0.3
PANC1	Pancreatic cancer	2.3	0.7
22B	Oral SCC	2.3	0.5
M21	Melanoma	2.6	0.5
U87	Glioblastoma	2.7	0.6
RKO	Colon carcinoma	3.0	0.4
HT-29	Colorectal adenocarcinoma	3.4	0
SNB75	Glioblastoma	3.8	1.4
MCF-7	Breast adenocarcinoma	4.0	0.5
A498	Renal cell carcinoma	4.9	1.3
IMR32	Brain neuroblastoma	5.1	0
A549	Non-small cell lung carcinoma	6.0	1.8

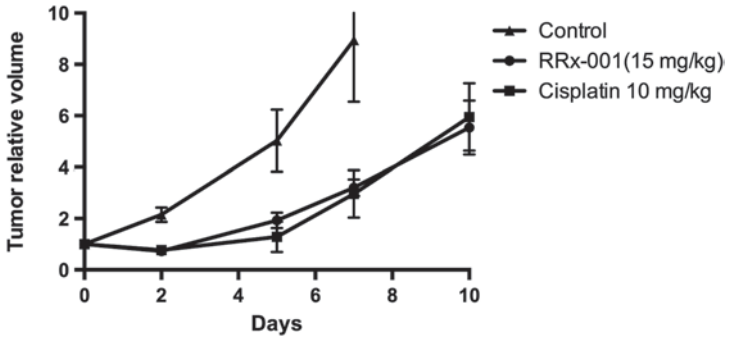


Fig. 16.3 Single dose antitumor activity of RRx-001 compared to cisplatin in the murine SCCVII tumor model

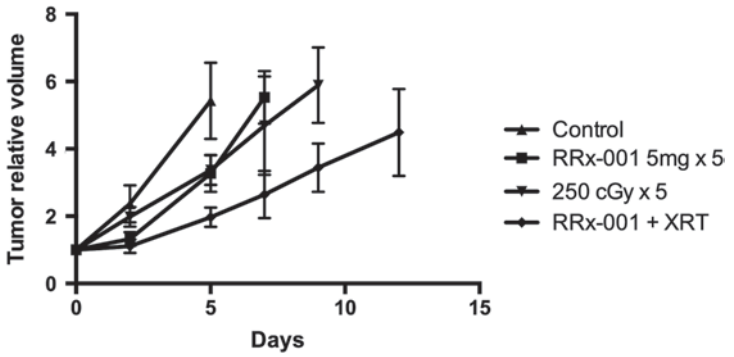


Fig. 16.4 Potentiation of the effect of radiation with RRx-001 in the murine SCCVII tumor model

daily at 250 cGy for 5 days (Fig. 16.4). RRx-001 was dosed IP at 5 mg/kg QD for 5 days. As a single agent, RRx-001 inhibited tumor growth. The mean tumor size exposed to the combination therapy was significantly smaller than the mean tumor size in animals treated with RRx-001 or radiation alone and the combined effect of RRx-001 and radiation was determined to be synergistic. During this study, animals dosed with RRx-001 as a single agent or in combination with radiation did not exhibit overt systemic toxicity as determined by weight loss. In addition, no significant hematological, biochemical, or histopathological changes were observed [12].

Metabolism of RRx-001

The development of drugs that actively alkylate biological targets is generally a medicinal chemistry strategy to be avoided [13]. However, alkylating agents are surprisingly common among approved products, which have led to a recent upsurge

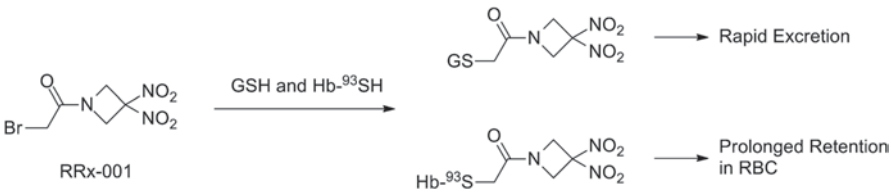


Fig. 16.5 Metabolism of RRx-001

of interest in these compounds [14–16]. In the context of anti-cancer field, the set of compounds typically referred to as alkylating agents is usually limited to those that irreversible bind nucleic acids, proteins, amino acids and other biomolecules that contain strong nucleophiles, leading to DNA damage, inactivation of repair functions and ultimately cell death. The major limitation of these compounds is a lack of selectivity leading to significant normal tissue toxicity. Binding to DNA leads to toxicity such as neutropenia and myelosuppression, however, additional non-selective, irreversible binding to protein thiols can result in unpredictable toxic side effects. These characteristics have stigmatized alkylating agents, resulting in a barrier to their development as therapeutic agents.

However, the key to their successful design and development is selectivity in binding. Strategies to minimize potential off-target effects that could lower the risk of unexpected toxicity, include the incorporation of a weakly alkylating moiety in a molecule that is optimized to fit precisely into the corresponding binding site on the target, like a lock and key, enabling the alkylating function to react with a nucleophile embedded there [16]. An alternative strategy is to combine rapid reactivity and permeability for faster binding kinetics.

RRx-001 possesses excellent permeability characteristics, while high reactivity enables rapid diffusion into RBCs and quantitative binding to glutathione and the cysteine 93 on the beta chain of hemoglobin (Fig. 16.5) [17]. The glutathione adduct is rapidly excreted, while the RRx-001 bound hemoglobin remains in circulation. Unlike typical anti-cancer alkylating agents, RRx-001 does not bind DNA and, hence, systemic toxicities, including myelosuppression, mucositis and neuropathy are not present.

Hemoglobin Binding and Local Release of Nitric Oxide

Studies on the metabolism of RRx-001 confirmed that, on infusion, the molecule permeates into RBCs and rapidly and irreversibly binds to glutathione and hemoglobin. Binding to glutathione via the bromoacetamide group leads to depletion of glutathione and glutathione precursors in RBCs, increasing oxidative stress. In addition, RRx-001 binds to the beta Cys-93 residue of hemoglobin, a highly conserved and critically important residue that modulates hemoglobin oxygen affinity and is implicated in nitric oxide transport [18, 19]. As shown in Fig. 16.6, modeling

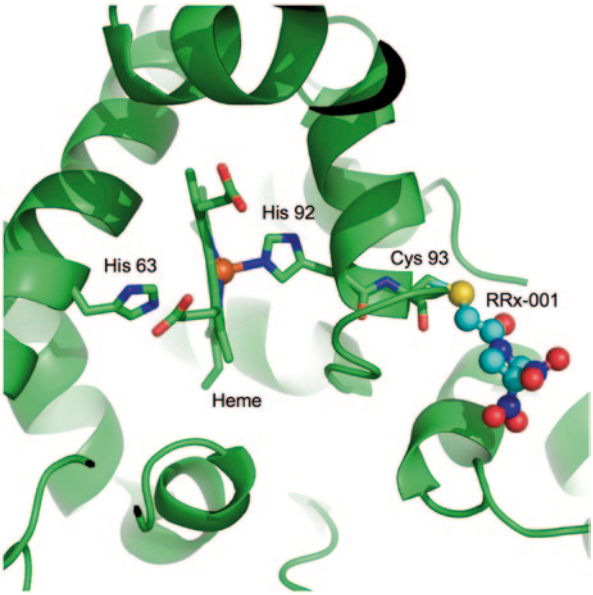


Fig. 16.6 Modeling demonstrates proximity of beta-Cys-93 binding to heme. Binding this residue changes heme oxygen affinity (Modeling based unbound Hb, no conformational changes modeled)

demonstrates the proximity of RRx-001 bound beta-Cys-93 to His 92 residue and Heme.

The binding of RRx-001 to this residue results in the immediate release of a nitric oxide (NO) burst, that is suggested by the presence of marked local vasodilation and transient infusion pain, which resolves immediately on stopping the infusion. Exhaled NO breath testing in hamsters before, during and after infusion (Fig. 16.7) confirmed a rise in nitric oxide levels. The release of NO was found to correlate strongly with infusion duration [20].

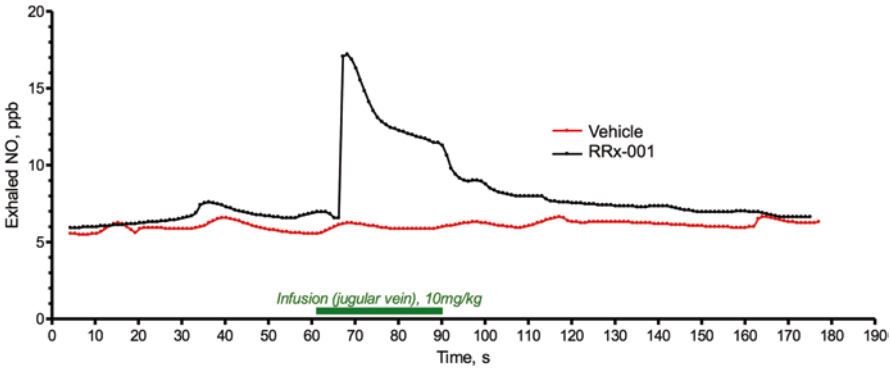


Fig. 16.7 Nitric oxide levels in hamster breath after jugular vein infusion

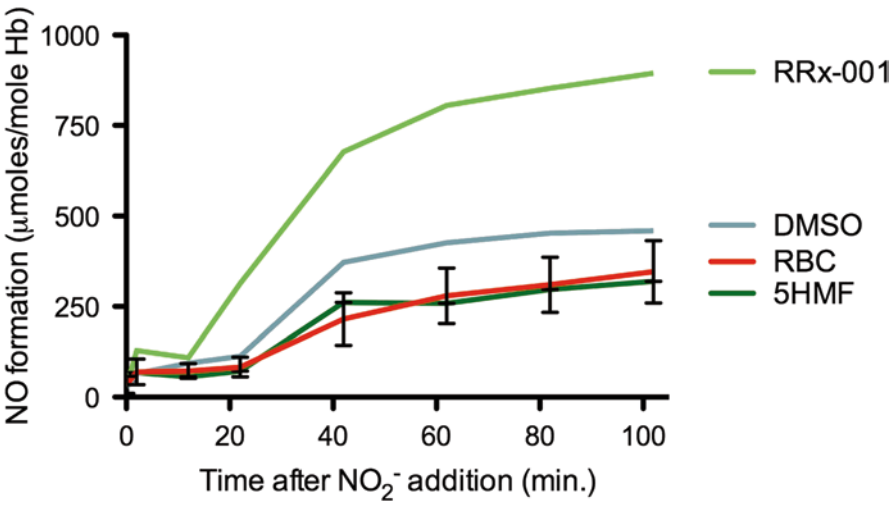


Fig. 16.8 Potentiated reduction of nitrite to nitric oxide by RRx-001-bound RBCs

The selective and specific modifications of hemoglobin in a small sub-population of RBCs are responsible for the catalytic overproduction of nitric oxide. Although deoxygenated hemoglobin can function as a nitrite reductase converting the inorganic anion nitrite into NO under hypoxic conditions [21–23], the binding of RRx-001 to this residue greatly amplifies and accelerates this catalytic reaction [24]. This *in situ* generation of ROS/RNS under hypoxia shifts the biocharacter of the tumor microenvironment from habitable to inhabitable, whereas the ultrashort lifetime of ROS and RNS confines their activity to the tumor, sparing normal tissues from toxicity.

The selective delivery of a high flux of nitric oxide specifically under hypoxic/ischemic conditions when oxygen-dependent nitric oxide synthases (NOS) are non-functional has great potential in several pathologic states such as hemorrhagic shock, sickle cell disease, malaria, as well as cancer, where tissue hypoxia plays an important role. It also differentiates RRx-001 from other nitric oxide donors, such as the nitrate esters, furoxans, benzofuroxans, NONOates, S-nitrosothiols, and metal complexes that release NO systemically rather than locally, thereby potentially leading to NO-induced systemic toxicity, such as headaches, hypotension and methemoglobinemia [25]. In contrast, in preclinical toxicology studies, RRx-001 was not associated with any systemic toxicity at doses that were considered to be therapeutic.

Moreover, unlike these small molecule NO donors, RRx-001 has an extended half-life on the order of days to months since RBCs, and therefore the deoxyhemoglobin contained in the RBCs, to which RRx-001 is bound, has a half life of approximately 60 days. Therefore, these RRx-001-loaded RBCs, which behave essentially as NO prodrugs, produce and deliver nitric oxide at a sustained but accelerated rate under hypoxia for the lifetime of the RBC (Fig. 16.9).

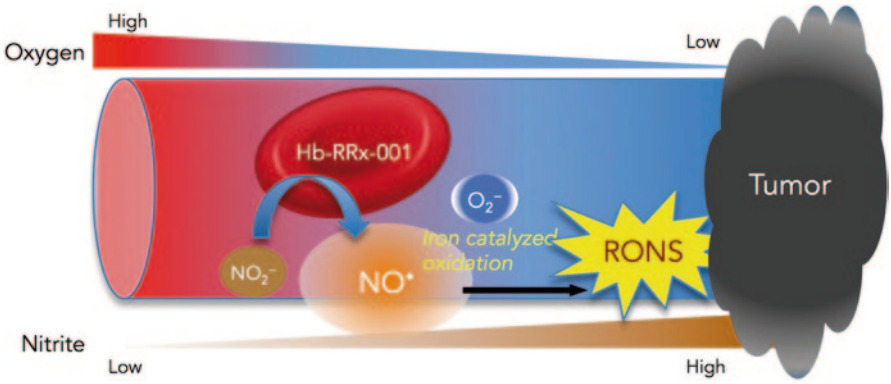


Fig. 16.9 Nitric oxide release "On Demand". Under hypoxia RRx-001 co-opted RBCs, produced nitric oxide which combines with superoxide and other reactive oxygen species (ROS) to produce the potent oxidant, peroxynitrite, and other reactive oxygen and nitrogen species (RONS)

RRx-001 Increases Nitrooxidative Stress In Tumors

The RBCs containing RRx-001 bound hemoglobin become oxidatively stressed as described above. This small subset of RRx-001 co-opted and oxidatively stressed RBCs act as delivery agents, shedding free radicals, diffusible oxidation products, chemokines and cytokines that are preferentially toxic and selectively target the tumor microenvironment. The basis for therapeutic selectivity is a controlled release of these endothelial cytotoxins under hypoxic conditions leading to excess free radicals in tumors pushing tumors into oxidative overload, DNA damage and cell death (Fig. 16.10).

Higher levels of oxidative stress compared to normal tissues are a hallmark of tumors. RRx-001 delivers nitric oxide together with RBC oxidation products to tumors under hypoxia, which leads to the formation of peroxynitrite, N_2O_3 and other reactive and toxic nitrogen oxides. These exert an additional oxidative burden on the already oxidatively susceptible tumor cells by transforming cellular stress from oxidative only to nitro-oxidative. The reactive nitrogen species such as peroxynitrite exert their harmful effects on the tumor directly and indirectly. They oxidize critical cysteine residues on the epigenetic regulators HDACs and DNA MTases, inhibiting them, which leads to p53 reactivation. $ONOO^-$ induced DNA damage can both lead to apoptosis and to further activation of repair processes that indirectly but eventually lead to ATP depletion and necrosis (Fig. 16.11).

In addition, preliminary data suggest that RRx-001 acts in a stress-response pathway, presumably through NO release, that promotes activation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 and the tumor suppressors p53 and p21, supporting the emerging idea that RRx-001 leads to the onset of replicative senescence, resulting in cell cycle arrest or apoptosis in addition to other mechanisms of cell death [26].

