Resistance to Targeted Anti-Cancer Therapeutics 1

Benjamin Bonavida
Editor

Molecular Mechanisms of Tumor Cell Resistance to Chemotherapy

Targeted Therapies to Reverse Resistance

Springer

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Preface

Patients with cancer are treated with conventional chemotherapeutic drugs and/or radiation. Initially, the majority of patients respond well to such therapies. However, there is a subset of patients who does not respond initially and another subset who no longer responds to further treatments. Clearly, in those two subsets of patients, the cancer cells exhibit mechanisms of resistance to apoptotic stimuli, resulting in cancer progression and metastasis and ultimately death of the patients. One of the main challenges facing us to date is to develop new therapies to treat patients with the resistant tumors in an effort to prolong their survival and to preferentially achieve long-term cures. The development of new effective therapies will be dependent on delineating the biochemical, molecular, and genetic mechanisms that regulate tumor cell resistance to cytotoxic drugs-induced apoptosis. Such mechanisms have revealed gene products that directly regulate resistance and, thus, one may develop new drugs that target these resistance factors. Such new drugs may be either selective or common to various cancers. If successful, doses used by such new drugs may not be toxic and may be used effectively in combination with subtoxic conventional drugs to achieve synergy and to reverse the resistance. Clearly, the development of sensitizing drugs in combination should lead to better clinical responses in patients with the resistant tumors and poor prognosis. Above efforts and approaches have resulted in several FDA-approved drugs that were able to overcome drug resistance and are successfully used clinically. They have been used as monotherapy or synergized with other therapies for the treatment of resistant tumors.

This volume assembles several selective reviews by scientist leaders in the field of cancer drug resistance. Specific mechanisms in drug resistance are reviewed and novel approaches for each mechanism are being proposed for intervention based on the targets that regulate resistance in different cancers. This volume consists of a total of 12 chapters each of which has been selected to cover a different topic in the field of drug resistance. Clearly, these chapters only review selected topics and it was not the intent of this volume to review the whole field of drug resistance. The highlights of each chapter are briefly summarized below.

Doctors Ambudkar and associates describe the multiple resistance phenotypes mediated by the multiple drug resistance (MDR) gene products that are exhibited by many cancer types. They discuss the mechanisms by which the three

ATP-binding cassettes (multiple drug transporters, namely, ABCB1, ABCC1, and ABCG2) prevent cytotoxic drugs to mediate their cytotoxic effects. How these gene products regulate the drug transport and how a large number of chemically unrelated drugs are regulated by the same ABC drug transporter have been thoroughly discussed. Of interest, the authors report single nucleotide polymorphisms (SNPs) in the ABC transporters in certain human populations. These findings open the door to possibly individualize specific treatments of such populations. Moreover, this SNP polymorphism and its relationship to the ABC transporter functions for drugs may also be used as biomarkers and screening to predict a priori the patients' response to certain drugs. The authors also suggest various means of intervention to reverse drug resistance by the use, for example, of tyrosine kinase inhibitors and various novel products. *Doctors Daniel and Rauch* extend the review by Dr. Ambudkar and colleagues on the biochemical and molecular mechanisms upon which the MDR functions in cancer cells. They describe how the physical properties of the cell membrane affect MDR functions and, primarily, discuss the pH changes responsible for altering the cell membrane physical properties and MDR activities. They also proposed an integrated approach unifying all reported studies and mechanisms of drug resistance. *Doctors Freeman and Morrison* discuss the role of the constitutively activated Raf/MEK/ERK pathways in cancer and how inhibitors of these pathways initially resulted in the reversal of resistance. However, tumor cells also develop resistance to such inhibitors. The constitutive activation of the above pathways is due, in part, in the acquisition of oncogenic mutations in components of this pathway. For instance, it has been reported that Ras mutation is found in approximately 20 % of cancers and in melanoma, Ras mutation is found in over 50 % of cases. The development of resistance to inhibitors of the Raf/MEK/ERK pathway, however, is a critical clinical problem for the treatment of patients with such resistance. The authors provide convincing evidence of how the resistance to inhibitors may develop and how interventions may obviate therapy in otherwise resistant patients. While there are currently many active clinical trials assessing the therapeutic drugs targeted MEK and Raf kinases in cancer, it is clear that a large body of data will be generated assessing clinical responses in relationship with the genotype of the tumor. Therefore, it may be possible to select the proper inhibitor for a particular cancer and prevent the development of resistance to the inhibitor. (2) Doctors Tantravahi, Hoffman, and Reddy extend the above studies by Doctors Freeman and Morrison and review published work by others and theirs on the mechanism of resistance of cancer cells to B-Raf therapies. They discuss the FDA-approved B-Raf inhibitor Vemurafinib (Plexxikon) in melanoma and the development of resistance to this drug. In this review, they describe how the B-Raf signaling pathway regulates the development of melanoma and how diverse mechanisms regulate resistance and suggest approaches to be considered for the reversal resistance. The above authors also suggested a different approach than the one used by conventional approaches whereby most of targeted therapies used ATP mimetic small molecules as competitive inhibitors of kinases. They proposed that cancer cells cytotoxicity takes precedence over affinity toward the targets. Hence,

ATP mimetics can be assayed in cultures of tumor cells for cytotoxicity and safety. *Doctors Mukherjee and colleagues* review the role of $\beta 1$ integrins in drug resistance in lung cancer. They discussed that the interaction between transmembrane integrin receptors and ligands in the extracellular milieu is of paramount importance in the pathogenesis of lung cancer. They review the primary role of $\beta 1$ integrins that are preferentially expressed in lung cancer and their involvement in cell proliferation, survival, invasion, and metastasis. β-integrins are also involved in drug resistance. They raise the issue whether targeting $\beta 1$ integrins can reverse drug resistance and may be of therapeutic importance.

Doctor Hara and colleagues reviewed the critical role of aldo-keto reductases (AKR) in colon cancer. In addition to the reported factors that regulate drug resistance such as P-glycoproteins, growth factors, proteasomes, Dr. Hara and colleagues emphasize the important role of aldo-keto-reductases as well. These are upregulated and are involved in both carcinogenesis and resistance. The AKR family consists of NAD(P)(H)-dependent enzymes that catalyzes the oxidoreductase of a variety of substrates. They report that three members only, AKR1B10, AKR1C1, and AKR1C3 are important in the regulation of drug resistance. The mechanism by which these enzymes regulate resistance are also discussed. They suggest that the above three enzymes are not only diagnostic/prognostic marker candidates, but are themselves therapeutic targets. *Doctor Maiti* reviews the role of elevated levels of ROS in cancer cells in relationship to drug resistance. Based on the observation in ovarian cancer that anti-cancer drugs initially increase ROS levels and induce cytotoxicity whereas long exposure of the same drug reduces ROS levels and leads to drug resistance; they suggest that the constant maintenance of high ROS levels is critical. They explain that since most drugs induce toxicity in the cells based on the induction of ROS, cancer cells that have low levels of ROS may be resistant to drugs-induced cytotoxicity. The underlying mechanism of ROS generation and maintenance of genes that regulate ROS in drug resistance may identify new targets for elevation of ROS levels and sensitization to drugs-induced cytotoxicity. *Doctors Nie and colleagues* review a clinically relevant question regarding the inherent resistance of cancer stem cells (CSCs) to cytotoxic drugs. They reviewed the putative underlying mechanisms of CSC resistance and various approaches to target CSCs to improve the clinical response to chemotherapy. *Doctors Maga and colleagues* review the potential use of DNA repair in the reversal of drug resistance and propose to target both the signal transduction pathways and DNA repair pathways. They review the differences that exist between normal cells and cancer cells with respect to underlying mechanisms with respect to cell proliferation and genetic stability in an effort to unravel novel targets for therapeutic intervention. Combination treatment targeting signal transduction and DNA repair pathways should lead to the cancer cells' demise by apoptosis. *Doctors Efferth and colleagues* review an important mechanism in drug resistance, namely, "collateral sensitivity." Collateral sensitivity is defined as the hypersensitivity of otherwise drug-resistant cells. Collateral sensitivity has not been examined in detail when compared to a large number of mechanisms of drug resistance. The mode of action of collateral sensitivity of

currently used new drugs has not been studied in detail. They suggest the development of drugs with high degree of collateral sensitivity to treat drug-resistant cancer.

Doctors Hao and Bellail reviewed the resistance of cancer cells to TRAIL-mediated apoptosis. Phase I and phase II clinical trials have examined recombinant TRAIL and agonist monoclonal antibodies to TRAIL DR4 and DR5 receptors. These clinical trials have not yielded any encouraging results, although, preclinically in mice, the results were highly encouraging. They suggest that it is possible that the previous analyses were done by using primarily tumor cell lines and that freshly derived tumor tissues may have either the absence of TRAIL apoptotic pathways or they may be defective. They propose to analyze freshly-derived human tumor tissues for analysis of TRAIL-mediated cytotoxicity. *Doctor Shekhar* reviews the paradoxical role of apoptosis in cancer cells. They propose that apoptosis results in the accumulation of genomic instability and the promotion of malignant progression of tumors, findings that may have been observed clinically. They suggest that rather than using combination of drugs to induce apoptosis, on the contrary, we have to use drugs that inhibit apoptosis.

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Multidrug Resistance in Cancer: A Tale of ABC Drug Transporters

Khyati Kapoor, Hong May Sim and Suresh V. Ambudkar

Abstract ATP-binding Cassette (ABC) drug transporters are present throughout prokaryotes as well as eukaryotes and have an important physiological role in detoxification and protection of sensitive tissues from toxic compounds. The overexpression of these transporters contributes to the development of multidrug resistance (MDR) by decreasing the intracellular concentration of therapeutic drugs. MDR is characterized by the ability of drug-resistant tumors to exhibit simultaneous resistance to a number of structurally and functionally unrelated chemotherapeutic agents. This chapter discusses the phenomenon of MDR and the proposed ways to overcome its challenges. It particularly focuses on how the three major ABC drug transporters, ABCB1, ABCC1 and ABCG2 hamper chemotherapy in the treatment of cancer. We provide an overview of the structural and functional aspects of these three transporters, discuss the role of single nucleotide polymorphisms in these transporters in the overall pharmacokinetics and pharmacodynamics of chemotherapeutic agents, and summarize current knowledge on the reversal of MDR mediated by these transporters using tyrosine kinase inhibitors and natural products as modulators.

Keywords ABC transporter · ABCG2 · Catalytic cycle · Drug efflux · Modulators · Multidrug resistance · MRP1 · P-glycoprotein · Tyrosine kinase inhibitors

Abbreviations

ABC	ATP-binding cassette
BCRP	Breast cancer resistance protein
CS	Collateral sensitivity
EGFR	Epithelial growth factor receptor
EPR	Electron paramagnetic resonance

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GSH	Glutathione
HDL	High-density lipoprotein
HER	Human epidermal growth factor receptor
LTC4	Leukotriene C4
MAP	Mitogen-activated protein
MDR	Multidrug resistance
MRP	Multidrug-resistance protein
NBD	Nucleotide-binding domain
PDGFR	Platelet-derived growth factor receptor
P-gp	P-glycoprotein
SNP	Single nucleotide polymorphism
TK	Tyrosine kinase
TKI	TK inhibitor
TM	Transmembrane
TMD	Transmembrane domain
VEGFR	Vascular endothelial growth factor receptor
Vi	Sodium ortho-vanadate

Introduction

Multidrug Resistance in Cancer

According to recent statistics from the American Cancer Society (http://www.cancer.org), the top three cancers in men are prostate, lung and colorectal cancers. In women, the lung, breast, colon and rectum are the cancer sites most frequently leading to death. About 45 % of all cancers are resistant to chemotherapy and the main hurdle to treating resistant cancer cells is the development of multidrug resistance (MDR). By definition, MDR is a phenomenon associated with the resistance of tumor cells to the cytostatic or cytotoxic actions of structurally dissimilar and functionally divergent drugs commonly used in cancer chemotherapy [1]. The mechanisms of MDR have been discussed in numerous reviews and can be generalized to the following mechanisms: (1) Over-expression of ATP-binding cassette (ABC) efflux transporters (2) decrease in drug uptake by solute carriers (3) inhibition of apoptosis (4) increase in DNA repair and (5) inactivation of chemotherapeutic drugs by metabolic enzymes [2–4]. In this chapter, we will focus on the role of ABC drug transporters in the development of MDR.

Intrinsic Versus Acquired Resistance

The clinical problem of MDR can be broadly classified as either intrinsic or acquired. Intrinsic MDR refers to pre-existing resistance present in the tumor cells at the time of diagnosis, hence giving rise to the MDR phenotype, while acquired MDR occurs when resistance develops during the course of chemotherapy. To differentiate the two mechanisms of MDR in clinical samples, it is usually necessary to identify the population of self-renewing cells (also referred to as cancer stem cells) present in the tumor. Cancer stem cells have the ability to initiate and sustain the growth of a heterogeneous cancer through self-renewal and differentiation [5]. Often, to isolate this population of cancer stem cells is a challenge, as the properties of these cells depend primarily on their niche environment and they may not be easily identified within the tumor population. Cancer stem cells are able to evade cancer chemotherapy through many avenues. A recent review includes a thorough discussion of the mechanisms of MDR in cancer stem cells. It ascribes the MDR phenotype of cancer stem cells to certain features of the tumor microenvironment and immunosuppression, in addition to the increased expression of ABC transporters [6]. An alternative way for tumor cells to acquire MDR is proposed by Levchenko et al. [7] who suggest that cancer cells can acquire the MDR phenotype by functional intercellular transfer of P-glycoprotein (P-gp). While this concept is interesting, the exact mechanism is not known and the physiological relevance of this mechanism remains to be determined [8].

MDR is multi-factorial and the over-expression of ABC transporters is one major contributor to MDR. Nonetheless, MDR can also develop with anticancer drugs that are not substrates of ABC transporters and clinical MDR can be mediated by other mechanisms besides the involvement of ABC transporters. An example is 'atypical MDR', a term which was applied to a structurally varied class of topoisomerase inhibitors including doxorubicin and etoposide. This type of MDR is mediated by the altered expression of topoisomerase II [9]. Other mechanisms of acquired MDR largely involve the suppression of signaling pathways that leads to the apoptosis of cancer cells.

Recently, the notion of cancer drug pan-resistance has emerged, referring to the state of a tumor when it has acquired resistance to all available chemotherapy treatments and often also to ionizing radiation [10]. According to a review by Piet Borst, pan-resistance should be differentiated from MDR, as its underlying mechanisms of resistance are more perplexing than MDR mediated by drug efflux pumps. This review also explains pan-resistance of cancer cells as mimicry, superior defense or compensation [10]. Cancer cells adopt the proliferation strategies of normal cells and thus mimic them. Also, cancer cells can set up a strong defense system by upregulation of expression of ABC efflux pumps (e.g., P-gp) or by compensation mechanisms by which the cancer cells adapt indirectly without affecting drug-target interaction.

To counteract MDR as well as pan-resistance of cancer cells mediated by the over-expression of efflux transporters such as P-gp, the use of agents that

specifically target cells with upregulated P-gp can be employed through a strategy referred to as collateral sensitivity (CS). CS is the ability of small molecules to selectively kill multidrug-resistant cells and not their parental cells [11, 12]. Examples of compounds that promote CS include verapamil [13], desmosdumotin B analog [14], NSC73306 [15] and tiopronin [16]. Although the development of CS agents to overcome MDR is a novel strategy, the precise mechanisms for the action of these CS agents have yet to be elucidated and warrant more research.

ABC Transporters and their Participation in Diseases

The ABC superfamily is one of the largest superfamilies of transporters, found in all living kingdoms from prokaryotes to eukaryotes. The transporters of this family perform a wide variety of functions. At present there are 48 known ABC members in the human genome and the over-expression of many of these results in various human diseases. These transporters can be subdivided into seven sub-families (A-G) based on structural and sequence similarities. Out of these seven, three subfamilies are commonly linked to MDR. They are the 'B' subfamily, which includes P-gp (MDR1 or ABCB1), the 'C' subfamily, which includes multidrug-resistance protein (MRP1 or ABCC1) and the 'G' subfamily, which includes breast cancer resistance protein (ABCG2 or MXR). P-gp, ABCG2 and MRP1 are the three most common ABC transporters reported to contribute to the development of MDR. Their contribution to resistance is still not well defined, largely because of the difficulties associated with the quantification of the expression level and the activity of these ABC transporters in tumor tissues compared to that in normal tissues in carefully designed trials [17]. Besides these three heavily investigated ABC transporters that are linked to MDR, there are at least 11 other members that can transport drugs [18]. They are ABCA2 [19], ABCA3 [20], ABCB4 [21], ABCB5 [22, 23], ABCB11 [24], ABCC2 [25], ABCC3 [26], ABCC4, ABCC5 [27], ABCC10 [28] and ABCC11 [29]. Defects in 14 ABC transporters cause 12 known genetic diseases, the most common of which are cystic fibrosis, Stargardt disease, adrenoleukodystrophy, Pseudoxanthoma elasticum and Tangier disease (Table 1).

Other than ABCC8 and ABCC9, which encode proteins not known to transport drugs, the ABCC family consists of members that are involved in the transport of various types of molecules. Some of the molecules are organic anions formed from conjugated drug metabolites. The MRPs also transport endogenous metabolites such as cysteinyl leukotrienes, prostaglandins, steroids and glucuronide conjugates. ABCC10 (MRP7) has a membrane topology similar to that of other MRPs but has the least sequence identity to them. The expression of ABCC10 has been seen in small-cell lung cancer cells after exposure to paclitaxel. *In vitro*, ABCC10 is known to confer resistance to taxols such as docetaxel and paclitaxel and a few other compounds such as vincristine, vinblastine and epothilone. Studies indicate MRP7 functions as a lipophilic anion transporter [30].

Table 1 Disease-associated human ABC transporters

ABC transporter	Associated disease[a]
ABCA1 (ABC1)	Tangier disease and familial HDL deficiency
ABCA4 (ABCR)	Stargardt disease, age-related macular degeneration, retinitis pigmentosa
ABCB1 (P-gp)	Cancer, inflammatory bowel disease
ABCB4 (MDR2)	Progressive familial intrahepatic cholestasis
ABCB7 (ABC7)	Sideroblastic anemia and ataxia
ABCB11 (SPGP)	Progressive familial intrahepatic cholestasis
ABCC1 (MRP1)	Cancer, chronic obstructive pulmonary disease, cystic fibrosis
ABCC2 (MRP2)	Dubin-Johnson syndrome
ABCC6 (MRP6)	Pseudoxanthoma elasticum
ABCC7 (CFTR)	Cystic fibrosis
ABCC8 (SUR1)	Persistent hypoglycemia of infancy, neonatal diabetes
ABCC9 (SUR2)	Persistent hypoglycemia of infancy, neonatal diabetes
ABCD1 (ALD)	Adrenoleukodystrophy
ABCG2 (BCRP)	Cancer, gout
ABCG5	Sitosterolemia
ABCG8	Sitosterolemia, gallstone disease

[a] In some instances association with disease condition is due to non-synonymous single nucleotide polymorphisms [125]

The localization of ABCC2, also known as MRP2, is exclusively on the apical membrane of polarized epithelial and endothelial cells in the liver, kidney and intestines. A mutation in the *ABCC*2 gene results in an autosomal recessive disorder known as Dubin-Johnson syndrome. Individuals harboring this mutation have increased serum-conjugated bilirubin levels, as the biliary elimination of bilirubin glucuronides is impaired [30]. Both ABCC1 and ABCC2 are able to confer resistance to a variety of natural product anticancer drugs. However, unlike ABCC1, ABCC2 is able to confer resistance to an important class of chemotherapeutics including platinum-containing drugs [17, 31–34]. Despite its role in conferring resistance to these drugs, the clinical significance of ABCC2 is limited, as there is no clear association between its expression and clinical outcome [17].

In 2003, ABCB5 was reported to regulate rhodamine transport in progenitor cells [35]. ABCB5 has also been identified in malignant melanoma-initiating cells (MMICs) and its expression was reported to be associated with the resistance of melanoma cells to a broad range of anticancer drugs and/or of melanocytes to toxic melanin intermediates and metabolites [36]. ABCB5-transfected HEK293 cells showed higher resistance to many anticancer drugs, which are also known substrates of P-gp. In addition, a reversal of this resistance in the transfectants was observed upon treatment with ABCB5 siRNA, thus indicating that the full-length ABCB5 may play a role in MDR in melanoma cells. However, additional work will be required to better understand the function of this transporter [22].

Tissue Localization and Substrate Specificity of ABC Transporters

Out of the 48 ABC proteins known in humans [37], only 14 appear to transport chemotherapeutic drugs [38]. Further, only three have been demonstrated to have an impact on drug resistance observed in the clinic. There are differences observed in their functions, substrate specificities, molecular mechanisms, and in vivo localizations. We will limit ourselves here to the discussion of only P-gp, ABCG2 and MRP1, which are known to be the major ABC drug transporters linked to MDR in cancer [39]. In addition to their role in drug resistance, they are expressed in non-malignant tissues and are believed to be involved in protecting those tissues from xenobiotic accumulation and resulting toxicity [31]. The localization of these three transporters in various human tissues is listed in Table 2.

One of the well-studied and best-characterized ABC transporters is P-gp, which was first discovered by Juliano and Ling because of its involvement in the development of MDR in cancer cells [40]. P-gp is expressed at the apical membranes of epithelial cells in kidney proximal tubules, the lungs, intestines, liver, brain microvascular endothelia, blood-nerve barrier, placenta, adrenal cortex, blood-testis barrier, uterus, lymphocytes, and in hematopoietic cells [41]. Its primary pharmacological function is to prevent the uptake of toxic compounds from the gut into the body, to expel them in the bile, urine or feces and to protect some very sensitive organs, such as the brain, from them [42–44]. P-gp is also involved in other physiologic processes, such as the control and regulation of apoptosis, stress, hypoxia, stem cell differentiation, cellular immune response and plasma membrane dynamics [45–47].

The second transporter that impacts the pharmacology of chemotherapeutic drugs and the latest to be discovered is ABCG2. Since this gene was isolated from a breast cancer cell line, it was named BCRP [48–50]. It is a half transporter, but functions as a homodimer or oligomer. In addition to its expression in stem cells, ABCG2 localizes mainly in the placental syncytiotrophoblast plasma membrane, the bile canalicular membrane of liver hepatocytes, the luminal membrane of villous epithelial cells in the small and large intestine, the apical side of ducts and lobules in the breast, and in the venous and capillary endothelial cells of almost all tissues [51, 52]. The tissue distribution of ABCG2 overlaps with that of P-gp, which indicates that it might have similar pharmacological functions as P-gp. The

Table 2 Tissue localization of three ABC drug transporters

ABC transporter	Tissue localization
ABCB1 (P-gp)	Intestine, liver, kidney, placenta, adrenal gland, blood–brain barrier, stem cells and hematopoietic cells
ABCG2 (BCRP)	Placenta, intestine, breast, liver, blood–brain barrier, liver, kidney and stem cells
ABCC1 (MRP1)	Ubiquitous and present in all tissues

localization of these three ABC drug transporters strongly suggests their importance in restricting the penetration of toxic substances in crucial tissues such as the central nervous system, intestines, and in the fetus and hematopoietic cells, thus playing a protective role.

Another ABC transporter, MRP1, is one of the best-characterized members of the MRP family and was identified by Cole et al. in 1992 [53]. The main physiological function of MRP1 is presumably leukotriene C4 transport. It is also known to maintain glutathione homeostasis in vivo. MRP1 is expressed in most tissues throughout the body, with relatively high levels found in the lung, testis, kidneys, skeletal muscle, and peripheral blood mononuclear cells. Unlike P-gp, it is mainly localized to the basolateral membrane of epithelial cells [54]. The endogenous expression of MRP1 can contribute to the basal resistance of cell lines to a wide range of chemotherapeutic drugs [55, 56].

Of the other ABCC subfamily members, ABCC4 (MRP4) has been found to be present at higher levels in the prostate and is implicated in the aggressiveness of prostate tumors and neuroblastomas [57, 58]. Depending on the tissue where MRP4 is found, it can be localized to either the basolateral or the apical membranes in polarized cells. In prostate cells and hepatocytes, MRP4 is localized to the basolateral membrane while in renal proximal tubules and brain capillaries, it is found on the apical side [59, 60]. MRP5 is detected at higher levels in brain capillary endothelial cells, pyramidal neurons and astrocytes and in smooth muscle cells of tissues in the genitourinary system [60, 61]. MRP5 can also be localized either on the apical side (in brain capillary endothelial cells) or the basolateral side (some polarized epithelial cells) [60, 62]. *ABCC11* (*MRP8*) is considered to be a non-essential gene, since no orthologous genes have been found in mammals, except in primates [17]. Full-length MRP8 is reported to localize to the apical membrane in stably-transfected polarized epithelial cells [17] and this protein has been detected in axons of neurons in human central and peripheral nervous systems [63].

Topologies of ABC Transporters

The topology of ABC transporters typically consists of four domains, which include two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs), located in the lipid bilayer and the cytoplasm, respectively. These four domains of ABC transporters form the minimum functional unit needed for transport of substrates across the lipid bilayer, powered by the energy from ATP binding and hydrolysis at the NBDs. ABC transporters have varying domain organizations and can exist as a complex of four separate single-domain polypeptides of two NBDs and two TMDs (e.g. bacterial maltose or histidine permease) or they can be two half-transporters with each half of the transporter having one NBD and one TMD, such as members of the ABCG subfamily (ABCG2/BCRP). They can also exist as a single four-domain polypeptide chain, such as P-gp, which has interconnecting NBDs and TMDs. Interestingly, in some of the ABCC subfamily members including MRP1,

besides the four critical domains; there is an additional N-terminal TMD (TMD0) that is smaller than the core TMDs. It has been shown that the deletion of TMD0 does not significantly affect function, but the linker region between TMD0 and TMD1 is important for activity [30, 64].

Substrate Specificity of ABC Drug Transporters

The most striking property of the ABC drug transporters is their broad substrate specificity, which leads to the efflux of a large number of chemically unrelated hydrophobic or amphipathic compounds from the cells. The substrate specificities of the three ABC drug transporters are notably overlapping, but each transporter can handle its own unique set as well (http://www.genecards.org/). P-gp can interact with a vast array of compounds including natural products, chemotherapeutic drugs, steroids, linear and cyclic peptides, fluorescent dyes and ionophores. Amphipathicity and hydrophobicity are a few of the common structural characteristics of P-gp substrates. Because of their hydrophobic nature, these compounds can easily cross the plasma membrane and penetrate into tissues and other pharmacological compartments. The half transporter ABCG2 also has a remarkable range of substrate specificity, and confers resistance to mitoxantrone, topotecan, methotrexate and many other drugs [50, 65]. On the other hand, MRP1 functions as co-transporter of amphipathic organic anions. Further, it extrudes drugs conjugated to glutathione (GSH), glucuronate, or sulfate. MRP1 also mediates the co-transport of unconjugated amphiphilic anions, together with free GSH. The high affinity substrates of MRP1 are leukotriene C4 (LTC4), 17 β-glucuronosyl estradiol and bis-glucuronosyl bilirubin [66–68]. The overlapping substrate specificity of the three drug transporters P-gp, ABCG2 and MRP1, as listed in Table 3, is very clear.

ABC Transporters: Polymorphisms and Pharmacogenomics

ABC transporters often have naturally occurring genetic polymorphisms, mainly single nucleotide polymorphisms (SNP), which result in changes in a single nucleotide (substitution, deletion or insertion) within the DNA sequence, some of which may or may not have an impact on the expression and function of the protein. Many SNPs, both synonymous (sSNP) as well as non-synonymous (nsSNP), have been identified in human ABC transporters. Although synonymous mutations are thought to have no effect on protein structure, there is a possibility that the secondary structure of the mRNA could be affected in such a way that its stability is altered, thus leading to a change in the protein expression levels as well as the functional properties [69]. Polymorphisms in P-gp and ABCG2 are widely

Table 3 Substrate specificity of three ABC drug transporters

Substrate	P-GP	ABCG2	MRP1
Apatinib	+	+	−
Bisantrene	+	+	−
Calcein	−	−	+
Calcein AM	+	−	+
Colchicine	+	−	−
Cyclosporine A	+	−	−
Daunorubicin	+	+	+
Dihydropyridines	+	+	−
Digoxin	+	−	−
Diltiazem	+	−	−
Docetaxel	+	−	−
Doxorubicin	+	+	+
Estrone-3 sulfate	−	+	+
Erythromycin	+	−	−
Etoposide	+	+	+
Flavopiridol	−	+	−
Fluo-3-AM	+	−	+
H33342	+	+	−
Indinavir	+	−	−
LTC_4	−	−	+
Losartan	+	−	−
Methotrexate	−	+	+
Mitoxantrone	+	+	+
Nelfinavir	+	−	−
NEM-GS	−	−	+
Nilotinib	+	+	−
Paclitaxel	+	−	−
Pheophorbide A	−	+	−
Prazosin	+	+	−
Sparfloxacin	+	−	−
Sulfasalazine	−	+	−
Topotecan	+	+	+
Uric acid	−	+	−
Verapamil	+	−	−
Vinblastine	+	−	+
Vincristine	+	−	+

+ indicates a substrate, − indicates a non-substrate

discussed, as they can alter the pharmacokinetics and pharmacodynamics of chemotherapeutic drugs. These SNPs can result in changes in substrate specificity, thus altering drug responses in certain individuals. Details on most of these polymorphisms are available in several databases (www.ncbi.nlm.nih.gov/projects/SNP and www.genecards.org).