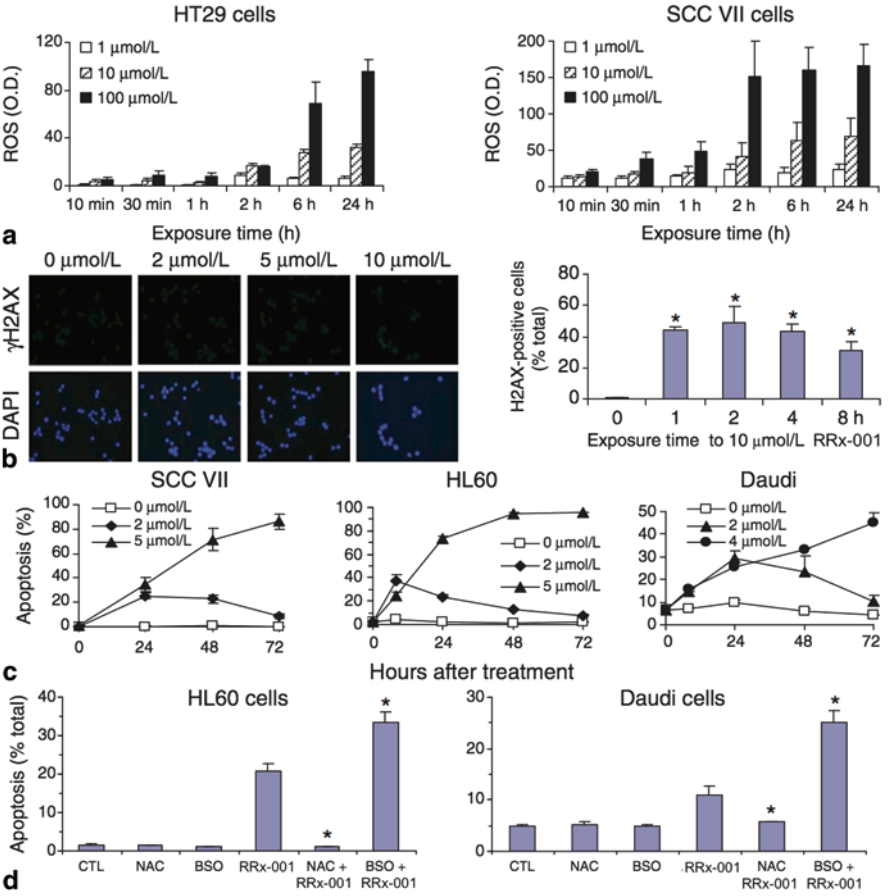


Fig. 16.10 RRx-001 induced ROS generation, DNA damage, and apoptosis of tumor cells. **a** Intracellular ROS in HT29 and SCC VII cells. O.D., optical density. **b** γH2AX expression in HT29 cells. *Left*, the γH2AX fluorescence in HT29 cells treated with 0 to 10 mmol/L RRx-001 for 2 h. *Right*, the quantification of γH2AX-positive cells as percentage of total cells counted in a time course. *, $P<0.01$ versus 0 h. **c** apoptosis for SCC VII, HL60, and Daudi cells after exposure to RRx-001 for up to 72 h. **d** Apoptosis of HL60 and Daudi cells treated with RRx-001 in the presence or absence of NAC (10 mmol/L) or BSO (200 mmol/L). 0, $P<0.01$ versus RRx-001 alone

Manufacture and Formulation of RRx-001

A particular advantage of medicinal compounds derived from energetic materials is that their synthesis proceeds from fairly advanced intermediates requiring a relatively small number of chemical steps to be undertaken under GMP. These advanced intermediates, which have well established and characterized synthetic routes, are readily available and obtainable in ton quantities.

Although RRx-001 is not an explosive molecule, the successful scale up and manufacturing of RRx-001 required careful consideration and due diligence given the inherently energetic *gem*-dinitroazetidine functional group of the molecule. ATK

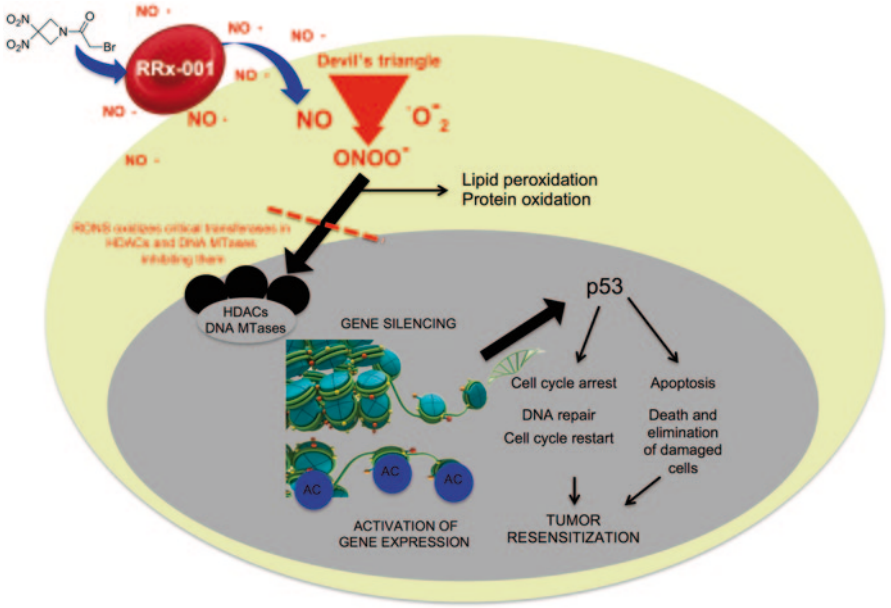


Fig. 16.11 Basic mechanism of RRx-001-induced cytotoxicity via a selective increase in oxidative stress in tumors resulting in inhibition of HDACs and DNMTs and induction of p53

Thiokol, which had initially prepared gram quantities of RRx-001 for preclinical studies, applied their expertise in handling and preparing energetic materials to the development of a synthetic process for the synthesis of multi-kilo quantities of the drug that simultaneously addressed the specific hazards posed by byproducts and intermediates. Accordingly, the development work to optimize product yield and purity and to improve the process efficiency and safety was guided by a comprehensive Critical Process Review [27], which determined that it was possible to conduct the entire process safely. Optimization resulted in the synthesis of multi-kilogram quantities of RRx-001 in high yield and purity; the process was transferred to a CMO for scale-up and preparation under GMP.

In the first synthetic stage, 1-tert-butyl-3- hydroxymethyl-3-nitroazetidine (HMNAZ) undergoes a retro-Henry reaction, followed by an oxidative nitration to yield 1-tert-butyl-3,3-dinitroazetidine (TBDNAZ) in an essentially quantitative yield. To avoid handling this hazardous product in a neat form, this material is not isolated but used directly in the next stage [28].

In the final stage, TBDNAZ is converted to RRx-001 by acylation with bromoacetylbromide in the presence of the Lewis acid, boron trifluoride etherate using TBDNAZ as a sacrificial base (Fig. 16.12). Purification by aqueous wash, followed by two crystallization steps gave RRx-001 in excellent purity and yield.

Development of a parenteral formulation was complicated by the highly reactive nature of RRx-001 coupled with very low aqueous solubility. The dipole moment, a measure of the difference of charge between two ends of a molecule can help

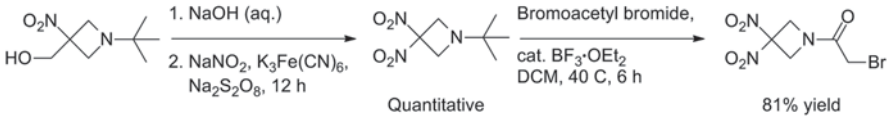


Fig. 16.12 Synthesis of RRx-001

guide formulation development, since the combination of high dipole moments and low solubility tends to promote aggregation in solution. Dipolar aprotic solvents that diminish and disperse these intermolecular precipitation forces are able to fully solubilize these micro-crystals. An excipient system, comprising the cosolvents, dimethylacetamide and PEG-400, already approved for use in the medication, busulfex [29], resulted in a sterile, stable and manufacturable formulation for RRx-001.

Clinical Experience with RRx-001: Resensitization, Epigenetics—A New Paradigm In Treating Cancer

Having successfully demonstrated preclinical activity and safety, a now-completed Phase 1 study of RRx-001 was initiated. The primary objectives of the trial were to assess the safety and tolerability, the pharmacokinetics and to determine the recommended Phase 2 dose of RRx-001 in patients with solid tumors who had previously failed conventional therapy. A total of 25 patients were treated intravenously with escalating doses of RRx-001 once or twice a week. Tumor types enrolled included colorectal (11 patients), head and neck (4 patients), pancreatic (3 patients), lung (2 patients), ovarian (1 patient), liver (1 patient), cholangiocarcinoma (1 patient), brain (1 patient), and melanoma (1 patient).

The most common adverse event related to RRx-001 was pain and vasodilation on infusion, ascribed to the displacement of nitric oxide from its binding site on the beta cysteine 93 hemoglobin residue after RRx-001 administration, as described above. However, other than the localized pain, typical chemotherapy side effects such as nausea/vomiting, alopecia, weight loss, fatigue, stomatitis, diarrhea and myelosuppression were notably absent. In fact, many patients anecdotally reported improvement in fatigue, appetite and quality of life. DLTs were not identified and an MTD was not reached and a safe and effective Phase 2 dose was established. RRx-001 had no effect on typical RBC parameters such as hemoglobin and hematocrit (Fig. 16.13) [30].

Although the primary objective of the Phase 1 study was not to determine efficacy, clinical benefit (measured by tumor regression or stable disease) was demonstrated in > 70 % of patients in multiple tumor types. One patient with head and neck cancer experienced a durable partial response for over a year. A subset of patients experienced prolonged stable disease independent of dose level. Two patients responded well to radiation while also receiving treatment with RRx-001, suggesting radiosensitizing properties [30].

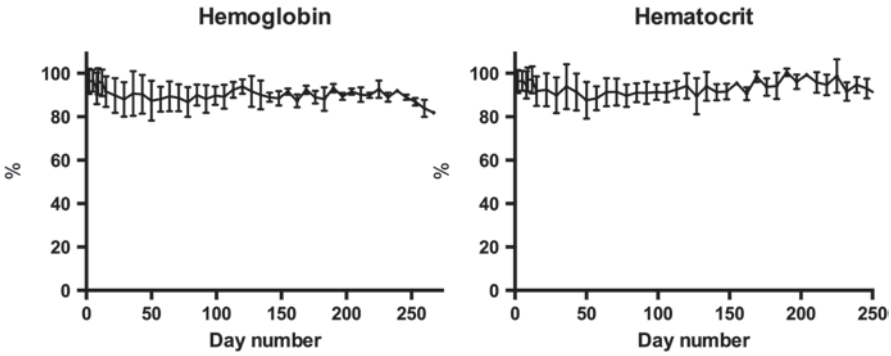


Fig. 16.13 Percent changes in hemoglobin and hematocrit for all 25 subjects over time. Day number = time from first RRx-001 treatment (Day 1)

The median duration of survival in the 11 colorectal patients in this trial was almost 17 months [30], more than 9 months greater than survival of 3rd line colorectal patients with regorafenib, the FDA-approved current standard of care for this population [31].

In an interesting observation, RRx-001 resensitized patients to previously failed therapies. After single agent therapy with RRx-001, four subjects became responsive to previously failed FOLFIRI, as shown by changes in CEA and by imaging (Fig. 16.14) [30, 32]. A further three subjects appeared to respond favorably to subsequent chemotherapy and radiation post RRx-001.

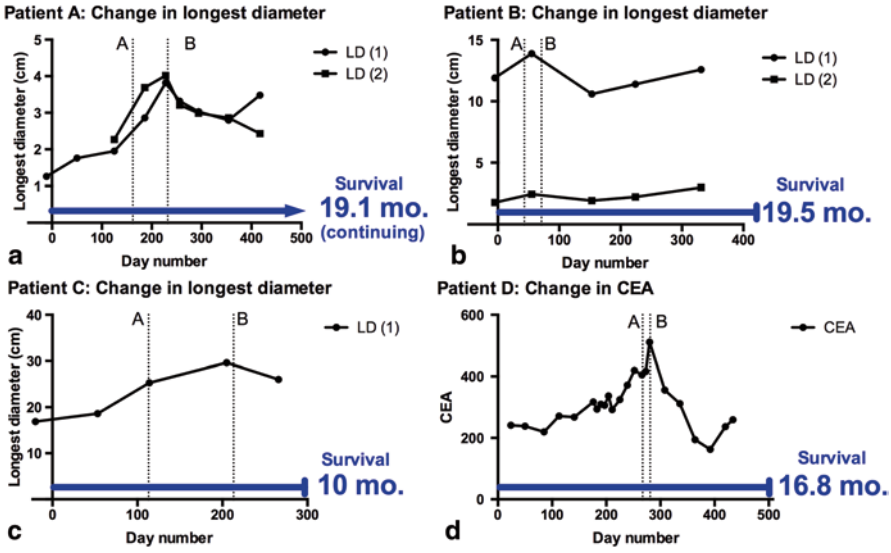


Fig. 16.14 Improved response to previously failed therapy post RRx-001: *Grey area* denotes a treatment gap between RRx-001 and subsequent therapy. **a** Last dose RRx-001; **b** First dose subsequent therapy. Patient A is still receiving FOLFIRI. Day numbers refer to days post first dose RRx-001

RRx-001—An Epigenetic Modulator: Perspectives and Future Plans

In addition to the other known mechanisms of action discussed above, the clinical observations of resensitization described suggested a pan-epigenetic mechanism with broad inhibition of DNA methyltransferases (DNMTs) and Histone Deacetylases (HDACs) [32], likely through NO-oxidation of critical cysteine residues, resulting in a variety of effects, including the possible reactivation of p53 expression [33, 34]. Such effects could help to explain the observed resensitization of resistant tumor cells to subsequent chemotherapy. Cysteine-dependent enzymes are targets of all forms of reactive oxygen and nitrogen species. Early data suggest that RRx-001-induced generation of nitric oxide, in combination with reduced glutathione depletion, leads to oxidative modification of catalytic cysteine thiols and inactivation of enzymes in the tumor microenvironment, which may provide a strategy for resensitizing resistant cancer cells in multiple tumor types [35].

RRx-001 was well tolerated in a Phase 1 clinical trial, demonstrating promising single agent survival and activity. As the first-in-class molecule, RRx-001 moves forward in Phase 2 in multiple tumor types, both clinical and preclinical studies are planned to highlight RRx-001's unique mechanisms of action and activity –validation of its one-of-a-kind potential to turn cancer into a chronic disease through resensitization and RadioRx's nontraditional 'non-me too' method of drug development.

Acknowledgements The authors greatly acknowledge the scientific foresight and ingenuity of the late Dr. Bednarski who was responsible for the genesis of RadioRx. We also gratefully acknowledge Dr. Arnold Oronsky and InterWest Partners for their willingness and courage to go out on a financial limb and fund an untested vision in the face of naysayers and sure criticism with novel, 'non-me too' materials as drug candidates. We also would like to acknowledge collaboration with Drs. R Wardle and L Cannizzo at ATK Aerospace Systems (ATK Thiokol) on the discovery of RRx-001. RadioRx also thank Dr. Tony Reid, our stalwart Principal Investigator as well as Drs. D Peehl, M Taylor, B Fitch, C Scribner, P Cabrales, F Kuypers and Mr. L Micheals for their invaluable contributions without which RRx-001 would not be where it is today.

Conflict Statement All authors have ownership interest in RadioRx, Inc. (including patents and stock option). SJ Knox and M Bednarski are founders of RadioRx, Inc.

References

1. American Cancer Society. Cancer facts & figures 2013. Atlanta: American Cancer Society; 2013.
2. Lipinski CA. Drug-like properties and the causes of poor solubility and poor permeability. J Pharmacol Toxicol Methods. 2000;44:235–49.
3. Walters WP, Murcko AA, Murcko MA. Recognizing molecules with drug-like properties. Curr Opin Chem Biol. 1999;3:384–7.

4. Virshup AM, Contreras-García J, Wipf P, et al. Stochastic voyages into uncharted chemical space produce a representative library of all possible drug-like compounds. J Am Chem Soc. 2013;135: 7296–303.
5. Swinney DC, Anthony J. How were new medicines discovered? Nat Rev Drug Discov. 2011;10:507–19.
6. Fried LE, Manaa MR, Pagoria PF, Simpson RL. Design and synthesis of energetic materials. Annu Rev Mater Res. 2001;31:291–321.
7. Mathieu J, Stucki H. Military high explosives. CHIMIA Int J Chem. 2004;58:383–9.
8. Watt D, Cliff M. Evaluation of 1, 3, 3-trinitroazetidine (TNAZ)-A high performance melt-castable explosive: Technical report No. DSTO-TR-1000; Aeronautical and Maritime Research Laboratory: Melbourne, Australia., 2000.
9. Genetic toxicity evaluation of 1, 3, 3—trinitroazetidine. Toxicon Corporation Report AL/OETR-1 Vol I–IV.
10. Sikder N, Sikder A, Bulakh N, Gandhe B. 1,3,3-Trinitroazetidine (TNAZ), a melt-cast explosive: synthesis, characterization and thermal behaviour. J Hazard Mater. 2004;113:35–43.
11. Hiskey MA, Johnson MC, Chavez DE. Preparation of 1-substituted-3,3-dinitroazetidines. J Energ Mater. 1999;17:233–54.
12. Ning S, Bednarski M, Oronsky B, et al. Dinitroazetidines are a novel class of anticancer agents and hypoxia-activated radiation sensitizers developed from highly energetic materials. Cancer Res. 2012;72:2600–8.
13. Oronsky BT, Reid T, Knox SJ, Scicinski JJ. The scarlet letter of alkylation: a mini review of selective alkylating agents. Transl Oncol. 2012;5:226–9.
14. Guterman L. Covalent drugs form long-lived ties—irreversible inhibitors may provide unique benefits in drug development. Chem Eng News. 2011;89:19–26.
15. Potashman MH, Duggan ME. Covalent modifiers: an orthogonal approach to drug design. J Med Chem. 2009;52:1231–46.
16. Singh J, Petter RC, Baillie TA, Whitty A. The resurgence of covalent drugs. Nat Rev Drug Discov. 2011;10:307–17.
17. Scicinski J, Oronsky B, Taylor M, et al. Preclinical evaluation of the metabolism and disposition of RRx-001, a novel investigative anticancer agent. Drug Metab Dispos. 2012;40:1810–6.
18. Gladwin MT, Ognibene FP, Pannell LK, et al. Relative role of heme nitrosylation and beta-cysteine 93 nitrosation in the transport and metabolism of nitric oxide by hemoglobin in the human circulation. Proc Natl Acad Sci U S A. 2000;97:9943–8.
19. Allen BW, Ca P. How do red blood cells cause hypoxic vasodilation? The SNO-hemoglobin paradigm. Am J Physiol Heart Circ Physiol. 2006;291:H1507–12.
20. Oronsky B, Ning S, Knox S, et al. RRx-001: a novel hypoxia activated nitric oxide generating vascular disrupting agent (VDA). 15th International Symposium on Anti-Angiogenic Therapy. La Jolla, 2013.
21. Fens MH, Larkin SK, Oronsky B, et al. The capacity of red blood cells to reduce nitrite determines nitric oxide generation under hypoxic conditions. PLoS One. 2014;9:e101626.
22. Grubina R, Huang Z, Shiva S, et al. Concerted nitric oxide formation and release from the simultaneous reactions of nitrite with deoxy- and oxyhemoglobin. J Biol Chem. 2007;282:12916–27.
23. Cosby K, Partovi KS, Crawford JH, et al. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. Nat Med. 2003;9:1498–505.
24. Fens M, Larkin S, Morris C, et al. NO or No NO, increased reduction of nitrite to nitric oxide by modified red blood cells. Blood (ASH Annual Meeting Abstracts). 2011;118:2125.
25. Oronsky B, Fanger GR, Oronsky N, et al. The implications of hyponitroxia in cancer. Transl Oncol. 2014;7:167–73.
26. Ning S, Sekar TV, Paulmurugan R, et al. Molecular imaging of RRx-001-induced oxidative stress in Nrf2-luciferase expressing SCC VII tumors in mice In: Proceedings of the 105th Annual Meeting of the American Association for Cancer Research; 2014 Apr 5–9; Philadelphia (PA): AACR; 2014. Abstract nr 906

27. Straessler NA, Lesley MW, Cannizzo LF. Development of a safe and efficient two-step synthesis for preparing 1-bromoacetyl-3, 3-dinitroazetidine, a novel clinical anticancer candidate. Org Process Res Dev. 2012;16:512–7.
28. Archibald T, Gilardi R, Baum K, George C. Synthesis and X-ray crystal structure of 1, 3, 3-trinitroazetidine. J Org Chem. 1990;55:2920–4.
29. Hempel G, Oechtering D, Lanvers-Kaminsky C, et al. Cytotoxicity of dimethylacetamide and pharmacokinetics in children receiving intravenous busulfan. J Clin Oncol. 2007;25:1772–8.
30. Reid T, Oronsky B, Infante J, et al. A phase 1 trial and pharmacokinetic study of RRx-001, a novel ROS-mediated pan-epigenetic agent. J Clin Oncol. 2014;32:(suppl; abstr 2578).
31. Grothey A, Van Cutsem E, Sobrero A, et al. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. Lancet. 2013;381:303–12.
32. Reid T, Dad S, Korn R, et al. Two case reports of resensitization to previous chemotherapy with the novel hypoxia-activated hypomethylating anticancer agent RRx-001 in metastatic colorectal cancer patients. Case Rep Oncol. 2014;7:79–85.
33. Hickok JR, Vasudevan D, Antholine WE, Thomas DD. Nitric oxide modifies global histone methylation by inhibiting Jumonji C domain-containing demethylases. J Biol Chem. 2013;288:16004–15.
34. Paradise WA, Vesper BJ, Goel A, et al. Nitric oxide: perspectives and emerging studies of a well known cytotoxin. Int J Mol Sci. 2010;11:2715–45.
35. Raghunand N, Scicinski J, Oronsky B, et al. RRx-001 Oxidation of redox sensitive protein thiols in tumors measured by Gd-LC7-SH enhanced MRI in preclinical tumor models. In: Proceedings of the 105th Annual Meeting of the American Association for Cancer Research; 2014 April 5–9; Philadelphia (PA): AACR; 2014. Abstract nr 2068.

Part V
NO Meditated Alterations in Gene Products

Chapter 17
Nitric Oxide, Coagulation and Cancer

Benjamin A. Derman, Hau C. Kwaan, Malak Elbatarny and Maha Othman

Abstract Nitric Oxide (NO) is a well-known potent and rapid vasodilator and inhibitor of coagulation. Synthesized from an L-arginine precursor, NO is produced via the Nitric Oxide Synthase enzyme which is expressed constitutively in endothelial cells. Nitric oxide has a wide range of biological properties that maintain vascular homeostasis and protection of the vessel from injurious consequences. The decreased production of NO in pathological states causes deleterious effects, creating an endothelial dysfunction state with a wide variety of subsequent diverse biological effects. There is now evidence of the link between hypoxia and/or reduction of NO availability and coagulopathies. NO is also a modulator of various cancer-related events and has anti-tumor properties. Cancer is a known hypercoagulable state and hypoxia is a typical feature of the tumor micro-environment. Cancer patients—particularly those with advanced or metastatic states—are at higher risk of developing venous and arterial thromboembolic events. The dichotomous nature of nitric oxide with regard to its tumorigenic and tumoricidal properties are at present under intense investigation. The transcendent field of nanotechnology has moved into the realm of NO donor therapy, though currently there are no commercially available carriers of NO. While nanotechnology is not quite at the translational research stage, it poses the greatest potential for storage and site-specific delivery of high concentrations of NO to tumors. In this chapter, we review the effects of NO on various hemostatic elements, the pro-tumoral and anti-tumoral effects of NO and finally shed some light on the link between NO, cancer and coagulopathies.

Keywords Cancer · Coagulation · Fibrinolysis · Nitric oxide · PAI-1 · Platelets

M. Othman (✉) · M. Elbatarny
Department of Biomedical and Molecular Sciences, Queen's University,
Boterell Hall Room 513, K7L 3N6 Kingston, ON, Canada
e-mail: othman@queensu.ca

School of Baccalaureate Nursing, St Lawrence College, Kingston, ON, Canada

B. A. Derman · H. C. Kwaan
Northwestern University Feinberg School of Medicine, Chicago, USA

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_17

Abbreviations

NO	nitric oxide
NOS	nitric oxide synthase
aPTT	activated partial thromboplastin time
vWF	von Willebrand factor
LPS	lipoproteinpolysaccharides
TEG	thromboelastography
TF	tissue factor
MPs	microparticles
PCa	prostate cancer
GTN	glyceryl trinitrate
tPA	tissue plasminogen activator
uPA	urokinase type plasminogen activator
PAI	plasminogen activator inhibitors
L-NMMA	L-NG-monomethylarginine
nNOS	neuro-isoform of NOS
eNOS	endothelial isoform of NOS
iNOS	inducible NOS
PGE2	prostaglandin E2
VEGF	vascular endothelial growth factor
COX-2	cyclooxygenase-2
EMT	epithelial-mesenchymal transition
MMP	matrix metalloproteinases
NODD	NO-donating drugs
NSAID	non-steroidal anti-inflammatory drug

"No potential conflicts of interest were disclosed."

Introduction

It is well known that nitric oxide (NO) is a potent and rapid vasodilator and inhibitor of coagulation. Synthesized from an L-arginine precursor, NO is produced via nitric oxide synthase (NOS), a calcium-calmodulin-dependent enzyme which is expressed constitutively in endothelial cells. The synthase enzyme is also known to be activated in the presence of inflammatory mediators as well as lipopolysaccharide (LPS) indicating the critical relationship between NO and inflammation. Studies have indicated that inducing NOS activity such as after an endotoxic shock challenge increases NO output detectable in 3–12 h [1–3].

NO has a wide range of biological properties that maintain vascular homeostasis, including modulation of vascular dilator tone, regulation of local cell growth, and protection of the vessel from injuries from activated platelets and white cells in the

blood, thereby playing a crucial role in the normal endothelial function. Hypertension, hypercholesterolemia, smoking, diabetes mellitus, heart failure and cancer are associated with diminished release of NO into the arterial wall either because of impaired synthesis or excessive oxidative degradation. NO is a modulator of various cancer-related events and has anti-tumor properties. The decreased production of NO in some pathological states causes deleterious effects in the endothelial equilibrium, resulting in endothelial dysfunction and a wide variety of subsequent diverse biological effects [4]. Evidence exists from pathological conditions such as obstructive sleep apnea [5] and placental alterations in rat models [6] of the relationship between hypoxia and coagulopathies.

NO is a core molecule in endothelial and blood vessel functions. The endothelial cell surface in an adult human is composed of approximately $1–6 \times 10^{13}$ cells, and spans a surface area of approximately 350 m^2 with a mass of about 110 g [7, 8]. The endothelium contains growth factors, coagulant and anticoagulant proteins, lipid transporting particles, metabolites and hormones; it also expresses proteins and receptors that control cell-cell and cell-matrix interactions [9]. Stimulation of the endothelium in conditions of shear stress or inflammation induces a prothrombotic and antifibrinolytic microenvironment. Given the wide distribution and heterogeneity of the endothelium, every organ system including blood vessels within these organs can be affected as result of endothelial damage or insult. Moreover, endothelial cells from diverse tissues are heterogeneous with respect to their surface phenotype and protein manufacturing, release and expression. This heterogeneity manifests itself differently in pathological states including thrombosis [10] and cancer [11–14]. In this chapter, we will review NO's specific effects on hemostatic parameters including its important role in physiological endothelium, platelets, coagulation factors, and other procoagulant and fibrinolytic elements (Fig. 17.1). We will also discuss tumoral and antitumoral effects of NO and shed some light on cancer therapeutics.

NO and Pro-Coagulant Factors

There have been no reports showing significant change in prothrombin time upon exposure to NO [15]. Inhaled NO in healthy males did not alter hemostasis significantly, as measured by the activated partial thromboplastin time (aPTT) and the bleeding time [16]. However, inhalation of NO in animals resulted in prolongation of the bleeding time [17], and the inhibition of NO synthesis shortened the prolonged bleeding time in uremic rats [18]. The reduction of NO did not significantly increase levels of factors II, V, or VII, but NOS inhibitors were associated with moderate increases in the plasma von Willebrand factor (vWF) [19], endothelial thrombogenicity, and vascular platelet thrombi [15, 20]. These effects indicate that this vascular damage can be causally linked to the increased vWF.

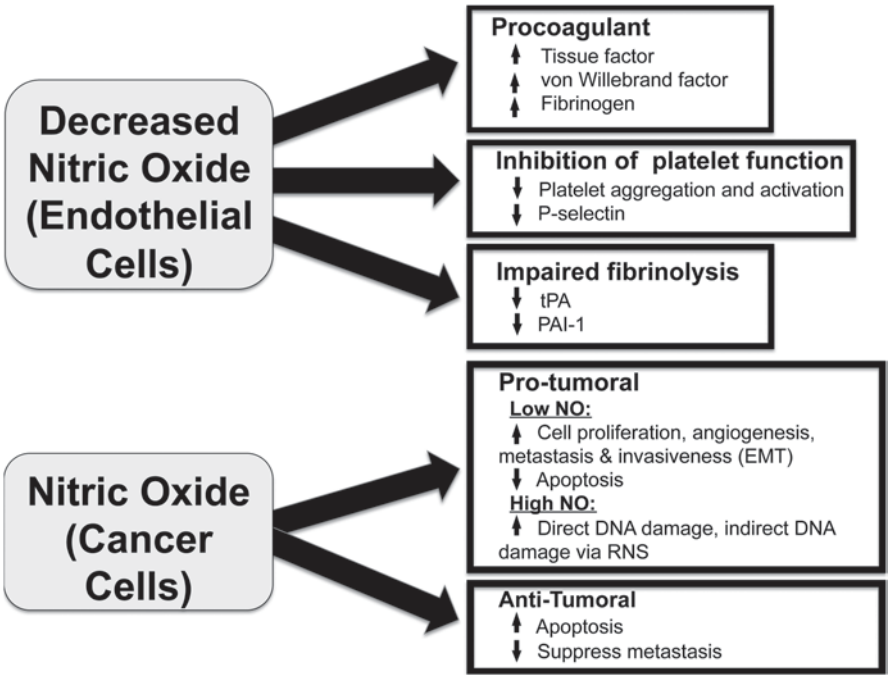


Fig. 17.1 NO is protective to endothelial cells; decreased NO results in a prothrombotic phenotype. In cancer cells, NO is both carcinogenic (pro-tumoral) and anti-tumoral. TF = tissue factor;TF-MPs = tissue factor bearing microparticles; TEG = thromboelastography; EMT = epithelial-mesenchymal transition; RNS = reactive nitrogen species

In a porcine model of endotoxin septic shock, a NOS inhibitor administration resulted in coagulation changes consistent with disseminated intravascular coagulation. In response to bacterial membrane lipoproteinpolysaccharides (LPS), there was a fourteen-fold elevation in thrombin and antithrombin complexes over the baseline; this increased further to twenty-seven-fold the baseline with the use of NOS inhibitors [15]. Moreover, prolonged inhibition of NOS significantly elevated fibrinogen levels in a dose-dependent manner in murine models [21].

Furthermore, the expression of tissue factor, the key trigger of coagulation, was inhibited in association with NO release in response to statin derivatives [22]. This inhibition was also associated with inhibition of collagen-induced platelet-aggregation, collagen-induced platelet P-selectin expression, platelet adhesion to collagen-coated coverslips under high shear stress indicating strong antithrombotic effects for these drugs, likely mediated through NO release.

NO and Global Hemostasis

Inhalation of NO has no major impact on hemostasis in healthy subjects, because no significant changes in platelet counts and levels of coagulation activation markers have been found in venous blood after drug administration [23]. However, in neonates with persistent pulmonary hypertension, inhalation of NO inhibited coagulation as evidenced by the more sensitive global hemostatic test thromboelastography (TEG) [24]. This was also supported by another study that showed an inhibitory effect for NO on all TEG parameters in platelet rich plasma and whole blood: it caused a longer reaction time (R), decreased angle, and reduced maximum amplitude (MA) in a dose-dependent manner [25].

Bradykinin is a potent stimulator of NO formation and prostacyclin release from the endothelium. Bradykinin binds to B1 and B2 receptors on endothelial cells, opens endothelial calcium channels, and activates NOS [26]. A study with bradykinin B receptor knockout mice showed reduction of thrombosis risk; this was shown to be mediated by an overriding mechanism involving angiotensin II which induced an elevation in NO and prostacyclin [27].