There are over 60 known SNPs in the coding region of human P-gp, which include both synonymous SNPs as well as non-synonymous SNPs. These SNPs

have been discussed in detail in a recent review by Wolf et al. [70]. Genetic polymorphisms in P-gp have been reported to change the mRNA expression, protein expression and function of P-gp [71]. Since P-gp plays a role in cellular defense against toxic substances, it is not surprising that polymorphisms in this transporter have been linked to inflammatory bowel diseases such as Crohn's disease and ulcerative colitis [72, 73] (Table 1). Some polymorphisms are also known to increase the risk of renal epithelial tumors. Most of the SNPs in the coding region of P-gp can be mapped to the homology model of human P-gp based on the crystal structure of mouse Abcb1 (mdr1a). Notably, the occurrence of SNPs in the transmembrane region of the protein is much lower in comparison to the extracellular or intracellular regions of the protein [70]. Two synonymous SNPs and 12 non-synonymous SNPs have been associated with differences in function or expression of P-gp. Further, none of these SNPs is known to result in non-functional P-gp. Thus, the exact functional consequences of most of the polymorphisms are not yet clear. Naturally occurring null mutations in P-gp have been reported in mice and dogs but not yet in humans [74, 75]. Expressing human ABC proteins and their variants in yeast allows the function of individual variants to be assessed directly [76].

Analysis of the *ABCG*2 gene has identified over 80 SNPs and other naturally occurring mutations in various ethnic groups. Individuals carrying these polymorphisms are predicted to show altered drug responses due to reduced activity of this efflux pump. Some polymorphisms even affect the stability of ABCG2 in cellular organelles, leading to increased degradation of the transporter [77, 78]. Most of the pharmacogenomic effects of ABCG2 polymorphisms are with respect to its substrates. One of the most characterized SNPs in ABCG2 is the Q141K mutation, which is located between the Walker A and signature motifs in the NBD. Similar to mutations in ABCC7 (CFTR), the Q141K mutation affects cell surface expression of ABCG2 [79]. This SNP has been shown to be associated with Gout disease [80] by genome-wide association studies [81]. The Q141K mutation is commonly found in Chinese and Japanese patients and affects their response to chemotherapy. ABCG2 mediates transport of urate and the Q141K mutation affects this function, which would lead to increased accumulation of uric acid in the kidneys of Gout patients. Another ABC transporter gene, *MRP1*, displays high haplotype diversity; a number of polymorphisms have been determined and many more are being investigated [82]. However, most of the polymorphisms in MRP1 do not appear to have any effect on drug disposition and only a very few have been associated with clinical disease or altered drug response.

Pharmacogenomic analysis is a way to predict the response of an individual to a certain drug, which can lead to improved treatment strategies. Drug therapy could be more effective if we were able to tailor drugs to an individual's genetic makeup. Polymorphisms in ABC drug transporters definitely affect the pharmacokinetics and pharmacodynamics of drugs. However, there are many other factors, such as age, race and diet, which will continue to influence the drug responses in different individuals.

Structure and Mechanism of ABC Drug Transporters

Despite their substrate diversity, ABC transporters share a basic modular structure. It has been well established that the TMDs harbor the drug-binding sites, while the more conserved NBDs are involved in the ATP binding and hydrolysis. We further explain the structure and mechanism of a typical ABC transporter in the sections below.

Drug-Binding Sites of P-gp

While the NBDs across the superfamily are highly conserved in primary sequence, the TMDs vary between the subfamilies of ABC transporters in both the primary sequence and protein folding. These differences in the TMDs lead to differences in the substrate specificities of the transporters. Some ABC transporters recognize very specific substrates, such as the *E. coli* vitamin B12 importer, BtuCD. Others, such as P-gp, are highly promiscuous and recognize a broad range of chemically and structurally diverse compounds, a property known as polyspecificity. Furthermore, the highly flexible nature of P-gp allows it to have a polyspecific nature, binding at least two chemically different drug molecules simultaneously. Identifying the residues in transmembrane (TM) helices that line the drug-binding pocket of P-gp has been intensively investigated by Loo and Clarke, who used cysteine scanning mutagenesis and cross-linking experiments, reporting that TM helices 4, 5, 6, 7, 10, 11 and 12 contribute to drug-binding [83–85]. Using methanethiosulfonate cross linkers and stipiamide derivatives of different lengths, the central putative drug-substrate binding region was proposed to be large enough to bind two drugs simultaneously [86–89]. With the determination of the structure of mouse P-gp, the structural basis for the polyspecificity of P-gp is explained by the large central cavity of 6,000 Å, involving π–π and cation-π stacking as well as hydrophobic and hydrogen bonding interactions between structurally diverse ligands and this transporter [90].

The crystal structures of some ABC exporters have been resolved, including those of mouse Abc1a (mdr1a) in the apo conformation [91] and bacterial Sav1866 in the ADP-bound conformation [92]. Most recently, a high resolution structure of *Caenorhabditis elegans* P-gp in the apo conformation was published [93]. In addition, the structure of the bacterial MsbA homodimer has been reported in various conformations [94]. The X-ray crystal structure of mouse P-gp was reported at resolutions of 3.8–4.4 Å [91], in the apo state in which the substrate is absent and in states in which stereoisomeric cyclic hexapeptide inhibitors (QZ59-RRR and QZ59-SSS) are bound to the transporter. The stereoselectivity of P-gp is based on differences in hydrophobic and aromatic interactions between the protein and the isomers. In the inward-facing apo conformation (in the shape of an inverted 'V'), the NBDs in the apo conformation are wide apart, separated by

approximately 30 Å. To convert from the inward facing to the outward-facing conformation (in the shape of a regular 'V'), the protein has to undergo drastic conformational changes involving the TMDs, following a bellows-like mechanism of action that is commonly proposed for ABC transporters [95]. The evidence for a regular 'V' shape of the outward conformation stems from the crystal structure of Sav1866 (a bacterial homolog of P-gp) [92], in which the NBDs are in a close arrangement with ADP in both the nucleotide-binding pockets and the TMDs are open on the extracellular side, forming a central cavity. The homology models of human P-gp using the structures of mouse P-gp in apo conformation and Sav1866 in ADP-bound conformation is given in Fig. 1.

The validity of the crystal structure of mouse P-gp is controversial [95] with regards to the large separation of the NBDs and whether the solved apo conformation is physiologically relevant [96]. Nonetheless, there is also a body of evidence that supports the mouse P-gp model. The crystal structure of the lipid A exporter, MsbA, in the apo state reveals a large distance of ~50 Å between the

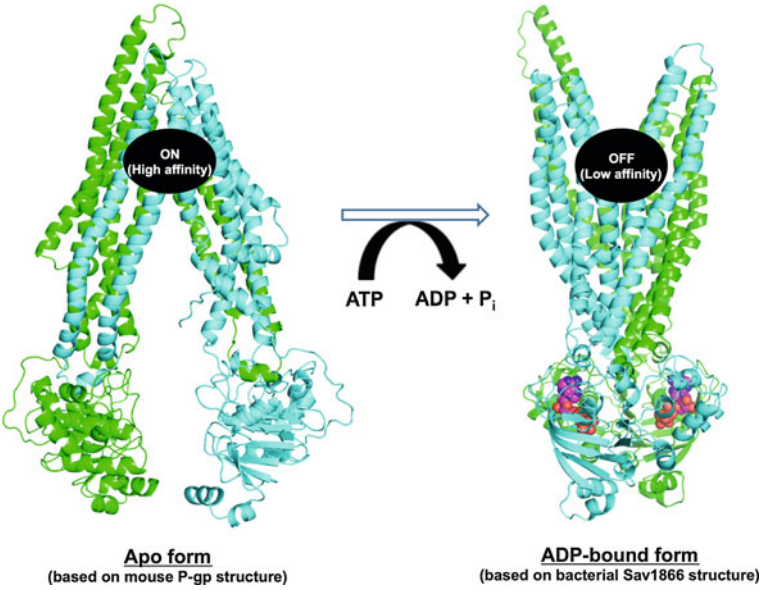

Fig. 1 *Conformation of P-gp before and after ATP hydrolysis.* The homology model of human P-gp in apo (open) form based on mouse P-gp structure [91] and in ADP-bound (closed) form based on Sav1866 structure [92] is shown. Binding of ATP *per se* has no effect on the conformation of either the ATP site or the transport substrate site(s). However, formation of the P-gp•ATP (E•S) reaction intermediate is accompanied by conformation changes in the drug binding site. These changes result in the high affinity (or "ON") site being transformed to the low affinity (or "OFF") site in the drug-binding site. "ON" site refers to the drug-binding site that is exposed to the cytoplasm and affinity for the drug substrate is high. "OFF" site refers to the drug-binding site that is exposed extracellularly with decreased affinity for the drug substrate

NBDs, although the resolution is poor. Using another technique known as electron paramagnetic resonance (EPR), the distance between the NBDs of MsbA are reported as 55 Å in the apo state and 27 Å in the ADP-Vi trapped state.

The crystal structure of *C. elegans* in the apo state was solved recently at a resolution of 3.4 Å [93]. *C. elegans* P-gp, like mouse P-gp, confers a multidrug-resistant phenotype in the insect cells [93]. Compared to mouse P-gp, which has 87 % sequence identity to human P-gp, *C. elegans* P-gp is only 46 % identical. However, similar to the mouse crystal structure, which was determined at the slightly lower resolution of 3.8 Å, *C. elegans* P-gp also showed an inward-facing conformation and the NBDs were seen to have a larger degree of separation as compared to the NBDs of mouse P-gp. In the apo form of *C. elegans* P-gp, the distance between the two serine residues located at the ABC signature motif and the Walker A motif is 35 Å. However, in the mouse P-gp, the same pair of serine residues would be separated by a shorter distance of 19 Å. This inward-facing conformation of the *C. elegans* P-gp in an inverted 'V' shape is similar to the crystal structure of mouse P-gp in the apo conformation, which is not consistent with the in silico model proposed by Jones and George [97]. The in silico model involves constant contact of the NBDs throughout the transport cycle, oscillating around the vertical axis to allow alternating access to the two nucleotide-binding pockets.

Furthermore, in the crystal structure of *C. elegans* P-gp, the drug transport pathway is open to the cytoplasmic surface and is continuous with the membrane inner leaflet. This structure is consistent with the hypothesis that drugs enter ABC exporters through the membrane's inner leaflet according to "hydrophobic vacuum cleaner" model instead of entering directly from the cytoplasm (the "flippase" model). An interesting structural feature observed in *C. elegans* P-gp that is different from the mouse P-gp is the discontinuity of both helices TM10 and TM12 lining the lateral opening of *C. elegans* P-gp. Between the discontinuous TM helices are extended loops that can potentially mediate drug binding. These loops flanking the lateral opening are also highly flexible and can function as hinges to gate the pathway of drug transport. These loop structures may account for the polyspecificity of P-gp. Although the crystal structures of mouse and *C. elegans* P-gp may help reveal the molecular basis of polyspecificity, there is still a significant gap in our understanding of the molecular complexity of multiple drug-binding sites in human P-gp.

Structure of NBDs of ABC Transporters

The transport of substrates by ABC transporters is driven by the binding and hydrolysis of ATP. Even within the ABC exporters, the catalytic and transport mechanisms are not fully conserved [98, 99]. However, the structure and function of NBDs is quite conserved among ABC transporters. Each NBD contains an A-loop, the Walker A and the Walker B motifs, the signature motif (also known as the C motif with consensus sequence LSGGQ), and the D- and H-loops. The two

NBDs are arranged in a head-to-tail manner with the two ATPs sandwiched between the Walker A and B motifs from one NBD and the ABC signature motif from the apposed NBD to form two nucleotide-binding sites. The signature motif as well as the Walker A motifs are involved in the binding of ATP, while the Walker B motif is involved in Mg^{2+} and water co-ordination at the catalytic sites. Other conserved motifs in the NBDs include the Q-loop, which has a role similar to that of Walker B, the A loop (upstream of the Walker A motif), which is a conserved aromatic residue that interacts with the adenine ring of the bound ATP to form π-π interactions [100], the D loop (downstream of Walker B motif), which is involved in indirect co-ordination of the γ-phosphate of ATP through a water molecule [101] and lastly the H-loop that participates in the hydrogen bonding of the γ-phosphate of ATP. An interesting feature to note is the presence of the X-loop (with consensus sequence TEVGERG), which is only present in ABC exporters. These are involved in interdomain communication and cross-talk between NBDs and TMDs.

Drug Transport Models for P-gp

Two main models that describe drug transport by P-gp are the "hydrophobic vacuum cleaner" and the "flippase" models (Fig. 2). These two models involve the hydrophobic or amphipathic drug partitioned in the lipid bilayer, unlike the normal action of a transporter that expels water soluble substrates directly from the cytoplasm to the extracellular medium (pump model on the left in Fig. 2). P-gp substrates are relatively hydrophobic or amphipathic and are readily partitioned in the lipid bilayer by diffusion. P-gp has been suggested to function as a "hydrophobic vacuum cleaner", binding nonpolar substances that are partitioned in the membrane and then expelling them into the extracellular medium [102, 103]. This model is supported by the recent X-ray crystal structure of mouse P-gp where two stereoisomeric cyclic peptides (QZ59-RRR and QZ59-SSS) are shown bound deep within the transmembrane helices of mouse P-gp. This suggests that these cyclic peptides can gain access to the protein from within the lipid bilayer [91]. Alternatively, P-gp has been suggested to function as a "flippase" for physiological substrates such as lipid-like drugs and platelet-activating factor [103–105], moving these substrates from the cytoplasm to the extracellular medium by flipping from the inner leaflet to the outer leaflet of the lipid bilayer. At present, the experimental data are lacking to distinguish between the vacuum cleaner and the flippase model for P-gp.

Transport Cycle of P-gp

As P-gp is the prototypical ABC exporter involved in MDR, there are extensive studies investigating the molecular features of its catalytic cycle and drug

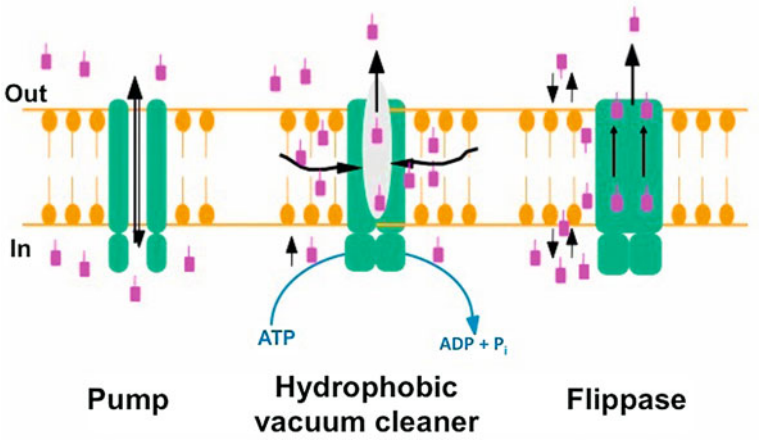

Fig. 2 *Models for drug transport by P-gp.* Substrates of P-gp are proposed to be transported via two models, which involve the partitioning of the amphipathic or hydrophobic substrate molecule such as an anticancer drug (↓) into the lipid bilayer. In the conventional pump model (*left*), a water soluble substrate such as calcium, sodium, potassium or proton is directly effluxed out of the cell from the cytoplasm to the extracellular medium by P-type ATPases [195]. In the "hydrophobic vacuum cleaner" model (*middle*), the drug molecule from the extracellular medium is partitioned into the lipid bilayer and is effluxed out of the cell by P-gp, with only a few molecules entering the cytoplasm [196]. In the "flippase" model (*right*), the drug molecule is partitioned into the lipid bilayer either from the extracellular medium or from the cytoplasm, and then it is flipped from the inner leaflet to the outer leaflet and subsequently effluxed out of the cell by P-gp [103]. In all cases, the energy for transport is provided by hydrolysis of ATP by the NBDs. *Black arrows* indicate the direction of movement of a drug molecule

transport. The functional NBDs were shown to be essential for the hydrolysis of ATP and the inactivation of either one of the NBDs, for example, by the trapping of an inorganic phosphate analogue, orthovanadate (V_i), which inhibits this hydrolysis [106]. It is recognised that in order to hydrolyze ATP, the NBDs must dimerize to form an 'ATP sandwich dimer' with two ATP molecules bound along the interface [107]. However, there are many aspects of the catalytic and transport cycle that are still not well understood. Molecular details on how ATP hydrolysis is co-ordinated between the two NBDs, and how energy from this hydrolysis is transmitted to the TMDs to drive drug transport and whether one or two ATP molecules is/are hydrolyzed per drug molecule transported, are still unknown [100, 108, 109].

In 1966, in his report on membrane pumps, Jardetzky introduced the current consensus view of the mechanism of ABC exporters to be that of the alternating-access switch model [110]. As proposed, a transport event should involve at least three steps: (1) binding of the transport substrate to the TMDs in the 'high-affinity inward-facing orientation' (2) the binding of ATP to the NBDs to form the 'ATP sandwich dimer' and (3) ATP hydrolysis, which leads to transmission of conformational changes from the NBDs to the TMDs to effect a high-affinity to

low-affinity switch at the substrate-binding site, as illustrated in Fig. 1. Notably, both nucleotide and the transport substrate can bind in the absence of each other. Different models have been proposed to identify the power stroke step which is used to drive the conformation from a high to a low affinity for drug-substrates. Is the power stroke obtained from ATP dimerization in the ATP sandwich or is it from ATP hydrolysis? Our work suggests that the power stroke for conformational change is provided only after formation of a pre-hydrolysis transition-like (E•S) state [111, 112].

Catalytic Cycle of P-gp

P-gp is energized by the hydrolysis of ATP to efflux substrates out of the cell. Senior et al. [113] proposed that there are two independent events of ATP hydrolysis in a single catalytic cycle and at a given time, only one NBD hydrolyzes ATP. Therefore, the NBDs work via an "alternating catalytic sites" system with no preference for either the N- or the C-terminal ATP site during ATP hydrolysis [114]. A simplified reaction scheme for ATP hydrolysis by P-gp is given in Fig. 3. The end products of ATP hydrolysis are inorganic phosphate (P_i) and ADP. Preceding the hydrolysis, there is evidence for the formation of an asymmetric

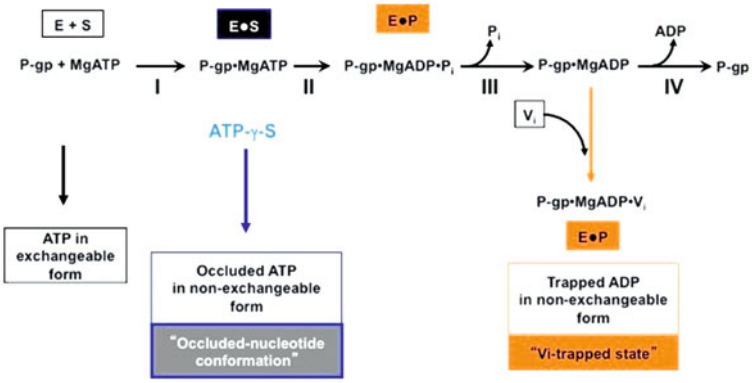

Fig. 3 *Reaction intermediates in the catalytic cycle of ATP hydrolysis by human P-gp.* A simplified scheme for the ATPase reaction of P-gp is shown. The P-gp•MgATP intermediate is equivalent to the E•S complex, which represents the "occluded-nucleotide conformation" that can be obtained with the use of ATP-γ-S, a non-hydrolyzable analog of ATP or by using the Walker B E556Q/E1201Q double mutant [112]. One of two ATPs is occluded at the ATP site and only the ATP that is occluded is committed to hydrolysis. Similarly, the P-gp•MgADP•P_i intermediate is equivalent to the E•P complex and can be captured using Vi, which is an analog of P_i, to form a "Vi-trapped state". The occluded pre-hydrolysis intermediate state at the NBD is proposed to be the "power stroke" for conformational change at the drug-binding site(s) in the TMDs [197]. The release of ADP at step IV (E + P) appears to be the rate-limiting step in the catalytic cycle [198]

occluded nucleotide as a reaction intermediate in the catalytic cycle of P-gp. This occluded reaction intermediate (E•S) is different from the nucleotide-bound state of P-gp. In the occluded nucleotide conformation, P-gp has reduced affinity for transport substrates. The role of this intermediate was characterized by Sauna et al. [112] using ATP-γ-S, which is a non-hydrolyzable analog of ATP. Those studies demonstrated that two ATP molecules initiate dimerization but only one is driven to an occluded pre-hydrolysis intermediate state at the NBD and this is the basis for the power stroke for conformational changes at the TMDs resulting in the movement of the drug substrate. Similarly, the ADP-Vi trapped post-hydrolysis conformation (E•P) also exhibits reduced affinity for drug-substrates [115].

Modulation of ABC Drug Transporters

Compounds having the ability to reverse resistance against anticancer drugs are termed chemosensitizers, MDR modulators or inhibitors. Modulators targeting P-gp function belong to a number of chemical classes and have been classified as first, second or third generation MDR reversal agents on the basis of their affinity for the transporter proteins and relative toxicity towards normal cells. Drugs that were used for other pharmacological functions and were coincidently found to be effective in sensitizing drug-resistant tumors towards chemotherapy are referred to as first generation modulators. These include compounds like verapamil (calcium channel blocker) and cyclosporine A (immunosuppressant) [116]. Obviously, these had lower affinity and were required in high doses and thus failed to manipulate MDR. Additionally, the higher dosage resulted in toxicity and other adverse effects towards normal cells [117]. Thus, these drugs were modified, giving rise to second generation modulators such as R verapamil (R-enantiomer of verapamil, a weaker calcium channel blocker). However, these drugs also failed to deliver the desired efficacy due to their low affinity for their target transporter proteins. Thus, third generation inhibitors were designed specifically for high transport affinity and low pharmacokinetic interaction. These included drugs such as tariquidar and mitotane, which exhibit high affinity and selectivity for target MDR transporter(s) at a low nanomolar range and subsequently low toxicity towards normal cells [118]. These molecules, when co-administered with the anticancer drug, act as competitive inhibitors for the drug-binding site of the transporter proteins, allowing the drug to accumulate inside the cells to effective lethal concentrations.

There are many alternative approaches to handle the problem of MDR, among which targeting RNA is the most popular. This is done by using antisense oligonucleotides, hammerhead ribozymes or siRNA. Other treatments include transcriptional regulators, agents to alter the plasma membrane and also compounds that selectively target MDR cancer cells [119]. All of these approaches, although effective, have not been rigorously tested in clinical trials. MDR reversal agents are known to inhibit the efflux activity of ABC transporters, thus leading to cytotoxicity and are, therefore, important alternative treatments to overcome MDR. Most modulators act

by binding to these ABC transporters in the drug-binding pocket, while others can influence gene expression; for example, the alkaloid piperine was found to lower the expression levels of *ABCB*1, *ABCC*1, and *ABCG*2 genes possibly by acting on the transcription factors [120].

Tyrosine Kinase Inhibitors (Small Molecule Targeted Therapies)

The practice of targeted therapies has significantly changed the treatment of cancer over the past decade. These therapies involve the use of monoclonal antibodies and small molecule inhibitors. Keeping in mind the scope of this chapter, we will discuss only small molecule inhibitors and natural products as modulators of MDR. Cancer treatment has undergone revolutionary changes with the introduction of tyrosine kinase inhibitors (TKIs) that selectively inhibit growth factor pathways critical for tumor growth. TKs can be further classified as receptor TKs (RTKs) and non-receptor TKs (NRTKs) and TKIs block the phosphorylation mediated by these TKs.

TKs initiate a downstream signaling response leading to cell growth, proliferation, migration or angiogenesis in normal as well as malignant tissues. Small-molecule inhibitors target these kinases by directly affecting the tumor cells, rather than by causing immune responses, as monoclonal antibodies do. These small molecule inhibitors typically interrupt cellular processes by interfering with the intracellular signaling of tyrosine kinases. These can be broadly divided into three categories: (1) Inhibitors that block the binding of ATP to the active site of kinases, such as the BCR-ABL kinase inhibitors imatinib, nilotinib and dasatinib, the epidermal growth factor receptor inhibitors gefitinib and erlotinib, and the cyclin-dependent kinase inhibitor, roscovitine. (2) The allosteric inhibitors block sites other than the catalytic sites. These sites are usually required for the activation of kinases, such as the p38 inhibitor BIRB 796, the RAF inhibitor BAY43-9006, and the MAP kinase inhibitor PD184352. (3) Other TKIs include drugs that may inhibit the activation of fusion of TKs by blocking their dimerization, antibodies against RTKs or their ligands, which interrupt TK signaling through neutralization of ligands, blockade of ligand binding, or receptor internalization. Most small-molecule inhibitors of TKs are ATP mimetics. These either block the active site or allosteric site within the intracellular catalytic domain of TKs, thereby inhibiting the subsequent downstream signaling processes (Table 4).

Imatinib or Gleevec, which was approved in 2001 by the FDA, was the first small molecule inhibitor targeted to the BCR-ABL kinase. It is used for the treatment of chronic myeloid leukemia and acts by inhibiting the continuously active BCR-ABL that results from the reciprocal translocation between chromosomes 9 and 22 (the Philadelphia chromosome). Unlike monoclonal antibodies, most small molecule inhibitors are metabolized by cytochrome P450 enzymes.

Table 4 Tyrosine kinase inhibitors, their kinase and ABC transporter targets

TKIs	Target kinase	ABC transporter	References
AG1478	EGFR	ABCB1, ABCG2	[126]
Apatinib	VEGFR2	ABCB1, ABCG2	[127]
Bortezomib	28s protease	ABCB1	[128]
Canertinib	EGFR, HER2, 3, 4	ABCG2	[129]
Cediranib	VEGFR2, PDGFR, c-KIT	ABCB1, ABCC1	[130]
Danusertib	BCR-ABL, Aurora kinases	ABCG2	[131]
Dasatinib	BCR-ABL, Src, c-KIT, PDGFR	ABCB1, ABCG2	[132]
Erlotinib	EGFR	ABCB1, ABCG2, ABCC10	[133, 134]
Gefitinib	EGFR	ABCB1, ABCG2	[135]
Imatinib	BCR-ABL, c-KIT, PDGFR	ABCB1, ABCG2, ABCC1, ABCC10	[121, 136]
Lapatinib	EGFR, HER2	ABCB1, ABCG2. ABCC10	[134]
Neratinib	EGFR, HER2	ABCB1	[137]
Nilotinib	BCR-ABL, c-SRC	ABCB1, ABCG2, ABCC10	[136, 138]
Ponatinib	PDGFR, VEGFR2, BCR-ABL and c-SRC	ABCG2	[139]
Saracatinib	c-SRC	ABCB1	[140]
SGI-1776	FLT-3, Pim	ABCB1, ABCG2	[141]
Sorafenib	EGFR, VEGFR 2, PDGFR, B-raf	ABCC2	[142]
Sunitinib	PDGFR, VEGFR2, c-KIT, FLT-3	ABCB1, ABCG2	[143]
Vemurafenib	B-Raf, MAP	ABCB1, ABCG2	[144]

But these TKIs such as imatinib (Gleevec), dasatinib (Sprycel), sorafenib (Nexavar), and sunitinib (Sutent) are multitargeting in nature, so they are much less specific as compared to the monoclonal antibodies used for treament. Gefitinib (Iressa) and erlotinib (Tarceva) selectively inhibit the epidermal growth factor receptor (EGFR), and both are efficacious against EGFR-expressing cancers. Erlotinib in combination with an antimetabolite, gemcitabine, is also approved for treating advanced pancreatic cancer. More recently, small molecule inhibitors targeting the EGFR pathway have been used in the treatment of solid tumors, such as non-small-cell lung cancer.

Emerging resistance towards the TKIs is a cause of major concern. Some of the most reported and studied mechanisms of resistance to TKIs are point mutations within the kinase domain resulting in decreased sensitivity of the kinases towards the TKIs. These mutations block the inhibitors from accessing the catalytic site and/or activation loop of the kinase. Another mechanism is the efflux of TKIs mainly by P-gp and ABCG2, which has been reported as a contributing factor to the development of resistance towards these inhibitors. A majority of TKIs are transported by P-gp and ABCG2 [121]. It is important to note that TKIs which inhibit ATP-binding to the active site of kinases interact at the drug-binding site in the TMDs of the ABC transporters. Such TKIs do not bind to the ATP sites in

these ABC transporters, suggesting that the ATP sites in kinases and those in the ABC transporters are structurally different. Taken together, an ideal TKI would be a molecule that can inhibit the activity of the target TK but would not be a substrate or modulator for ABC drug transporters. The development of such a molecule will lead to novel TKIs that will be effective and will be able to kill tumor cells at much lower concentrations.

Natural Products as Modulators of ABC Drug Transporters

It is widely known that natural compounds found in vegetables, fruits, plant-derived beverages and herbal dietary supplements not only have anticancer properties, but may also modulate the activity of ABC transporters. Such compounds extracted from natural sources are now referred to as "fourth generation inhibitors". The secondary metabolites of plants, which include alkaloids, phenolics and terpinoids, can interfere with the activity of the well-known efflux pumps [122]. These may either interact directly with the protein, disturbing the tertiary structure, or might function as competitive inhibitors for therapeutic drugs. They can also be used in combination with a cytotoxic agent, resulting in reversal of MDR. Out of these numerous natural products, flavonoids are the most well-known and well-studied. Over 6,000 flavonoids have now been identified and are the most abundant polyphenols in the human diet. They are found in foods such as fruits, vegetables, food supplements, tea and wine. This class of secondary metabolites includes flavonols (quercetin, myricetin), flavones (luteolin, apigenin, and chrysin), isoflavones (genistein, diadzein), flavonones (naringenin, hesperetin), flavonolols, chalcones and aurones. In addition to the antitumor, antimitotic and antiviral properties of flavonoids, they are also able to inhibit kinases, and are active in radical scavenging and metal ion chelating. Most of these natural product modulators act by binding to the drug-binding pocket of ABC transporters. These compounds can thus change the absorption, distribution, metabolism and excretion (ADME) properties of chemotherapeutic drugs by modulating the activity of ABC transporters. A few of them might affect the ATP-binding or hydrolysis at the NBDs or alter the cell-surface expression of ABC transporters. Since most of these natural compounds are components of the human diet, it may be presumed that they would be non-toxic even at higher doses. A list of important natural product modulators for the three ABC drug transporters is given in Table 5. Although many innovative approaches are currently available, studies to design and find a modulator that is selective, relatively less toxic and highly potent appear to be the most likely way to resolve the problem of MDR [123, 124].