NO and Pro-Coagulant Microparticles

Microparticles (MPs) are membrane vesicles with procoagulant and proinflammatory properties released during cell activation or apoptosis. MPs can be released from all types of cells and carry phenotypic markers of these cells. Tissue factor (TF)-bearing MPs can serve as novel signaling elements and trigger hemostasis. Studies have shown that LPS administration leads to an increase in the numbers of MPs released from platelets, monocytes, and endothelium but inhalation of NO did not influence them [28].

However, patients with prostate cancer (PCa) have shown increased plasma procoagulant MPs. Moreover, hypoxia was recently shown to induce the release of TF-MPs by human PCa cell lines *in vitro,* which was reduced by the NO mimetic nitroglycerin (glyceryl trinitrate, GTN) [29]. In a pregnancy rat model of abnormal inflammation, inflammation-induced systemic coagulopathies were associated with placental hemostatic alterations and impaired placental hemodynamics [6]. In a closely similar model, GTN prevented inflammation-associated coagulopathies and fetal death, indicating a role for NO in triggering inflammation-induced coagulopathy [30]. These data add to the evidence of the link between hypoxia, nitric oxide, and coagulation.

NO and Natural Coagulation Inhibitors

Little is known about the relationship between of NO and natural coagulation inhibitors. Antithrombin III pretreatment was shown to reduce NO levels and improve survival in a rat model of heat stress-induced acute inflammation [31]. Activated protein C administration in rat model of experimental septic shock improved hemodynamics and myocardial efficiency by downregulating the inducible nitric oxide synthase pathway and reducing myocardial oxidative stress [32]. These data indicate once again the close link of hypoxia, inflammation and coagulation.

NO and the Fibrinolytic System

The plasminogen-plasmin system consists of plasminogen, a precursor of the active protease plasmin, which is then converted to plasmin by plasminogen activators, tissue plasminogen activator (tPA) and urokinase type plasminogen activator (uPA). The process is inhibited by several plasminogen activator inhibitors (PAI), of which PAI-1 has been shown to play an important role in both physiologic and pathologic conditions [33, 34]. NO is involved in modulating the fibrinolytic activity in blood. Sodium nitroprusside given to rabbits results in increase in tPA [35], believed to be due to the inhibition of clearance of tPA by PAI-1. This was confirmed by findings in human volunteers when after administration of a NO donor, mobsidomine, PAI-1 was reduced concomitantly with an increase in tPA [36] Conversely, the inhibition of NOS by L-N^G-monomethylarginine (L-NMMA) can block the increase in tPA [37].

On the other hand, tPA can also affect NO production. In the central nervous system, NO is an important modulator in mediating neurogenesis [26, 38] synaptic plasticity [35], and neuronal signaling [36]. Under physiologic conditions, tPA reduces NO by dislocating the neuro-isoform of NOS (nNOS) in neuronal cultures and, through the activation of plasminogen to plasmin, by proteolysis of NOS [41]. In stressed conditions with excitotoxicity, NO modulates neurodegeneration [42]· tPA regulates NO production through its proteolytic action on NOS [43, 44]. Much less is known of tPA in the other organs. Notably, hypertension, aortic arteriosclerosis, and coronary perivascular fibrosis developed in experimental animals given long-term treatment with N^{Ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS. These changes are not seen in PAI-1 knock-out animals 45. In these animals, there is upregulation of PAI-1. PAI-1 is known to have a role in the pathogenesis of a number of vascular pathologies, including hypertension and atherosclerosis and in pulmonary fibrosis. It is, thus, hypothesized that this interaction with the endothelial isoform of NOS (eNOS) may account for the role of PAI-1 in the vasculopathy and fibrosis. This concept was verified in experiments when an inhibitor of PAI-1, TM5441, was able to prevent hypertension and vascular changes

in L-NAME-treated animals [46, 47]. Such experimental therapeutic approaches pave the way for further exploration of the NO system for many vasculopathies.

NO and Platelets and Thrombosis

The NO derived from endothelial cells also affects platelet function. It impairs platelet aggregation [48, 49] and activation [50]. It also down-regulates P-selectin, thus preventing platelet-endothelial adhesion [51]. By the same token, inhibition of NO results in platelet adhesion [51–53] and aggregation [53].

Thus, the enhancement of NO may be of potential therapeutic benefit. This has been explored in sepsis, where platelet adhesion to endothelium can impair the microcirculation [54]. In sepsis, there is increased oxidative stress leading to platelet adhesion and microcirculatory blockage. It has been shown that the administration of the antioxidant ascorbic acid inhibits this series of events and that the action of ascorbic acid is dependent on local NO produced by eNOS [54].

Recently, studies showed that NO also participates in the pathology of the antiphospholipid syndrome, in which the presence of high levels of anti-beta2 glycoprotein I antibodies is predictive of thrombotic complications [55]. This antibody inhibits eNOS and attenuates the production of NO on the endothelial surface [56], leading to a prothrombotic endothelial phenotype. A correlation between low plasma NO levels and the titer of antiphospholipid antibodies as well as the number of vascular occlusive lesions had been observed in patients with antiphospholipid antibody syndrome [57].

NO and Cancer

NO is a modulator of various cancer-related events with additional anti-tumor properties [58]. Among the isotypes of NOS, inducible NOS (iNOS) has been found to be activated in various types of tumors, including breast, colon, head and neck, esophagus, lung, prostate, bladder, and pancreatic carcinomas, brain tumors, mesothelioma, Kaposi's sarcoma, and hematologic malignancies [59]. iNOS is induced by cytokines and lipopolysaccharides within the inflammatory milieu [60]. NO production is particularly important in these inflammatory states, as it can form reactive nitrogen species (RNS) nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$), which in turn cause DNA and lipid damage via oxidative and nitrosative stress. Moreover, RNS are involved in inducing a tumor specific immune response that ultimately inhibits T-cell penetration and function in the tumor microenvironment that aids in tumor growth [61].

Pro-Tumoral Effects of NO

Under normal physiologic circumstances, NO in low concentrations performs many vital functions including regulating blood flow, iron hemostasis, and neurotransmission. At higher concentrations, it acts as an immune regulator; iNOS generates large amounts of NO in macrophages and neutrophils in order to eliminate a variety of pathogens [60].

Nonetheless, in the setting of inflammation and/or cancer, both low and high NO concentrations can have deleterious pro-tumoral properties. Low NO concentrations can act to increase cell proliferation, angiogenesis, metastasis and invasiveness, and decrease apoptosis. High levels—though it has the effect of increasing apoptosis—cause extensive DNA damage, but more importantly cause oxidative and nitrosative stress. However, the direct modification of DNA and inactivation of DNA repair enzymes by NO alone are simply not enough to lead to carcinogenesis [62].

The indirect effect of NO—the production of RNS—is the true pathogenic and carcinogenic culprit. Excess NO, particularly in the setting of inflammation, reacts with superoxide anion to form the powerful oxidant peroxynitrite ($ONOO^-$) [58, 60, 63]. Peroxynitrate is involved in several carcinogenic pathways, including genotoxic mechanisms (inducing DNA damage, suppressing DNA repair enzymes such as p53, and modifying post-translational proteins), antiapoptotic effects, promotion of angiogenesis, promotion of metastasis, inhibition of antitumor immune responses, and promotion of the epithelial-mesenchymal transition [58, 62–65].

NO alone can cause direct DNA damage by way of strand breaks, oxidation, and deamination of nucleic acids. The more potent derivative peroxynitrite induces DNA damage by forming 8-nitroguanine, in addition to inducing lipid peroxidation that ultimately creates more reactive species to form DNA adducts [62]. Chronic nitrosative stress is further genotoxic via its post-translational alteration of proteins involved in intracellular signal transduction; this in turn leads to abnormal growth and proliferation of cells [62]. NO can cause further genome infidelity by inhibiting DNA repair processes via nitrosylation of DNA alkyl-transferase, xeroderma pigmentosum-A, and 8-oxoguanine glycosylase-1 [59].

But it is truly the antiapoptotic effects exerted by NO and its derivatives that allow for the aforementioned mutations to escape repair or inactivation. NO has been shown to cause loss-of-function mutations in p53 in HPV [65], inhibit caspase activation via S-nitrosylation [66], activate cyclooxygenase, inhibit release of cytochrome C, and increase bcl-2 expression [67]. All of these actions serve to create a permissive milieu for cancer cells to thrive.

The metabolically active cancer cells need a robust vascular network in order to maintain growth. NO produced by eNOS dilates arterioles that assist in augmenting tumor blood flow, in addition to decreasing leukocyte endothelial adhesion and increasing vascular permeability via increased prostaglandin E2 (PGE2) production [68, 69]. NO is perhaps most influential in angiogenesis by mediating up-regulation of the vascular endothelial growth factor (VEGF) by cGMP pathways [69, 70].

Production of cyclooxygenase-2 (COX-2) by NO also stimulates proangiogenic factors and prostaglandins that lead to neovascularization to increase a tumor's invasiveness and metastatic potential [68, 70]. To achieve effective invasiveness and metastasis, however, the tumor environment must undergo the epithelial-mesenchymal transition (EMT). EMT refers to a complex set of events that are responsible for the rapid changes in the epithelial cell phenotype, during which they assume mesenchymal cellular properties. EMT essentially enables transformed cells to travail through the basement membrane that is encapsulating a tumor, and invade lymphatic or blood vessels so as to gain access to other organs [63, 71, 72]. The effect of NO on EMT is controversial, with evidence supporting its ability to both augment and attenuate the EMT dedifferentiation and hence tumor invasiveness and metastasis. In gastric carcinomas, for example, a significant correlation was found between iNOS expression in tumor cells and loss of differentiation. iNOS expression was most notable in 'EMT-like' dedifferentiation areas with loss of cohesion and an invasive phenotype [73]. Similarly, a study involving colorectal adenocarcinoma cells expressing iNOS (HRT-18 cells) versus those not expressing iNOS (HRT-29 cells) were shown to be nearly three times more invasive; further supporting this result was the increased invasiveness of the HRT-29 cells in the presence of an NO donor and inflammatory cytokines [74]. The mechanism by which NO increases tumor invasiveness is thought to be related to up-regulation of matrix metalloproteinases (MMP-2 and MMP-9) and downregulation of tissue inhibitors of MMPs such as TIMP-2 and TIMP-3 [75].

Anti-Tumoral Effects of NO

The dichotomous nature of NO with regard to its tumorigenic and tumoricidal properties is not to be understated. Low concentrations of NO promote tumor cell survival and angiogenesis, whereas high levels of NO (typically > 500 nM) have a cytotoxic propensity, particularly as it pertains to inducing apoptosis [63, 76]. The concept of a dose threshold for NO is key to understanding NO-induced cytotoxicity.

NO has the potential to induce apoptosis through various mechanisms, including S-nitrosylation of NF-kappa-B, glyceraldehyde-3-phosphate dehydrogenase, Fas receptor and Bcl-2 [77, 78]. Furthermore, NO ignites the caspase cascade that is responsible for releasing mitochondrial cytochrome C into the cytosol that is ultimately responsible for initiating the chain of apoptotic events [67, 79]. NO and its relationship to p53 has proven to be baffling. Wild-type p53 appears to be activated by low-dose NO, which in turn exerts a negative feedback loop to inhibit further NO generation. High NO concentrations appear to inactivate p53 via peroxynitrite tyrosination, causing mutations in the *p53* gene itself that lead to the loss of repressor activity. This, in turn, leads to increased iNOS expression, which feeds a cycle of NO generation, DNA damage, and additional mutations [59].

NO has also been shown to suppress metastasis by inhibiting the EMT at high concentrations. Indeed, *in vitro* studies have shown that treatment of human metastatic prostate cell lines with NO donors is able to inhibit EMT and reverse the mesenchymal phenotype and cell invasive properties [80]. Given that most studies investigating the anti-tumoral role of NO have been *in vitro*, it is difficult to extrapolate its potential *in vivo* effects. In particular, the question remains whether high enough NO concentrations can be accomplished *in vivo* so as to facilitate apoptosis without up-regulating pro-tumoral pathways.

NO and Cancer Therapeutics

It should follow that NO is a strong candidate and target for anticancer therapeutics, albeit with two drastically different approaches to achieve the same desired effect. Anti-NO cancer therapies have been investigated as a means to modulate the deleterious effects of RNS. NO scavengers have shown promising results, from reducing cancer-related vascular hyperpermeability [81] to inhibiting colon cancer development [82]. NOS enzyme inhibitors, especially iNOS-specific inhibitors, have been shown to decrease the rate of premalignant lesion development in colon cancer [83]. However, NOS inhibitors require long-term administration, as early cessation of therapy can result in tumor regrowth [84].

As it pertains to radiation, NO appears to have both radiosensitizing and radioprotecting properties. NO has the ability to act as a radiosensitizer of hypoxic tumor cells, mimicking the effects of oxygen on fixation of radiation-induced DNA damage [85, 86]. NO can also exert radioprotective effects as it is a free radical that can scavenge other free radicals induced by radiation and inhibit further DNA damage. Moreover, NO can decrease blood flow to bone marrow via the vascular steal phenomenon, which paradoxically causes hypoxia in the marrow and protects those cells from radiation damage [87].

The concept behind NO-based drugs is that its therapeutic consequence is heavily dependent on the concentration and duration of NO delivered. Indeed, effective NO-based drugs must be able to accomplish three tasks: (1) store NO doses for a specific duration, (2) deliver finite amounts of NO over a specific amount of time (i.e. rate), and (3) selectively deliver NO to the tissue of interest given its short half-life [84]. Pro-NO cancer therapies follow this recipe by increasing NO concentrations locally at the tumor site to exert its anti-tumoral (i.e. proapoptotic) effects while sparing healthy cells [88].

Pro-NO strategies include iNOS gene therapy and NO donor drugs. NOS gene therapy was thought to be a workaround to increase NO delivery in the setting of malignancy. However, this strategy has been met with substantial obstacles initially due to the hazard from the viral vectors required to deliver the gene therapy [89], but also secondary to early death of the NOS transfectants. The constitutive expression of NOS can paradoxically lead to the death of the transfectant, thereby decreasing the amount of time that NO can be generated [59].

There are many NO donor therapies (also known as NO-donating drugs, NODD), but the most studied and best understood is NO-NSAID, a NSAID (non-steroidal anti-inflammatory drug) with a NO-donor covalently bound to it [59, 90]. NO appears to enhance the anticancer potential of NSAIDs, though the mechanism by which this occurs is unclear. Some have suggested that NO-NSAIDs accomplish this via direct inhibition of hypoxia-inducible factor-1alpha, a VEGF transcriptional activator [91]. Other evidence suggests that NO-NSAIDs induce apoptosis and modulate the Wnt and NF-kappa-B signaling pathways to achieve its anticancer effect [90]. Regardless, NO-NSAIDs such as NO-acetylsalicylic acid (NO-ASA) exhibit inhibition of colon cancer cell lines *in vitro* and in animal models as well [92]. NO-NSAIDs have also been shown to have proapoptotic and anti-invasive properties in prostate cancer [93, 94]. Other NO-donor drugs have been studied as well, including NONOates DEA/NO and PAPA/NO, SNAP and GSNO, with varying efficacy in numerous cancer cell lines.

The transcendent field of nanotechnology has moved into the realm of NO donor therapy, though there are currently no commercially available carriers of NO. The theory behind nanoparticles is to load high amounts of NO onto a stable material that can be photoactivated. While nanotechnology is not quite at the translational research stage, surely it poses the greatest potential for storage and site-specific delivery of high concentrations of NO to tumors.