Table 5 Plant natural products as modulators of ABC transporters

Natural product	ABC transporter			References
	P-gp	ABCG2	MRP1	
3′-4′-7′-trimethoxyflavone	–	+	–	[145]
5-bromotetrandrine	+	–	–	[146]
6-prenylchrysin	–	+	–	[124]
Abietane diterpene	+	–	–	[147]
Acacetin	+	+	–	[148]
Afrormosin	+	–	+	[149]
Agosterol A and derivatives	+	–	–	[150]
Alisol B 23-acetate	+	–	–	[151]
Amooranin	+	–	–	[152]
Apigenin	+	+	+	[153, 154]
Asarum heteropoides var. mandshuricum	+	–	–	[155]
Ayanin	–	+	–	[156]
Baicalein and derivatives	+	–	–	[124]
Biochanin A	+	+	–	[157]
Bitter melon extract	+	–	–	[158]
Cantharidin	+	–	–	[159]
Cannabinoids	+	+	+	[124]
Carotenoids	+	+	–	[160]
Catechins	+	–	–	[161]
Cepharanthine	+	–	+	[124]
Chrysin	+	+	–	[149, 153]
Citronellal	+	–	–	[162]
Coumarins	+	–	–	[163]
Curcumin	+	+	+	[124]
Cycleanine	+	–	–	[164]
Cycloartanes	+	–	–	[165]
Cyclopamine	+	–	–	[166]
Deoxyschizandrin	+	–	–	[167]
Dimethoxyaurone	+	+	ND	[168, 169]
Diadzein	–	+	–	[145]
Eudesmin	+	–	–	[170]
Eupatin	–	+	–	[171]
Euphocharacins A-L	+	–	–	[172]
Formononetin	+	–	+	[149]
Fumitremorgin C	–	+	–	[124]
Galangin	–	+	–	[122]
Genistein	+	+	+	[148]
Ginkgo biloba extract	+	–	+	[124]
Ginsenoside Rg	+	–	–	[173]
Ginsenosides	–	+	–	[174]
Grapefruit juice extracts	+	–	–	[175]
Hapalosin	+	–	–	[176]
Harmine	–	+	–	[177]

(continued)

Table 5 (continued)

Natural product	ABC transporter			References
	P-gp	ABCG2	MRP1	
Hesperetine	+	+	−	[145]
Hypericin	+	−	−	[124]
Hyperforin	+	−	−	[124]
Ibogaine	−	+	−	[178]
Isoquinoline alkaloid	+	−	−	[124]
Isotetrandrine	+	−	−	[124]
Jatrophanes	+	−	−	[179]
Kaempferol	−	+	+	[148]
Kaempferia parviflora extracts	+	−	+	[124]
Kavalactones	+	−	−	[124]
Kendarimide A	+	−	−	[180]
Limonin	+	−	−	[181]
Luteolin	+	+	+	[153]
Morin	+	−	−	[157]
Myricetin	−	−	+	[182]
Naringin, Naringenin and derivatives	+	+	+	[122]
Ningalin B and derivatives	+	−	−	[124]
Opiates	+	−	−	[183]
Phloretin	+	+	−	[122]
Piperine	+	−	−	[184]
Plumbagin	−	+	−	[185]
Polyoxygenated steroids	+	−	−	[124]
Protopanaxatriol ginsenosides	+	−	−	[124]
Pyranocoumarins	+	−	−	[186]
Quercetin	+	+	+	[124]
Resveratrol	−	+	−	[145]
Retusin	−	+	−	[156]
Rotenoids	−	+	−	[187]
Rutaecarpine	+	−	−	[122]
Sanguinarine	+	−	−	[188]
Schisandrol A	+	−	−	[189]
Sesquiterpines	+	−	−	[190]
Silymarin	+	+	−	[124]
Sinensetin	+	−	−	[191]
Stemona curtissi root extract	+	−	+	[124]
Stilbenoids	−	+	−	[192]
Taxane derivatives	+	−	−	[124]
Tectochrysin	−	+	−	[187]
Terpenoids	+	+	−	[124]
Tetrahydrocurcumin	−	+	−	[122]
Tryprostatin A	+	−	−	[193]
Tryptanthrin	+	−	−	[194]
Vitamin E TPGS	+	−	−	[124]

+ modulation; − no effect; ND Not determined

Conclusion

With the advent of targeted therapies and improved drugs, the treatment of cancer has made remarkable progress in the past few decades. However, the MDR phenomenon limits the benefits of chemotherapy. Studies deciphering the structural and functional aspects of ABC transporters help us to better understand the mechanism of these transporters and assist in designing targeted drugs as modulators for these efflux proteins. Furthermore, understanding changes in the pharmacokinetics and pharmacodynamics of drugs owing to SNPs in these transporters in certain ethnic populations helps us to design treatment plans for specific populations or patients, which is a step towards individualized medicine. This area is being recognized due to variation in the genetic makeup of individuals and, hopefully, soon we will be able to predict the patient-specific efficacy of certain drugs. Clinical MDR is multifaceted and the broad and overlapping substrate specificity of ABC drug transporters adds another dimension to this problem. Recently characterized targeted-therapy drugs including TKIs and fourth generation natural product modulators will have to be tested in clinical trials along with conventional chemotherapeutic drugs to overcome MDR in the clinic and to increase the efficiency of chemotherapy for cancer patients.

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Conflicts of Interest No potential conflicts of interest were disclosed.

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Multidrug Resistance: A Role for Membrane Physics, pH and Drug Transporters

Chloe Daniel and Cyril Rauch

Abstract Cancer is the second cause of mortality worldwide (~8 million death a year) with a cost amounting to ~$900b a year. Early treatment is of paramount importance, and tissue susceptible to becoming cancerous is a target for preventative treatment. Multi-drug resistance (MDR) is a common cause of chemotherapeutic failure in the case of 90 % of metastatic cancer. Different valid theories have been suggested about MDR (drugs transporters, pH, membrane and so on) but there is now a need to provide a unified model concerning all these observations. Herein we show how the alteration in the membrane physical properties mediated by pH changes and the expression of drug transporters are paramount in MDR.

Keywords MDR · NHE1 · pH · Warburg's hypothesis

Abbreviations

ABC	ATP-binding cassette
MDR	Multi-drug resistance
NHE1	The sodium-hydrogen exchanger 1
Pgp	P-glycoprotein

Introduction

The number of cases of cancer in the year 2030 has been estimated, via extrapolation of 2007 statistics [1], and is expected to surpass 430,000 new clinical cases. With a projected rise in all cancers of 45 % by the year 2030 [1], despite taking into the account the predicted increase in screening for breast and prostate cancer,

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research into potential therapeutic techniques is becoming more important than ever. A major obstacle in the treatment of cancer is the development of Multi-drug resistance (MDR) in both metastatic and benign tumours. This transformation, from a cell susceptible to treatment by drugs to a state of resistance has long been the focus of cancer research and continues to be to this day.

MDR involves necessarily the plasma membrane as drugs need to cross the plasma membrane leading to interactions between their physico-chemical properties and those of the bilayer structure. Furthermore, pH [2–4], proton pumps/transporters [5], Pgp [6, 7] and sodium channels [8] are just a few examples of other cancer cell features which have been discovered to have a role in tumour cell survival and/or MDR. All things considered, an interaction must exist between all these biological and physico-chemical components and the ability of a cancer cell to escape drugs treatment.

Understanding how the usual mechanisms of apoptosis are avoided, and how MDR is achieved, should facilitate our research into reversing these characteristics of cancer cells. Whilst the ultimate aim would be a cure for cancer, it is crucial that all researchers have the patient in mind; therefore, it is more realistic that research will enable us to provide palliative treatment options rather than a cure.

Multi-Drug Resistance and the Fluid Mosaic Model of the Plasma Membrane

The plasma membrane of a cell is composed of a phospholipid bilayer, as well as many other lipids and proteins. Its structural integrity is maintained by the electrostatic forces between amphipathic phospholipids; outward facing hydrophilic heads protect the hydrophobic fatty acid tails. The membrane creates a selectively impermeable barrier to most water-soluble solutes. However, it is by no means a rigid, static structure; the many membrane proteins and lipids can laterally traverse the surface, and the numbers of these cell membrane features can alter dramatically, even between the inner and outer leaflet. This constant movement of the constituents, and the varying composition, of the plasma membrane has led to the term ‘Fluid Mosaic Model’ [9] being used to describe the cell membrane structure. In addition to the make-up of the membrane, the presence, or absence, of certain membrane proteins can radically modify the permeability of a cell to a range of molecules.

In the treatment of cancer, as in many other diseases, a drug molecule must first negotiate the cell membrane before acting on its target inside the cell. For that reason, the phospholipid bilayer can be seen as the first barrier a drug must encounter at the level of the cell. It therefore follows, that alterations in the composition, fluidity and, as a result, the permeability of the membrane can have a significant effect on the efficacy of a drug.

Multi-drug resistance can be characterised by cells ability to avoid apoptosis when treated with a substance which aims to bring about its' demise. MDR is known to occur at the level of the cell membrane, it has been shown to manifest itself through increased efflux of a drug as well as through complex interactions with the components of the membrane itself, leading to decreased permeability [10–12]. This suggests that part of the MDR cause may reside in a change of the plasma membrane physical properties.

MDR and Lipid Packing Theory

The density of phospholipids in the membrane has been shown to have a noticeable effect on membrane biomechanics [13, 14] such as fluid-phase endocytosis [15, 16] and the overall permeability of the membrane.

A densely populated cell membrane leads to a more compact packing of phospholipids and this, in turn, augments the rigidity of the membrane [17]. The stiffer the membrane, the less permeable it is to drugs, for example [18].

A higher density of phospholipids in the inner membrane of a cell, compared to the outer leaflet, has been shown to increase the rate of endocytosis [15, 16]. In an intracellular vesicle, the number of phospholipids in the outer membrane is greater than that in the inner membrane, causing a spherical structure to form. This means, therefore, that more lipid was present in the inner leaflet of the plasma membrane at the onset of vesiculation. Increasing the number of phospholipids in the inner leaflet of a cell membrane causes compression, and an out-pouching must occur to alleviate the pressure and rectify the imbalance between the two layers.

Endocytosis of anticancer drugs envelops them in intracellular compartments where they are not free to interact with other cytosolic components of the cell. This can be easily observed in culture conditions [19]. It therefore follows that an increased rate of endocytosis will render more of the drug futile. It has been shown that in cancer cells that are resistant to drugs, the inner leaflet of the membrane is more densely compacted [11, 20], thus a level of MDR can be achieved through the packing of lipids within the cell membrane impeding the transverse movement of drugs. Let us think 1 min about the changes that an increase in membrane tension (i.e. lipid packing) can generate. To interact with its intracellular target a drug needs to traverse the membrane but this event can take some time usually defined by classical rules of thermodynamics and energy barriers involved. If the membrane is "stiffer", the drug will remain trapped in the lipid bilayer for longer period of time. During its residency time, the drug is not static but does diffuse. This residency time will likely increase the likelihood of a drug interacting with any partner on the membrane. Here comes an essential aspect as MDR is also related to the expression of drug transporters among those the archetypal Pgp. Interaction between drugs and Pgp may thus fundamentally rely on the membrane physical state.

Pgp

P-glycoprotein (Pgp) is a member of the ATP-binding cassette (ABC) transporter family, and is also commonly known as the Multi-drug resistance protein 1 (MDR1). As the name suggests, the protein is involved in controlling what substances cross the membrane. A low level of Pgp is present in many tissues of the body [21], however, higher levels of expression occur in tissues such as the blood–brain barrier [22] and excretory luminal epithelium [21, 23]. Distribution of this protein suggests a role in protection of tissues from xenobiotics and toxins of the circulatory system. In cancer, Pgp is responsible for the efflux of xenobiotic drugs [6], and hence has an important role to play in the development of multi-drug resistance in cancer cells. Pgp actively transports the drug back out of the cell and acts as a "flippase" to eject the drug from the membrane [24, 25].

Pgp expression is known to be upregulated in the MDR phenotype [7, 26, 27] in cancer cells and as such is a key target for improving the efficacy of chemotherapy. Pgp inhibitors, such as guggulsterone and CJX1, are capable of re-sensitising cells to drugs [28, 29]. They are known to achieve this through inhibiting Pgp drug expulsion mechanisms in addition to preventing expression of the channel protein in the lipid bilayer.

The Pgp theory looks adequate on its own but there is one point that needs further investigation. Why would drugs interact with a transporter like Pgp and be expelled efficiently? Remind that by definition MDR violates the law of enzyme specificity. The question is to envision a mechanism whereby chemical efficiency without the need for chemical affinity occurs.

The Role of Space Dimensions in MDR

There is one beautiful mathematical theorem known as Polya's theorem that shows that when a particle diffuses in a space with a dimension smaller or equal to two, all the space will be visited and this possibly more than one time [30]. This means that a drug diffusing in the membrane over a long period of time will repeatedly collide with drug transporters. According to Polya's theorem the amount of collision should be far greater in 2D than 3D. In a sense the dimensionality of the membrane (as a 2D object) is paramount for MDR. What we see here is that the low dimensionality of a membrane plays a significant role similar (but certainly not identical) to the one that the temperature plays. This brings in an interesting point that is that if we were living in a different universe with a different number of space dimensions, say 4D for example, MDR would be "physically" impossible.

In any case, Polya's theorem reinforces the role of membrane stiffness in MDR with the presence of drug transporters. The next question is to find out the main biological process that by changing the membrane physical properties increases its

stiffness to allow repeated collisions between drugs and transporters (as dictated by Polya's theorem). An important contributor to membrane stiffness is the pH.

pH

Intracellular pH is intricately regulated by all animal cells, with the exception of erythrocytes, and a small change in $[H^+]$ can dramatically change the ionisation state of all weak bases and acids, affecting most internal cellular processes. Although the actual mechanisms for pH_i regulation are complex and diverse, the concept of pH management is relatively simple. As reviewed by Boron et al. [31] transporters known as "Acid extruders" and "Acid loaders" are responsible for the alkalinisation and acidification, respectively, of the cytosol. For a competent pH_i regulation mechanism to exist, there must be a pH sensor, a signal-transduction system and effector proteins—the 'extruders' and 'loaders'. In this review the membrane-bound protein NHE1 is extensively reviewed as an example.

A reversal in the pH gradient of the cell membrane is now recognised as the foundation for the neoplastic phenotype. Pioneering the research into the involvement of pH in MDR, Otto Warburg's 'Theory of Cancer' of 1927 [32], more commonly known as 'Warburg's Hypothesis', is to this day, the most widely accepted explanation for the aforementioned reversal of the pH gradient. Intracellular pH is known to increase from 6.2–6.9 to 7.3–7.4, alongside a decrease in extracellular pH from 7.12–7.7 to 6.99–7.05 [33]. pH is known to be regulated via many membrane proteins [34], including NHE1 [35], Carbonic anhydrases [36], Monocarboxylic transporters [37], V-ATPase [38, 39] and Cl^-/HCO_3^- [40]. However, the role of NHE1 in particular, has been thoroughly examined [5, 33, 35, 41] and its importance validated in the absence of other membrane transporters. The role of NHE1 will be considered in more detail later in this review. Both intracellular and extracellular pH have a role to play in cancer cell survival, metastasis and invasion.

Warburg's Hypothesis

In 1924, Otto Heinrich Warburg was the first to propose that tumorigenesis is driven by the upregulation of glycolysis [32], an anaerobic mechanism for respiration which promotes cell survival in the often hypoxic environment in the centre of a solid tumour. However, Warburg discovered that this switch to glycolysis is observed even under aerobic conditions. The level of proliferation in cancer cells is much higher than in other cells [42], and therefore they have an increased metabolic rate and subsequent demand for ATP. Given the relative inefficiency of glycolysis, a yield of just 2 ATP molecules in contrast to the 36–38 ATP molecules generated via oxidative phosphorylation, upregulation would appear to be counter-intuitive. This is indeed the case when the cells are under aerobic conditions,

however, as previously mentioned, when cells are subjected to the hypoxic conditions typical of solid tumours. Aerobic respiration via oxidative phosphorylation can therefore not take place and glycolysis must provide the ATP necessary. Warburg demonstrates that the upregulation of glycolysis as seen in cancer cells is initiated by damage to the mitochondria [32], this alleviates mitochondrial inhibition of glycolysis [43]. Bonnet et al. [44] reversed the damage to mitochondrial respiration in cancer cells and thus observed a decline in growth and proliferation, validating the role of the mitochondria in the switch to glycolysis.

The Warburg Effect, not to be mistaken for the Warburg hypothesis, is a measurement of this switch to glycolysis, and is calculated as the ratio of glycolysis to respiration. The impact of this switch to glycolysis is the build-up of lactate and its dissociation products: lactic acid and H^+. Accumulation of H^+ ions intracellularly is detrimental to cell survival and so the cell must extrude excess H^+ ions via proton pumps and transporters. As is described earlier, the mechanism for this externalisation of H^+ ions is via membrane proteins, especially NHE1. It therefore follows that it is the reversal of the pH gradient which must occur first, or indeed in parallel, in order to permit tumour cells to undergo a switch to glycolysis.

NHE1: The Archetypal Example in Cancer

The Sodium-Hydrogen Exchanger 1 (NHE1) is an antiporter which is involved in the regulation of cell pH and volume; it achieves this through mediating the influx and efflux of the cations Na^+ and H^+ respectively, across the cell membrane [34, 35]. NHE1 is incredibly pH sensitive [45]. Its activity is triggered by a drop in intracellular pH, whereby the membrane-bound protein causes the efflux of H^+ ions, to bring the intracellular pH back towards normal. An allometric binding-site for protons is able to detect the smallest in changes of pH_i. In normal cells, NHE1 is calibrated to the physiological pH of the cytosol and remains dormant until such a time as a fall in pH_i is measured [35]. This is then quickly rectified via extrusion of positively-charged hydrogen ions. Present in virtually all tissues [46], the Na^+/H^+ exchanger also plays a particularly important role in the cardiomyocyte action potential.

The role of NHE1 is crucial to the development of the reversed pH gradient and subsequent cancer cell survival. Tumour cells undergo a transformation propelled by the oncogenes ras and v-mas [47, 48]. Activity of the transmembrane protein, NHE1, is amplified through increasing the affinity for its proton substrate [33]. In addition, increased transcription of the gene *SLC9A1* upregulates the expression of NHE1 in tumour cells, one factor implicated in the increased transcription of this gene is epidermal growth factor (EGF) [49]. It has recently been hypothesised that, for NHE1 to be functioning at full capacity and efficiency, the sodium-ion channel $Na_V1.5$ must also be present [50, 51]. The theory behind this describes the role of

$Na_V1.5$ in replenishing the intracellular Na^+ stock which NHE1 uses in exchange for H^+ ions to bring about intracellular alkalinisation [35].

The combination of increased expression and activity of NHE1 augments the efflux of hydrogen ions, acidifying the external microenvironment with the excess of positively charged ions. The resulting depletion of hydrogen ions from the cytosol creates an intracellular alkalinisation. This reversal of the pH gradient across the membrane is necessary for the further transformations a tumour cell must undergo, including, and arguably most importantly, the metabolic switch to glycolysis.

pH and Tumour Cell Survival

Once a reversed pH gradient has been established, tumour cells are free to invade surrounding tissues and commence metastasis. The efflux of H^+ ions, mediated by transporters such as NHE1, not only permits glycolysis to take place at an elevated rate, but also enhances tumour cell aggressiveness [52, 53]. Extruded H^+ ions contribute to sufficiently lowering the pH_e such that the immediate tumour microenvironment is toxic to surrounding tissues [54, 55]. Indeed, proteases secreted by the tumour cell itself have been suggested to become activated by acidic pH_e and therefore bring about the destruction of the extracellular matrix [56]. Furthermore, specific proteases such as Cathepsin B and metalloproteinases have been implicated in cancer cell invasion [57]. Death of adjoining cells creates space for tumour cell advancement, and therefore permits metastasis.

The role of pH in cancer cell survival has been thoroughly researched. Reviewed recently by Harguindey et al. [41] it can be summarised as follows. An increase in pH_i alkalinity drives cells into the S-phase of the cell cycle, promoting a state of perpetual disorganised cell division, and therefore tumour growth. Inhibition of NHE1, an important pH regulator, can prevent tumour cell growth by reducing cancer cell proliferation. Neoangiogenesis is an important feature of tumours, as it allows oxygen and other important nutrients to perfuse solid tumours. Peptides which promote angiogenesis are known to be mediated via NHE1, therefore upregulation and intracellular alkalinisation promote the growth of new blood vessels. pH_i has also been implicated in the functioning of p53, tumour suppressor gene; alkaline pH inhibits p53 therefore promoting cell survival. Suppression of p53 is widely recognised as being an important factor implicated in MDR; loss of function is key to resistance to anticancer drugs.

The effect of intracellular and extracellular pH in cancer cell invasion and metastasis is summarised in Fig. 1.

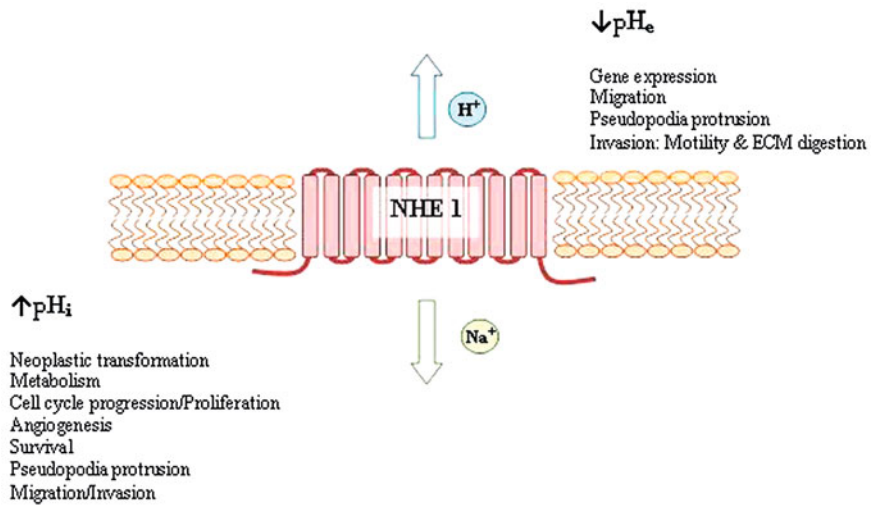

Fig. 1 This figure highlights the role of NHE1 in the acidification of the extracellular tumoural microenvironment and alkalinisation of the cellular cytosol. It also summarises the effects of the aforementioned reverse in membrane potential [35]

pH and MDR

To consider the role of pH in MDR, both intracellular and extracellular pH must be reviewed in turn. Whilst the role of extracellular pH has been examined in detail, intracellular pH has only recently been considered a more significant participant in MDR [58].

The pH partition theory is a widely accepted model which explains how pH_e affects weakly acidic and weakly basic drug entry into a cell. From this theory, it is understood that acidic pH_e has the effect of reducing uptake of weakly basic drugs, such as Doxorubicin, due to protonation making the compound impermeable to the cell membrane barrier [59].

The significance of pH_i has an impact on current potential anticancer therapies. Re-establishing a normal pH gradient across cells would decrease pH_i, with the potential to increase weak base drugs binding with their intracellular targets.

In addition to its effect on the drug itself, it has been proposed that pH can have an effect on the lipid-packing of the cell membrane, which will contribute towards MDR [20]. In a drug-sensitive cell, the fluidity of the inner leaflet of the membrane is maintained via accumulation of protons in the cytosol in close proximity to the membrane. It is hypothesised that these positively-charged ions could have the effect of diminishing the electrostatic repulsion between neighbouring, negatively-charged, phospholipids. However, in a drug-resistant cell, as previously discussed, a proton-efflux has been established which leads to depletion of intracellular protons.

Therefore, neutralisation of the repulsive charges between the phospholipids could not be achieved to the same extent, resulting in a more rigid and less permeable membrane.

Conclusion

MDR is of paramount importance when considering treating a patient with any disease. This is especially so in cancer where often palliative care, and not a cure, is the prevailing objective. MDR is responsible for anticancer drug therapy failure, and the focus of research today is to reverse this phenomenon with the aim of re-sensitising cells to current therapeutic treatments.

Research into the influence of the cell membrane in MDR is extensive and related now to the pioneering research of Otto Warburg and his discovery of the switch in cancer cell metabolism. It is now widely accepted that MDR can be characterised at the level of the cell membrane, and that the key determining factor in the neoplastic phenotype relies predominantly on the pH gradient across the membrane. The Na^+/H^+ antiporter NHE1 is perhaps one of the most important membrane-bound transporters involved in the maintenance of the reversed pH gradient, and therefore it has been the focus of many studies.

Research into MDR will continue indefinitely, with the aim being: to identify other parameters involved in MDR, and ultimately to discover therapeutic methods that disrupt the mechanisms cancer cells employ to achieve resistance to multiple drugs.

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Mechanisms and Potential Therapies for Acquired Resistance to Inhibitors Targeting the Raf or MEK Kinases in Cancer

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Abstract Aberrant activation of the Ras/Raf/MEK/ERK signaling pathway often occurs in human cancer through the acquisition of oncogenic mutations in key pathway components. In particular, Ras mutations are found in about 20 % of human cancers, and B-Raf mutations occur in more than half of all melanomas. Thus, this pathway has become an attractive target for cancer therapies. Inhibitors targeting either the Raf or MEK kinases have shown initial success in treating cancers that depend on the Ras/Raf/MEK/ERK signaling pathway; unfortunately, resistance to the inhibitors eventually develops. For both sets of inhibitors, resistance most commonly occurs via reactivation of the Ras/Raf/MEK/ERK pathway or upregulated signaling from an alternate pathway, such as the PI3K/AKT pathway. Here, we discuss the mechanisms for acquired resistance to inhibitors targeting the Raf or MEK kinases and possible combination therapies to overcome or delay drug resistance.

Keywords Raf inhibitor · MEK inhibitor · Inhibitor resistance · Combination therapy · Signaling pathways

Abbreviations

ATP	Adenosine-5′-triphosphate
AML	Acute myeloid leukemia
CML	Chronic myelogenous leukemia
ERK	Extracellular regulated kinase
EGFR	Epidermal growth factor receptor
FGFR3	Fibroblast growth factor receptor 3
HGF	Hepatocyte growth factor
IGF-1R	Insulin-like growth factor 1 receptor
MEK	MAP or ERK kinase

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NSCLC	Non-small-cell lung carcinoma
PDGFR-β	Platelet-derived growth factor receptor-β
PI3K	Phosphatidylinositide 3-kinase
PTEN	Phosphatase and tensin homolog
RTK	Receptor tyrosine kinase
SAHA	Suberoylanilide hydroxamic acid
STAT3	Signal transducer and activator of transcription 3
VEGFR	Vascular endothelial growth factor receptor

Introduction

The Ras/Raf/MEK/ERK Pathway

The Ras/Raf/MEK/ERK pathway is a critical regulator of cellular processes that include proliferation, differentiation, and senescence [1]. Upon receptor tyrosine kinase (RTK) activation, Ras (K-Ras, N-Ras, or H-Ras) cycles to its active GTP-bound form and recruits the Raf kinases (A-Raf, B-Raf, and C-Raf) to the membrane. At the cell surface, the Rafs dimerize and become active, thus initiating a phosphorylation cascade in which Raf activates MEK, and MEK activates ERK [2, 3]. Once activated, ERK phosphorylates numerous substrates that are then needed for the desired cellular response. Active ERK also instigates a negative feedback loop, whereby ERK phosphorylates the Rafs at several sites, thus attenuating Raf signaling by disrupting the Ras–Raf interaction and dissociating the Raf dimers [4, 5]. ERK activation also results in the upregulation of protein phosphatase activity, which in combination with ERK's inhibitory phosphorylation on Raf as well as the Ras guanine exchange factor SOS1, provides tight control of pathway signaling [6, 7].

The biological importance of proper Ras/Raf/MEK/ERK pathway regulation is highlighted by the frequent mutation of its components in human cancer. Ras is mutated in 20 % of all human cancers, and *B-Raf* is mutated in $\sim$50 % of melanomas, with a single valine-to-glutamic acid substitution at position 600 (V600E) accounting for 90 % of the B-Raf mutations in melanoma [8–10]. Two recent reviews have examined in detail how activating mutations in Ras and Raf can affect various hallmarks of cancer [11, 12]. Given the role of the Ras/MEK/ERK pathway in cancer, the kinase components of this signaling cascade have become attractive targets for pharmaceutical inhibition [13]. A number of Raf inhibitors as well as MEK inhibitors have been developed and are in clinical use. Although these inhibitors are showing promise in the treatment of certain cancers, as has been observed with other kinase-targeted therapeutics, drug-resistance to the inhibitor eventually develops [14, 15].

Raf Inhibitors

Given that B-Raf is a prominent cancer driver and that signaling initiated by activated RTK or Ras proteins is dependent on the Rafs, considerable effort has been focused on the development of Raf kinase inhibitors (Table 1). These include drugs that target all three Raf family members (B-Raf, C-Raf, and A-Raf) as well as those with specificity towards the V600E-B-Raf mutant. Most of the reported Raf inhibitors are ATP-competitive kinase inhibitors, including sorafenib (BAY 43-9006), vemurafenib (PLX4032 and PLX472, a tool compound for in vitro studies), dabrafenib (GSK2118436), SB590885, AZ628, and RAF265. Of these, sorafenib, vemurafenib, and dabrafenib have been studied most extensively in clinical trials.

Sorafenib is a multi-kinase inhibitor that exhibits activity towards wild-type Raf kinases and V600E-B-Raf, as well as the RTKs VEGFR and PDGFR-β, among others [16]. Because B-Raf is so highly mutated in melanoma, several clinical trials have investigated sorafenib's effects in treating this cancer. Unfortunately, this drug appears ineffective at treating melanoma, with a beneficial effect only observed in a subset of patients that have yet to be characterized [17]. Nonetheless, sorafenib has been approved as a standard therapy for hepatocellular carcinoma and shows potential for the treatment of renal cancer [18, 19].

Table 1 Kinase inhibitors that target either the Raf or MEK kinases

	Generic name	Target(s)	Mechanism	References
Raf inhibitors				
BAY 43-9006	Sorafenib	C-Raf, VEGFR-2, VEGFR-3, B-Raf, V600E-B-Raf, PDGFRb, Flt-3, c-KIT	ATP competitive	[16]
PLX4032/PLX4720	Vemurafenib	V600E-B-Raf, BRK, B-Raf	ATP competitive	[20]
GSK2118436	Dabrafenib	V600E-B-Raf, B-Raf, C-Raf	ATP competitive	[21]
SB590885		V600E-B-Raf, C-Raf	ATP competitive	[88]
AZ628		V600E-B-Raf, B-Raf	ATP competitive	[89]
RAF265		C-Raf, B-Raf, V600E-B-Raf, VEGFR-2	ATP competitive	[90]
MEK inhibitors				
AZD6244/ ARRY142886	Selumetinib	MEK1/2	Allosteric	[91]
GSK1120212/ JTP-74057	Trametinib	MEK1/2	Allosteric	[92]
RO4927350/RG7167		MEK1/2	Allosteric	[93]
PD0325901		MEK1/2	Allosteric	[94]
CI-1040/PD184352		MEK1/2	Allosteric	[94]

Unlike sorafenib, vemurafenib and dabrafenib primarily target the highly active V600E-B-Raf [20, 21]. Both of these inhibitors have exhibited clinical efficacy for the treatment of metastatic melanomas that express the B-Raf V600E mutant [14, 15]. However, a frequently reported adverse effect has been the appearance of cutaneous squamous-cell carcinomas and keratoacanthomas that, in many cases, develop because the patient's skin cells harbor an oncogenic mutation in H-Ras [22–25]. In addition, vemurafenib treatment of one melanoma patient was found to promote the proliferation of a previously undetected Ras-mutant leukemia [26]. Surprisingly, all ATP-competitive Raf inhibitors generated to date promote and stabilize Raf dimer formation in the presence of activated Ras, and in these secondary cancers, inhibitor treatment causes a paradoxical activation of the pathway, which results in tumor progression [27–29].

MEK Inhibitors

Although MEK mutations are rare in human cancer, MEK kinases, like the Rafs, are requisite mediators of Ras/Raf/MEK/ERK signaling. Thus, numerous MEK inhibitors have also been developed (Table 1), all of which are allosteric inhibitors and bind to a site other than the ATP-binding pocket. These drugs include selumetinib (AZD6244/ARRY-142886), trametinib (GSK1120212/JTP-74057), RO4927350 (RG7167), PD0325901, CI-1040 (PD184352), and U0126 (a tool compound). Of the MEK inhibitors, trametinib and selumetinib have progressed the furthest in clinical trials, as discussed below.

During inhibitor development, several groups set out to determine predictive markers for cellular responsiveness to MEK inhibition in a variety of Ras pathway-dependent tumor types. Overall, the presence of *Ras* or *B-Raf* mutations was found to correlate with sensitivity to various MEK inhibitors when tested in cancer cell lines of diverse lineages [30–35]. Although one study examining colorectal cancer lines did not find a significant correlation between the presence of *K-Ras* or *B-Raf* mutations and sensitivity to the MEK inhibitor selumetinib, the seven lines they deemed sensitive did have mutations in *K-Ras* or *B-Raf* [36]. Interestingly, sensitivity to the MEK inhibitors has not been found to correlate with levels of phosphorylated ERK or AKT in several studies [32, 33, 36]. In addition, some cell types may be inherently more sensitive to MEK inhibition than others. For example, basal-type and triple-negative breast cancer lines are quite sensitive to MEK inhibition as are the hematologic malignancies AML and CML, whereas breast cancer lines of luminal-origin and cancer cells that have undergone an epithelial-to-mesenchymal transition are less sensitive [35, 37].

MEK inhibitors have shown mixed responses in clinical trials. Similar to Raf inhibitor therapy, one study found that trametinib treatment led to improved overall and progression-free survival in melanoma patients containing a V600E or V600K *B-Raf* mutation [38]. One important difference, however, was that unlike the Raf inhibitors, treatment with trametinib did not induce secondary squamous

cell carcinomas [38]. In regard to selumetinib, although several clinical trials indicate that it has no significant benefit as a general cancer therapy, other studies find that it may be useful for the treatment of melanoma or other cancers that contain oncogenic *Ras* or *B-Raf* mutations [39–45].

Raf Inhibitor Resistance

Despite the reported success of the Raf inhibitors vemurafenib and dabrafenib in the treatment of metastatic melanoma, drug resistance typically develops within months [14, 15]. As a consequence, much effort has been focused on determining the mechanism(s) of acquired resistance. Vemurafenib is the best characterized of these drugs and has been approved by the Food and Drug Administration for use in unresectable or metastatic melanoma, many of which contain activating *B-Raf* mutations [46]. Therefore, much of the work investigating Raf inhibitor resistance has been conducted with vemurafenib and melanoma cell lines or patient tumors samples expressing V600E-B-Raf. To date, the majority of resistance mechanisms identified involve either reactivation of the Ras/Raf/MEK/ERK pathway or sustained upregulation of an alternative pathway, such as the PI3K/AKT pathway (Fig. 1).

Reactivation of the Ras/Raf/MEK/ERK Pathway in Raf Inhibitor Resistance

A common mechanism conferring resistance to numerous ATP-competitive kinase inhibitors is mutation of the gatekeeper residue in the protein kinase domain, which disrupts drug binding [47]. Although experimentally-induced mutation of the B-Raf gatekeeper site can confer the V600E-B-Raf or V600K-B-Raf proteins resistance to many Raf inhibitors [48], mutation of the gatekeeper site does not appear to be a naturally occurring mechanism of Raf inhibitor resistance, in that DNA sequencing has failed to identify any B-Raf gatekeeper mutations in patient samples or melanoma lines exhibiting drug resistance [49–55]. However, a B-Raf alteration that has been identified in patient samples and melanoma lines resistant to vemurafenib involves a splice variant of V600E-B-Raf [56]. This mutant protein lacks exons coding for the Ras binding domain, and it dimerizes in a Ras-independent manner [56]. Thus, due to its enhanced dimerization potential, this mutant increases ERK signaling in the presence of Raf inhibitor [56].

Another mechanism of Raf inhibitor resistance involves the upregulation of Raf protein levels. In a report by Shi et al. [57], whole exon sequencing of tumor samples from four patients with disease progression following vemurafenib treatment revealed copy number gains in the mutant *B-Raf* gene. Increased

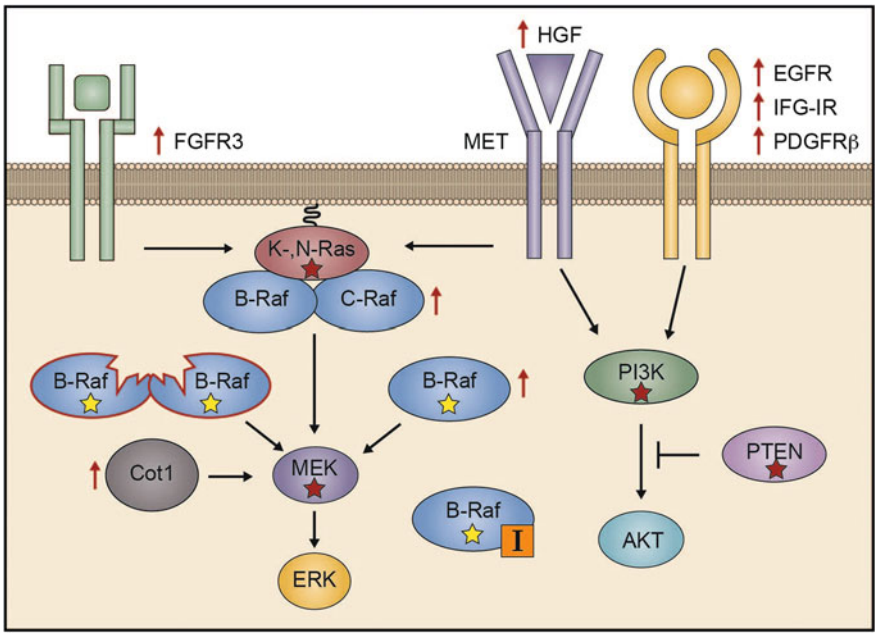

Fig. 1 *Mechanisms of Raf inhibitor resistance.* Several mechanisms of Raf inhibitor resistance are highlighted in *red*, all of which can increase signaling through the Ras/Raf/MEK/ERK or PI3K/AKT pathways. These include increased signaling through FGFR3; higher levels of HGF causing increased signaling through MET; an increase in the number of EGF, IGF-1, or PDGF receptors; mutations (indicated by *stars*) in K-Ras, N-Ras, MEK, PI3K, or PTEN; an increase in C-Raf, V600E-B-Raf, or COT1 protein levels; and a splice variant of V600E-B-Raf

expression of the V600E-B-Raf mutant has also been observed in a vemurafenib-resistant melanoma cell line [51]. Interestingly, further investigation by Shi et al. [57] revealed that the vemurafenib-resistance caused by upregulation of V600E-B-Raf could be overcome by increasing the dose of the Raf inhibitor. An increase in C-Raf protein levels has also been reported to promote drug resistance in melanoma cell lines treated with the Raf inhibitors PLX4720, AZ628 or SB590885 [50, 51, 58]. Moreover, another study using shRNAs to deplete each Raf family member independently and then simultaneously showed that, although the sustained ERK activation in drug-resistant cells required the Rafs, the signal could be generated by any of the Raf family members [53]. Taken together, these studies indicate that, in the presence of a Raf inhibitor, increased signaling from mutant B-Raf or through A-Raf or C-Raf can increase the activation of ERK, thus conferring drug resistance.

Reactivation of the Ras pathway despite inhibitor treatment can also occur via increased signaling from components upstream of Raf, such as RTKs and Ras. A recent study found that FGFR3 showed heightened activity in vemurafenib-resistant melanoma cell lines, potentially due to an autocrine loop generated by an increase in cellular bFGF protein and secreted FGF2 ligand [55]. Upregulated signaling from the

PDGFR and the IGF-1R has also been observed in resistant melanomas [49, 53]. In addition, a case study of a patient whose cancer progressed after vemurafenib treatment reported two different activating mutations in N-Ras at metastatic sites: Q61K and Q61R [49]. Several other studies have linked mutations at N-Ras Q61 to vemurafenib and dabrafenib resistance [49, 52, 57, 59], which may be dependent, in part, on C-Raf and the SHOC2/Sur8 scaffold protein, which contributes to C-Raf activation [60]. Two additional Ras mutations, N-Ras A146T and K-Ras K117N, have also been implicated as mediators of vemurafenib resistance in B-Raf mutant melanomas [51].

Alterations in Ras pathway components that function downstream or parallel to Raf can also confer Raf inhibitor resistance. For example, activating mutations in the downstream MEK1 kinase, such as C121S, Q65P, and K59del, have been reported to mediate PLX4720/vemurafenib resistance in melanoma cells [61–63]. In a study conducted by Shi and colleagues [61], pre-existing MEK1 mutations, P124S or I111S, were identified in baseline tumors from five melanoma patients. However, when a doxycycline-repressible promoter was used to regulate the expression of these MEK1 mutants in cells, neither mutation was found to alter ERK signaling or confer vemurafenib-resistance [61]. In contrast, a different group found that MEK1 P124S or P124L could confer modest resistance to vemurafenib [62]. The cause for this difference is currently unknown, but may be related to different experimental conditions or the influence of other cellular factors.

Raf inhibitor resistance has also been observed in cells with increased expression of Cot1/Tpl2, a kinase that functions parallel to Raf to phosphorylate and activate MEK. By stably expressing cDNA clones encoding various protein kinases in a melanoma cell line, Johannessen et al. [58] found that increased Cot1 protein levels could confer resistance to PLX4720 by increasing MEK and ERK phosphorylation in a Raf-independent manner. High Cot1 expression levels and increased ERK signaling have also been observed in a dabrafenib-resistant melanoma cell line [59]. Moreover, in the study by Johannessen et al. [58], two out of three samples from patients treated with vemurafenib showed increased expression of the mRNA for Cot1, and in a specimen from a patient whose cancer had recurred, the expression was further increased.

Raf Inhibitor Resistance Through Alternative Pathways

Another major mechanism for resistance to Raf inhibitor therapy is the upregulation of signaling through the PI3K/AKT pathway. In particular, an increase in phosphorylated AKT and AKT activity appears to play a role in promoting vemurafenib resistance in melanoma lines [51, 64]. The mechanism is likely to be due, in part, to the pro-survival/anti-apoptotic activity of activated AKT, given that the expression of a constitutively active AKT can protect melanoma cells from PLX4720-induced apoptosis in collagen growth assays [65]. In further support of this mechanism, melanoma cells lacking PTEN, a negative regulator of the PI3K/

AKT pathway, showed reduced apoptosis in response to PLX4720 treatment [66]. Moreover, a failure to upregulate the pro-apoptotic factors Bim and Bmf was found to accompany the reduction in apoptosis, indicating that increased AKT signaling may block the initiation of the apoptotic pathway [65–67].

One manner by which the PI3K/AKT pathway can be engaged is through enhanced RTK signaling. In one study, upregulation of IGF-1R led to increased PI3K/AKT signaling in SB590885-resistant melanoma cells, with the upregulation of IGF-1R due, at least in part, to the downregulation of its negative regulator, IGFBP-3 [53]. In addition, increases in IGF-1R expression and phosphorylated AKT levels were observed in a tumor biopsy from a post-relapse melanoma patient treated with vemurafenib [53]. The upregulation of PDGFR-β can also promote increased levels of phosphorylated AKT and reduced apoptosis in vemurafenib-resistant melanoma lines, and in one study, four of eleven resistant tumors from patients showed higher levels of PDGFR-β expression as compared to the baseline tumor [49, 68].

Although activation of the PI3K/AKT pathway via EGFR upregulation has not been reported in drug resistant melanoma cells, activation of EGFR appears to contribute to the efficacy of the Raf inhibitors in certain cancer types. More specifically, while vemurafenib is beneficial in the treatment of metastatic melanoma harboring the B-Raf V600E mutation, it has little effect on colon cancers with the same mutation [69]. The inhibition of V600E-B-Raf in colon cancer cells reportedly causes feedback activation of EGFR, thus increasing AKT activation and reducing the tumor's dependence on mutant B-Raf to ERK signaling [70]. Interestingly, melanoma cells express relatively low levels of EGFR, and thus cannot deploy this mechanism of inhibitor resistance, at least at the time of initial treatment [70].

Finally, signals from the tumor microenvironment can also impact Raf inhibitor therapy. For example, melanoma cells exposed to the HGF ligand in a cell culture setting had increased resistance to the PLX4720 tool compound and showed elevated ERK and AKT signaling that was initiated by the MET RTK [71, 72]. Similarly, melanoma patients with high plasma levels of HGF or whose stromal cells secreted HGF, showed poorer responses to vemurafenib than patients lacking these characteristics [71, 72].

MEK Inhibitor Resistance

MEK inhibitor resistance is mediated by many of the same mechanisms as Raf inhibitor resistance. These include reactivation of Ras/Raf/MEK/ERK pathway signaling, via MEK mutations or increases in Ras or Raf protein levels, as well as increased signaling through alternative pathways, including the PI3K/AKT and STAT3 pathways (Fig. 2).

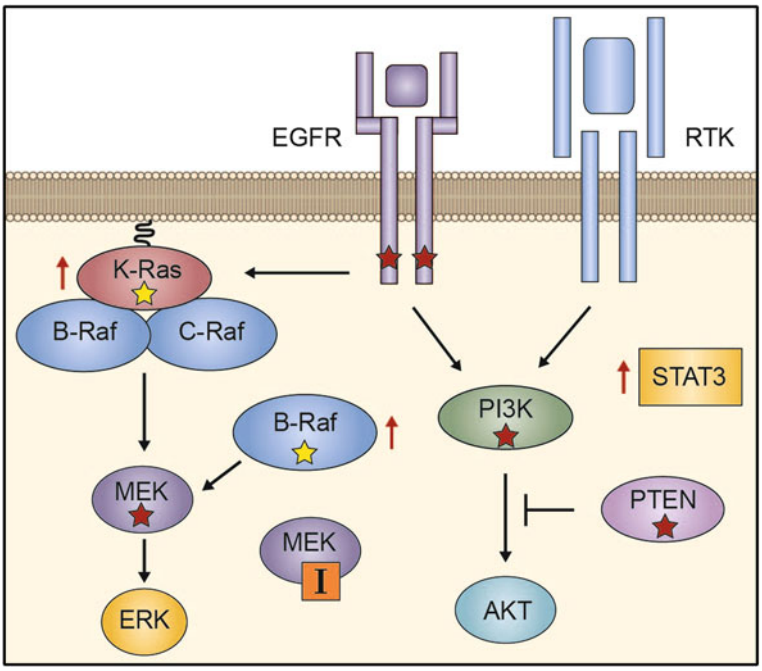

Fig. 2 *Mechanisms of MEK inhibitor resistance*. Several mechanisms of MEK inhibitor resistance are highlighted in *red*. These include mutations (indicated by *stars*) in EGFR, MEK, PI3K, or PTEN, upregulation of STAT3 expression, and an increase in mutant K-Ras or mutant B-Raf protein levels. These alterations can all cause increased signaling through either the Ras/Raf/MEK/ERK, PI3K/AKT, or STAT3 pathways

MEK Inhibitor Resistance Via Reactivation of the Ras/Raf/MEK/ERK Pathway

Although secondary mutations that disrupt drug binding to oncogenic B-Raf have not been identified as a naturally occurring mechanism of Raf inhibitor resistance, this type of mutation does promote acquired resistance to the MEK inhibitors in tumor cell lines and patients. By expressing a saturating cDNA library of MEK1 mutations in melanoma cells containing V600E-B-Raf, a study by Emery et al. [62] identified two classes of mutations that conferred resistance to the MEK inhibitors selumetinib or CI-1040: one set that may enhance MEK1 activity (Q56P and P124S) and another set that alters binding of the drug to MEK1 either through direct mutation of the binding site or by altering the conformation of the αC helix. Mutations in the MEK allosteric binding pocket, including L115P, F129L, and V215E, have been found to promote resistance against the MEK inhibitors RO4927350 or PD0325901 in breast cancer cells harboring an activated K-Ras mutation and in colorectal cells with a B-Raf mutation [73, 74]. Of note, the MEK

F129L mutant also showed increased kinase activity over wild-type MEK1 in vitro, as well as stronger binding to C-Raf [74].

MEK inhibitor resistance can also arise from gene amplification that upregulates the expression of mutant Ras or Raf proteins. In colorectal cancer lines expressing V600E-B-Raf, an increased copy number of mutant *B-Raf* has been found to upregulate mutant B-Raf protein levels, and thereby confer selumetinib resistance [72, 75]. An increase in *K-Ras* gene copy number has also been observed in selumetinib or CI-1040 resistant colorectal lines that contain a mutant K-Ras [73]. Strikingly, when the driver oncogene in these cases was *K-Ras*, *K-Ras* amplification increased both ERK and PI3K/AKT signaling; however, when V600E-B-Raf was the amplified driver, only the ERK signaling was activated [72].

Finally, upregulation of RTK signaling can affect tumor susceptibility to MEK inhibition. More specifically, intrinsic/*de novo* resistance to MEK inhibition (via PD0325901 or CI-1040) has been observed in non-small-cell lung carcinoma (NSCLC) cell lines with mutations in the EGFR kinase domain [31], and in several breast cancer cell lines where increased EGFR and AKT activation were detected [37]. As with the Raf inhibitors, further analysis of patient samples and tumor cell lines will be required to determine whether increased EGFR signaling will be a mechanism of acquired resistance to MEK inhibition.

Upregulation of Alternative Pathways Can Cause MEK Inhibitor Resistance

Several studies have focused on the role of increased signaling through the PI3K/AKT pathway in MEK inhibitor resistance. In one report, four selumetinib-resistant lung cancer cell lines exhibited high levels of phosphorylated AKT and high AKT activity [76]. In another study, increased signaling through the PI3K/AKT pathway in a panel of colorectal cancer cell lines was found to correlate with a decrease in sensitivity to the MEK inhibitor selumetinib [77]. Moreover, in diverse cancer cell lines, K-Ras mutant cells that lacked PTEN or contained activating mutations in PI3K exhibited resistance to MEK inhibition by PD0325901 or selumetinib treatment [78, 79]. Taken together, these findings suggest that increased signaling through the PI3K/AKT pathway can mediate both *de novo* and acquired resistance to MEK inhibitors.

Additional factors may also contribute to MEK inhibitor resistance. In particular, an increase in STAT3 activation has been reported to promote selumetinib resistance in NSCLC cell lines by upregulating miR-17, a microRNA cluster that modulates the expression of certain genes including the pro-apoptotic gene *Bim* [80]. As a result, STAT3 activation prevented apoptosis by blocking the upregulation of Bim and, in turn, PARP cleavage [79, 80]. Treatment of colorectal cancer and melanoma cells with TNFα has also been shown to block cell death in response to the MEK inhibitor CI-1040 [81]. In one study, a 13-gene signature that

predicts selumetinib resistance mediated by Ras effectors other than PI3K was identified [30]. In addition, gene array analysis has revealed that upregulation of the Wnt pathway can contribute to selumetinib resistance in K-Ras mutant colorectal cancer cell lines [36]. Finally, intrinsic resistance to selumetinib in a panel of NSCLC or colorectal cancer cell lines has also been linked to cAMP-dependent PKA activation [82].

Treatment Strategies to Overcome RAF or MEK Inhibitor Resistance

Current studies with cancer cell lines and patient samples indicate that the administration of two or more targeted inhibitors may be more effective than single-drug therapies in treating human malignancies. In particular, combination therapies are proving beneficial for the treatment of tumors that have developed resistance to a particular inhibitor. Moreover, using combination therapy from the start of treatment may prevent or delay the development of drug resistance. Selecting combination therapies to treat Raf or MEK inhibitor resistance has largely focused on the two most common mechanisms of resistance, namely, reactivation of the Ras/Raf/MEK/ERK pathway and upregulation of the PI3K/AKT pathway (for details, see above sections).

Dual Therapies Co-Targeting Raf and MEK

Dual therapies that co-target the Raf and MEK kinases are proving successful for suppressing growth in cancer lines that have become resistant to either Raf or MEK inhibition via reactivated ERK signaling. In melanoma cells, the combination of vemurafenib and selumetinib has been reported to act synergistically and to inhibit the proliferation of vemurafenib-resistant cells that have increased FGFR3 activation or the amplification of mutant *N-Ras* or *B-Raf* genes [55, 57]. Vemurafenib and the MEK inhibitor RO5068760 were also found to promote apoptosis in B-Raf mutant melanoma cells with acquired resistance to vemurafenib and to have significant antitumor activity in mouse xenograft assays [51]. In dabrafenib-resistant melanoma cells containing *N-Ras* or *B-Raf* mutations, the combination of dabrafenib and trametinib was found to inhibit growth and induce apoptosis, although it may only partially restore sensitivity to dabrafenib [52]. Combined treatment with PLX4720 and a MEK inhibitor (CI-1040 or selumetinib) has also been reported to be more effective at reducing cell growth than either single treatment in melanoma cells with elevated Cot1 expression [58, 59].

It is likely that dual targeting of Raf and MEK will also be useful for the treatment of some colorectal cancers. Studies indicate that colorectal cell lines resistant to a MEK inhibitor (RO4927350 or selumetinib) due to a MEK1 mutation

or amplification of the mutant *B-Raf* gene are sensitive to combination therapy with the MEK inhibitor and a Raf inhibitor (RG7204 or AZ628) [72, 74, 75]. In some cases, both the resistant and parental cells were shown to display increased apoptosis with dual treatment [75]. These findings indicate that combination therapy can be effective even in MEK-inhibitor-naïve cells, and that dual treatment may prevent inhibitor resistance from developing. Indeed, the emergence of resistant colonies was inhibited when B-Raf mutant melanoma cells with or without a MEK1 mutation were treated with both Raf and MEK inhibitors (PLX4720 and selumetinib or U0126) [62, 83].

Dual therapies with Raf and MEK inhibitors are currently ongoing in the clinic. Recently, the results of a phase 1/2 combined trial revealed that patients receiving dual dabrafenib and trametinib therapy had significantly improved progression-free survival when compared with dabrafenib monotherapy (an average of 9.4 vs. 5.8 months) [84]. Moreover, the percentage of progression-free survival at one year was also greater with the combined therapy (41 % as opposed to 9 %) [84]. Of note, the secondary squamous cell carcinomas seen in single-therapy trials with dabrafenib, but not with trametinib, also occurred with the dual therapy [84]. Should resistance to the dual inhibition of Raf and MEK develop, it will be interesting to determine whether the mechanisms are similar to those seen in single inhibitor therapies.

Combination Therapies Co-Targeting Raf and RTKs

In certain cases, the co-inhibition of Raf and an upstream RTK may also be an effective cancer therapy. For example, treatment of NSCLC and colorectal cancer cell lines containing *K-Ras* or *B-Raf* mutations with a Raf inhibitor (sorafenib or vemurafenib) plus an EGFR inhibitor (erlotinib, cetuximab, or gefitinib) caused synergistic anti-growth effects and enhanced apoptosis [70, 85]. This combined approach was also effective in reducing tumor growth in xenograft mouse models [70, 85]. The combined inhibition of Raf and MET (using PLX4720 and crizotinib or gefitinib) also exhibited a synergistic effect in the treatment of B-Raf mutant non-melanoma cell lines that secret HGF [71]. Finally, melanoma cells with vemurafenib resistance through increased FGFR3 activity were re-sensitized to vemurafenib via treatment with the FGFR inhibitor LY2874455 [55].

Combination Therapies Co-Targeting Raf and the PI3K/AKT Pathway

Simultaneously targeting the Ras/Raf/MEK/ERK and PI3K/AKT pathways may be an additional method for combating Raf-inhibitor resistance. Studies have found that B-Raf mutant melanoma cells resistant to the Raf inhibitor vemurafenib/

PLX4720 can be re-sensitized to drug by co-inhibition of Raf and PI3K (using LY294002 or GDC-0941), Raf and AKT (using MK-2206), or Raf and mTOR (a kinase component of both the mTORC1 and mTORC2 complexes in the PI3K/AKT pathway) [51, 64, 66, 68]. In addition, the dual PI3K and mTOR inhibitor BEZ235 has been reported to have a strongly synergistic anti-growth effect when combined with vemurafenib to treat resistant melanoma cells expressing V600E-B-Raf [68]. Villanueva et al. [53] also observed increased cell cycle arrest and apoptosis when Raf inhibitor (SB590885)-resistant melanoma cells were treated with a MEK inhibitor (trametinib or selumetinib) and the PI3K inhibitor GSK2126458. Two more studies have provided further support that treatment with inhibitors for PI3K/AKT and either Raf or MEK may be effective against Raf inhibitor-resistant cancers. In one study, combination treatment with trametinib or dabrafenib and the PI3K/mTOR inhibitor GSK2126458 synergistically inhibited cell growth in dabrafenib-resistant melanoma cells expressing mutant N-Ras or B-Raf [52]. In a second study, the addition of an AKT inhibitor to treatment with vemurafenib or selumetinib synergistically blocked the growth of patient-derived melanoma cell lines previously exposed to vemurafenib [54]. Finally, Staussman et al. [71] found that HGF-induced PLX4720 resistance in melanomas could be suppressed by the inhibition of MEK and AKT (using PD184352 and MK-2206).

As well, cells resistant to MEK inhibitors may be sensitive to the co-targeting of MEK and PI3K or AKT. U0126 and the PI3K inhibitor LY294002 were found to have an additive effect when used on a cell line derived from a metastatic melanoma that was partially resistant to these inhibitors alone [86]. Some breast cancer cell lines resistant to the MEK inhibitor CI-1040 have also shown sensitivity to dual MEK/PI3K inhibition using CI-1040 and PIK90 or PI103 [37]. However, targeting MEK and the PI3K/AKT pathway may not work in all MEK inhibitor-resistant cells, given that selumetinib-resistant colorectal cancer cells with upregulation of the mutant *K-Ras* gene were also resistant to the combination of selumetinib and a PI3K/mTOR inhibitor, AZ12321046 [72].

Other Potential Therapeutic Combination Strategies

The use of other targeted inhibitors is also being explored as therapies for cancers resistant to Raf or MEK inhibition. For example, HSP90 inhibitors may be of value in dual therapy strategies, given that AZ628-resistant melanoma cells expressing elevated C-Raf protein levels were reported to be significantly more sensitive to the HSP90 inhibitor geldanamycin than were parental cells [50]. In addition, the HSP90 inhibitor XL888 could effectively induce apoptosis in a variety of vemurafenib-resistant melanoma cell lines, regardless of their mechanism of resistance [87]. In a study by Shao et al. [67], treating PLX4720-resistant melanoma cells with the histone deacetylase inhibitor SAHA (suberoylanilide hydroxamic acid) and either PLX4720 or selumetinib re-sensitized cells and inhibited the re-emergence of PLX4720-resistant colonies in a long-term survival assay. The combined

inhibition of MEK using selumetinib and STAT3 with JSI-124 has also been found to re-sensitize selumetinib-resistant NSCLC cell lines to selumetinib and increase the level of selumetinib-induced apoptosis [79, 80]. Finally, other combination therapies that may be beneficial, depending on the mechanism of resistance, include treatment with a Raf or MEK inhibitor and a Cot1, IGF-1R, or ERK inhibitor [53, 58, 73].

Conclusions

There are currently hundreds of active clinical trials evaluating the use of drugs targeting the MEK or Raf kinase in cancer treatment (for details, see www.clinicaltrials.gov). New inhibitors are also in development, and may prove even more effective. With the knowledge coming from these trials, we are moving ever forward into the era of personalized medicine. It is now clear that knowing the genotype of a tumor prior to treatment is critical for selecting the proper kinase inhibitor therapy and for predicting the possible mechanisms of inhibitor resistance. With time, it may be possible to prevent kinase inhibitor resistance from developing, which may end the danger of recurrence for at least some tumor types.

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Conflicts of Interest No potential conflicts of interest were disclosed.