References

1. Moncada S, Palmer RMJ, Higgs A. Nitric oxide: physiology, pathology and pharmacology. Pharmacol Rev. 1991; 43:109–42.
2. Geller Da Nusseler AK Di Silvo. Cytokines, endotoxin and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. Proc Natl Acad Sci USA. 1993; 90:522–6.
3. Killbourn RG, Belloni P: Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. J Natl Cancer Inst. 1990; 82:772–6.
4. Tousoulis D1, Kampoli AM, Tentolouris C, Papageorgiou N, Stefanadis C. The role of nitric oxide on endothelial function. Curr Vasc Pharmacol. 2012;10:4–18.
5. Toukh M, Pereira EJ, Falcon BJ, Liak C, Lerner M, Hopman WM, Iscoe S, Fitzpatrick MF, Othman M. CPAP reduces hypercoagulability, as assessed by thromboelastography, in severe obstructive sleep apnea. Respir Physiol Neurobiol. 2012;183:218–23.
6. Falcón BJ, Cotechini T, Macdonald-Goodfellow SK, Othman M, Graham CH. Abnormal inflammation leads to maternal coagulopathies associated with placental haemostatic alterations in a rat model of fetal loss. Thromb Haemost. 2012; 107:438–47.
7. Augustin HG, Kozian DH, Johnson RC. Differentiation of endothelial cells: analysis of the constitutive and activated endothelial cell phenotypes. Bioessays. 1994;16:901.
8. Pries AR, Kuebler WM. Normal endothelium. Handb Exp Pharmacol. 2006;176:1–40.
9. Cines DB, Pollak ES, Buck, CA et al. Endothelial Cells in Physiology and in the Pathophysiology of Vascular Disorders. Blood. 1998;91: 3527–6.

10. Kwaan HC, Samama MM. The significance of endothelial heterogeneity in thrombosis and hemostasis. Sem Thromb Hemost. 2010;36:286–300.
11. Tousoulis D1, Kampoli AM, Tentolouris C, Papageorgiou N, Stefanadis C. The role of nitric oxide on endothelial function. Curr Vasc Pharmacol. 2012 Jan;10:4–18.
12. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins: Identification by morphologic criteria. J Clin Invest. 1973;52:2745.
13. Gimbrone MA, Cotran RS, Folkman J. Human vascular endothelial cells in culture. Growth and DNA synthesis. J Cell Biol. 1974;60:673.
14. Lewis LJ, Hoak JC, Maca RD, Fry GL. Replication of human endothelial cells in culture. Science. 1973;181:453.
15. Jourdain M1, Tournoys A, Leroy X, et al. Effects of N omega-nitro-L-arginine methyl ester on the endotoxin-induced disseminated intravascular coagulation in porcine septic shock. Crit Care Med. 1997;25:452–9.
16. Goldstein B, Baldassarre J, Young JN. Effects of inhaled nitric oxide on hemostasis in healthy adults treated with heparin: a randomized, controlled, blinded crossover study. Thromb J. 2012; 10:1.
17. Högman M, Frostell C, Arnberg H, Sandhagen B, Hedenstierna G. Prolonged bleeding time during nitric oxide inhalation in the rabbit. Acta Physiol Scand. 1994;151:125–9
18. Remuzzi G, Perico N, Zoja C, Corna D, Macconi D, Vigano G. Role of endothelium-derived nitric oxide in the bleeding tendency of uremia. J Clin Invest. 1990;86:1768–71.
19. Radomski MW, Palmer RMJ, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. Lancet. 1987;2:1057
20. Radomski MW, Palmer RMJ, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. Biochem Biophys Res Commun. 1987;148:1482–9.
21. Kawabata A. Evidence that endogenous nitric oxide modulates plasma fibrinogen levels in the rat. Br J Pharmacol. 1996; 117: 236–7.
22 Rossiello MR, Momi S, Caracchini R et al. A novel nitric oxide-releasing statin derivative exerts an antiplatelet/antithrombotic activity and inhibits tissue factor expression. J Thromb Haemost. 2005;3:2554–62.
23. Krejcy K, Schmetterer L, Johannes Kastner, J et al. Role of nitric oxide in hemostatic system activation in vivo in humans. arterio, thromb, Vas Biol. 1995; 15: 2063–7
24. Tanriverdi S1, Koroglu OA, Uygur O, Balkan C, Yalaz M, Kultursay N. The effect of inhaled nitric oxide therapy on thromboelastogram in newborns with persistent pulmonary hypertension. Eur J Pediatr. 2014;173:1381–5
25. Tanriverdi S1, Koroglu OA, Uygur O, Balkan C, Yalaz M, Kultursay N. Inhibitory effect of nitrite on coagulation processes demonstrated by thrombelastography Nitric Oxide. 2014;40:45–51.
26. Packer MA, Stasiv Y, Benraiss A, et al. Nitric oxide negatively regulates mammalian adult neurogenesis. Proc Natl Acad Sci USA. 2003;100:9566–9571.
27. Shariat-Madar Z, Mahdi F, Warnock M, et al. Bradykinin B2 receptor knockout mice are protected from thrombosis by increased nitric oxide and prostacyclin. Blood. 2006; 108: 192–199.
28. Soop A, Hållström L, Frostell C, Wallén H, Mobarrez F, Pisetsky DS. Effect of lipopolysaccharide administration on the number, phenotype and content of nuclear molecules in blood microparticles of normal human subjects. Scand J Immunol. 2013;78:205–13.
29. Sterniczuk B, Toukh M, Kaur H, et al. Pro-coagulant properties of prostate cancer cell lines and the effect of androgen deprivation. Abstract at ISTH 2014 June 24-27 Milwakuee, USA.
30. Cotechini T1, Othman M, Graham CH. Nitroglycerin prevents coagulopathies and foetal death associated with abnormal maternal inflammation in rats. Thromb Haemost. 2012;107:864–74.
31. Hagiwara S1, Iwasaka H, Shingu C, Matsumoto S, Uchida T, Noguchi T. High-dose antithrombin III prevents heat stroke by attenuating systemic inflammation in rats. Inflamm Res. 2010;59:511–8.

32. Lemarie J, Blet A, Bouazza Y, Boisrame-Helms J, Meziani F, Levy B. Dexamethasone and recombinant human activated protein C improve myocardial function and efficiency during experimental septic shock. Shock. 2014;41:522–7.
33. Kwaan HC. The biologic role of components of the plasminogen-plasmin system. Prog Cardiovasc Dis. 1992;34:309–16.
34. Kwaan HC. The plasminogen-plasmin system in malignancy. Cancer Met Rev. 1992;11:291–311.
35. Lidbury PS, Korbut R, Vane JR. Sodium nitroprusside modulates the fibrinolytic system in the rabbit. Brit J Pharm. 1990;101:527–350.
36. Grodzinska L, Hafner G, Darius H. Effect of molsidomine on t-PA and PAI activity in man: a double blind, placebo controlled study. Thromb Hemost. 1990;64:485.
37. Newby DE, Wright RA, Dawson P, et al. The L-arginine/nitric oxide pathway contributes to the acute release of tissue plasminogen activator in vivo in man. Cardiovasc Res. 1998;38:485–92.
38. Zhu XJ, Hua Y, Jiang J, et al. Neuronal nitric oxide synthase-derived nitric oxide inhibits neurogenesis in the adult dentate gyrus by down-regulating cyclic AMP response element binding protein phosphorylation. Neuroscience 2006;141:827–36.
39. Bon CL, Garthwaite J. On the role of nitric oxide in hippocampal long-term potentiation. J Neurosci. 2003;23:1941–8.
40. Araujo IM, Carvalho CM. Role of nitric oxide and calpain activation in neuronal death and survival. Curr Drug Targets CNS Neurolog Disord. 2005;4:319–24.
41. Baron A, Hommet Y, Casse F, Vivien D. Tissue-type plasminogen activator induces plasmin-dependent proteolysis of intracellular neuronal nitric oxide synthase. Bio Cell. 2010;102:539–47.
42. Zhou L, Zhu DY. Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. Nitric Oxide. 2009;20:223–30.
43. Parathath SR, Parathath S, Tsirka SE. Nitric oxide mediates neurodegeneration and breakdown of the blood-brain barrier in tPA-dependent excitotoxic injury in mice. J Cell Sci. 2006;119:339–49.
44. Park L, Gallo EF, Anrather J, et al. Key role of tissue plasminogen activator in neurovascular coupling. Proc Natl Acad Sci USA. 2008;105:1073–8.
45. Kaikita K, Fogo AB, Ma L, Schoenhard JA, Brown NJ, Vaughan DE. Plasminogen activator inhibitor-1 deficiency prevents hypertension and vascular fibrosis in response to long-term nitric oxide synthase inhibition. Circulation. 2001;104:839–44.
46. Boe AE, Eren M, Murphy SB, et al. Plasminogen activator inhibitor-1 antagonist TM5441 attenuates Nomega-nitro-L-arginine methyl ester-induced hypertension and vascular senescence. Circulation. 2013;128:2318–24.
47. Eren M, Boe AE, Murphy SB, et al. PAI-1-regulated extracellular proteolysis governs senescence and survival in Klotho mice. Proc Natl Acad Sci USA. 2014;111:7090–5.
48. Radomski MW, Palmer RM, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. Lancet. 1987;2:1057–8.
49.. Azuma H, Ishikawa M, Sekizaki S. Endothelium-dependent inhibition of platelet aggregation. Brit J Pharmacol. 1986;88:411–5.
50. Busse R, Luckhoff A, Bassenge E. Endothelium-derived relaxant factor inhibits platelet activation. Naunyn-Schmiedeberg's Arch Pharmacol. 1987;336:566–71.
51. Armstead VE, Minchenko AG, Schuhl RA, Hayward R, Nossuli TO, Lefer AM. Regulation of P-selectin expression in human endothelial cells by nitric oxide. Am J Physiol. 1997;273:740–6.
52. Minamino T, Kitakaze M, Sanada S, et al. Increased expression of P-selectin on platelets is a risk factor for silent cerebral infarction in patients with atrial fibrillation: role of nitric oxide. Circulation. 1998;98:1721–7.
53. Takajo Y, Ikeda H, Haramaki N, Murohara T, Imaizumi T. Augmented oxidative stress of platelets in chronic smokers. Mechanisms of impaired platelet-derived nitric oxide bioactivity and augmented platelet aggregability. J Am Coll Cardiol. 2001;38:1320–7.

54. Tyml K. Critical role for oxidative stress, platelets, and coagulation in capillary blood flow impairment in sepsis. Microcirculation. 2011;18:152–62.
55. Mineo C. Inhibition of nitric oxide and antiphospholipid antibody-mediated thrombosis. Curr Rheumatol Rep. 2013;15:324.
56. Ramesh S, Morrell CN, Tarango C, et al. Antiphospholipid antibodies promote leukocyte-endothelial cell adhesion and thrombosis in mice by antagonizing eNOS via beta2GPI and apoER2. J Clin Invest. 2011;121:120–31.
57. Ames PR, Batuca JR, Ciampa A, Iannaccone L, Delgado Alves J. Clinical relevance of nitric oxide metabolites and nitrative stress in thrombotic primary antiphospholipid syndrome. J Rheum. 2010;37:2523–30.
58. Choudhari SK, Chaudhary M, Bagde S, Gadbail AR, Joshi V. Nitric oxide and cancer: a review. World J Surg Onc. 2013;11:118.
59. Mocellin S, Bronte V, Nitti D. Nitric oxide, a double edged sword in cancer biology: searching for therapeutic opportunities. Medicinal Res Rev. 2007;27:317–52.
60. Habib S, Ali A. Biochemistry of nitric oxide. Indian J Clin Biochem. 2011;26:3–17.
61. De Sanctis F, Sandri S, Ferrarini G, et al. The emerging immunological role of post-translational modifications by reactive nitrogen species in cancer microenvironment. Front Immunol. 2014;5:69.
62. Kundu JK, Surh YJ. Emerging avenues linking inflammation and cancer. Free Radic Bio Med. 2012;52:2013–2037.
63. Burke AJ, Sullivan FJ, Giles FJ, Glynn SA. The yin and yang of nitric oxide in cancer progression. Carcinogenesis. 2013;34:503–12.
64. Hussain SP, He P, Subleski J, et al. Nitric oxide is a key component in inflammation-accelerated tumorigenesis. Cancer Res. 2008;68:7130–6.
65. Wei L, Gravitt PE, Song H, Maldonado AM, Ozbun MA. Nitric oxide induces early viral transcription coincident with increased DNA damage and mutation rates in human papillomavirus-infected cells. Cancer Res. 2009;69:4878–84.
66. Brune B. Nitric oxide: NO apoptosis or turning it ON? Cell Death Differ. 2003;10:864–9.
67. Choi BM, Pae HO, Jang SI, Kim YM, Chung HT. Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. J Biochem Molec Bio. 2002;35:116–1126.
68. Ziche M, Morbidelli L. Nitric oxide and angiogenesis. J Neuro-onc. 2000;50:139–48.
69. Morbidelli L, Donnini S, Ziche M. Role of nitric oxide in the modulation of angiogenesis. Curr Pharm Des. 2003;9:521–30.
70. Xu W, Liu LZ, Loizidou M, Ahmed M, Charles IG. The role of nitric oxide in cancer. Cell Res. 2002;12:311–20
71. Klymkowsky MW, Savagner P. Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. Am J Pathol. 2009;174:1588–93.
72. Savagner P. The epithelial-mesenchymal transition (EMT) phenomenon. Ann Oncology Off J Euro Soc Med Onc/ESMO. 2010;21(Suppl 7):89–92.
73. Karadayi N, Kandemir NO, Yavuzer D, Korkmaz T, Gecmen G, Kokturk F. Inducible nitric oxide synthase expression in gastric adenocarcinoma: impact on lymphangiogenesis and lymphatic metastasis. Diagn Pathol. 2013;8:151.
74. Siegert A, Rosenberg C, Schmitt WD, Denkert C, Hauptmann S. Nitric oxide of human colorectal adenocarcinoma cell lines promotes tumour cell invasion. Brit J Cancer. 2002;86:1310–5.
75. Lala PK, Orucevic A. Role of nitric oxide in tumor progression: lessons from experimental tumors. Cancer Metastasis Rev. 1998;17:91–106.
76. Krischel V, Bruch-Gerharz D, Suschek C, Kroncke KD, Ruzicka T, Kolb-Bachofen V. Biphasic effect of exogenous nitric oxide on proliferation and differentiation in skin derived keratinocytes but not fibroblasts. J Invest Dermatol. 1998;111:286–91.
77. Marshall HE, Stamler JS. Nitrosative stress-induced apoptosis through inhibition of NF-kappa B. J Bio Chem. 2002;277:34223–8.

78. Bonavida B, Baritaki S. Dual role of NO donors in the reversal of tumor cell resistance and EMT: Downregulation of the NF-kappaB/Snail/YY1/RKIP circuitry. Nitric Oxide. 2011;24:1–7.
79. Shahani N, Sawa A. Protein S-nitrosylation: role for nitric oxide signaling in neuronal death. Biochimica et biophysica acta. 2012;1820:736–42.
80. Baritaki S, Huerta-Yepez S, Sahakyan A, et al. Mechanisms of nitric oxide-mediated inhibition of EMT in cancer: inhibition of the metastasis-inducer Snail and induction of the metastasis-suppressor RKIP. Cell Cycle. 2010;9:4931–40.
81. Wu J, Akaike T, Maeda H. Modulation of enhanced vascular permeability in tumors by a bradykinin antagonist, a cyclooxygenase inhibitor, and a nitric oxide scavenger. Cancer Res. 1998;58:159–65.
82. Kawamori T, Lubet R, Steele VE, et al. Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. Cancer Res. 1999;59:597–601.
83. Rao CV, Kawamori T, Hamid R, Reddy BS. Chemoprevention of colonic aberrant crypt foci by an inducible nitric oxide synthase-selective inhibitor. Carcinogenesis. 1999;20:641–44.
84. Carpenter AW, Schoenfisch MH. Nitric oxide release: part II. Therapeutic applications. Chemical Soc Rev. 2012;41:3742–52.
85. De Ridder M Verellen D Verovski V Storme G. Hypoxic tumor cell radiosensitization through nitric oxide. Nitric Oxide. 2008;19:164–9.
86. Aranda E, Lopez-Pedrera C, De La Haba-Rodriguez JR, Rodriguez-Ariza A. Nitric oxide and cancer: the emerging role of S-nitrosylation. Curr Mol Med. 2012;12:50–67.
87. Oronsky BT, Knox SJ, Scicinski JJ. Is nitric oxide (no) the last word in radiosensitization? a review. Translational Onc. 2012;5:66–71.
88. Hirst D, Robson T. Nitric oxide in cancer therapeutics: interaction with cytotoxic chemotherapy. Current Pharm Design. 2010;16:411–20.
89. Lehrman S. Virus treatment questioned after gene therapy death. Nature. 1999;401:517–8.
90. Rigas B, Kashfi K. Nitric-oxide-donating NSAIDs as agents for cancer prevention. Trends Mol Med. 2004;10:324–30.
91. Semenza GL. Targeting HIF-1 for cancer therapy. Nature Rev Cancer. 2003;3:721–32.
92. Williams JL, Nath N, Chen J, et al. Growth inhibition of human colon cancer cells by nitric oxide (NO)-donating aspirin is associated with cyclooxygenase-2 induction and beta-catenin/T-cell factor signaling, nuclear factor-kappaB, and NO synthase 2 inhibition: implications for chemoprevention. Cancer Res. 2003;63:7613–8.
93. Ishiguro H, Kawahara T. Nonsteroidal anti-inflammatory drugs and prostatic diseases. BioMed Res Internatol. 2014;2014:436123.
94. Royle JS, Ross JA, Ansell I, Bollina P, Tulloch DN, Habib FK. Nitric oxide donating nonsteroidal anti-inflammatory drugs induce apoptosis in human prostate cancer cell systems and human prostatic stroma via caspase-3. J Urology. 2004;172:338–44.