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Mechanisms of Resistance to Targeted B-Raf Therapies

Ramana V. Tantravahi, Benjamin Hoffman and E. Premkumar Reddy

Abstract *Targeted Cancer Therapies and Resistance* Our modern search for effective cancer therapies began after the First World War and the discovery of the myelosuppressive properties of nitrogen mustards. Since that time, adjuvant and chemo-radiotherapy have become the standard of care for many cancers. These non-targeted therapies have produced remissions in many patients. As our technical capacity to target radiation to tumors and our ability to perform surgical interventions improves, it is likely that these approaches will gain in effectiveness. Nevertheless, the dose limiting toxicities of chemotherapy drugs and ionizing radiation have served to expose vulnerabilities in this clinical approach. Modern translational research has sought to develop novel, targeted approaches to cancer therapy. These new approaches are based upon our understanding of cellular growth control, and bring with them the promise of greater potency and safety. The concept of targeted cancer therapy began with the identification and characterization of growth regulatory proteins in normal cells. These proteins were initially identified in acute transforming retroviruses. During the 1970s and 1980s, investigators working in a number of laboratories learned that the transforming genes of these viruses were actually derived from the host genome. In subsequent years, investigators came to understand that biochemical or genetic inactivation of these deleterious proteins in cultured cell lines leads to tumor cell death. This new understanding led to the search for specific proteins, whose activities drive the malignant transformation process. These proteins are now referred to as *druggable targets.* Chemical and molecular biologists are translating their understanding of the cell proliferation control into molecular therapeutics directed at these targets. Targeted therapy development for cancer is still in its infancy. Despite this,

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clinical trials for a number of small molecules have provided positive outcomes in patients who may have been given dire prognoses only a few years ago. Two FDA-approved drugs, Gleevec (Novartis) and Vemurafinib (Plexxikon) have provided some of the most dramatic results. Unfortunately, these drugs have also revealed a new challenge in cancer therapy—the development of resistance and relapse. Here, we describe the B-raf growth signaling pathway associated with the development of melanoma, summarize the mechanisms associated with resistance to targeted B-raf therapies, and discuss the future of this field of inquiry.

Keywords B-Raf mutations · Melanoma · Vemurafenib · Resistance

Abbreviations

BCR	Breakpoint cluster region
CML	Chronic myelogenous leukemia
Src	The transforming gene of the Rous Sarcoma Virus (RSV), a non-receptor tyrosine kinase that represents a family of such kinases that regulate cell proliferation and play a prominent role in malignant transformation
Abl	The transforming gene of Abelson Murine Leukemia Virus (A-MuLV). Abl is a src family kinase associated with transformation of the lymphoid compartment of the hematopoietic system
MAP	Originally microtubule-associated protein, now also mitogen-activated protein. MAP kinases refer to a group of cytoplasmic protein kinases that act downstream of growth factor receptors to transmit proliferative signals to cells
FTI	Farnesyl transferase inhibitor
JNK	c-Jun N terminal kinase
PI-3K	Phosphatidyl inositol 3′ kinase
MEK	Mitogen activated/extracellular regulatory kinase
ERK	Extracellular regulatory kinase

Introduction: Growth Associated Signaling Pathways

The development of cancer has long been understood to have a genetic basis. Investigators have come to understand the clonal nature of malignancy from the earliest observations of tumors arising in avian and murine systems. Studies in human populations led Nordling [1] and later Knudson [2] to assert that a minimum of two mutational events within a single cell is necessary for cancer development [1, 2]. Our modern understanding of cancer is now considerably more sophisticated than it was at the turn of the 20th century. Since then, we have assembled and tested elegantly constructed experimental model systems, identified numerous growth control genes and elucidated complex regulatory pathways that control cell growth, differentiation and death.

The characterization of complex signal transduction networks lies at the heart of our current understanding of cancer; it shapes many of the strategies with which therapeutics are conceived and developed. One of the most transformative developments in modern cancer therapy was the development of Gleevec® (Imatinib mesylate, STI57, Novartis), a targeted treatment for chronic myelogenous leukemia (CML) [3]. The synthesis of Gleevec followed on from the accumulated understanding of decades of basic science research into the identity and biological function of a subset of growth control proteins known as the *src* family tyrosine kinases. The oncogenic activation of the *src* family kinase c-Abl via chromosomal translocation was determined to be the critical step in CML development [4]. Gleevec is the result of structure-based design of a molecule that could effectively inhibit the kinase activity of the deleterious BCR-Abl protein [5]. The structure-based design paradigm led to a drug that acts specifically and with great efficacy in the clinic.

The development of Gleevec is a useful case study in the current development of targeted cancer therapies. CML is treated with Gleevec routinely across the world where once less effective and more highly toxic interventions served as the only therapeutic avenues [6]. Despite its widespread success, Gleevec does not cure CML. Many patients, after years in remission, develop resistance to the drug and relapse [7]. So while our greater understanding of the molecular events underlying the malignant transformation has led to significant advances in therapeutics development, the enthusiasm over these advances must be tempered with the understanding that much work remains to be done.

The present chapter addresses the identification and development of novel therapies directed at a different growth control protein, B-raf, as well as the observed mechanisms of therapeutic resistance to B-raf inhibitors. B-raf sits at the center of a highly complex series of regulatory nodes that control proliferation, differentiation, and programmed death (apoptosis). As was the case with the Abl protein in CML, the B-raf protein, and one of its mutant isoforms, B-raf^{V600E}, is associated with a particular form of cancer, melanoma [8]. Here, we discuss the identification and function of B-raf and its role in cellular homeostasis. We will also discuss the mechanistic effects of B-raf mutation on cell proliferation control and the current state of therapeutics development.

MAP Kinase Pathway Signaling

For many decades, investigators in a variety of disciplines have tried to come to an understanding as to how extracellular mediators can guide changes in patterns of gene expression. Molecular biologists addressed this issue within the past 30 years through identification, and structure–function characterization of gene products associated with cell growth and proliferation. These gene products were found to form interdependent regulatory networks. The key proteins involved in these networks were found to have discrete biochemical functions, and their dysregulation in a

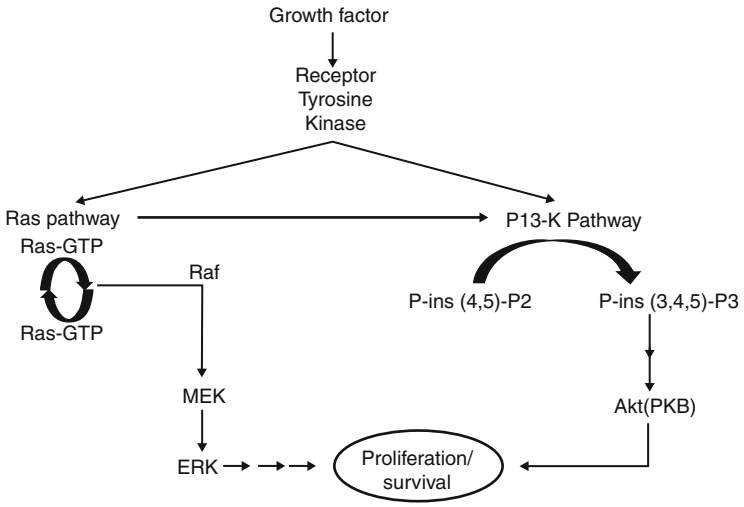

Fig. 1 Signaling Pathways that control Proliferation and Survival–Extracellular mediators such as soluble growth factors bind with high affinity to their cognate receptors, leading to the activation of downstream kinase cascades. Certain receptor tyrosine kinases (*RTK*)

variety of cellular contexts was often found to contribute to malignant transformation.

Figure 1 is a representation of the Mitogen-Activated Protein (MAP) kinase network, which serves to couple extracellular signals in the form of soluble growth factors with changes in gene expression associated with proliferation and survival. Growth factor receptors—cell surface proteins with extracellular ligand-binding and intracellular tyrosine kinase domains initiate this process. Ligand binding results in receptor dimerization coupled with auto- and trans-phosphorylation of receptor dimers. Phosphorylated dimers are then primed to bind intracellular mediators, including single subunit GTP-binding proteins of the *ras* family, and cytoplasmic protein kinases. The MAP kinase cascade plays a central role in cellular growth dynamics because of the association of its terminal kinases, $p38^{MAPK}$, c-Jun N-Terminal Kinase (JNK), Extracellular Regulatory Kinase (ERK), and Phosphatidyl Inositol 3′ kinase (PI-3 kinase) with DNA-binding transcription factors. These transcription factors regulate genes involved in the control of proliferation and survival. The ERK1/2 pathway is the most commonly involved in the proliferative response. ERK1/2 activation is also associated with malignant transformation, and the components of the MAP kinase pathway most frequently involved in dysregulated activation occur upstream of ERK1/2. Constitutively upregulated activity of RTK, or overexpression of growth factors can lead to upregulation of ERK1/2. Also, mutations in Ras and Raf isoforms can similarly lead to constitutive proliferation signals mediated by ERK1/2.

Ras

The Ras proteins comprise a subset of small membrane-associated single polypeptides that bind to and hydrolyze GTP. Four isoforms of Ras have been identified in humans, Ha-ras, N-ras, Ki-ras 4A and Ki-Ras 4B [9]. Each Ras isoform has a distinct pattern of expression and a characteristic ability to affect specific downstream signaling pathways, such as the MAP kinase cascade, or the PI-3 kinase/protein kinase B (Akt) pathway via differential interaction with Raf isoforms [10]. Each Ras isoform is encoded on a single gene, except for Ki-ras 4A and 4B, which arise from differential splicing of a single parental transcript [9].

History

The Ras genes were identified in isolates from three acute transforming retroviruses. The Harvey and Kirstein sarcoma viruses (named for their discoverers) were able to induce ***RA***t ***S***arcomas, and were thus named *ras* genes (Ha-ras, and Ki-ras) [11, 12]. A third virus that transduced the mouse ortholog of *ras* was isolated from a BALB/c mouse tumor and was named as BALB-MSV [13]. Sequence analysis of these three viral oncogenes revealed that BALB-MSV encodes the mouse homolog of the *Ha-ras* oncogene [14–16]. Human cellular Ras orthologs were discovered later by several groups [17]. As is the case with many of the oncogenes first identified in the genomes of sarcoma viruses, the *ras* gene has been the subject of intense scrutiny for the past 30 years. *Ras* is the most commonly mutated gene in all human cancers, and mutation of the coding sequence has been observed in a variety of human malignancies. The identification of specific cellular *Ras* mutations at codons 12 and 61 remains a fundamental contribution to our understanding of the neoplastic process [18–24].

Structure and Function

The human genome encodes three *Ras* genes, termed H-, K- and N-*Ras*. These three *Ras* genes encode proteins of approximately 200 amino acids characterized by a GTP-binding domain. This domain is encoded on 5 so-called G box elements spread throughout the primary amino acid sequence [25]. The C-terminus of Ras encodes a membrane-association sequence, CAAX. Either farnesyl or geranylgeranyl transferase enzymes covalently modify the cysteine residue within the CAAX sequence [26]. These two isoprenoid lipid moieties enable Ras association with the plasma membrane. Ras isoforms that cannot associate with membranes fail to transmit mitogenic signals as efficiently as does wild type Ras [27]. Consequently, inhibition of lipid modification has been considered a viable strategy for

therapy development. Many attempts have been made to develop small molecules that target farnesyl transferases [28, 29]. Some of these molecules have been assessed in clinical trials, although their efficacy has not had the dramatic effect that had been originally hoped. Since certain Ras proteins can be modified by both farnesylation and geranylgeranylation, inhibition of one process does not necessarily prevent Ras association with the plasma membrane. Farnesyl transferase inhibitors (FTI) are largely unable to inhibit geranylgeranyl transfer. Consequently, Ras can associate with the plasma membrane even in the presence of these compounds. Also, FTI affect H-Ras disproportionately. K-*Ras* and N-*Ras* are, however, more commonly mutated in human cancer [27]. While activated Ras remains a high profile target for drug design and development, it has become clear in recent years that the Ras downstream effector, Raf, can also contribute to the neoplastic process.

Raf

The Raf proteins function as intracellular conduits through which growth, differentiation and apoptosis signals are transmitted. Raf proteins are serine/threonine protein kinases that reside proximal to the membrane and participate in complex multi-protein kinase relays that ultimately activate powerful programs of gene expression. Raf proteins are now known to participate in membrane-associated signaling events, such as transduction of growth factor receptor signals via Ras. Recent studies have tied mutations in *raf* genes, particularly *B-raf*, with melanoma as well as carcinomas of the thyroid, ovary and colon [30–32].

History

The v-*raf* oncogene was first identified in the murine acute transforming retrovirus, 3611-MSV by Ulf Rapp in 1983 [33, 34]. The name *raf* derives itself from the ability of 3611-MSV to induce ***RA***pid murine ***F***ibrosarcomas. Jansen et al. [35] working in avian systems, isolated the MH2 acute transforming virus in the same year. The MH2 transforming gene was named *v-mil*. In the following year, two groups would report that *v-mil* and *v-raf* are sequence orthologs derived from murine and avian genomes and that both genes encode serine/threonine protein kinase activities [36, 37]. Cellular *raf* genes were later cloned, and the first isoform cloned was named *c-raf* [38]. C-Raf is highly conserved in evolution, with orthologs reported in *D. melanogaster*, and *C. elegans* [39, 40]. There are 2 mammalian *c-raf* genes, *c-raf-1* and *c-raf-2* (c-*raf*-1 is now referred to as *Raf*-1). Raf-1 is a protein coding gene, while *c-raf-2* is a pseudogene [32, 37]. In 1986 and 1987, two different groups reported the sequence of A-*raf,* a highly similar, but not identical isoform of *Raf*-1 [35, 40] Humans possess two A-*raf* gene sequences,

A-*Raf-1* and A-*Raf-2*. As was the case with *Raf-1*, A-*Raf-2* is a pseudogene [41]. The B-*raf* ortholog was identified by Marx et al. [42] in 1988. The original study was designed to identify acute transforming viruses following infection with the Rous-associated virus-1 (RAV-1) in chicken neuroretinal cells. The isolation of proliferating cells revealed the presence of a novel virus isolate, Ic10, that had transduced the coding sequence from the host genome between the RAV-1 *gag* and *env* genes. When sequenced, the host-derived gene bore significant homology to *Raf-1*, but was considered to be a related gene due to sequence divergence at the N and C termini of the predicted protein and the presence of the genomic sequence in an unrelated area of the genome to the avian *Raf-1* gene.

Structure and Function

Raf proteins share significant structural homology befitting their similar roles in cell homeostasis and their similar biochemical functions. Raf isoforms differ in size and patterns of expression. Human A-Raf is a 68 kD protein. Raf-1, with a slightly longer N-terminus, is approximately 74 kD. B-Raf protein sizes are heterogeneous, appearing anywhere from 75 to 100 kD. Multiple B-Raf species arise due to alternative splicing of the B-*raf* message [43]. *Raf-1* is expressed ubiquitously both during development and in the adult organism; B-*Raf* expression predominates in neuronal tissues. A-*Raf* expression predominates in the epididymis and ovary [44].

Each Raf protein isoform contains three principle regions of homology denoted as *conserved regions* (CR) 1, 2 and 3 [45]. Each of these regions contains structural and/or regulatory properties of interest. CR1 contains two sub-regions, the RAS-binding domain, and the cysteine-rich region, both of which are responsible directly or indirectly for the movement of Raf toward the plasma membrane. Upstream of the CR1 region, Raf-1 contains a regulatory serine (S43), which acts as an inhibitor of Ras/Raf association [45]. CR2 contains docking sites for the 14-3-3 class of adapter proteins. This further reinforces the notion that Raf signaling occurs against the backdrop of formation of large multifactorial protein complexes that can regulate activity both spatially and temporally. CR2 also contains the protein's N region, a site of overall negative charge. Phosphorylation of the N region is considered to be a mechanism by which Raf kinase activity is primed to overcome its intramolecular negative regulation by its C-terminus [45]. The Raf CR3 contains the ATP-binding site and kinase domain. Serine and threonine residues within CR3, especially those near the ATP binding activation segment, are of particular interest from a regulatory perspective. In the case of B-raf, mutations within this region are particularly informative with regard to cancer development [45].

BRAF Mutations in Cancer

In 1988, *BRAF* was identified as an oncogene through the NIH/3T3 assay system [46]. Following this discovery, attempts were made to characterize *BRAF* mutations in human tumor samples. Yet, it was not until 2002 when a team of researchers were able to identify specific abnormalities in the gene encoding B-*Raf* [47]. In this seminal study, investigators used high-throughput sequencing technology on 923 cancer samples and ultimately identified 43 presumptive mutations. Strikingly, they also found that 70 % of the melanoma samples tested harbored mutations in the *BRAF* gene.

BRAF Mutations in Melanoma

A preponderance of the lesions, first identified by Davies and colleagues [47], were found to occur in exons 11 and 15 of the *BRAF* gene. At the structural level, nearly all of the mutations were immediately adjacent to the B-Raf kinase domain, and one particular mutation, a V600E substitution (initially called V599E), was highly represented in many of the melanoma samples tested. In the same study, it was found that the V600E lesion caused a mutated version of B-Raf that had increased enzymatic activity [47].

The V600E lesion occurs in a critical coding region and is the result of a single point mutation involving a thymine to adenine switch (T1796A) [47]. This point mutation alters the coding sequence and leads to a glutamate −> valine substitution (at the peptide level) causing a structural change in the activation segment of the B-raf catalytic domain [48]. Ultimately, this structural change introduces a negative charge that mimics critical activation events such as the phosphorylation of threonine 598 and serine 601 [48, 49]. In this way, the V600E mutation allows for a B-Raf mutant, that is independent of Ras and, thus, can signal through its downstream effectors in the absence of any upstream stimuli (see Fig. 2).

The added kinase activity of V600E B-raf has been well characterized and its functionality in establishing and maintaining the melanoma phenotype has been studied by a number of groups including Pollock et al. Their work, in particular, was the first to provide evidence that V600E B-Raf was critical for all of the steps in melanoma pathogenesis [50]. By examining benign pre-melanocytic nevi, primary malignant melanoma, and metastatic disease, Pollock and colleagues were able to demonstrate that the V600E mutation was pervasive in each step in the natural history of melanoma. While this study validated the importance of the B-Raf/MEK pathway in disease maintenance, it also suggested a role for additional oncogenic signals in the transformation process.

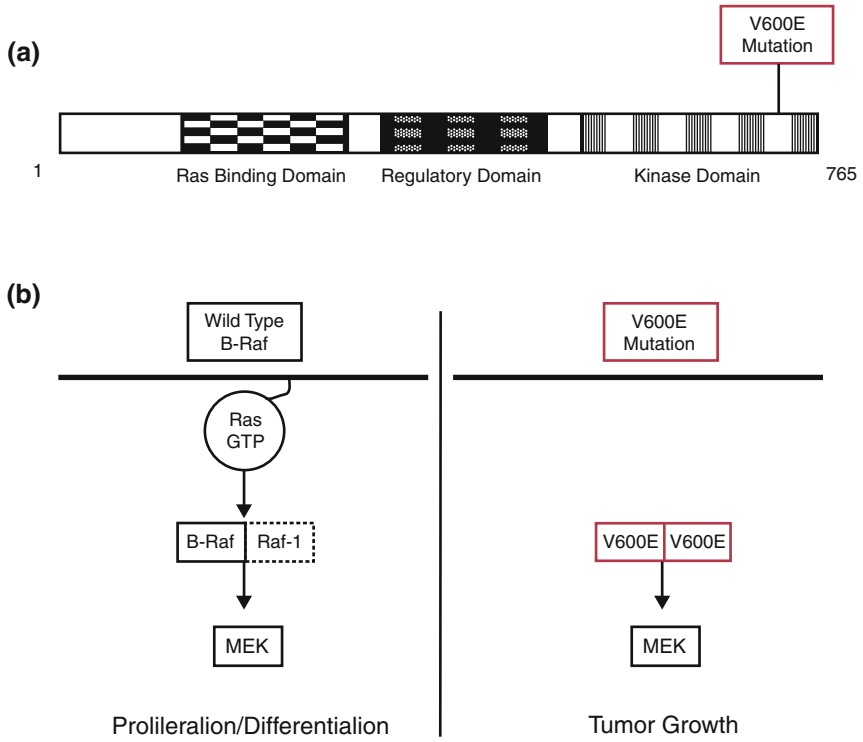

Fig. 2 B-Raf^{V600E} stimulates tumor cell proliferation independently of Ras

Prevalence of BRAF Mutations in Melanoma

According to the SEER database (SEER.cancer.gov), 76,250 people are expected to be diagnosed with melanoma in the United States in 2012. Nearly 4 % of newly diagnosed melanoma patients have distal metastases and 9 % have disease that has spread regionally to their lymph nodes (SEER.cancer.gov). Therefore, almost 10,000 new cases of un-resectable melanoma are expected to be diagnosed this year in the US alone. In a recent clinical study, 56 % of un-resectable melanoma cases tested positive for the V600E mutation [51].

BRAF Mutations in Other Malignancies

Mutations in *BRAF* have been identified in other human malignancies. In addition to melanoma, *BRAF* is mutated in other solid tumors such as: papillary thyroid carcinoma, non-small cell lung cancer, and colorectal cancer [52–55]. Also, a recent a clinico-pathological report suggested a novel role for *BRAF* in hematological

malignancies [56]. In this study, a team of investigators used whole exome sequencing technology to test paired malignant/normal tissues from 47 patients that had been diagnosed with Hairy Cell Leukemia (HCL). Using the advanced sequencing technology, they were able to positively identify V600E mutations in 100 % of the patients that were tested. Interestingly, this mutation was not present in any of the other leukemias or B cell disorders that were studied. In light of this report, more work will be required to fully characterize the role of *BRAF* mutations in the many different types of human hematological malignancies.

B-Raf as a Target for Anti-Cancer Agents

The discovery that *BRAF* was highly mutated in human cancer spurred an intense effort by both industry and academia to develop targeted B-Raf agents. To date, a number of different B-Raf agents with varying molecular mechanisms of action have been tested in the clinic for the treatment of both solid and hematological malignancies.

Sorafenib

Bay 43-9006 (sorafenib or Nexavar) was the first Raf-targeting agent to achieve clinical success in treating human cancers. Sorafenib is a biaryl urea ATP-competitive protein kinase inhibitor [57]. It was first identified as a potent small molecule inhibitor of the Raf family of kinases and in particular as a C-Raf inhibitor [58]. However, it was later shown that sorafenib also had a high affinity for wild type B-Raf, B-Raf^{V600E}, and a number of other tyrosine kinases including some of the VEGFR and the PDGFR family members [59]. The elucidation of this dual mechanism has helped to explain both the anti-angiogenic and pro-apoptotic effects of sorafenib [58, 60]. Moreover, the *in vitro* activities and molecular mechanism of sorafenib may also help explain its strong clinical activity. To date, sorafenib has been approved for use in the treatment of advanced renal cell carcinoma (RCC) as well as primary hepatocellular carcinoma (HCC). While sorafenib has shown clinical efficacy in the treatment of HCC and RCC, it lacked substantial activity when treating malignant melanoma [61]. While this ended the development of sorafenib for this indication, it did provide critical information regarding the class of B-Raf inhibitors.

PLX-4032

The failure of sorafenib in melanoma provided an important data point for both scientists and physicians to build upon. Many in the field hypothesized that sorafenib's promiscuous nature or lack of selectivity for B-Raf and B-Raf^{V600E} explained the nominal clinical efficacy in malignant melanoma [62]. The first test of this principle occurred when researchers, using crystallographic techniques, identified a 7-azaindole derivative (PLX4270) which was highly selective for the active B-Raf kinase or the V600E mutant [63]. Not only was this molecule shown to be active in the test tube, but it was also active against melanoma cell lines in the laboratory and in melanoma-based xenograft models. Moreover, the high degree of selectivity for the active B-Raf kinase allowed for a minimal amount of preclinical toxicity. Ultimately, an analogue of PLX4270, called PLX4032 (vemurafenib), was carried into the clinic for testing in human subjects.

The translation into the clinic followed a unique development strategy. To provide proof of principle, developers designed clinical trials to enrich for malignant melanoma patients that harbored the V600E B-Raf mutation. This strategy allowed clinicians to observe efficacy at a very early point in clinical development, and the proof of principle was provided in the first of these trials [64]. Vemurafenib is now approved for the treatment of mutant B-Raf^{V600E} metastatic melanoma.

Other B-Raf Agents in Development

Sorafenib and vemurafenib provided direct evidence that inhibition of the Raf family of proteins, and in particular B-Raf, was a highly effective strategy for the development of targeted anti-tumor agents. A number of investigational agents has added to this class of molecules. Follow-on-drugs such as dabrafenib are being advanced in the clinic, and while these agents should provide differentiation with respect to toxicity profile and general tolerability, they do not alleviate any of the clinical resistance that has been seen with the first-in-class agents [65]. Therefore, strategies need to be developed that deal with the mechanisms which are associated with resistance to the B-Raf class of inhibitors.

Clinical Observations of Resistance to B-Raf Inhibitors

Although clinical trials have shown that select tumors are extremely sensitive to mutant B-Raf inhibition, resistance has also been observed [66]. In the first clinical publication of PLX4032, Flaherty et al. [64] demonstrated complete or partial regressions in 81 % of V600E melanoma patients. While this response was

observed to last 2–18 months, ultimately patients were desensitized to B-Raf inhibition and their disease progressed. Moreover, 19 % of the patients tested in these trials had no significant clinical response (indicated as >30 % tumor regression).

Mechanisms of Resistance

Understanding the mechanisms that underlie desensitization and resistance have become an area of intense interest. A number of molecular mechanisms have been proposed and subsequently tested in the laboratory setting. Many of the proposed mechanisms have been studied using cultured melanoma cell lines and melanoma patient samples, and therefore these mechanisms may well be cell-type specific. Ultimately, other tissue-specific resistance mechanisms may be identified, but for the scope of this review, we will focus on the resistance mechanisms that have hitherto been elucidated in melanoma.

The canonical Ras-Raf-MEK-ERK1/2 pathway is well characterized. The result of Raf activation is phosphorylation of ERK1/2, and the translocation of activated ERKs into the nucleus to activate substrates that are involved in cellular proliferation. Melanoma cells, in which the predominance of signaling is mediated through V600E B-Raf, are highly dependent on the MEK/ERK signaling, and mutant B-Raf inhibitors can affect this signaling in a mutation-specific manner [67]. Given the critical nature of the MEK/ERK signaling, it is not surprising that all of the resistance mechanisms, hitherto identified, involve the reactivation of the tumorigenic MEK/ERK signaling.

Reactivation of the MEK-ERK Signaling Pathway by Overexpression of RAF Isomers

In 2008, Monagut et al. [68] were the first to demonstrate a specific resistance mechanism. Their research efforts predated any observations of clinical resistance and involved the establishment of drug-insensitive clones using human melanoma cells that harbored the V600E mutation. While the investigators did not test for resistance to PLX4032, they did use a similar compound (AZ268 a V600E inhibitor) for selection. Monagut and colleagues followed clonal selection with DNA sequencing and discovered that resistance and desensitization resulted from an increase in CRAF expression and reactivation of the MEK-ERK signaling axis.

Reactivation of the MEK-ERK Signaling Pathway by Upstream Activation

Resistance and desensitization can also occur in response to activation of signals that are upstream of the Raf proteins [69]. In 2010, Nazarian and colleagues used PLX-4032 drug-resistant cell lines, as well as tumor-matched short term cultures from melanoma patients, to identify two mechanisms for acquired resistance. The first was an activating mutation in NRAS (Q61 K), and in a series of elegant experiments, the investigators demonstrated that constitutive Ras potentiated MEK-ERK signaling in a canonical pattern through the WT RAF. The second mechanism involved overexpression of PDGFRβ, activation downstream through WT RAF and MEK-ERK phosphorylation. While these mechanisms were found to be mutually exclusive, they both acted to promote resistance through WT Raf dimerization and the reactivation of MEK-ERK signals. Moreover, both molecular lesions were confirmed in patients that were either resistant or insensitive to PLX4032.

Reactivation of MEK-ERK Signaling by COT

Overexpression of COT is a third resistance mechanism that has been identified. Importantly, COT overexpression leads to Raf-independent reactivation of MEK-ERK. Johannessen et al. [70] were the first to suggest this new paradigm. Using a cDNA library that encoded 75 % of the human kinome, the investigators introduced activated kinases into a V600E melanoma cell line and then analyzed for resistance to PLX4032. In addition to COT, Johannessen and colleagues identified C-Raf as a mediator for the resistance phenotype, thus providing an internal validation for their approach; however, the discovery that COT also mediated resistance was a breakthrough. COT, as reviewed earlier, activates MEK-ERK in a Raf-independent manner. Furthermore, its expression is inversely correlated to V600E expression, suggesting that mutant BRAF activation may downregulate COT. It is therefore feasible to hypothesize that when this pressure is removed (through pharmacological inhibition of V600E) COT can mediate resistance by directly activating MEK-ERK.

Other Mechanisms of Resistance

In addition to the above described mechanisms of resistance, additional acquired resistance mechanisms have been reported; these include upregulation of insulin-like growth factor-1 receptor (IGF1R) signaling and acquisition of activating mutations in *MEK1* [71–73], high level expression of epidermal growth factor

receptor (EGFR) [74] and activation of PI3 Kinase pathway as a result of loss of PTEN expression [72, 73].

Moreover, alternative splicing of BRAFV600E has also been described in a subset of melanoma tumor samples from patients who relapsed following BRAF inhibitor therapy [75]. The protein encoded by this alternatively spliced mRNA lacked the regulatory elements of BRAF, including the RAS binding site, resulting in a kinase that is capable of dimerizing and activating MEK even in the presence of a BRAF inhibitor. It is interesting to note that most of these resistance mechanisms involve the reactivation of the MEK/ERK pathway. However, increased insulin growth factor receptor 1 (IGF-1R) signaling or acquired loss of PTEN which correlated with increased activation of AKT [71–73] represent examples of MAPK-independent bypass mechanisms. Most of these mechanisms have been corroborated in small numbers of tumor samples from patient biopsies.

The discovery that RAS mutations promote resistance to RAF inhibitors led to a series of studies showing that RAF inhibitors often induce hyperactivation of MEK–ERK1/2 signaling in cells harboring N-RAS mutations [76]. Thus, ectopic expression of mutant N-RAS in a mutant B-RAF colorectal cancer cell line resulted in resistance to PLX4720 [77], a result that could also be seen in B-RAFV600E melanoma cells [69, 70]. As mentioned earlier, another possible mechanism to by-pass the activity of RAF inhibitors is through mutations in MEK1 and 2 which are downstream effectors of B-RAF. Through a random mutagenesis screen, P124L mutation in MEK1 was identified to confer cellular resistance to the MEK inhibitor, AZD624478. MEK1P124L expression also conferred resistance to PLX4720 in cell-based assays and was identified in a metastasis from a patient with acquired resistance to AZD6244, indicating the clinical relevance of this mutation [78]. Interestingly, the combination of PLX4720 plus AZD6244 overcame the resistance conferred by MEK1P124L expression. These and other data have prompted the use of MEK inhibitors in clinical trials enrolling mutant B-RAF melanoma patients who were previously treated with or without a BRAF inhibitor. Thus, phase I/II trials of the BRAF inhibitor dabrafenib in combination with the MEK inhibitor trametinib (GSK1120212, NCT01072175) and vemurafenib combined with the MEK inhibitor GDC-0973 (NCT01271803) are underway and results from the phase I/II trial of the trametinib-dabrafenib combination was reported to be associated with objective response rates of 77 % [79, 80]. Other clinical studies combining BRAF inhibitors with inhibitors of the mTOR/PI3K/AKT pathway are due to commence in the near future.

Conclusions

The introduction of targeted therapies such as Gleevec and Vemurafinib represent a historical shift in our approach to cancer treatment. For the first time, serious malignancies have been attacked by therapeutic agents that account for our new

and specialized understanding of the nature of cancer and the controls on cellular proliferation in normal cells. While the initial results in laboratory studies and in the clinic have proved promising, they have also provided cancer biologists and oncologists with a cautionary tale. Resistance to targeted therapy reveals a level of plasticity in the response of the cancer cell to the agents that seek its destruction. In some cases, that response is genetic selection, as is revealed by the allele specific suppressor mutations revealed in the BCR-Abl protein and the B-raf protein. In other cases, the response manifests itself by compensatory changes in the regulation of co-linear signaling pathways, such as growth factor receptor upregulation upstream of Raf, or constitutive activation of the MAP kinase cascade.

Strategies for the development of targeted therapies, such as Gleevec and Vemurafinib, rely on the identification of *druggable* targets. Those targets can be involved in what Weinstein and Joe described as *oncogene addiction* [81]. Oncogene addiction scenarios, such as the expression of the *bcr-abl* fusion gene, transform normal cells into cancer cells through creation of growth signaling dysequilibria. In the laboratory, restoration of the equilibrium in cultured cells through inactivation of these deleterious proteins results in the induction of apoptosis. This critical observation has provided the rationale for structure based rational drug design. Using molecular modeling and x-ray crystallography, teams of chemical biologists designed small molecules that inactivated the biochemical properties of druggable target proteins. As we have witnessed, the initial results in the clinic provided impressive results. Nevertheless, the difficulty of resistance remains. In the future, it may be possible to marshal the vast bioinformatics resources now available to design individualized therapies that account for signaling plasticity. Perhaps, using combinations of targeted therapies will limit the probability of resistance reactions.

A different therapy strategy may also prove more efficacious. At the present time, most targeted therapies rely on the deactivation of ATP-dependent reactions using ATP-mimetic small molecules that act as competitive inhibitors. Almost all of the available ATP mimetic compounds seek to inhibit kinase reactions in much the same manner that both Gleevec and Vemurafinib do. In the future, we may abandon structure-based rational drug design, and move to a development strategy in which cancer cell cytotoxicity takes precedence over affinity toward a target determined *a priori*. In that way, ATP mimetic scaffolds and their derivatives can be assayed in culture for both their ability to kill cultured tumor cell lines and their safety. Because of the highly conserved tertiary structure of kinase ATP-binding sites, these compounds will likely affect a combination of growth control kinases with varying affinity. In so doing, compounds developed by this method will likely affect multiple routes of aberrant signaling within cancer cells. By using cancer cell cytotoxicity as a first measure of efficacy, we should be able to accomplish two goals simultaneously: (1) the development of effective therapeutics and (2) the identification of novel targets or target combinations that limit the possibility of resistance.

The future of targeted therapy for cancer remains bright. Our continuing accumulation of knowledge from basic research into cellular growth control will

serve to shed light on our already well-developed understanding of malignant transformation. In recognizing the phenomena of non-response and resistance, basic and applied researchers will learn and adapt to these challenges.

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Role of β1 Integrins in the Complication and Drug Resistance Against Lung Cancer: Targeting β1 Integrins to Eradicate Lung Cancer

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Abstract Lung cancer is a major global concern of inevitable mortality and morbidity in modern times. In the context of lung carcinogenesis, bi-directional cell signaling events mediated by the interaction of transmembrane integrin receptors with the extracellular matrix (ECM) is highly critical. Structurally, the integrin superfamily comprises of a large family of receptors consisting of heterodimeric α and β chains, noncovalently linked with each other. Eighteen different α subunits combine with 8 different β subunits to emerge 24 known integrin family members. By far, β1 integrins combine with their 12 different α subunits to yield the largest family members within the integrin superfamily. β1 integrins are preferentially expressed in lung cancer cells and are significantly involved in lung carcinogenesis in terms of cell proliferation, survival, invasion and metastasis. Besides, the β1 integrin family has a prime role in imparting chemoresistance to the lung cancer cells. The present discussion entitles a brief overview of the integrin superfamily, the cross-talk of β1 integrins with ECM proteins, the importance of β1 integrins as prognostic factors for lung cancer, the role of β1 integrins in the complication of lung cancer and the associated drug resistance and, finally, targeting β1 integrin functions to control lung cancer.

Keywords Non-small cell lung cancer (NSCLC) · Small cell lung cancer (SCLC) · β1 integrins · Extracellular matrix (ECM) · Cell signaling · Drug resistance

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Abbreviations

BAD	$Bcl_2/Bclx_L$-associated death promoter
ECM	Extracellular matrix
EGFR TKIs	Epidermal growth factor receptor tyrosine kinase inhibitors
FAK	Focal adhesion kinases
mTOR	Mammalian target of rapamycin
NSCLC	Non-small cell lung cancer
PKB	Phosphatidyl inositol 3-kinase [PI(3)K]-protein kinase B
RGD	Arginine-(R)-glycine-(G)-aspartic acid-(D)
SCLC	Small cell lung cancer
ZO-1	Zonula-occludensa

Introduction

Lung cancer is one of the prominent reasons of mortality in recent times owing to the cause of lung being the major vital organ that needs ultimate care and specialized supervision. There are mainly two types of lung cancer, namely, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). SCLC is further subdivided into adenocarcinoma and large cell differentiated carcinoma. In addition, carcinoid, Kaposi's sarcoma and melanoma are other types of lung cancers. The development of carcinogenesis in lung is a life long process, therefore, in all possibilities cancer cells from other organs may eventually metastasize and subsequently grow and develop in the lungs [1].

Normally, cells interact with their extracellular matrix (ECM) either by transmitting signals to the ECM (inside out signaling) or receive signals from ECM into the cell (outside in signaling) via cell surface integrins. Thus, integrins act as bidirectional signal transduction molecules [2, 3]. Biochemically, integrins are transmembrane glycoprotein receptors [4] that bind specifically with their corresponding ECM ligands, namely, fibronectins, collagens, vitronectins and laminins [5]. The integrin-ligand interaction and activation of bidirectional signal transduction are mediated through the activation of a number of so called proteins (e.g. talin) and the engagement of various kinases (e.g. focal adhesion kinases) (FAK) [6–11]. However, the integrins themselves lack any intrinsic kinase activity.

In practice, integrins promote cancer in two distinct ways; firstly, by preventing the apoptosis and, secondly, by imparting resistance of cancer cells to chemotherapeutic agents. Collectively, the inside out and outside in signaling mediated by integrins are key limiting steps in the regulation of antiapoptosis (pro-survival), growth, proliferation and the invasive properties of tumor cells into the surrounding tissues including blood vessels, and finally metastasizing to distant organs. Integrins are therefore regarded as the targets of cancer chemotherapy [12–14].

β1 integrins are the largest family among the integrin superfamily members [15]. Various members of the β1 family are preferentially expressed in the lung cancer tissues [16, 17]. The present review describes the central role of β1 integrins in the survival, proliferation, invasion, metastasis and drug resistance of SCLC and NSCLC. In addition, it also summarizes various drugs that target β1 integrins to combat various cancers including lung cancers.

Expression of Various Integrin Superfamily Members in Lung Cancers

The integrin superfamily consists of 24 family members. These integrin family members are transmembrane receptors consisting of heterodimeric noncovalently linked different α and β chains. Based on their specific functions, integrins can be commonly subdivided into three main types: cell–cell adhesion integrins, vascular integrins and cell-ECM adhesion integrins. Commonly, integrins having the β1 subunit constitute the majority of receptors for the ECM components and, therefore, hold an immense potential for cancer chemotherapy. The β1 integrin family is largely composed of 12 different α subunits members (α1–α12). Various α subunit members have the capacity to bind with the β1 subunit and, therefore, form 12 different β1 integrin subfamilies. Various subfamily members of the β1 integrins family are expressed in pulmonary tissues and, therefore, assume a very important role in the complication of lung cancer [15–18].

Basically, integrin expression is noticed in most of the nontransformed (normal) cells as well as cancer cells [18]. Comprehensive review articles by Gogali et al. [16] and Mizejewsk [17] described the distribution and action of various integrins in the lung and other cancer tissues. In general, SCLC cell lines fail to interact with the adhesive proteins in serum as well as with most ECM proteins, although, some SCLC cell lines can attach themselves onto the laminin substrates [19–21]. Feldman et al. [22] have shown that SCLC lung cancer cells viz, NCI-H187, H345, H146, H209, and N417 express sufficiently high levels of α3β1. Similarly, Pellegrini et al. have demonstrated that the α6β1 integrin is expressed by SCLC lung cancer cells [23]. In the following years, by using 19 human lung cancer cell lines, Hirasawa et al. have noticed that the main integrin expressed by SCLC cells is β1 and combined with α2, α3, α5 and α6, respectively [24]. In the same year, Falconi et al. have described that β1, β3, β4 and β5 integrins expressed by human lung carcinoma cells [25]. Using various SCLC cell lines, Sethi et al. have again reconfirmed that α2β1, α3β1, α6β1 and α5β1 are abundantly expressed in these cell lines [26]. Recently, Roman et al., by using mouse Lewis lung carcinoma cells, further demonstrated that the α5β1 integrin is highly essential for adhesion with fibronectin and lack of the α5 subunit of this integrin drastically affects cell proliferation, migration and colony formation [27]. Another recent study by

Dingemans et al. have indicated that the major integrin expressed by the NSCLC cells is $\alpha5\beta1$ [28]. Thus, the major $\beta1$ integrins expressed by various lung cancer cells/tissues are $\alpha5\beta1$, $\alpha3\beta1$, $\alpha2\beta1$, and $\alpha6\beta1$ integrins, respectively.

Cross Talk of Integrins with Extracellular Matrix Proteins

ECM-cell interactions occur locally in specialized structural foci called focal adhesions (FA). FA are the specific focal contact zones between ECM and the cytoskeleton of the cell [8, 9], permitting the physical attachment of the integrins in membrane to their ECM ligands (e.g. fibronectins, vitronectins, laminins and collagen IV) and the intracellular transduction of downstream signals [29]. Other adhesion complexes mediated by integrins are focal complexes, fibrillar complexes, podosomes, etc. [30]. Critchley et al. have proved that the cytoplasmic domain of the β subunit of the integrin is important in the localization of integrins to focal adhesions, and for integrin-mediated cell adhesion/spreading. The β-subunit of the integrins can be linked to F-actin via the actin-binding proteins talin, α-actinin, filamin and vinculin [31]. Thus, these intracellular signaling proteins are highly essential for the connections of the integrins with the cytoskeleton.

The $\beta1$ integrins preferentially interact with various ECM proteins. For example, $\alpha1\beta1$ and $\alpha2\beta1$ integrins are major collagen receptors, whereas $\alpha4\beta1$ and $\alpha5\beta1$ integrins bind to fibronectins and $\alpha3\beta1$ and $\alpha6\beta1$ are receptors for laminins [3]. In SCLC lung cancer cells viz, NCI-H187, H345, H146, H209, and N417 cells, laminin is the major ligand which binds with the $\alpha3\beta1$ integrin [22]. Similarly, the $\alpha6\beta1$ integrin of the lung tissues binds with laminin [23]. Immunohistochemical analyses have confirmed the high level expression of collagen IV, which binds to $\alpha2\beta1$ or $\alpha3\beta1$ integrins and fibronectins bind to the $\alpha5\beta1$ integrin [32, 33] (Fig. 1 describe the various ligands of $\beta1$ integrins).

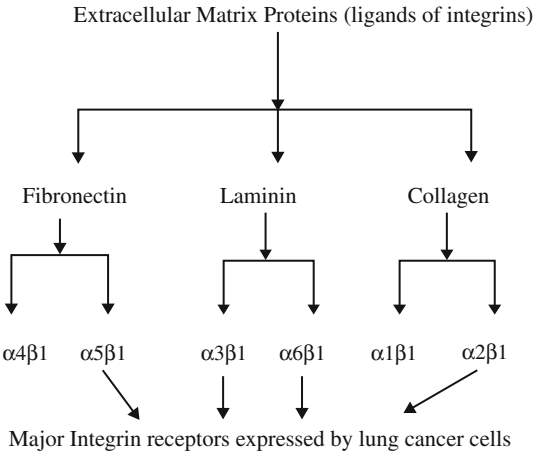

Fig. 1 Cell surface integrin receptors binds with various extracellular matrix proteins namely fibronectins, laminins and collagen IV. $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$ and $\alpha6\beta1$ are the major integrins involved in lung cancer complication

Moreover, the SCLC cells express the functional CXCR4, the chemokine receptor for the stromal cell-derived factor 1 (SDF1). Expression of SDF-1 increases SCLC cell adhesion to collagen 1 and fibronectin via $\alpha 2\beta 1$ and $\alpha 5\beta 1$, respectively [34]. Of the various ECM molecules, the level of expression of fibronectins and collagens are unusually high in NSCLC cells and tissue samples [35, 36]. In A549 NSCLC cells, radiation-induced apoptosis or the cytotoxic drug ukrain-induced cell death are prevented by laminins and fibronectins, respectively [37]. In a comprehensive review article, Cacavari et al. have described the mechanism through which integrins-ECM interactions affects TGFβ and EGFR signaling in lung cancer cells [38]. Another study by Han et al. has demonstrated that the presence of fibronectin activates the survival of lung cancer cells by activating cyclooxyginase 2 (COX-2) through $\alpha 5\beta 1$ integrin [39].

Interactions of Integrins with Extracellular Matrix Proteins

At the cellular level, several integrins bind with their corresponding ECM ligands (proteins), having a highly conserved amino acid sequence of the tripeptide arginine-(R)-glycine-(G)-aspartic acid-(D) [RGD]. $\alpha v\beta 3$, $\alpha 5\beta 1$ and $\alpha IIb\beta 3$ are the examples of this kind of integrins that recognize the RGD sequence of the ECM proteins. However, other integrins may recognize a different amino acid sequence of the ECM proteins. For example, the $\alpha 4\beta 1$ integrins recognize EILVD and REVD amino acid sequences in the alternatively spliced CS-1 fibronectin. In a comprehensive review article, Ruoslahti described RGD and other recognition sequences of various ECM proteins/ligands that interact with the integrins [40]. Mizejewsk [17] has tabulated the various ligands of integrins with respect to their structural motifs, recognition sites and functional activities. Identifying and understanding these unique binding sequences is important for targeting integrin-mediated therapy since the know-how of these amino acid sequences helps to develop therapeutic peptides or antibodies that could disrupt integrins-ECM interactions and regulate integrin-directed proliferation, growth, survival and metastasis of the cancer cells [41–44].

β1 Integrins as a Prognostic Factor in Small Cell Lung Cancer and Non Small Cell Lung Cancer

The level of expression of β1 integrins is related to the prognosis of both SCLC and NSCLC. A clinical study of transbronchial biopsies revealed that the increased expression of β1 integrins is directly correlated with chemoresistance and poor prognosis in SCLC [45]. Another study by the same group has shown that an

association of β1 integrin and oncogenic p53 gene expression together can act as a prognostic factor in SCLC [46]. In a recent in vivo study, by using samples from SCLC patients, Lawson et al. have shown that the levels of expressions of β1 integrin and Bcl_2 are independent prognostic factors for the development of SCLC [47]. A number of other studies have also confirmed the elevated expression of β1 integrins in SCLC cells but not of β3, β4, or β5 integrins [48, 49]. However, in another very recent study using SCLC patients neither β1 integrin nor its downstream signaling molecule rac1 are confirmed as a prognostic indicator [50].

In NSCLC, the expression of α5 integrins has been suggested to play a role in predicting the clinical course and prognosis of this disease [51, 52]. Okamura et al. [53] have shown that elevated β1 integrin expression is a prognostic factor for overall survival in NSCLC patients. During early stages of the disease, the NSCLC patients suffer with a high risk of recurrence, therefore, determination of the level of $\alpha5\beta1$ expression may predict the prognosis of the disease in the individual patient [28]. A number of other studies also confirm the importance of $\alpha5\beta1$ as a prognostic factor against NSCLC. Thus, it is essential to check the level of expression of β1 integrins and ECM proteins involved in various lung cancer cells.

Role of β1 Integrins in Prosurvival of Small Cell Lung Cancer Cells and Non Small Cell Lung Cancer: Implication on Anoikis

In normal cells, the interaction of cell surface integrins with various ECM proteins leads to pro-attachment signals. These pro-attachment signals generate prosurvival signals, whereas detachment from the ECM triggers many non-cancerous (normal) cell types to undergo "anoikis", a form of cell death due to cellular detachment [54, 55]. Understanding anoikis is very important for cancer cells since the acquisition of a resistance to anoikis constitutes a critical step in tumor progression and immortality, particularly in the emergence of invasive and metastatic cells [56]. Cancer cells have a unique potential of growth that enable them to have an anchorage-independent growth signaling which again signifies that cancer cells are not dependent on integrin-mediated cell-ECM interactions. Accordingly, cancer cells have a unique feature that allows them to escape and evade anoikis as they can survive without anchorage-dependent growth and, therefore, may rapidly metastasize to other body parts.

It must be mentioned that cancer cells exhibit major alterations in their repertoire of expressed integrins as well as in their surrounding ECM [57–59]. Moreover, metastatic cells display a marked resistance to anoikis [60–62]. For example, unlike normal cells, cell detachment-induced anoikis does not occur in cells expressing the oncogenes *Src* or *Ras*. These oncogenes must therefore express themselves properly with the active participation of specific integrins for the unlimited and infinite survival of associated tumor cells [63, 64].

Not only the enhanced expression of integrins and ECM proteins, the ability of integrins to prevent aniokis and to allow the cells to survive depends upon integrin-dependent activation of a number of prosurvival pathways [64]. However, unlike growth factors, integrins have no intrinsic kinase activity. Interestingly, integrins have the capacity to cocluster with kinases and adaptor proteins in focal complexes, thereby transmitting downstream prosurvival signals. A number of review articles describe in details integrin-dependent cell survival and inhibition of aniokis both in transformed cells and nontransformed cells [12]. At present, intensive attention has been given to FAK and Src in tumor progression [65–67], as well as two main downstream signaling pathways engaged by them, such as the PI(3)-K/AKT and MEK/ERK pathways, respectively [68, 69]. The most important signaling pathways that are involved in generating a prosurvival signal and eventually drug resistance in the lung cancer cells are discussed below.

SCLC is particularly the more aggressive form of lung cancer. In SCLC cells, the interaction of β1 integrins with the ECM proteins activate the phosphatidyl inositol 3-kinase [PI(3)K]-protein kinase B (PKB) pathway, a prosurvival pathway and a key regulator of cell cycle progression [70, 71]. Of note, PKB is also known as AKT, a serine-threonine kinase. In SCLC cells, the PI(3)K-PKB prosurvival pathway thus inhibits the expression of p21 and p27, the critical inhibitors of cell cycle. It has also been observed that this prosurvival pathway is active even in the presence of DNA damage, indicating that DNA damage cannot prevent the cell cycle to proceed forward [72]. The integrins $\alpha5\beta1$ and $\alpha5\beta6$ are also involved in the elevated expression of the anti-apoptotic Bcl_2 protein [73]. Further studies showed that AKT activation actually leads to the generation of anti-apoptotic mechanisms such as the phosphorylated inactivation of the $Bcl_2/Bclx_L$-associated death promoter (BAD), a key negative regulator of cell survival that binds and blocks the anti-apoptotic Bcl_2 family proteins [74].

NSCLC is the most common type of lung cancer. Sometimes, the word NSCLC is also used as a generic term when a particular lung cancer cannot be properly classified. β1 integrins play a very important role in NSCLC cells growth, angiogenesis, invasion, and metastasis to specific organs [75]. Han et al. have shown that in NSCLC cells, fibronectin activates the AKT-mammalian target of rapamycin (mTOR)-S6 kinase and inactivates the liver kinase B1 (LKB1)-5′-adenosine monophosphate-activated protein kinase (AMPK) pathway. The net effect of this dual action results in the enhanced proliferation of NSCLC cells [76]. In a recent study using NSCLC cells A549, β1 integrin gene-silencing results in defective activity of the epidermal growth factor receptor (EGFR). This phenomenon leads to reduced in vitro proliferation, enhanced sensitivity towards the chemotherapeutic agents cisplatin and gefitinib and leading to impaired migration and invasive behavior of the A549 cells [77]. The integrin-linked kinase (ILK)-AKT pathway also provides a significant advantage to NSCLC cells for proliferation, survival, and invasion to distant organs (metastasis) by the protein kinase Cε (PKCε)-dependent signaling pathway [78].

β1 Integrins in the Migration and Metastasis of Small Cell Lung Cancer Cells and Non Small Cell Lung Cancer Cells

The involvement of integrins in cancer cell invasion and migration are explained in a number of studies [79–81]. Normally, SCLC cells have a high tendency to metastasize in the bone marrow and bone marrow cells secrete a high amount of the chemokine stromal cell derived factor-12 (CXCL12) [82]. Of note, CXCL12 is a chemokine constitutively expressed by bone marrow stromal cells. CXCR4 is a lone receptor for the CXCL12 chemokine, which plays an important role in the housing of cancer cells within the bone marrow microenvironment [83–86]. The role of CXCL12-CXCR4 interaction is, thus, a very critical and an important driving factor for lung cancer onset as it drives the cytoskeletal organization of tumor cells involving the activation of vital cell surface receptors. This phenomenon also results in the attachment of cancer cells to the ECM via β1 integrins [86]. Therefore, the adhesion of SCLC cells through β1 integrins to fibronectin and collagen ligands is increased by the CXCL12 chemokine.

Likewise, studies using the NSCLC cell line NCI-H1299 have shown that integrin signaling induces various proteolytic enzymes (proteases) that are co-localized within the integrins regulating the interface between integrins and the intracellular cytoskeleton. In one study, urokinase, a protease, promotes the ligand-like binding of its receptor (urokinase receptor is a GPI linked membrane adaptor protein) to a set of β1 integrins. This binding, in turn, affects integrin-dependent cell invasion [87]. Similar findings by Takenaka et al. have shown that a highly metastatic variant of PC9 cells has increased $\alpha5\beta1$ integrin expression and that the metastatic potential of these cells can be reduced to more than half by treatment with an anti-β1 integrin antibody [88]. Interestingly, studies by Han et al. have demonstrated that increased expression of $\alpha5\beta1$ integrin is associated with lymph node metastasis in NSCLC patients [35]. An association between increased integrin $\alpha5$ or $\beta1$ expression and poor prognosis in NSCLC patients are reported previously [35].

A number of studies indicate the central role of PKCs in invasion and migration of NSCLC. For example, PKCε has been recently identified as a key regulator of a molecular network controlling the migration of NSCLC cells through the active coupling of zonula-occludensa-1 (ZO-1) proteins and $\alpha5\beta1$ integrin [78]. Protein kinase inhibitors prevent junction dissociation-induced by low extracellular calcium in MDCK epithelial cells [89]. Moreover, the clinical diagnosis of NSCLC cancer patients has revealed the presence of ZO-1-$\alpha5\beta1$ complex, being actively involved in the metastasis of the related disease. These clinical reports summarize the key signaling events brought about by the PKCε-$\alpha5\beta1$-ZO-1 pathway suggesting its extremely sensitive role in the metastasis of NSCLC in humans. Although the molecular mechanism responsible for ILK over-expression in NSCLC cells is still not well understood, it is a highly likely perception that the integrin-ILK-AKT signaling pathway may provide a big boost for NSCLC cell proliferation, survival and invasion. So, inhibition of ILK expression to attenuate

the onset of NSCLC presents an exciting approach to prevent the lung cancer occurrence. Some initial successes in this regard have been achieved following the use of KP-392 and cisplatin, a chemical inhibitor (drug) that prevents the growth and malignancy of NSCLC cells [90]. In a most recent study using A549 and H1299 cell lines, a novel histone deacetylase inhibitor HTPB significantly suppresses tumor metastasis, partly, through inhibition of the β1 integrin-focal adhesion kinase (FAK)-matrix metallo proteinase (MMP)-RhoA-F-actin pathway [91]. These observations obviously lead to the conclusion that α5β1 integrin is actively involved in promoting both NSCLC proliferation as well as its metastatic invasion.

Role of Integrins Other than β1 in the Complication of Lung Cancer

While β1 integrins are the major integrin subfamily involved in lung cancer survival, invasion, metastasis and drug resistance, a few other β integrin family members are also involved in the complication of lung cancers. Two such integrin members of this type are αvβ5 integrins and αvβ3 integrins respectively, as discussed below.

Role of αvβ5 Integrins in Lung Cancer Complication

In a significant attempt, Zhu et al. have investigated the combined role of the protease-activated receptor (PAR-1) and αvβ5 integrin during the onset of thrombin-mediated lung cancer invasion, both in immobilized as well as in native state. They have found an active involvement of αvβ5 integrins in both native as well as in the immobilized thrombin-mediated tumor cell invasion. αvβ5 integrins promote lung cancer cell adhesion, migration and extracellular signal-regulated kinase (ERK) phosphorylation, in both native as well as in immobilized thrombin configuration. This study indicates that targeting of αvβ5 integrins or the αvβ5 integrin complex with PAR-1 can be an excellent therapeutic strategy in the treatment of lung cancer invasion [92].

Role of αvβ3 Integrins in Lung Cancer Complication

Recently, Meng et al. have predicted that there is an active cooperative signaling between αvβ3 integrins and the estrogen receptor alpha (ERα), which in turn, is involved in the proliferation of lung cancer via thyroid hormone induction. In their study, Meng et al. have comprehensively analyzed the signaling mediated by ERα-

positive human lung cancer cells and have successfully concluded that the proliferative action of the thyroid hormone is mediated by the corresponding activity of ERα. Their analysis has further shown that the lung cancer cell proliferation has an active dependence on the corresponding integrin receptors and that the thyroid hormone crosstalks with αvβ3 integrins and is an active endogenous factor supporting the proliferation of lung cancer [93].

Very recently two more studies have also described the importance of the contributory role of the $\alpha v\beta 3$ integrin in the adhesion, invasion and metastasis of B6 melanoma cells in the lungs. In one of these studies, Lonsdorf et al. have observed a reduction in the hematogenous metastasis of B16 melanoma cells to the lungs following treatment with a monoclonal antibody aimed specifically at blocking the expression of α subunit of the $\alpha v\beta 3$ integrin. This study further concludes that there is an active role of engagement of GPIIb/IIIa (platelet receptors) with $\alpha v\beta 3$ integrin interaction that mediates the interaction of tumor cells with platelets and also explains how this interaction is involved in hematogenous tumor metastasis [94].

In another comprehensive study, Goldeman et al. have suggested crucial roles of $\beta 3$ integrins in the progression and metastasis of lung cancer and have proposed these $\beta 3$ integrins to be the potential targets in the treatment of lung cancer via therapeutic agents. They have used the RNA interference (siRNA) technology to study the consequence of $\beta 3$ integrins impairment in the B16 murine melanoma cells. They have also screened their expression for tumor proliferation, metastasis, invasion, adhesion and migration. They have shown through their analysis that siRNA against $\beta 3$ causes a significant reduction in the progression, metastasis, invasion and adhesive nature of the lung cancer. However, the proliferation rate of the lung cancer cells remains unaltered. These findings concluded that targeting the $\beta 3$ integrins expression at the molecular level can be an excellent therapeutic approach in the treatment of lung cancer [95].

Interestingly, another study has demonstrated that $\alpha 5\beta 1$ integrin is the major integrin expressed in the lung cancer cells and it regulates $\alpha v\beta 3$-dependent angiogenesis by modulating the activity of the protein kinase A (PKA) [96]. Another study has also explained the signaling cooperation between $\beta 1$ and $\beta 3$ integrins [97]. Thus, it can be solely predicted that a combined inhibition of $\alpha 5\beta 1$ and $\alpha v\beta 3$ may be more effective to attenuate lung cancer angiogenesis instead of targeting $\alpha 5\beta 1$or $\alpha v\beta 3$ alone.

Role of $\beta 1$ Integrins in Drug Resistance in Small Cell Lung Cancer and Non-Small Cell Lung Cancer

Cancer cells are inherently different from their normal counterparts and have differential expression of integrins. While a particular cancer cell sufficiently expresses a few particular types of integrins, the same integrins may not be

sufficiently expressed by some other cancer cells. Moreover, any particular cancer cell may express more than one type of integrins. Therefore, the task of understanding the role of integrins in the regulation of drug resistance of metastatic cancer cells seems to be a daunting subject. The rapid metastasis (for example to the bone marrow) and the tendency to develop resistance towards chemotherapeutic drugs are the two main reasons for the extreme malignancy of SCLC [98].