Chapter 18
Neutral Sphingomyelinase 2: Structure, Function, and Regulation with Emphasis on Nitric Oxide Involvement and Potential Implications for Cancer Therapy

Bei Lei Sun and Bulent Mutus

Abstract Mammalian neutral sphingomyelinase 2 is encoded by the gene *smpd3* and belongs to the family of hydrolases which catalyze the breakdown of sphingomyelin to form ceramide and phosphocholine. The bioactive ceramide can then act as the second messenger molecule capable of mediating an array of cellular events, such as growth arrest and apoptosis. Recent studies have revealed that the expression and activity of neutral sphingomyelinase 2 are selectively regulated and this regulation can take place at the transcriptional level as well as at the post-translational level. Upon exposure to oxidative stress, endoplasmic reticulum stress, tumour necrosis factor alpha stimulation or anti-cancer drugs, altered neutral sphingomyelinase 2 activity directly translates into changes in ceramide levels which help cells mount an appropriate response. On the other hand, inappropriate activation or inhibition of neutral sphingomyelinase 2 could contribute to the development of pathological conditions such as cancer and endothelial dysfunction. In this chapter, we focus on current knowledge regarding neutral sphingomyelinase 2 structure, the regulation of its activity, its function and potential involvement in stress response and cancer genesis.

Keywords Cancer · Ceramide · Neutral sphingomyelinase 2 · Nitric oxide · Post-translational modifications · Stress response

Abbreviations

APL	Anionic phospholipid
ATRA	All-trans retinoic acid
BAEC	Bovine aortic endothelial cells
EED	Embryonic ectodermal development
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase

B. Mutus (✉) · B. L. Sun
Department of Chemistry & Biochemistry, University of Windsor, 273-1 Essex Hall, 401 Sunset Ave., Windsor, ON N9B 3P4, Canada
e-mail: mutusb@uwindsor.ca

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_18

nNOS	Neuronal nitric oxide synthase
ER	Endoplasmic reticulum
HAEC	Human airway epithelial cells
HEK	Human embryonic kidney
HSP	Heat shock protein
MAPK	Mitogen activated protein kinase
PKC	Protein kinase C
ROS	Reactive oxygen species
SM	Sphingomyelin
SMase	Sphingomyelinase
TNF	Tumour necrosis factor

Introduction

Sphingolipids are a major component of the plasma membrane in eukaryotic cells. This class of lipids typically consists of a sphingosine backbone, a long chain fatty acid molecule, and a variable polar head group. Originally considered to serve only structural roles, these lipids are now recognized as important players in a wide range of signal transduction pathways [1–3]. In particular, sphingomyelin (SM)-based pathways have received considerable attention in recent years.

The SM molecule has a polar phosphorylcholine head group and the composition of the long chain fatty acid varies from tissue to tissue and can be either saturated or mono-unsaturated with 14 to 24 carbons [4–5]. Results based on cell fractionation studies and degradation experiments suggest that more than half of the cellular SM mass is confined to the plasma membrane [6]. The exact percentage may vary from one cell type to another, though it has been reported that cells with extensive plasma membrane recycling have a larger fraction of SM in the intracellular compartments [7].

The hydrolysis of SM yields ceramide, which is an important second messenger molecule capable of modulating a variety of cellular events, such as cell cycle arrest, differentiation, inflammation and apoptosis [8–10]. SM hydrolysis is specifically catalyzed by a group of enzymes known as sphingomyelinase (EC.3.1.4.12). SMase can be further classified into three groups (acid, alkaline and neutral) based on their distinct pH optimum. Acid SMase is responsible for the catabolism of SM within the lysosomes and a deficiency of this enzyme leads to the human Niemann-Pick disease [11, 12]. In recent years, acid SMase has also been reported to be an important player in stress-induced ceramide generation and subsequent signaling pathways [13–16]. For more detailed information on acid SMase, we recommend the reviews by Smith and Schuchman [17]; and Zeidan and Hannun [18]. On the other hand, alkaline SMase is found in the intestinal tract, bile and liver, and it plays a crucial role in SM digestion [19, 20]. Recent findings by Zhang et al. also point toward the potential involvement of alkaline SMase in regulating mucosal growth as well as the function of alkaline phosphatase [21].

Neutral magnesium-dependent SMase activity was first described in 1967 by Scheider and Kennedy [12]; since then, several mammalian forms have been identified and characterized. Neutral SMase1 was identified and cloned based on remote sequence similarity to known bacterial sphingomyelinases in 1998 [22]. A year later, results from overexpression and radiolabeling experiments suggested that this 423 amino acid integral membrane protein acts as lyso-platelet activating factor phospholipase C rather than sphingomyelinase in cells [23]. Additional studies are needed to further determine the physiological roles of neutral SMase1. More recently, neutral SMase3, a C-tail anchored protein, was identified using peptide sequence from purified bovine SMases [24]. A study by Cororan et al. suggested this 97 kDa protein may be linked to tumorigenesis and cellular stress response [25]. Neutral SMase2 is the most studied member of the neutral SMase family and has been implicated in a number of pathological conditions. This short chapter will review current knowledge regarding the structure and function of neutral SMase2, as well as regulation of its expression and activity.

Neutral SMase2 Structure and Subcellular Localization

In 2000, Hofmann and colleagues identified the mammalian neutral SMase2 based on remote similarity to bacterial sphingomyelinases using a bioinformatics based gene discovery approach coupled with phylogenetic analysis [26]. This membrane protein consists of 655 amino acid residues with an overall predicted molecular weight of 71 kDa. Neutral SMase2 was reported to be magnesium dependent and can be activated by unsaturated fatty acids as well as anionic phospholipids, such as phosphatidylserine [26, 27]. Unlike neutral SMase1, neutral SMase2 exhibits SMase activity both *in vitro* and *in vivo* with overexpression of this enzyme resulting in accelerated SM catabolism and an increase in ceramide levels [27]. The proposed domain structure of neutral SMase2 (Fig. 18.1) consists of two hydrophobic segments near the N-terminus, followed by a 200-residue collagen-like triple heli-

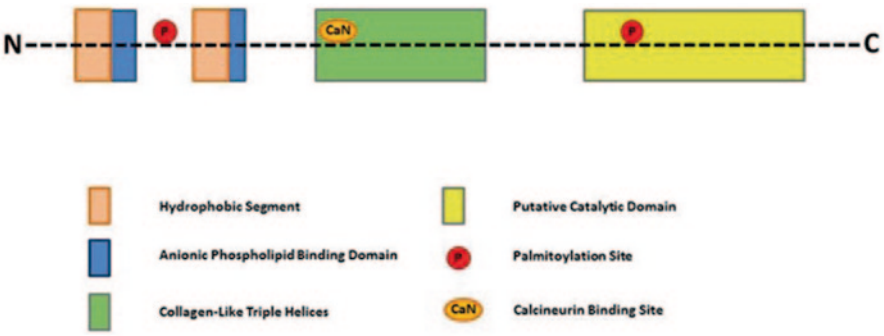


Fig. 18.1 Schematic representation of human neutral SMase2 (GenBank accession number Q9NY59.1). Structural features and putative domains are indicated

ces and a catalytic domain near the C-terminus [26]. Although the two hydrophobic segments were initially proposed to be transmembrane domains, subsequent analysis of neutral SMase2 membrane topology by Tani and Hannun suggested that these segments do not actually span the entire membrane [28]. Two discrete anionic phospholipid (APL) binding domains were identified near the N-terminus which allow neutral SMase2 to interact specifically with certain APLs including phosphatidylserine and phosphatidic acid [29]. The two APL binding domains partially overlay with the two hydrophobic segments and mutagenesis studies revealed that Arg-33, Arg-45 and Arg-48 are essential for interaction with APL in the first domain while Arg-92 and Arg-93 are critical for the second domain [29]. Neutral SMase2 can also be palmitoylated in two cysteine clusters via thioester bonds [30]. Site directed mutagenesis of cysteine to alanine uncovered that this palmitoylation is important for protein stability, as well as its localization with palmitoylation deficient mutants showing rapid degradation and reduced membrane association [30].

The subcellular localization of neutral SMase2 has been reported mainly in two organelles. Hofmann et al. observed localization predominantly at the Golgi in several cell lines derived from the brain [26]. In contrast, Marchesini et al. reported localization at the plasma membrane in confluence arrested MCF7 cells a few years later [31]. Subsequent studies suggest neutral SMase2 is transported between the Golgi and plasma membrane and this intracellular trafficking may be important for its catalytic regulation [32, 33].

Neutral SMase2 Function

During recent years, neutral SMase2 has emerged as an important mediator of cellular stress response, mainly through the production of ceramide. In human airway epithelial cells (HAEC), exposure to oxidative stress (H_2O_2, cigarette smoke) selectively induces the activation of neutral SMase2; and the resultant increase in cellular ceramide leads to HAEC apoptosis and lung injury. This response to oxidative stress is lost upon siRNA silencing of neutral SMase2 [33, 34]. The expression level of neutral SMase2 is also significantly higher in lung tissues obtained from patients with pulmonary emphysema (smokers) as compared to normal control subjects [35]. Together, these studies suggest that neutral SMase2 plays a critical role in ceramide generation following oxidative stress both *in vitro* and *in vivo*. Oxidant exposure has been shown to affect the subcellular localization of neutral SMase2, such that preferential trafficking to the plasma membrane is observed under conditions of oxidative stress; and exposure to the antioxidant glutathione (GSH) leads to the trafficking of neutral SMase2 to the nucleus, where both ceramide generation and apoptosis appear to be attenuated [33]. Clement et al. demonstrated that certain types of neuronal cells could adapt to chronic oxidative stress by down-regulating neutral SMase activity [36]. These cells exhibit increased intracellular cholesterol levels and are resistant to apoptosis. Extracellular treatment of the stress resistant cells with neutral SMase reverses the stress-resistant phenotype; while treatment

of oxidative stress sensitive neuronal cells with neutral SMase2 inhibitors elevated cellular cholesterol and made the cells more resistant to oxidative stress [36].

Endoplasmic reticulum (ER) stress has been shown to inhibit the activity of neutral SMase2 in bovine aortic endothelial cells (BAEC) [37]. When treated with the ER stressor palmitate or tunicamycin, reduced neutral SMase2 activity in BAEC leads to less ceramide generation, which results in a decrease in NO production as endothelial nitric oxide synthase (eNOS) activation is ceramide-dependent [38]. Similarly, siRNA mediated knock-down of neutral SMase2 also results in a decrease in NO generation [37]. This reduced bioavailability of NO promotes the dominance of vasoconstriction over vasodilation. In this way, decreased neutral SMase2 activity could be a contributing factor in the induction of endothelial dysfunction. Neutral SMase2 is involved in the activation of inducible nitric oxide synthase (iNOS). In C6 rat glioma cells, inhibition of neutral SMase2 prevents the induction of iNOS by lipopolysaccharide, whereas inhibition of acid SMase or ceramide *de novo* synthesis had no effect, suggesting that the ceramide produced by neutral SMase2 is critical in the regulation of iNOS expression [39]. Similarly, treatment with GW4869, a specific neutral SMase inhibitor, decreases iNOS expression in cultured human retinal pigment epithelial cells and protects these cells from ER stress-induced apoptosis [40].

Neutral SMase2 also participates in the regulation of cell growth and cancer genesis. Marchesini and colleagues demonstrated that neutral SMase2 is involved in confluence-induced growth arrest in MCF7 cells [31]. Specifically, endogenous neutral SMase2 mRNA is up-regulated when cells become confluent and this up-regulation is associated with G_0/G_1 cell cycle arrest as well as an increase in the level of ceramide. While neutral SMase2 is distributed throughout the cells in sub-confluent, proliferating cultures, its localization is limited to the plasma membrane in growth arrested cultures at confluence [31]. Nucleotide sequencing in a panel of human cancers revealed mutations in the *smpd3* gene, which encodes neutral SMase2, were present in a subset of human leukemia [41], suggesting that neutral SMase2 may have a functional role in cancer initiation or progression.

A number of anti-cancer drugs can act through neutral SMase2. Ito et al. showed that treatment with daunorubicin increases neutral SMase2 mRNA and protein levels in MCF7 cells, placing neutral SMase2 in an important role in daunorubicin-induced cell death [42]. In oligodendrocytes, neutral SMase2 over-expression leads to increased ceramide generation and enhances apoptosis induced by staurosporine or C(2) ceramide [43]. Furthermore, the Sonic-hedgehog inhibitor cyclopamine has been shown to induce apoptosis in Daoy human medulloblastoma cells by selectively activating neutral SMase2 in a nNOS/NO-dependent fashion. siRNA knock-down of neutral SMase2 protected these cells from drug induced apoptosis [44]. Protopanaxadiol, from the root extract of Panax ginseng, can exert cytotoxicity against 5 different cancer cell lines through neutral SMase2 activation and disruption of membrane lipid rafts [45]. Although much more work is needed to deduce all relevant mechanisms, neutral SMase2 could potentially be used to improve the efficiency and selectivity of chemotherapeutic treatments.

Regulation of Neutral SMase2

Neutral SMase2 is a redox sensitive enzyme and the antioxidant GSH inhibits its upregulation [33, 46]. Pre-treatment of MCF7 cells with GSH has been shown to prevent diamide-induced neutral SMase activation [47]. Similarly, treatment with GSH can also protect HAEC from oxidative stress-induced ceramide generation and apoptosis [33, 34]. Further experiments are needed to deduce the specific mechanisms of this inhibition.

All-trans retinoic acid causes G_0/G_1 growth arrest in many cell types. Using MCF7 cells as a model system, Clarke et al. showed that this growth arrest was mediated by an increase in neutral SMase2 activity [48]. This increase in activity was later found to be mostly due to enhanced transcription [49]. Promoter analysis revealed the importance of the 5' promoter region of neutral SMase2 which contains 3 Sp1 sites. Mechanistically, Ito et al. suggested that ATRA treatment activates PKCδ which then phosphorylates Sp1. The phosphorylated Sp1 transcription factor binds to the neutral SMase2 promoter, resulting in increased level of transcription [49].

HSP60 has been shown to interact with neutral SMase2 using proximity ligation assay and immunoprecipitation. Treatment with HSP60 siRNA leads to an increase in neutral SMase2 protein levels in neutral SMase2 overexpressing HEK293 cells, suggesting that HSP60 could be a negative regulator of neutral SMase2 [50].

Long-term as well as acute stimulation with the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) can activate neutral SMase2 in a number of cell lines including MCF7, A549, HUVEC and smooth muscle cells [27, 38, 51, 52]. In A549 cells, exposure to TNF-α results in the translocation of neutral SMase2 to the plasma membrane in a time- and dose-dependent manner [51]. Interestingly, both the activation and translocation of neutral SMase2 following TNF-α stimulation appear to be dependent upon p38 MAPK [51]. These results suggest that neutral SMase2 activity could be modulated by intracellular trafficking and the major site of action is likely at the plasma membrane. In 2012, Barth and colleagues have also shown that TNF-α activates neutral SMase2 in both neurons and non-neuron cells, causing ceramide accumulation, ROS formation and apoptosis [53]. The polycomb group protein EED has been identified as an interaction partner for neutral SMase2 and physically couples neutral SMase2 to the [RACK1-FAN-TNF receptor] complex allowing the transduction of signals initiated by TNF [54].