It is speculated that adhesion molecules are somehow related to drug resistance [99]. Interestingly, adhesion of SCLC lung carcinoma cells to fibronectins enhances tumorigenicity and confers resistance to apoptosis-induced by standard chemotherapeutic agents against cancers [33]. A similar study has reported that the adhesion of SCLC cells to fibronectin enhances their viability and cytoskeletal organization mainly by activating the PI(3)K-FAK pathway [100]. Kraus et al. [101] have demonstrated that chemoresistance of SCLC cells correlates with adhesion to ECM and constitutive activation of the Akt and MAPK-dependent prosurvival and proproliferative pathways. Moreover, Tsurutani et al. have shown that laminin-mediated activation of the PI(3)K-Akt pathway enables SCLC cells to escape from imatinib-induced apoptosis [102]. In a comprehensive work by Sethi et al., it is further discussed that adhesion of SCLC cells via β1 integrins to ECM components promotes cell survival and conferring resistance to chemotherapeutic agents. The chemotherapeutic resistance is possibly due to the activation of the PI(3)K-PKB prosurvival pathway which inhibits the expression of p21 and p27, the critical inhibitors of cell cycle. Interestingly, this prosurvival pathway is active even in the presence of DNA damage, indicating DNA damage cannot prevent the cell cycle to move forward [26]. Krystal et al. have described that inhibition of the PI(3)K-Akt pathway attenuates growth, promotes apoptosis and enhances sensitivity of SCLC cells to chemotherapy [70]. Similarly, Hartmann et al. have reported that in SCLC cells the interaction of α2, α4, α5, and β1 integrins with the chemokine receptor CXCR4 induces adhesion and chemoresistance [33]. Contemporary studies have also reported that over expression of β1 integrins leads to chemoresistance, thus affecting the diagnosis of SCLC [45]. In another significant attempt, Ritzenthal et al. have demonstrated that there is an elevated expression of matrix glycoprotein fibronectins in most of the chronic lung disorders. They have explained that the increased expression of fibronectins in the lung cancer cells leads to an increased signaling with the cell surface integrins in these cells that further activate and stimulate the intercellular signals involved in the pathobiology of lung carcinogenesis and lung tumor chemoresistance including those of the MAPKs, GTPases and the PI3-kinase/Akt/mTOR pathways [103]. In yet another significant study, Kohmo et al. have explained the extreme aggressiveness of the malignant and metastatic nature of SCLC cells, citing chemoresistance as the primary cause after the very early initial trials. They have suggested that the signaling by ECM proteins is very acutely involved in imparting of the chemoresistance towards the mostly used cytotoxic drugs. They have also confirmed that there is an unusual and extraordinary subcellular signal transduction ability mediated via the activity of the tetraspanin family member CD9 which expresses itself in SCLC tumors and leading to the development of resistance against

cisplatin and etoposide. They have further analyzed that the higher expression of CD-9 in SCLC cell lines continues even after 48 h of their exposure to the potential anticancerous drugs. In their study, Kohmo et al. have found that chemoresistant SCLC cell lines express the CD-9 receptor in an uncharacteristically higher concentration and are also bound to the fibronectins via $\beta 1$ integrins far more tightly than their non-chemoresistant counterparts. However, they are less metastatic due to lower motility. They have further analyzed that the secretion of cell surface CD-9 receptor on the chemoresistant cells are decreased significantly following treatment with the chemokine CXCL12 and this has also resulted in the restoration of the motility. To support their conclusive evidence, they have also found that selectively treating with either specific anti-CD-9 monoclonal antibody or the incorporation of CD-9 siRNA they resulted in the initiation of apoptosis in the chemoresistant SCLC cell lines [104]. Another attempt by Hoddkinson et al. have concluded that there is an extraordinary role of SCLC-ECM-proteins interactions via integrin activation and PI-3 kinase activity, which is responsible for the generation of a high degree of ECM protein mass around the SCLC cells. Therefore, ECM-integrin signaling becomes a hot target pathway for chemotherapy of lung cancers [72]. Thus, $\beta 1$ integrin expression is directly related to the metastatic potential and drug resistance of SCLC.

In the NSCLC cell line PC9/AB2, overexpression of $\beta 1$ integrin leads towards the resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) such as gefitinib and erlotinib [92]. Notably, $\beta 1$ integrins control EGRF signaling and tumorigenic properties of lung cancer cells [77]. Altogether, these studies emphasize the critical role of $\beta 1$ integrins in both the malignancy and chemoresistance in lung cancer, in particular to SCLC cells.

Modulation of Interactions Between $\beta 1$ Integrins and Extracellular Matrix Proteins

A number of molecules regulate the interactions between $\beta 1$ integrins and ECM proteins. Palecek et al. [105] have described in details the various molecules (e.g. COX-2) that may affect the integrin-ligand binding. Transforming growth factor beta (TGFβ) is a potent regulator of integrin-ligand interactions [106]. In fact, the latent form of TGFβ acts as a ligand for $\alpha 5\beta 1$ integrins [107]. A recent study has shown that the chief tobacco alkaloid nicotine stimulates lung cancer growth via the elevated synthesis of the ECM protein fibronectin. Truly, $\alpha 5\beta 1$ integrins are the major receptors involved in the synthesis of the fibronectin ligand and its activation. Again, silencing the functional activity of $\alpha 5\beta 1$ integrins impairs the mitogenic effect of nicotine on lung cancer cells [108]. Thus, COX-2, TGFβ and nicotine are some of the molecules that affect the engagement of $\beta 1$ integrins with the ECM protein ligands and, thereby, regulate several cell signaling pathways both in SCLC and NSCLC cell types.

β1 Integrins as a Drug Target Against Lung Cancer

Several studies have been tried in the last decade to establish the role of integrins as suitable therapeutic targets against various cancers. Some of these strategies are focused on the regulation of integrin-dependent angiogenesis. Notably, integrins have a very distinct role in cancer angiogenesis [109, 110]. In a comprehensive review, Brook has described various anti-angiogenic strategies for the treatment of lung cancer [111]. Similarly, Jin et al. have described the role of integrins in cancer development and as therapeutic targets [14]. They have described several clinical trials of various cancers by using anti-$\alpha5\beta1$ or anti-$\alpha v\beta3$ antibodies or peptides and concluded that these anti-integrin antibodies or peptides have promising anti-antiangiogenic effects against these cancers. Lately, Desgrosellier in a comprehensive review article has described various strategies utilized for anti-integrin therapy and thereby attenuating cancer complications [112]. In another comprehensive review, Chen has also described various synthetic peptides and proteins that targets the RGD sequence of integrin-ligands and integrin-targeted delivery of nanoparticles carrying therapeutic agents to regulate integrin ligand-integrin interactions [113]. All these above studies are limited to different phases of clinical trials. More importantly, in a study by Reynolds et al., it is shown that deletion of Itgb3 (which encodes $\alpha v\beta3$) or deletion of both Itgb3 and Itgb5 (which encodes $\alpha v\beta5$) failed to inhibit angiogenesis and actually potentiated angiogenesis [114]. Therefore, more studies are necessary with other integrins to conclusively determine the success of anti-integrin therapy to inhibit vascular angiogenesis.

Noteworthy, attention must also be given on several in vitro experiments using various lung cancer cell lines. For example, Takenake et al. have shown the involvement of the $\beta1$ subunit during attachment and this result is consistent with data showing reduced metastasis by a lung carcinoma cell line after treatment with blocking antibodies to the $\beta1$ integrin subunit [88]. In another attempt, the CXCL12-CXCR4 binding-axial orientation has been targeted with BKT140, an antagonist of the CXCR4 chemokine. This has helped in the inhibition of the growth of NSCLC cells and also helped in augmenting the remedial effects of chemotherapy and radiotherapy [115].

BRL 49653 and GW1929, the ligands of peroxisome proliferator-activated receptor gamma (PPARγ), inhibit lung carcinoma cell growth by inhibiting the expression of $\alpha5$ subunit of $\alpha5\beta1$ integrins [116]. Tsai Y et al. have indicated that glabridin, a flavonoid, inhibits migration, invasion, and angiogenesis of in vitro cultured A549 cells by inhibiting the FAK/rho signaling pathway. Inhibition of this signaling pathway is possible due to proteasomal degradation of $\alpha v\beta3$ integrins [117]. The study by Aixinjueluo et al. has further elaborated the mechanism of anti-GD2-ganglioside-dependent apoptosis of the SCLC cell lines SK-LC-17, NCI-417, ACC-LC-171, and ACC-LC-96. This study also claimed that $GD2^+$ SCLC cells undergo anoikis through the conformational changes of integrin molecules and subsequent FAK dephosphorylation [118]. In SBC-5 and SBC-3 SCLC cell lines, siRNA-dependent down regulation of $\beta3$ integrins may prevent

the metastatic potential of the cancer cells [119]. In a recent experimental metastasis study, a powerful subcellular in vivo imaging model is used to demonstrate how an anti-integrin antibody affects seeding and growth of osteosarcoma cells on the lungs. However, treatment with anti-β1 integrin monoclonal antibody, AIIB2, greatly inhibited the seeding of cancer cells on the lungs. This study has concluded that AIIB2 significantly inhibited the spontaneous lung metastasis but not the primary tumor growth [120]. In addition, in the human lung cancer cell line A549 and H1299, a novel histone deacetylase inhibitor, HTPB, significantly suppressed tumor metastasis partly through inhibition of the integrin-β1/FAK/MMP/RhoA/F-actin pathways [91].

Conclusion and Future Perspective

The studies and findings collected in this review highlight the importance of the β1 integrin-ECM signaling in tumorigenesis and drug resistance, with particular reference to lung cancer. β1 integrins have been correctly implicated as very important mediators of the pathogenesis of lung cancer including proliferation, invasion and metastasis. Targeting integrins, particularly β1 integrins, could be one of the most crucial breakthroughs in the successful treatment of lung cancers. Studies aimed at inhibiting the expression of integrins and the weakening of their interaction with the ECM protein ligands are being investigated at the present time for the treatment of lung cancer in different parts of the world.

Conflict of Interest No conflict of interest.

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Aldo-Keto Reductases as New Therapeutic Targets for Colon Cancer Chemoresistance

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Abstract The aldo-keto reductase (AKR) superfamily comprises NAD(P)(H)-dependent enzymes that catalyze the oxidoreduction of a variety of substrates, including prostaglandins, steroids, toxic aldehydes and drugs. Among members of this superfamily, AKR1B10, AKR1C1, AKR1C2 and/or AKR1C3 are overexpressed in several types of cancers. Out of the four AKRs, AKR1B10, AKR1C1 and AKR1C3 are also significantly up-regulated with acquisition of resistance to several anticancer drugs in colon cancer, although the up-regulated enzyme species differ among themselves depending on the drug types. Studies with cell-based experiments have proposed multiple mechanisms leading to the drug resistance through regulation of cell proliferation and detoxification of lipid-derived toxicants by the up-regulated enzymes. Thus, the three enzymes have been recognized not only as potential diagnostic and/or prognostic markers, but also as potential therapeutic targets for the prevention and treatment of the colon cancer chemoresistance. Recently, potent and selective inhibitors of AKR1B10, AKR1C1 and AKR1C3 have been reported, and experimentally used for reversal of the colon cancer chemoresistance. In this chapter, we describe the current literature focusing mainly on the expression profiles of the three AKRs in chemoresistance of colon cancer cells and availability of the inhibitors for overcoming the anticancer drug resistance.

Keywords Aldo-keto reductase · AKR1B10 · AKR1C1 · AKR1C3 · Colon cancers · Chemotherapy · Chemoresistance · Proliferation

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Abbreviations

AKR	Aldo-keto reductase
ARE	Antioxidant response element
BPSA	3-Bromo-5-phenylsalicylic acid
CDDP	Cisplatin
CPSA	3-Chloro-5-phenyl salicylic acid
DOX	Doxorubicin
EGF	Epidermal growth factor
FAL	Farnesal
FOH	Farnesol
GGAL	Geranylgeranial
GGOH	Geranylgeraniol
HAHE	3-(4-Hydroxy-2-methoxyphenyl)acrylic acid 3-(3-hydroxyphenyl) propyl ester
HNE	4-Hydroxy-2-nonenal
HSD	Hydroxysteroid dehydrogenase
HT29/CDDP	HT29 phenotype resistant to cisplatin
Keap1	Kelch-like ECH-associated protein 1
LOHP	Oxaliplatin
MAPK	Mitogen-activated protein kinase
MMC	Mitomycin C
NSAID	Non-steroidal anti-inflammatory agent
NFκB	Nuclear factor-κB
Nrf2	Nuclear factor-erythroid 2-related factor 2
ONE	4-Oxo-2-nonenal
PHPC	(*Z*)-2-(4-Methoxyphenylimino)-7-hydroxy-*N*-(pyridin-2-yl)-*2H*-chromene-3-carboxamide
PG	Prostaglandin
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
VEGF	Vascular endothelial growth factor

Introduction

Colon cancer is one of the most common cause of death from gastrointestinal malignancy and the first leading cause of cancer-related mortality in Japanese women [1]. A first-line of treatment for metastatic colon cancer is chemotherapy, in which chemotherapeutic drugs, such as 5-fluorouracil, leucovorin, oxaliplatin (LOHP), irinotecan, tegafur, doxifluridine, carmofur, capecitabine, doxorubicin (DOX) and mitomycin C (MMC), are clinically utilized for retracting the tumor

prior to surgery or averting the recurrence after the surgery [2]. Since the cancer cells easily acquire tolerance toward the drugs during the long-term administration, most patients with metastatic colon cancer receive the combined chemotherapy and irradiation therapy [3] despite of the appearance of severe side effects including neutropenia and alopecia. Several different mechanisms underlying the resistance induction in colon cancer cells were previously reported. For example, one major mechanism underlying the chemotherapeutic therapy-induced resistance is the overexpression of P-glycoprotein [4], which secretes the chemotherapeutic agents from the cells before exerting the genotoxic efficacy. Other mechanisms of the resistance appear to be due to the reinforcements of the proliferative and tumorigenic potentials through hypersecretion of growth factors, e.g. epidermal growth factor (EGF) [5, 6] and vascular endothelial growth factor (VEGF) [6], and activation of the proteasome [7, 8], a multi-catalytic proteolytic apparatus for degrading damaged proteins, and an up-regulation of cell surface markers, CD133 [9, 10] and CD44 [6, 10], some of which could jointly lead to lowering the sensitivity to chemotherapy-based therapy. Some effective inhibitors for the above target molecules were developed [11–15], and have been in part approved for clinical use as the adjuvant drugs against the inducible chemoresistance of colon cancer.

The aldo-keto reductase (AKR) superfamily is a rapidly growing group of NAD(P)(H)-dependent oxidoreductases that metabolize carbohydrates, steroids, prostaglandins (PGs), and other endogenous aldehydes and ketones, as well as xenobiotic compounds [16–18]. Members of this superfamily are classified into 15 families and each individual family is further subdivided into several subfamilies based on their amino acid sequence similarities and substrate specificities. Structural and enzymatic features of all AKR members are collected and listed at an AKR superfamily homepage (http://www.med.upenn.edu/akr/). In humans, 15 genes for AKRs have been identified. Ten of the human AKRs belong to the AKR1 family, which include aldehyde reductase (AKR1A1), aldose reductase (AKR1B1) and its related enzymes (AKR1B10 and AKR1B15), and hydroxysteroid dehydrogenases (HSDs: AKR1C1, AKR1C2, AKR1C3 and AKR1C4). Among them, four AKR members (AKR1B10, AKR1C1, AKR1C2 and AKR1C3) have been reported to be up-regulated in several types of cancer and cultured cancer cells [19–21]. Therefore, the four AKRs act as potential markers for the corresponding overexpressing cancers. In addition, AKR1B10, AKR1C1 and AKR1C3 have been recently shown to be involved not only in facilitating proliferation of some cancer cells [16, 19, 21, 22], but also in developing chemoresistance of the cells [21, 23–25]. Thus, these AKRs have also received considerable attention as potential therapeutic targets for chemoresistance of colon cancer cells. Here, we provide an overview of the pathophysiological roles of the AKRs in the development of colon cancer cell resistance to anticancer drugs and the usefulness of their inhibitors in overcoming the chemoresistance.

Possible Factors Involved in Colon Cancer Chemoresistance

The tumor microenvironment has been formerly accepted as a major mechanism that leads to the induction of drug resistance [4, 26, 27]. That is because the environment surrounding the tumor limits accessibility of antitumor drugs to the tumor cells and reduces the oxygen radicals generated during the treatment by the drugs [4, 26]. Recent reports provided substantial evidence indicating that the cancer chemoresistance is heterogenously developed by endogenous defensive responses to lethal stress, including sources derived from the microenvironment, and the possible mechanisms encompassing a variety of factors including the P-glycoprotein [4], EGF [5, 6], VEGF [6], CD133 [9, 10], CD44 [6, 10] and the proteasome [7, 8]. Here we outline the factors that are believed to be responsible for inducible chemoresistance of colon cancers, except for AKRs.

P-Glycoprotein

The P-glycoprotein, encoded by the gene for human multidrug-resistance protein 1 [28], is a transporter ATP-binding cassette family protein, which is expressed in the apical membrane of epithelial cells in the small and large intestines and functions as a part of the gastrointestinal barrier for protecting against xenobiotics, bacterial toxicants and drugs [29, 30]. It also plays central roles in the absorption, distribution and excretion of chemotherapeutic drugs and actively extrudes xenobiotic substances including the cytotoxic drugs due to the overexpression in colon cancer cells, displaying drug resistance [4]. The aberrant cellular transportability by the overexpression is considered to be a major causal factor for mechanisms underlying drug resistance of colon cancer cells. Recently, glucosylceramide synthase, that converts ceramide into glucosylceramide and then reduces the ceramide-induced apoptosis, has been reported to be coincidently overexpressed with P-glycoprotein in drug-resistant cells [31, 32]. Based on these studies, the glucosylceramide synthase appears to modulate expression of the transporter protein through c-Src and β-catenin signaling pathways and confers the cell resistance to DOX, daunorubicin, and tumor necrosis factor-α [32]. Administration of zosuquidar, a potent and specific inhibitor of P-glycoprotein, reverses P-glycoprotein-mediated resistance in acute myeloid leukemia and breast cancer [11, 12], but little is known about the effect of the inhibitor on the colon cancer resistance.

EGF Receptor and VEGF

The EGF receptor is expressed in a large proportion (30–85 %) of patients with colorectal cancer, and its signaling pathway is always activated in the cancer cells [5, 33]. Experiments show that persistent activation of the signaling through the EGF receptor promotes cell growth [34] and metastatic transformation [35], and inhibits apoptosis of the cells [36]. Upon EGF receptor stimulation by the binding of its ligand, including EGF and transforming growth factor, the ligand-receptor complex triggers a chain of the down-stream signaling pathways such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase [37]. Therefore, potent blocking agents of the EGF receptor are expected to suppress the colon cancer growth and resistance to certain drugs. Cetuximab is a monoclonal antibody that targets the extracellular domain of the EGF receptor and inhibits the ligand binding to the receptor [13]. Based on a randomized phase II clinical trial, the antibody was approved for use in combination with irinotecan or as monotherapy in EGF receptor-positive irinotecan-refractory colorectal cancer [38]. Thus, the clinical use is limited for treatment of patients with overexpression of the EGF receptor.

Angiogenesis is an essential event for progression and metastasis of tumors [39]. The anti-angiogenic therapy interferes with the supply of oxygen and nutrients that are required for the tumor growth and is, therefore, utilized for the treatment of a variety of cancers as well as colon cancer [39]. A major target of the therapy is VEGF, which is known to promote several steps in angiogenesis including proliferation, migration and tube formation of endothelial cells. Bevacizumab, a monoclonal antibody that binds to VEGF, is approved for use in the treatment of metastatic colorectal cancer [14] and its application is expanded to non-small-cell lung carcinoma and breast cancer because of the clinical availability [40].

CD133 and CD44

Significant increase in a cancer stem cell marker CD133-positive subpopulations of colorectal cancer was found in human colorectal cancer HT29 cells resistant to high doses of 5-fluorouracil [41]. A recent study also revealed that up-regulation of CD133 is associated with prognosis in patients bearing the drug-resistant colorectal cancer [42], suggesting that CD133 is a potential prognostic marker for the resistant cells. The drug tolerance seemed to be sensitized to 5-fluorouracil- or LOHP-mediated damage by using an anti-interleukin-4 neutralizing antibody [43]. In addition to the marker CD133, CD44 is recently proposed as the new potential marker, based on the data showing the positive relationship of its expression in colon SW620 cells with high potentials concerning colony formation, proliferation and chemoresistance development [44, 45].

Proteasome

The proteasome is a large multi-subunit assembly that degrades unwanted proteins, which include oxidized and damaged proteins formed during exposure of cells to chemotherapeutic drugs, as well as proteins involved in the regulation of the cell cycle and transcription [46–48]. Compared to the normal cells, the proteolytic activity of the proteasome in colon cancer cells is considerably higher, but is lower than that in chemoresistant phenotypes of colon cancer cells [25]. The proteasome is considered an important potential therapeutic target for chemoresistance. One potential proteasome inhibitor that reverses the chemoresistance is bortezomib, and its availability is supported by the clinical trials [49] as well as data from the cell-based experiments [50]. The bortezomib-mediated sensitization of the cancer cells to drugs has been shown to be mediated by multiple regulations of cell cycle progression including the p21 cyclin-dependent kinase, checkpoint kinase and cyclins [51], and by controlling turnover of transcriptional factors including nuclear factor-κB (NFκB) and hypoxia-inducible factor-1 [15, 49]. Currently, bortezomib is approved for treating only refractory and recurrent multiple myeloma in Japan and has been tested against other cancers such as lymphoma [52], lung cancer [53] and colorectal cancer [54].

Up-Regulation of AKRs Related to Development of Cancer and Chemoresistance

High Expression of AKRs in Tumors and Carcinomas Cells

AKR1B10 is a 36-kDa cytosolic NADPH-dependent reductase that is originally identified as aldose reductase-like 1 or small intestine aldose reductase [55]. The enzyme is expressed in human gastrointestinal tract and adrenal gland, but its expression levels in other tissues including lung and liver are low [56, 57]. Clinical investigation exhibited that high expression of AKR1B10 is found in airway of healthy smokers compared to those of healthy non-smokers [57–62], suggesting that the enzyme is up-regulated due to components of tobacco smoke. The significant overexpression is also reported in the malignant tumors such as squamous cell lung carcinomas [63–66], hepatocellular carcinomas [67, 68], uterine carcinomas [69], cholangiocarcinomas [67], and gastric [70] and esophageal tumors [71], albeit at different expressions depending on their stage and sites examined. Thus, AKR1B10 is the tumor marker, and is suggested that its overexpression is implicated in carcinogenesis of the above types of cancers.

Four human members (AKR1C1, AKR1C2, AKR1C3 and AKR1C4) in the AKR1C subfamily are cytosolic HSDs with different positional and stereochemical preferences for steroid substrates [16]. AKR1C1 (20α-HSD), AKR1C2 (type 3 3α-HSD), AKR1C3 (type 2 3α-HSD and type 5 17β-HSD) and AKR1C4 (type 1 3α-HSD)

share >84 % amino acid sequence identity, and collectively called AKR1C isoforms. With the exception of liver-specific AKR1C4, the other three isoforms are expressed in many human tissues, in which the extents of their expressions are different from each other [16–18]. The three isoforms are overexpressed in cancer tissues, but their expression levels are different depending on types of cancer. For example, AKR1C1 is overexpressed in lung [63, 72, 73], uterine cervix [74] and colon cancers [23, 48]. Expression of AKR1C2 is elevated in lung [72], bladder [75] and esophageal cancers [71]. In contrast, AKR1C3 is up-regulated in leukemia [76], lung cancers [77], squamous cell carcinomas of head and neck [78], and carcinomas of hormone-sensitive tissues such as the prostate [79] and the mammary gland [80]. The differences in the expression levels of AKR1C1, AKR1C2 and AKR1C3 may reflect their various roles in carcinogenesis and/or cancer cell proliferation [19–22].

Alteration in the AKR Expression in Colon Cancer Chemoresistance

Resistance to chemotherapy is a principal problem in treating most common solid tumors. Like the solid tumors, colon cancers are frequently resistant to the chemotherapy and irradiation. One of the major problems is that the resistance leads to malignant transformation and metastasis of tumors [2, 3]. Despite progressive improvement of the therapeutic approach for colorectal cancer, a high proportion of patients carrying the tumors eventually succumb to the metastatic transformation resulting from the resistance. Therefore, it is of great importance to discover new cardinal factors that are involved in the development of colon cancer chemoresistance, besides the above factors mentioned in section Possible Factors Involved in Colon Cancer Chemoresistance.

There is growing evidence that some members of the AKR superfamily are induced during the development of chemoresistance in a variety of cancers. The alteration in expression of the AKR members (AKR1B10, AKR1C1, AKR1C2 and AKR1C3) in various cancer cells resistant to anticancer drugs has been reviewed [21]. In colon cancers, there are four papers that describe the changes in expression of the AKRs caused by chemoresistance [21, 23–25], which are summarized in Table 1. Although other AKR species were not examined, AKR1C1 is up-regulated in methotrexate-resistant HT29 cells, and is suggested to abolish the cell cycle arrest at S-phase and apoptotic response provoked by the drug [23]. In HT29 phenotypes resistant to MMC [24] and 2 μM LOHP (low-grade) [21], the AKRs, other than AKR1C2, are overexpressed, and the level is the highest in AKR1B10, followed by AKR1C1 and AKR1C3. However, the expressions of the four enzymes are decreased in the HT29 phenotype resistant to 20 μM LOHP (high-grade) [21]. Table 1 includes our unpublished data on the expression levels of AKR1B10, AKR1C1, AKR1C2 and AKR1C3 in the HT29 phenotypes resistant to

Table 1 Expression of AKRs in chemoresistant colon cancer cells

Cell	Drug[a]	Concentration[b] (μM)	Expression of AKR[c]				Ref.
			1B10	1C1	1C2	1C3	
HT29	MTX	10	nd	↑	nd	nd	[23]
	MMC	0.5	↑↑	↑	→	↑	[24]
	LOHP	2 (low-grade)	↑↑	↑	→	↑	[21]
		20 (high-grade)	↓	↓	↓	↓	[21]
	DOX	0.5	↑↑	↑	→	↑	Unpublished
	CDDP	10	↓	↓	↓	↓	Unpublished
HCT15	CDDP	10	ne	↑↑	→	↑↑	[25]
RKO	CDDP	5	ne	↑↑	→	↑↑	Unpublished

[a] *MTX* methotrexate; *MMC* mitomycin C; *LOHP* oxaliplatin; *DOX* doxorubicin; and *CDDP* cisplatin

[b] Drug concentration that is used to establish the resistant cells

[c] Change compared to the parental cells: ↑↑ Highly up-regulation; ↑ up-regulation; → no change; ↓ down-regulation. *nd* not determined. *ne* no expression is observed

DOX and CDDP (HT29/CDDP). Gain of DOX resistance increases the expression of AKR1B10, AKR1C1 and AKR1C3, and the expression patterns of the four AKRs are similar to those in the MMC- and low-grade LOHP-resistant cells. In contrast, the CDDP resistance resulted in significant decreases in the expression levels of the four AKRs, which are similar to those in the high-grade LOHP-resistant cells. The diversity in the AKR expression may be due to different toxic mechanisms toward HT29 cells among the two platin-based drugs (CDDP and LOHP), DOX and MMC. Presumably, the difference is also explained by the results that the HT29/CDDP phenotype had high cross-resistance to toxicity induced by LOHP, but the cross-resistance to DOX or MMC was low (unpublished data). On the other hand, our recent study revealed that significant overexpressions of AKR1C1 and AKR1C3 result from acquisition of CDDP resistance of other colon cancer HCT15 and RKO cells, in which the mRNA for AKR1B10 is hardly expressed [25]. Thus, alteration in the AKR expression due to the chemoresistance is affected by both the enzyme distribution and cell types employed. Considering that the four AKR members (AKR1C1, AKR1C2, AKR1C3 and AKR1B10) are up-regulated with the development of chemoresistance of cancer cells derived from other tissues [19–21], these enzymes are suggested to be closely involved in cancer cell chemoresistance, except in some cases using the two platin drugs.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a key transcriptional regulator of many enzymes including AKR1B10, AKR1C1, AKR1C2 and AKR1C3 (Fig. 1) [61, 81–83]. Under normal conditions, Kelch-like ECH-associated protein 1 (Keap1) recruits Nrf2 into the Cul3 containing ubiquitin ligase E3 complex for ubiquitin conjugation and subsequent proteasomal degradation. When cells receive various stimuli including reactive oxygen species (ROS) [84] and electrophiles [85], Nrf2 is dissociated from Keap1 for translocation into the nuclei, and binds with Maf to genes of antioxidant response element (ARE) to transcriptionally induce proteins including the above AKRs. Ciaccio et al. showed that the

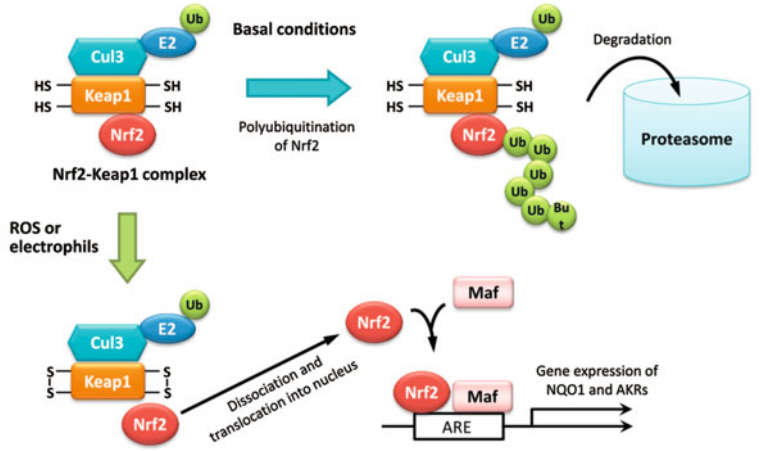


Fig. 1 Induction of AKRs by Nrf2-dependent mechanism. Under basal conditions, Nrf2 complexed with Keap1-Cul3-ubiquitin (Ub) ligase E3 is subjected to polyubiquitination, and the modified proteins are degraded in the Ub-dependent proteasome system. Upon stimulation with oxidants such as ROS and electrophiles, Nrf2 is dissociated from Keap1 through its structural modification by formation of disulfide bonds. Nrf2 is translocated to the nucleus, forms a heterodimer with its obligatory partner Maf, and ultimately induces the expression of ARE-dependent proteins such as NADPH: quinone oxidoreductase (NQO) 1 and AKRs

acquisition of resistance to ethacrynic acid, a potent AKR inducer and Nrf2 activator, up-regulates the expression of AKRs in HT29 cells, and the resistant cells also exhibit cross-resistance to adriamycin and MMC [86, 87]. These findings apparently indicate that the AKR overexpression in cancer showing chemoresistance is conceivably due to the Nrf2 activation. Induction of chemoresistance in cancer cells frequently requires some somatic mutations [88, 89], which disrupt the interaction between Nrf2 and Keap1. Therefore, constitutive up-regulation of these AKRs may be triggered by an abnormality of the Nrf2-Keap1 system that occurred during acquisition of chemoresistance. The mechanistic base for hyperactivation of Nrf2 in the chemotherapy-resistant cancer cells is also explained by down-regulation of Cul3 ubiquitin E3 ligase [90]. Thus, the constitutive activation of Nrf2 is considered as a crucial event in the development of chemoresistance.