Recently, Filosto and colleagues reported that neutral SMase2 is a phosphoprotein with phosphorylation occurring exclusively at serine residues [55]. This phosphorylation event has been suggested to occur downstream of p38 MAPK and PKC [55]. In human airway epithelial cells, exposure to oxidative stress enhances the phosphorylation of neutral SMase2, which leads to increased activity [55, 56]. In addition, the phosphatase calcineurin has been reported to bind directly to neutral SMase2, and when the binding site was mutated away, neutral SMase2 would exhibit constitutively elevated phosphorylation and activity [55]. A subsequent publication by the same research group identified five serine residues which were phosphorylated. Three of those residues (Ser-289, Ser-292 and Ser-299) are positioned

near the catalytic domain, while the other two (Ser-173 and Ser-208) are next to the calcineurin binding site [56]. Overall, the phosphorylation of these five serine residues plays a critical role in neutral SMase2 activation under oxidative stress. In addition, neutral SMase2 protein stability could also be regulated post-translationally by phosphorylation; specifically, the phosphorylation of Ser-208 leads to increased protein stability [56].

Another reported post-translational modification of neutral SMase2 is palmitoylation. Two palmitoylated Cys. clusters were identified by Tani and Hannun based on site directed mutagenesis [30]. One of those two clusters is located between the hydrophobic segments, while the other one is found within the catalytic domain. This modification is important for the plasma membrane localization of neutral SMase2 as well as its stability with the palmitoylation deficient mutants being directed to lysosomes and rapidly degraded [30].

Unpublished work in the Mutus lab points toward the possibility that protein S-nitrosylation could be an additional type of post-translational modification capable of down-regulating the activity of neutral SMase2. With reduced activity, the corresponding decrease in ceramide generation can lead to evasion of apoptosis by cancer cells in order to exhibit continued survival and proliferation following exposure to stressors such as oxidative stress and chemotherapeutic drugs. Investigation is currently ongoing to determine whether inappropriate S-nitrosylation of neutral SMase2 could confer a survival advantage to cancer cells.

Conclusion

Since its identification and cloning in 2000, neutral SMase2 has emerged as an important regulator of ceramide generation and sphingolipid signaling. Upon exposure to oxidative stress, anti-cancer drugs or TNF-α stimulation, the activity of neutral SMase2 is selectively up-regulated, resulting in elevated levels of cellular ceramide, which then activate pathways leading to programmed cell death. This increase in neutral SMase2 activity could be attributed to an up-regulation of transcription and/or post-translational modifications. The subcellular localization of neutral SMase2 also affects its activity. Certain types of stimuli, such as hydrogen peroxide and TNF-α, cause the preferential trafficking of neutral SMase2 to the plasma membrane, where there is an enrichment of the substrate SM and the neutral SMase2 activating lipid phosphatidylserine [57]. Determination of trafficking mechanisms will allow us to gain a deeper understanding of neutral SMase2 physiology. In addition to stress response, neutral SMase2 is also implicated in cell growth and cancer genesis. Elucidation of relevant pathways will determine if neutral SMase2 could be used as a therapeutic target for cancer treatments.

Acknowledgements BM research related to this chapter is supported by grants from Seeds4Hope and Natural Sciences and Engineering Research Council (NSERC).

Conflict of interest No potential conflicts of interest are disclosed.

References

1. Zheng W, Kollmeyer J, Symolon H, Momin A, Munter E, Wang E, Kelly S, Allegood JC, Liu Y, Peng Q, Ramaraju H, Sullards MC, Cabot M, Merrill AH Jr. Ceramides and other bioactive sphingolipid backbones in health and disease: lipidomic analysis, metabolism and roles in membrane structure, dynamics, signaling and autophagy. Biochim Biophys Acta. 2006;1758:1864–84.
2. Morales A, Lee H, Goni FM, Kolesnick R, Fernandez-Checa JC. Sphingolipids and cell death. Apoptosis. Int J Programmed Cell Death. 2007;12:923–39.
3. Igarashi Y. Functional roles of sphingosine, sphingosine 1-phosphate, and methylsphingosines: in regard to membrane sphingolipid signaling pathways. J Biochem. 1997;122:1080–7.
4. O'Brien JS, Blankenhorn DH. Fatty acid composition of sphingomyelin and lecithin in normal human serum. Proc Soc Exp Biol Med. 1965;119:862–6.
5. Kishimoto Y, Agranoff BW, Radin NS, Burton RM. Comparison of the fatty acids of lipids of subcellular brain fractions. J Neurochem. 1969;16:397–404.
6. Koval M, Pagano RE. Intracellular transport and metabolism of sphingomyelin. Biochim Biophys Acta. 1991;1082:113–25.
7. Allan D, Kallen KJ. Transport of lipids to the plasma membrane in animal cells. Prog Lipid Res. 1993;32:195–219.
8. Hill PA, Tumber A. Ceramide-induced cell death/survival in murine osteoblasts. J Endocrinol. 2010;206:225–33.
9. Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol. 2008;9:139–50.
10. Hannun YA. The sphingomyelin cycle and the second messenger function of ceramide. J Biol Chem. 1994;269:3125–8.
11. Otterbach B, Stoffel W. Acid sphingomyelinase-deficient mice mimic the neurovisceral form of human lysosomal storage disease (Niemann-Pick disease). Cell. 1995;81:1053–61.
12. Schneider PB, Kennedy EP. Sphingomyelinase in normal human spleens and in spleens from subjects with Niemann-Pick disease. J Lipid Res. 1967;8:202–9.
13. Kirschnek S, Paris F, Weller M, Grassme H, Ferlinz K, Riehle A, Fuks Z, Kolesnick R, Gulbins E. CD95-miated apoptosis in vivo involves acid sphingomyelinase. J Biol Chem. 2000;275:27316–23.
14. Komatsu M, Takahashi T, Abe T, Takahashi I, Ida H, Takada G. Evidence for the association of ultraviolet-C and H(2)O(2)-induced apoptosis with acid sphingomyelinase activation. Biochim Biophys Acta. 2001;1533:47–54.
15. Santana P, Pena LA, Haimovitz-Friedman A, Martin S, Green D, McLoughlin M, Cordon-Cardo C, Schuchman EH, Fuks Z, Kolesnick R. Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. Cell. 1996;86:189–99.
16. Li X, Gulbins E, Zhang Y. Oxidative stress triggers Ca-dependent lysosome trafficking and activation of acid sphingomyelinase. Cell Physiol Biochem. 2012;30:815–26.
17. Smith EL, Schuchman EH. The unexpected role of acid sphingomyelinase in cell death and the pathophysiology of common diseases. FASEB J. 2008;22:3419–31.
18. Zeidan YH, Hannun YA. The acid sphingomyelinase/ceramide pathway: biomedical significance and mechanisms of regulation. Curr Mol Med. 2010;10:454–66.
19. Duan RD. Alkaline sphingomyelinase: an old enzyme with novel implications. Biochim Biophys Acta. 2006;1761:281–91.
20. Duan RD, Nyberg L, Nilsson A. Alkaline sphingomyelinase activity in rat gastrointestinal tract: distribution and characteristics. Biochim Biophys Acta. 1995;1259:49–55.
21. Zhang Y, Cheng Y, Hansen GH, Niels-Christiansen LL, Koentgen F, Ohlsson L, Nilsson A, Duan RD. Crucial role of alkaline sphingomyelinase in sphingomyelin digestion: a study on enzyme knockout mice. J Lipid Res. 2011;52:771–81.
22. Tomiuk S, Hofmann K, Nix M, Zumbansen M, Stoffel W. Cloned mammalian neutral sphingomyelinase: functions in sphingolipid signaling? Proc Natl Acad Sci U S A. 1998;95:3638–43.

23. Sawai H, Domae N, Nagan N, Hannun YA. Function of the cloned putative neutral sphingomyelinase as lyso-platelet activating factor-phospholipase C. J Biol Chem. 1999;274:38131–9.
24. Krut O, Wiegmann K, Kashkar H, Yazdanpanah B, Kronke M. Novel tumor necrosis factor-responsive mammalian neutral sphingomyelinase-3 is a C-tail-anchored protein. J Biol Chem. 2006;281:13784–93.
25. Corcoran CA, He Q, Ponnusamy S, Ogretmen B, Huang Y, Sheikh MS. Neutral sphingomyelinase-3 is a DNA damage and nongenotoxic stress-regulated gene that is deregulated in human malignancies. Mol Cancer Res. 2008;6:795–807.
26. Hofmann K, Tomiuk S, Wolff G, Stoffel W. Cloning and characterization of the mammalian brain-specific, Mg2+-dependent neutral sphingomyelinase. Proc Natl Acad Sci U S A. 2000;97:5895–900.
27. Marchesini N, Luberto C, Hannun YA. Biochemical properties of mammalian neutral sphingomyelinase 2 and its role in sphingolipid metabolism. J Biol Chem. 2003;278:13775–83.
28. Tani M, Hannun YA. Analysis of membrane topology of neutral sphingomyelinase 2. FEBS Lett. 2007;581:1323–8.
29. Wu BX, Clarke CJ, Matmati N, Montefusco D, Bartke N, Hannun YA. Identification of novel anionic phospholipid binding domains in neutral sphingomyelinase 2 with selective binding preference. J Biol Chem. 2011;286:22362–71.
30. Tani M, Hannun YA. Neutral sphingomyelinase 2 is palmitoylated on multiple cysteine residues. Role of palmitoylation in subcellular localization. J Biol Chem. 2007;282:10047–56.
31. Marchesini N, Osta W, Bielawski J, Luberto C, Obeid LM, Hannun YA. Role for mammalian neutral sphingomyelinase 2 in confluence-induced growth arrest of MCF7 cells. J Biol Chem. 2004;279:25101–11.
32. Milhas D, Clarke CJ, Idkowiak-Baldys J, Canals D, Hannun YA. Anterograde and retrograde transport of neutral sphingomyelinase-2 between the Golgi and the plasma membrane. Biochim Biophys Acta. 2010;1801:1361–74.
33. Levy M, Castillo SS, Goldkorn T. nSMase2 activation and trafficking are modulated by oxidative stress to induce apoptosis. Biochem Biophys Res Commun. 2006;344:900–5.
34. Levy M, Khan E, Careaga M, Goldkorn T. Neutral sphingomyelinase 2 is activated by cigarette smoke to augment ceramide-induced apoptosis in lung cell death. Am J Physiol Lung Cell Mol Physiol. 2009;297:L125–33.
35. Filosto S, Castillo S, Danielson A, Franzi L, Khan E, Kenyon N, Last J, Pinkerton K, Tuder R, Goldkorn T. Neutral sphingomyelinase 2: a novel target in cigarette smoke-induced apoptosis and lung injury. Am J Respir Cell Mol Biol. 2011;44:350–60.
36. Clement AB, Gamerdinger M, Tamboli IY, Lutjohann D, Walter J, Greeve I, Gimpl G, Behl C. Adaptation of neuronal cells to chronic oxidative stress is associated with altered cholesterol and sphingolipid homeostasis and lysosomal function. J Neurochem. 2009;111:669–82.
37. Chaube R, Kallakunta VM, Espey MG, McLarty R, Faccenda A, Ananvoranich S, Mutus B. Endoplasmic reticulum stress-mediated inhibition of NSMase2 elevates plasma membrane cholesterol and attenuates NO production in endothelial cells. Biochim Biophys Acta. 2012;1821:313–23.
38. De Palma C Meacci E Perrotta C Bruni P Clementi E. Endothelial nitric oxide synthase activation by tumor necrosis factor alpha through neutral sphingomyelinase 2, sphingosine kinase 1, and sphingosine 1 phosphate receptors: a novel pathway relevant to the pathophysiology of endothelium. Arterioscler Thromb Vasc Biol. 2006;26:99–105.
39. Won JS, Im YB, Khan M, Singh AK, Singh I. The role of neutral sphingomyelinase produced ceramide in lipopolysaccharide-mediated expression of inducible nitric oxide synthase. J Neurochem. 2004;88:583–93.
40. Kucuksayan E, Konuk EK, Demir N, Mutus B, Aslan M. Neutral sphingomyelinase inhibition decreases ER stress-mediated apoptosis and inducible nitric oxide synthase in retinal pigment epithelial cells. Free Radic Biol Med. 2014;72:113–23.
41. Kim WJ, Okimoto RA, Purton LE, Goodwin M, Haserlat SM, Dayyani F, Sweetser DA, McClatchey AI, Bernard OA, Look AT, Bell DW, Scadden DT, Haber DA. Mutations in the neutral sphingomyelinase gene SMPD3 implicate the ceramide pathway in human leukemias. Blood. 2008;111:4716–22.

42. Ito H, Murakami M, Furuhata A, Gao S, Yoshida K, Sobue S, Hagiwara K, Takagi A, Kojima T, Suzuki M, Banno Y, Tanaka K, Tamiya-Koizumi K, Kyogashima M, Nozawa Y, Murate T. Transcriptional regulation of neutral sphingomyelinase 2 gene expression of a human breast cancer cell line, MCF-7, induced by the anti-cancer drug, daunorubicin. Biochim Biophys Acta. 2009;1789:681–90.
43. Goswami R, Ahmed M, Kilkus J, Han T, Dawson SA, Dawson G. Differential regulation of ceramide in lipid-rich microdomains (rafts): antagonistic role of palmitoyl:protein thioesterase and neutral sphingomyelinase 2. J Neurosci Res. 2005;81:208–17.
44. Meyers-Needham M, Lewis JA, Gencer S, Sentelle RD, Saddoughi SA, Clarke CJ, Hannun YA, Norell H, da Palma TM, Nishimura M, Kraveka JM, Khavandgar Z, Murshed M, Cevik MO, Ogretmen B. Off-target function of the Sonic hedgehog inhibitor cyclopamine in mediating apoptosis via nitric oxide-dependent neutral sphingomyelinase 2/ceramide induction. Mol Cancer Ther. 2012;11:1092–102.
45. Park B, Lee YM, Kim JS, Her Y, Kang JH, Oh SH, Kim HM. Neutral sphingomyelinase 2 modulates cytotoxic effects of protopanaxadiol on different human cancer cells. BMC Complement Altern Med. 2013;13:194.
46. Liu B, Hannun YA. Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione. J Biol Chem. 1997;272:16281–7.
47. Okamoto Y, Obeid LM, Hannun YA. Bcl-xL interrupts oxidative activation of neutral sphingomyelinase. FEBS Lett. 2002;530:104–8.
48. Clarke CJ, Mediwala K, Jenkins RW, Sutton CA, Tholanikunnel BG, Hannun YA. Neutral sphingomyelinase-2 mediates growth arrest by retinoic acid through modulation of ribosomal S6 kinase. J Biol Chem. 2011;286:21565–76.
49. Ito H, Tanaka K, Hagiwara K, Kobayashi M, Hoshikawa A, Mizutani N, Takagi A, Kojima T, Sobue S, Ichihara M, Suzuki M, Tamiya-Koizumi K, Nakamura M, Banno Y, Nozawa Y, Murate T. Transcriptional regulation of neutral sphingomyelinase 2 in all-trans retinoic acid-treated human breast cancer cell line, MCF-7. J Biochem. 2012;151:599–610.
50. Ahn KH, Kim SK, Choi JM, Jung SY, Won JH, Back MJ, Fu Z, Jang JM, Ha HC, Kim DK. Identification of Heat Shock Protein 60 as a regulator of neutral sphingomyelinase 2 and its role in dopamine uptake. PloS One. 2013;8:e67216.
51. Clarke CJ, Truong TG, Hannun YA. Role for neutral sphingomyelinase-2 in tumor necrosis factor alpha-stimulated expression of vascular cell adhesion molecule-1 (VCAM) and intercellular adhesion molecule-1 (ICAM) in lung epithelial cells: p38 MAPK is an upstream regulator of nSMase2. J Biol Chem. 2007;282:1384–96.
52. Tellier E, Negre-Salvayre A, Bocquet B, Itohara S, Hannun YA, Salvayre R, Auge N. Role for furin in tumor necrosis factor alpha-induced activation of the matrix metalloproteinase/sphingolipid mitogenic pathway. Mol Cell Biol. 2007;27:2997–3007.
53. Barth BM, Gustafson SJ, Kuhn TB. Neutral sphingomyelinase activation precedes NADPH oxidase-dependent damage in neurons exposed to the proinflammatory cytokine tumor necrosis factor-alpha. J Neurosci Res. 2012;90:229–42.
54. Philipp S, Puchert M, Adam-Klages S, Tchikov V, Winoto-Morbach S, Mathieu S, Deerberg A, Kolker L, Marchesini N, Kabelitz D, Hannun YA, Schütze S, Adam D. The Polycomb group protein EED couples TNF receptor 1 to neutral sphingomyelinase. Proc Natl Acad Sci U S A. 2010;107:1112–7.
55. Filosto S, Fry W, Knowlton AA, Goldkorn T. Neutral sphingomyelinase 2 (nSMase2) is a phosphoprotein regulated by calcineurin (PP2B). J Biol Chem. 2010;285:10213–22.
56. Filosto S, Ashfaq M, Chung S, Fry W, Goldkorn T. Neutral sphingomyelinase 2 activity and protein stability are modulated by phosphorylation of five conserved serines. J Biol Chem. 2012;287:514–22.
57. Vance JE, Steenbergen R. Metabolism and functions of phosphatidylserine. Prog Lipid Res. 2005;44:207–34.

Erratum to:

Front Matter in: Benjamin Bonavida, *Nitric Oxide and Cancer: Pathogenesis and Therapy,*

DOI 10.1007/978-3-319-13611-0

The publisher regrets to have incorrect surname for Dr. Cian McCrudden and affiliation for Dr. McCarthy and Dr. McCrudden in the Preface. The correct version of Preface is mentioned below:

Preface

The recent developments on understanding the challenging topic of nitric oxide (NO) and its derivatives in the field of cancer have yielded significant advances in the potential therapeutic use of NO-related donors in the fight against cancer, used either alone or in combination with other therapies to achieve synergy. The first book in this field "Nitric Oxide and Cancer: Prognosis, Prevention, and Therapy" was published in 2010 by Springer, New York. This book consisted of many reviews by authoritative scientists/ clinicians and included topics as follows: (1) The role of NO in the pathogenesis of cancer (2) The dual roles of NO in protecting or inducing cell death (3) The role of NO in metastasis (4) The chemo-immunosensitizing activities of NO (5) The prognostic significance of NO and (6) The therapeutic applications of NO. Hence, this first book provided a general introduction regarding the important role NO may play in cancer, a taboo subject that has not seriously been considered in the past.

This new book "Nitric Oxide and Cancer: Pathogenesis and Therapy" extends and adds several relevant advances that have been made in the last several years with a thorough understanding of the current status of NO in cancer and its potential therapeutic translational application in the clinic. This book has assembled contributions from experts in this field and reports on up to date reviews on novel findings on various topics of interest to both the scientific and non-scientific communities.

Part I deals with the "*Molecular cell signaling by NO in cancer*." Five contributions cover this topic. Doctors Du and Geller (University of Pittsburgh) reviewed the Wnt/β-catenin signaling pathways modulated iNOS/NO signaling in inflammation-induced carcinogenesis. They used transgenic animal models for their studies. Signals by iNOS/NO result in loss of heterozygosity of adenomatous polyposis colon (APC) and activate the Wnt/β-catenin signaling pathway and contribute to the development of cancer. In fact, inhibition of iNOS decreases the Wnt/β-catenin signaling and cancer growth. These investigators established three pathways for the

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy,*
DOI 10.1007/978-3-319-13611-0_19

interaction between iNOS and the Wnt signaling, namely (i) a positive feedback loop in which iNOS causes APC and β-catenin mutations and by Wnt-inducing iNOS expression (ii) a negative feedback between Wnt and Dichhoff-1 (DKK1) in which iNOS inhibits DKK1 gene expression and (iii) cross-regulation between the NF-κB and Wnt/β-catenin pathways through iNOS/NO genes. They suggested that combining iNOS inhibitors with NSAIDs may synergize for more potent anticancer effects. Dr. Yakovlev (Virginia Commonwealth University) reviews "*Nitric Oxide and Genomic Stability*." It is well established that inflammation induces iNOS and results in the overproduction of NO and reactive nitrogen species (RNS) that participate in carcinogenesis by different mechanisms. Dr. Yakovlev discusses the NO/ RNS-dependent mechanisms of genomic instability and bystander effects.He reviews the mechanisms of "*Synthetic lethality*" of NO-RNS donor/PARP inhibitor combination in sensitizing cancer cells to DNA-mediated damage effects. Dr. Scicinski and colleagues (Mountainview, California) review "*Targeting hyponitroxiain cancer therapy*." Hyponitroxia is a pro-neoplastic effector and they review strategies to reverse this effect by increasing NO and killing the tumor cells. NOS is inhibited due to hypoxia and stimulation under oxic conditions. They discuss attempts to manipulate hypoxia in cancer treatments and they postulate that manipulation of NO levels may represent a potential conversion form hypoxia to enoxia as a function of mutually reinforcing the relationship of NO and oxygen. Dr. Glynn and colleagues (National University, Ireland) discussed "*The role of NO in tumor invasion and metastasis*." They proposed that NOS expression in tumor epithelia has a tumor promoting activity, while NOS expression in tumor-associated macrophages has an anti-tumor activity. Hence, tumor progression/regression depends on the balance between these two NO-associated activities. They present a challenging complexity of the cellular source of NO, the direct exposure and the amount of NO, all of which, form NO-mediated suppressive or stimulating activities in the tumor microenvironment. They proposed that more research is warranted to achieve a highly selective application to favor anti-tumor activity over pro-tumor activity by NO. Dr. Postovit (University of Alberta) reviews "*The role of NO in the regulation of the pro-tumorigenic and hypoxic phenotype*." Clearly, tumor hypoxia correlates with poor clinical prognosis. Dr. Postovit discusses how NO can mimic and mitigate the effect of hypoxia in tumors as a function of the NO concentration. Several clinical trials have been used as examples in which NO-mediated anti-tumor effects correlated with inhibition of hypoxia. Furthermore, a report is discussed in which patients were treated with GTN and resulting in an increased response rate and decreased time to progression in stages IIIb/IV NSCLC treated with cis platinum and vinorelbine. Further, a retrospective study showed that GTN increases the response rate of patients with lung cancer treated with docetaxel and carboplatin. The patients treated with GTN showed decreased levels of VEGF and HIF-1α, corroborating the role of NO in mitigating the pro-tumorigenic effects of hypoxia. Dr. Postovit cautions of NO-mediated treatments to consider the paradoxical role of NO in the regulation of hypoxia-induced manifestations.

Part II covers three reviews on "S-nitrosylation and cancer." Dr. Brown and colleagues (Columbia State University) discussed "The signaling mediated by NO and

through its S-nitrosylation of various proteins and their impact on the tumor cells." S-nitrosylation is reversible and involves the attachment of a nitroso moiety to the reactive thiol/cysteine residues and producing S-nitrothiol (SNO). Several proteins have been reported to be S-nitrosylated that are involved in transcription, DNA repair, and apoptosis, and also proteins involved in tumorigenesis. These investigators have summarized in a table format several proteins that are S-nitrosylated and that are involved in either the progression or inhibition of cancer. Clearly, a better understanding of the mechanisms of signaling and consequences of S-nitrosylation is needed to enable the selective anti-tumorigenic activity over the pro-tumorigenicactivity. Dr. Jeannin and colleagues (Burgundy University, Dijon, France) review "S-nitrosylation of cancer cells." They discussed several protein targets of chemotherapy that are S-nitrosylated. They discuss the potential role of activation of death receptor signaling pathways by NO to treat tumors. They elaborated on the role of NO in the regulation of death receptors, directly or indirectly, and how in combination with chemotherapeutics result in synergistic anti-tumor effects. Clearly, the potential clinical application of suitable NO donors in combination with chemotherapeutic drugs may result in an improved clinical response in cancer patients. Doctors Luanpitpong and Rojanasakul (West Virginia University) discussed "The role of S-nitrosylation in cancer metastasis." They reviewed the roles of S-nitrosylation incancer focusing on anoikis, resistance, cell invasion, migration, and angiogenesis, all of which are key events in metastasis. In their review, they discuss the role of theconstitutively activated PI3K-AKT signaling pathway, in turned on or off, via S-nitrosylationof the phosphatase PTEN. PTEN activity is inhibited by S-nitrosylationand, thus, enhances PI3K-AKT activity and cell survival. The mechanism of anoikis resistance in cancer and S-nitrosylation were well discussed. In addition, they also discuss the S-nitrosylation of various proteins involved in metastasis and apoptosis, including FLIP, Bcl2, cavolin 1, c-Src, EGFR, Ras, MMP-9, etc. These various Snitrosylated proteins are shown to play an important role in the metastatic cascade and resistance to apoptosis.

Part III deals with the "*Modulation of anti-tumor immune responses by NO*." Three contributions are presented. Dr. Garban (University of California, Los Angeles) reviews "*The role of NO on the anti-tumor immune response*." He discusses the reported literature on the role of NO in the sensitization of drug-resistant tumor cells to immune-mediated cytotoxic activities. In addition, he discusses the role of NO-mediated modification of proteins that results in the potentiation of antigenicity. For instance, he refers on the role of NO in IL-2-mediated activation of anti-tumor response. He summarizes the role of NO on the inhibition of the constitutively activated NF-κB pathway and downstream inhibition of anti-apoptotic gene products as well as pro-inflammatory cytokines. In addition, he discusses the role of NO on the inhibition of the resistant factor Yin-Yang 1 (YY1) and FOXP3. Dr. Siesjo (Lund University, Sweden) reviews "*The regulation of anti-tumor immune response by NO*." He discusses the contrasting roles of NO by direct cytotoxicity and by inhibition of anti-tumor immune reactivity. He elaborates on the role of NO on the regulation of both central and peripheral tolerance. Among the topics discussed, he reviews the regulation of T-cells activated by NO, the immune-suppressive role of

NO, the potentiation of anti-tumor cytotoxic cells by NO, the role of dendritic cells and suppressor cells modulated by NO and the mechanism of NO-mediated immune suppression. He attributes the obstacle in manipulating NO in cancer therapy due to the lack of clinically approved NO donors or NO inhibitors. He also suggests the potential of combination of immunotherapy and NO-modulating agents on the fight against cancer. Dr. Doctors Janakiram and Rao reviewed "*Nitric Oxide: Immune modulation of tumor growth*." It is well known that in the tumor microenvironment NO is generated by tumor cells, infiltrated cells, and tissue cells in the microenvironment. Hence, the generation of NO and its levels play a pivotal role in the regulation of tumor growth, both as an enhancer and as a repressor. Clearly, this complexity of the tumor microenvironment and the interaction of tumor cells with the infiltrating immune cells create a system that is not predictable and thus, difficult to establish the best approach to favor NO anti-cancer effects through the use of NO donors or NO inhibitors in clinical therapy.

Part IV deals with "*Therapeutics and overcoming resistance*." Dr. Bonavida reviews the "*Role of NO in chemo-immune resistance*." In this review, he focused on the underlying mechanisms that regulate resistance and how NO treatment results in the reversal of resistance. Emphasis was placed on a dysregulated loop in cancer cells, namely, the NF-κB/Snail/YY1/RKIP loop, that was reported to regulate both drug and immune resistance. Each gene product in the loop was reported to regulate resistance as assessed by the use of specific inhibitors. Treatment with NO donors leads to the modification of the dysregulated loop resulting in the inhibition of NF-κB, Snail, and YY1 and concomitantly with the derepression and the upregulation of RKIP. The mechanism of inhibition of NO was examined and was found to be, in part, due to the direct S-nitrosylation of NF-κB (p50 and p65) and also by S-nitrosylation of Snail and YY1. The direct inhibition of NO as well as indirect inhibition of YY1 and Snail through NF-κB inhibition resulted in upregulation of RKIP and reversal of resistance. In addition, evidence was presented on the activity of NO donors in chemo-immunosensitization of resistant cells. A discussion was provided on the role of NO on inhibiting EMT via inhibition of the loop since inhibition of Snail, a metastasis inducer, was responsible, in part, to the inhibition of EMT and metastasis. Doctors McCarthy and McCrudden (Queen's University, Belfast, UK) discuss "*Emerging role of NO-mediated therapeutics*." They reviewed the emerging strategies of utilizing NO-mediated therapeutics for cancer. They also review the role of *iNOS* gene therapy and its limitations, which was not effective *in vivo*. These investigators have reported a novel inducible and tumor-specific activation of the *iNOS* gene for therapy. Using inducible promoters, they were able to deliver the *iNOS* gene for therapy and observed a delay in tumor growth. They pointed out that, in combination, treatments with high concentrations of NO may not result in antitumor activity. For example, NO can react with some chemo-therapeutic drugs such as etoposide and abolishes its activity. Other examples of *iNOS* upregulation for promoting tumor growth were presented. Doctors Rapozzi and Della Pietro (University of Udine, Italy) reviewed "*The role of NO in photodynamic therapy (PDT)*." PDT is clinically used therapeutically for treatment of early stages of cutaneous tumors. As PDT may induce apoptotic effects, these investigators have examined the role of

NO in mediating PDT anti-tumor response. They discussed the induction of *iNOS*/NO by PDT and NO-mediated tumor cell death by PDT. They also discussed the alternatives to delivering NO-releasing compounds to enhance PDT anti-tumor response. They present the possibility of conjugating NO with a photosensitizer in PDT. This is a new application of PDT which has significant clinical ramifications. Dr. Muntane and colleagues (University of Sevilla, Spain) reviewed "*The inhibition of cell death signaling by NO in cancer cells*." They discuss the role of NO in anti-tumor activity by the regulation of stress response mediated by HIF1-α and p53 that lead to cell growth arrest and apoptosis. They also discuss the induction of DNA damage by NO, the increase of p53 and cell death. They report that NO nitrosylates critical thiols in DNA repair enzymes in hepatoma cells that results in chemo-sensitization. Dr. Scicinski and colleagues (Mountainview, California) review "*Discovery and development of RRX-001*." They have developed a new compound, RRx-001, the first of a class of NO-mediated epigenetic anti-cancer agents. They reported that RRx-001 (designed by combining two structural components, a dinitroazetidine derived from TNAZ [tri-nitroazetidine] and α-bromoacetate) was active as a single agent *in vitro* and *in vivo* against tumor cell lines. They describe the mechanisms by which RRx-001 mediates its activity via NO. They have completed a phase I study with RRx-001 and, aside from phase I end point, they also found clinical benefits in 70 % of patients with multiple tumor types. Of interest, RRx-001 sensitized patients who previously failed therapy. Based on these positive findings, a phase II is being considered.

Part V deals with "*NO-mediated alterations in gene products*." Dr. Othman and colleagues (Northwestern University) review "*The role of NO in coagulation in cancer*." It is well known that NO is a rapid vasodilator and inhibitor of coagulation. Cancer patients are at high risk of developing venous and arterial thrombo-embolic events. They discuss the relationship of pro-coagulant factors and NO, the role of NO and global hemostasis, the link among hypoxia, NO, and coagulation, NO as a fibrinogen system, and NO and thrombosis. They also discuss both the pro and antitumorigenic effects of NO-mediated therapies. Doctors Mutus and Sin (University of Windsor, Canada) review "*The relationship between neutral sphingomyelase 2 and NO and their implications in cancer therapy*." Neutral sphingomyenase 2 is a regulator of ceramide and sphingolipid signals. Under oxidative stress, such as anticancer drugs, the level of SMase2 is upregulated resulting in increased levels of cellular ceramide, which lead to activate pathways that lead to apoptosis.

Clearly, the above contributions have added a new dimension in understanding the complex roles of NO in cancer and have presented several mechanisms of its multiple effects and its potential for therapy when used under optimal conditions. It is, noteworthy, that current studies are aimed at developing novel NO donors that will be effective in the treatment of highly resistant cancer as well as preventing metastasis, when used alone or in combination with sub-toxic therapeutics. In addition, current studies are also exploring the development of complexes consisting of NO and other agents for targeted delivery to enhance specificity and reduce toxicity.

Benjamin Bonavida

Index

B. Bonavida (ed.), *Nitric Oxide and Cancer: Pathogenesis and Therapy*,
DOI 10.1007/978-3-319-13611-0