Possible Roles of AKRs in Colon Cancer Chemoresistance

Cell Proliferation

Retinoids function as major regulators for proliferation, differentiation and morphogenesis of various types of cells (Fig. 2) [91]. Retinols are oxidized into retinoic acids via retinals, and the resulting retinoic acids (all-*trans*-retinal, 9-*cis*-retinal and

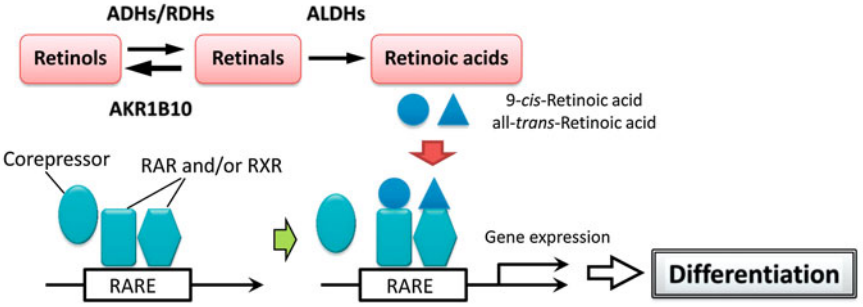


Fig. 2 Retinoid metabolism and cell proliferation. Retinols are oxidized to retinoic acids via retinals by alcohol dehydrogenases (ADHs) or retinol dehydrogenases (RDHs), and aldehyde dehydrogenase (ALDHs). 9-*cis*- and all-*trans*-Retinoic acids bind to nuclear retinoic acid receptor heterodimers composed of RAR and/or RXR, and promotes the expression of genes involved in cell differentiation through binding of the heterodimer to retinoic acid response element (RARE) and consequently dissociating a corepressor protein. AKR1B10 efficiently reduces retinals back to retinols, leading to cell proliferation through cellular low concentrations of the active retinoic acids

13-*cis*-retinal) promote cell differentiation through bindings to retinoic acid receptors or retinoid X receptors. In cancer cells, retinoic acids are maintained at low concentrations, resulting in the high growth potentials [91]. AKR1B10 efficiently reduces the retinals to their corresponding retinols, as its catalytic efficiency is much higher than those of other enzymes including AKR1B1, AKR1C1 and AKR1C3 [92,93]. AKR1B10 overexpressed in cancer cells is thought to contribute to cancer development by lowering the cellular levels of retinoic acids by reducing retinals back to retinols [92–94].

Three AKR1C isoforms (AKR1C1, AKR1C2 and AKR1C3) are involved in the synthesis of active androgen and estrogen, whose hyperproduction is recently considered to promote proliferation of prostate and breast cancer cells [16, 19]. In addition, the metabolism of PGs by the AKR1C isoforms is likely to be one potential mechanism of cellular proliferation (Fig. 3). AKR1C1 and AKR1C2 convert PGD_2 into its 11β-OH form, 9α,11β-PGF_2, but have low $PGF_{2\alpha}$ synthase activity [95]. As AKR1C3 was originally identified as a PGF synthase, it efficiently catalyzes both the formation of $PGF_{2\alpha}$ from PGH_2 and the reduction of PGD_2 into 9α,11β-PGF_2 [96]. The produced PGs ($PGF_{2\alpha}$ and 9α,11β-PGF_2) are ligands for the PGF receptor, and their binding to the receptor activates signaling pathways including MAPK and NFκB, finally promoting cellular proliferation [97, 98]. At the same time, the up-regulation of the enzymes decreases the formation of 15-deoxy-PGJ_2, an endogenous ligand for peroxisome proliferator-activated receptor (PPAR) γ, resulting in inactivation of the pro-apoptotic signaling pathway [95].

In addition to the PG-dependent signaling, we have recently proposed the contribution of two isoprene derivatives to the proliferative potential of cells (Fig. 4) [21, 99]. Isoprenyl aldehydes, farnesal (FAL) and geranylgeranial (GGAL), are the intermediates in the metabolism of farnesol (FOH) and geranylgeraniol (GGOH),

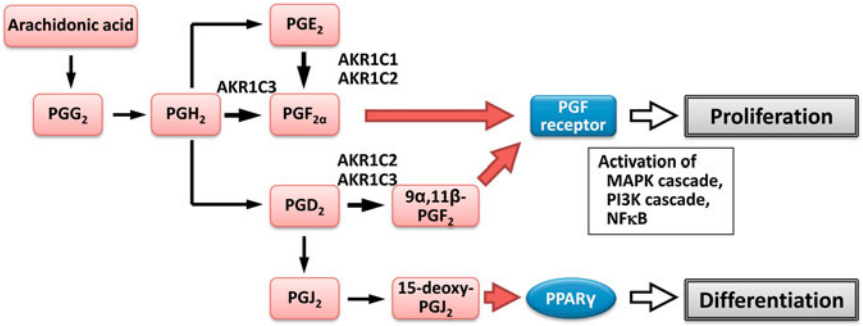


Fig. 3 PG metabolism by AKRs related to cell proliferation. $PGF_{2\alpha}$ and $9\alpha,11\beta$-PGF_2 are formed from PGH_2 and PGD_2, receptivity, by AKR1C1, AKR1C2 and/or AKR1C3, and are ligands for PGF receptor, leading to cell proliferation through activation of signal cascades including MAPK, PI3K and/or NFκB. PGD_2 is also metabolized to 15-deoxy-PGJ_2, which is assumed to link to the initiation of differentiative process via its binding to PPARγ and/or inactivation of NFκB. The reduction of PGD_2 by the AKRs decreases the cellular level of 15-deoxy-PGJ_2, and the low level of 15-deoxy-PGJ_2 also promotes cell proliferation

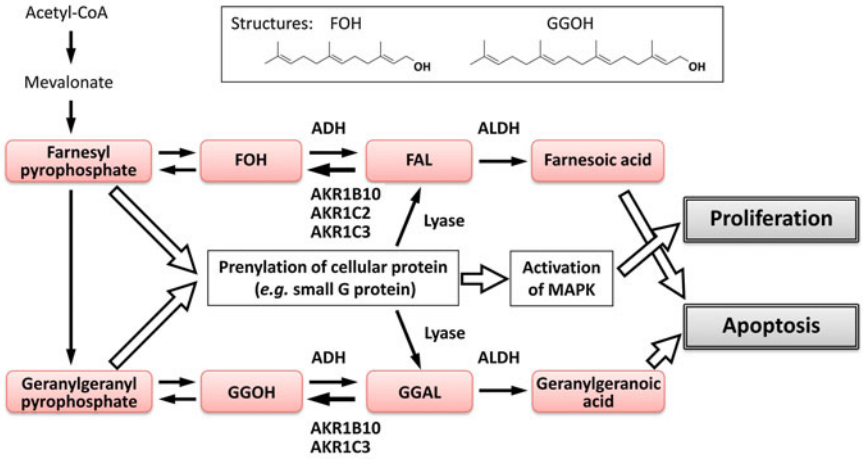


Fig. 4 Metabolic pathway of isoprenoids. Two prenyl pyrophosphates (farnesyl and geranylgeranyl pyrophosphates), intermediates of the mevalonate pathway, are dephosphorylated to the corresponding alcohols (FOH and GGOH), which are oxidized to the carboxylic acids by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) via the aldehydes (FAL and GAL). FAL and GAL are also released from prenylated proteins by lysosomal prenylcysteine lyase (Lyase). The carboxylic acid metabolites possibly lead to activation of apoptotic signaling pathway. Like the retinoid metabolism, the reduction of FAL and GAL by AKRs decreases the cellular concentrations of the apoptotic carboxylic acid metabolites, and increases those of FOH and GGOH. FOH and GGOH are converted to their pyrophosphates, which are thought to provoke both prenylation of cellular proteins including Ras and activation of MAPK cascade, resulting in cell proliferation

respectively, into their carboxylic acids (farnesoic acid and geranylgeranoic acid) by the catalysis of dehydrogenases. The two isoprenyl alcohols (FOH and GGOH) are phosphorylated to their pyrophosphates, which are rendered to prenylation of cellular proteins including small G-proteins and activation of the downstream MAPK signaling, leading to promotion of cell proliferation [100]. AKR1B10 efficiently reduces FAL and GGAL into FOH and GGOH, respectively [99]. Comparison of the kinetic constants (k_{cat}/K_m values) involved in reduction of the aldehydes among seven AKR members (AKR1A1, AKR1B1, AKR1B10, AKR1C1, AKR1C2, AKR1C3 and AKR1C4) reveals that AKR1B10 and AKR1C3 are predominant enzymes involved in the reduction of FAL and GGAL, respectively [99]. Consistent with the enzymatic activities, enhancement of growth potential by forced overexpressions of AKR1B10 and AKR1C3 was observed in human leukemia U937 [101] and prostate PC3 cells [102], and is in accordance with data in similar experiments using other cell lines [103–105]. In addition, it should be stated that the proliferation rate in colon cancer HT29 cells is positively correlated with the AKR1B10 expression, and enhanced by the addition of low concentrations of FAL [21]. These findings clearly indicate that the AKR1B10-mediated reduction of FAL into FOH promotes the cell proliferation. When compared with the proliferation rates between AKR1B10- and AKR1C3-overexpressing U937 cells prepared under the same transfection conditions, the overexpression of AKR1B10 resulted in a 1.5-fold higher growth rate than that of AKR1C3 (unpublished data). Although the precise mechanisms underlying the difference remain uncertain, elevation of the proliferative capacity by the overexpression of AKR1B10 may be due to promotion of fatty acid and lipid synthesis through acetyl-CoA carboxylase α [106, 107], in addition to the above mentioned metabolisms of retinoids and isoprenoids.

Antioxidant Capacity

Exposure of some chemotherapeutic agents (DOX, MMC and CDDP) to cancer cells generates ROS. The accumulation of ROS into the cells evokes the oxidative damage of biomolecules, such as proteins, lipids and nucleic acids, and consequently forms highly reactive and toxic carbonyl compounds including lipid peroxidation-derived 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE). It has been previously proposed that glutathione *S*-transferase [108], AKRs (1B1, 1B10 and 1C1) [107, 109–111] and aldehyde dehydrogenases (ALDH) [112] are the major detoxifying enzymes of the lipid peroxidation-derived aldehydes and their glutathione-conjugated derivatives. Among them, one of the most effective enzymes is AKR1B10, because overexpression of AKR1B10 lowers damage induced by lipid aldehydes including HNE, ONE and acrolein in non-resistant and resistant cancer cells [24, 103]. In addition, knockdown of *AKR1B10* gene by small interference RNAs sensitizes colon cancer HCT-8 cells to acrolein and crotonaldehyde [103]. Thus, the detoxification of lipid peroxidation-derived carbonyl compounds is a role of AKR1B10 in tumor development. In colon cancer

showing chemoresistance, up-regulation of AKR1B10 is responsible for inducing MMC resistance in HT29 cells, and the major molecular basis is suggested to be due to the enzyme-mediated detoxification of the lipid aldehydes resulting from the MMC treatment [24]. In HCT15 cells that do not express AKR1B10, AKR1C1 and AKR1C3 are overexpressed and detoxify the aldehydes in the CDDP resistant cells [25]. This study also shows that the AKR-mediated detoxification of the toxic aldehydes and proteolytic activity of the proteasome toward the oxidatively damaged proteins formed during the CDDP treatment collaborate to confer the colon cancer chemoresistance. Since the treatment with proteasome inhibitors bortezomib and MG132 enhances the expression levels of AKR1C1, AKR1C3 and AKR1B10 in four colon cancer cells (SW480, HT29, Caco2 and HRT-18) [47, 48], it is very intriguing to evaluate the relationship between AKR expression and proteasomal activation.

Metabolism of Anticancer Drugs

AKR1B10 and AKR1C3 are suggested to be involved in the metabolic inactivation of anticancer drugs, such as daunorubicin, idarubicin, epirubicin, oracin and DOX, by reducing the carbonyl groups of the drugs into their less active alcohol metabolites [113–119]. Recently, we have also found high NADPH-linked reductase activity towards MMC in the drug-resistant HT29 cells (unpublished data), in which AKR1B10 is significantly overexpressed compared to the parental cells [24]. Indeed, AKR1B10 exhibits the MMC reductase activity, as it efficiently reduces several other *p*-quinones [120]. In addition to the above two mechanisms of AKR1B10-overexpression in the development of MMC resistance, the ability of AKR1B10 to metabolize this drug may be in part responsible for the drug resistance [24].

Reversal of Colon Cancer Chemoresistance Due to Inhibitors Specific to Individual AKRs

As mentioned above, AKR1B10, AKR1C1 and AKR1C3 are predominant factors for the gain of colon cancer chemoresistance, and their specific inhibitors are therefore expected to be potential adjuvants to suppress the cancer tolerance to anticancer drugs, leading to the persistence of the therapeutic efficacy. Since the enzymes are recognized to be targets for the prevention and treatment of several types of cancers described in the Introduction, various kinds of their inhibitors have been reported [20, 21, 121, 122]. However, there are few studies that show the selectivity of the inhibitors to the target enzyme in comparison with their inhibitory potencies for the structurally similar enzymes with distinct functions. The amino acid sequence identity between AKR1B10 and AKR1B1 is 73 %, and

that among the four AKR1C isoforms (1C1-1C4) is >84 %. In particular, AKR1C1 and AKR1C2 differ only by seven amino acids. Recently, several specific inhibitors of the respective enzymes have been found by molecular docking and virtual screening based on the crystal structures of the enzymes and databases of compounds that may show anti-tumor properties. In this section, we briefly review recent progress on specific inhibitors of AKR1B10, AKR1C1 and AKR1C3, in relation to prevention of resistance to anticancer drugs.

AKR1B10 Inhibitors

Various synthetic and natural compounds show inhibitory effects on AKR1B10, as reviewed previously [21]. Table 2 summarizes the inhibitory potency and selectivity (ratio of AKR1B10 to AKR1B1) of the inhibitors including those reported recently. Among the natural compounds, γ-mangostin is the most potent inhibitor of AKR1B10, and oleanoic acid, followed by bisdemethoxycurcumin, show high selectivity. The natural compounds listed in Table 2 have been described as being effective in the chemoprevention of cancer or having potential antitumor actions, and their inhibitory activities of AKR1B10 are believed to be responsible for their anti-cancer properties [101, 123–125]. In the synthetic inhibitors, (*Z*)-2-(4-methoxyphenylimino)-7-hydroxy-*N*-(pyridine-2-yl)-*2H*-chromene-3-carboxamide (PHPC) and 3-(4-hydroxy-2-methoxyphenyl)acrylic acid 3-(3-hydroxyphenyl)propyl ester (HAHE) are the most potent inhibitors. HAHE is highly selective, because it is designed based on molecular docking of caffeic acid phenthyl ester in AKR1B10, for synthesis of potent and selective inhibitors [101]. PHPC and 9-methyl-2,3,7-trihydroxy-6-fluorone were found by virtual screening of chemical databases based on the crystal structure of AKR1B10 [126, 127], but like an AKR1B1 inhibitor tolrestat, they lack the selectivity to AKR1B10.

Table 2 Natural and synthetic inhibitors of AKR1B10

Inhibitor	AKR1B10		AKR1B1	IC_{50} ratio	Ref.
	IC_{50} (μM)	K_i (nM)	IC_{50} (μM)	(1B1/1B10)	
Natural compounds					
γ-Mangostin	0.018	5.6	0.29	16	[123]
Bisdemethoxycurcumin	0.060	22	5.1	85	[124]
CAPE[a]	0.080	46	0.57	7	[101]
Oleanolic acid	0.090	72	124	1,370	[125]
Synthetic compounds					
PHPC	0.0060	2.7	0.011	2	[126]
HAHE	0.0062	2.6	4.9	790	[101]
Tolrestat	0.054	46	0.014	0.3	[128]
MTF[b]	0.40	200	1.6	4	[127]

[a] *CAPE* caffeic acid phenthyl ester; [b] *MTF* 9-methyl-2,3,7-trihydroxy-6-fluorone

The efficacies of bisdemethoxycurcumin [124], oleanolic acid [125], PHPC [126], HAHE [101] and tolrestat [128] as the inhibitors were demonstrated in cellular AKR1B10-mediated metabolism. In addition, PHPC [21] and HAHE [101] suppress the proliferative potential in AKR1B10-overexpressing U937 cells. The proliferation capacity of HT29 cells resistant to MMC and oxaliplatin is also decreased by the addition of oleanolic acid [125] and PHPC [21], respectively. Furthermore, an endogenous AKR1B10 inhibitor, PGA_1, exhibits anti-tumoral activity, and is also suggested to counteract the DOX-induced resistance of lung cancer A549 cells through the inhibition of DOX metabolism by this enzyme [129].

Inhibitors of AKR1C1 and AKR1C3

In 2011, three elegant reviews on the inhibitors have been published [20, 121, 122]. Recently, new inhibitors with high potency and/or selectivity have been reported, and are focused in this section. The potent inhibitor of AKR1C1 is 3-chloro-5-phenyl salicylic acid (CPSA), which shows an inhibition constant (K_i) value of 0.86 nM [130]. The inhibitory potency and selectivity are compared with those of several known inhibitors [20, 131–135] in Table 3, in which the IC_{50} values are determined under the same assay conditions using *S*-1-tetralol as the substrate. CPSA shows the highest selectivity to AKR1C1 over other AKR1C isoforms, but its selectivity versus AKR1C2 is still low (only 30-fold). This low selectivity is due to the structural similarity between AKR1C1 and AKR1C2, which differs only by one active-site residue (Leu54 versus Val54). Table 3 also includes two flavonoids, naringenin and 7-hydroxyflavone, which are reported to inhibit AKR1C1 potently and selectively [134, 135]. The inhibitory potency and selectivity of the two flavonoids are much lower than those of the synthetic inhibitors, CPSA and its bromo derivative, 3-bromo-5-phenylsalicylic acid (BPSA).

AKR1C3 is inhibited by several structurally different compounds, which are divided into steroidal and non-steroidal inhibitors, as previously reviewed [121, 122]. Representative steroidal inhibitors are medroxyprogesterone acetate and steroidal

Table 3 Inhibitory potency and selectivity of AKR1C1 inhibitors

Inhibitor	IC_{50} (μM)[a]				IC_{50} ratio (1C2/1C1)	Ref.
	AKR1C1	AKR1C2	AKR1C3	AKR1C4		
CPSA	0.0037	0.11	>50	>50	30	[130]
BPSA	0.018	0.38	>50	33	21	[131]
3,5-Dichlorosalicylic acid	0.044	0.39	>50	>50	9	[132]
Benzbromarone	0.048	0.35	2.2	0.73	7	[133]
Naringenin	0.31	4.0	0.91	0.62	13	[134]
7-Hydroxyflavone	0.74	2.5	1.0	12	3	[135]

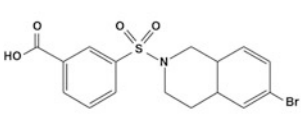
[a] The values are determined with substrate (*S*-1-tetralol) concentrations of 0.1 mM (for AKR1C1) and 1.0 mM (for other enzymes) in the presence of 0.25 mM $NADP^+$ at pH 7.4

lactones, and the non-steroidal inhibitors are non-steroidal anti-inflammatory agents (NSAIDs) and their analogues, benzodiazepines, prostaglandin analogues, flavonoids, cyclopentane derivatives, and cinnamic acids. These inhibitors lack AKR1C3 selectivity or their inhibitory effects on other AKR1C isoforms have not been tested. Among the previously known inhibitors, a highly selective inhibitor is an indomethacin analogue, N-(4-chlorobenzoyl)-melatonin, but its IC_{50} value is 7.8 μM [136]. Recently, more potent and selective AKR1C3 inhibitors have been reported and are listed in Table 4. Two NSAIDs, tolfenamic acid and *R*-flurbiprofen, are more potent and/or selective inhibitors of AKR1C3 than N-(4-chlorobenzoyl)-melatonin [99]. Using a NSAID, flufenamic acid, as lead compound, several highly potent and selective inhibitors of AKR1C3 have been synthesized [137]. The selectivity of a representative inhibitor, compound 40, is shown in Table 4. The remarkable feature of this inhibitor is the lack of cyclooxygenase inhibition, which is an undesirable action for AKR1C3 inhibitors derived from NSAIDs. In addition, more selective inhibitors that are structurally distinct from NSAIDs have been found by biological evaluation of natural compounds and virtual screening of chemical libraries. Baccharin [3-prenyl-4-(dihydrocinnamoyloxy)cinnamic acid], a component of honeybee propolis, is a potent competitive inhibitor (K_i 56 nM) with high selectivity to AKR1C3 [102]. Compounds, 38 and 26, are actually specific inhibitors of AKR1C3 [138, 139], and compound 38 shows the highest inhibitory potency, and inhibits cellular AKR1C3-mediated metabolism at low nM concentration [138].

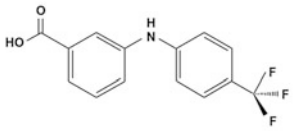
Table 4 Selective AKR1C3 inhibitors

Inhibitor	IC_{50} (μM)				IC_{50} ratio			Ref.
	AKR1C3	AKR1C1	AKR1C2	AKR1C4	1C1/1C3	1C2/1C3	1C4/1C3	
Compound 38[a]	0.0061	>30	>30	>30	>4,918	>4,918	>4,918	[138]
Compound 40[a]	0.062	22.7	15.4	62.7	368	249	1,015	[137]
Tolfenamic acid	0.017	0.71	0.57	53	42	34	3,118	[99]
Baccharin	0.11	>100	>100	>100	>909	>909	>909	[102]
Compound 26[a]	0.213	>300	206	>300	>1,408	967	>1,408	[139]
R-Flurbiprofen	1.5	>50	>50	act	>30	>30	–	[99]
CBM[b]	7.8	>100	>100	nd	>13	>13	–	[136]

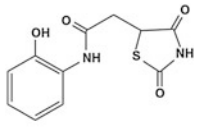
[a] Structures



Compound 38



Compound 40



Compound 26

[b] Abbreviations *CBM* N-(4-chlorobenzoyl)-melatonin; *act* activation; and *nd* not determined

In the cell-based experiments, the AKR1C1 inhibitors BPSA [131] and CPSA [130] effectively inhibit cellular progesterone metabolism by AKR1C1, showing low IC_{50} values of 0.46 and 0.10 μM, respectively. The AKR1C3 inhibitors, tolfenamic acid [99] and baccharin [102], are capable of mitigating facilitation of proliferation potential of U937 and PC3 cells, respectively, by overexpression of AKR1C3, in addition to almost completely inhibiting the cellular metabolism of the preferred substrates (FAL and 17-ketosteroids). Treatment with tolfenamic acid is also found to promote U937 cell differentiation through conceivably lowering the proliferative effect of AKR1C3 [140]. Moreover, our recent study shows that combination of BPSA and tolfenamic acid increases the sensitivity of CDDP-resistant HCT15 cells to the anti-cancer drug [25]. This result may confer direct evidence that AKR1C1 and AKR1C3 are key enzymes responsible for CDDP resistance of the colon cancer cells. However, there is limited evidence in the literature regarding the suppressive effects of the inhibitors on colon cancer chemoresistance. It is therefore necessary to further determine efficacy of the adjuvant therapy using the inhibitors in various malignant stages, such as invasion, metastasis and tumorigenesis of cancer cells, caused with the chemoresistance.

Conclusion

Treatment with chemotherapeutic agents against colon cancer is currently limited by appearance of the therapy-resistant cancer cells. Previous studies for evaluating the molecular basis have proposed some candidates of the adjuvant therapy targets, which include P-glycoprotein, growth factors, cell membrane antigens and proteasomes. Besides them, other rationale candidates are three AKRs (AKR1B10, AKR1C1 and AKR1C3), because they are up-regulated and play roles in not only developing carcinogenesis of cells, but also acquiring colon cancer chemoresistance. Therefore, accumulation of such experimental data using the inhibitors specific to the enzymes would lead to the development of a new strategy, which makes combination of anticancer drugs and the inhibitors applicable to colon cancer chemotherapy. Since the cancer tolerance to chemotherapy is the most significant problem for other carcinomas including lung cancer, we believe that exploration of potent and specific inhibitors of the respective AKRs directly leads to the development of adjuvant drugs useful for the treatments of various kinds of cancers.

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Overcoming Drug Resistance Through Elevation of ROS in Cancer

Amit K. Maiti

Abstract Drug resistance is the most devastating problem in treating cancer and drug-resistant cells are harder to kill with the same drug. The mechanism of drug resistance is believed to differ in various cancers for different anticancer drugs. Most of the anticancer agents induce the generation of Reactive Oxygen Species (ROS) to kill cancer cells by apoptosis. However, prolonged treatment with the same drug reduces the ROS level and this reduced ROS level causes drug-sensitive cancer cells to become drug-resistant. Exogenous ROS in conjunction with the same drug resensitizes these drug-resistant cells. Thus, the apoptosis of cancer cells by inducing ROS generation or drug resistance by lack of ROS could be the principle mechanisms of drug sensitivity or drug resistance in various cancer cells. The genetic mechanism of ROS-induced drug sensitivity and ROS depletion leading to drug resistance in various cancer cells with most of the anticancer drugs could involve 'common molecular pathways'. Understanding the molecular mechanism of ROS generation and maintenance could identify distinct targets for subsequent manipulation to elevate ROS levels in cancer cells. Thus, a 'combinational chemotherapy' could be designed using an anticancer drug while maintaining an elevated level of ROS in the cell during the drug treatment for developing a successful chemotherapy.

Keywords Apoptosis · ROS · Drug sensitivity · Drug resistance · Chemotherapy · Gene network

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Abbreviations

ABCB1	ATP-binding cassette sub-family B member 1
APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1
CASPs	Caspases
CAT	Catalase
CDDO-ME	C-28 methyl ester derivative methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate
ERK1/2	Extracellular-signal-regulated kinases
IPA	Ingenuity Pathway Analysis
GST	Glutathione-S-transferase
HIF 2-alpha	Hypoxia-inducible factor 2, alpha subunit
hTERT	Human telomerase reverse transcriptase
JUN	Jun proto-oncogene
KEAP1	Kelch-like ECH-associated protein 1
MAPK8	Mitogen-activated protein kinase 8
MCTS	Multi-cellular tumor spheroids
MDR1	Multi drug resistance protein 1
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PEITC	Phynyl isothiocyanate
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase

Introduction

Cancer, a complex disease, arises as a result of a progressive accumulation of genetic and epigenetic changes that lead to the breakdown of normal cell division checkpoints. The World Health Organization (WHO 2008) estimated that 10 million patients worldwide die of this disease every year [1]. The remarkable fact is that most deaths occur after subsequent medical intervention with anticancer therapy that includes radio, chemo, targeted, immune and gene therapy. Despite initial high response rates, a large proportion of patients relapse, resulting in a therapeutic challenge. Many of these patients are not curable with current chemotherapeutic strategies, so the goal of therapy is only to improve the quality and length of life [2]. Consequently, more focus is needed on understanding drug resistance in chemotherapy.

Chemotherapy is the most commonly used therapy that randomly kills rapidly growing cancer cells. These chemotherapeutic drugs are classified in different families such as DNA damaging agents (cisplatin, carboplatin), alkylating agents (chlorambucil, cyclophosphamide, temozolomide, carmustine), topoisomerase inhibitors (irinotecan, etoposide,), anti-metabolites (5-fluorouracil, methotrexate,

capecitabine) and oncoprotein targeting antibodies and small molecule inhibitors [3]. These anticancer agents generally affect various cellular functions leading to rapid cell death. Although anticancer agents kill cancer cells, biological barriers make it difficult for anticancer drugs to access specific targets in cancer cells. The major problems are that efflux pumps (p-glycoprotein, encoded by ABCB1 or MDR1) that expel the drugs from these cells into the blood may lead to adequate levels of the drug in the bloodstream with only small concentrations actually reaching the targeted area and cells. Sometimes, drugs accumulate in isolated cellular areas, known as reservoirs, and never reach the targeted cells [4].

Therefore, improvements in the successful therapeutic management of cancer require an understanding of multi drug resistance (MDR) mechanisms and the identification of the underlying drug resistant pathways to successfully manipulate genes for all classes of tumors. This process is believed to be dependent on the cellular and microenvironmental context that include oncogene expression, apoptosis mechanisms, cell cycle control and regulation, DNA repair and mutation, vascularization, and many others.

The cellular signaling leading to these above mentioned molecular processes depends on redox signaling in the cell. Altered redox signaling affects the stability and integrity of cellular proteins, DNA, RNA and other small molecules and modifies normal cellular pathways. Thus, understanding the redox regulation and its role in developing drug resistance in cancer cells is immensely important in overcoming chemotherapeutic challenges.

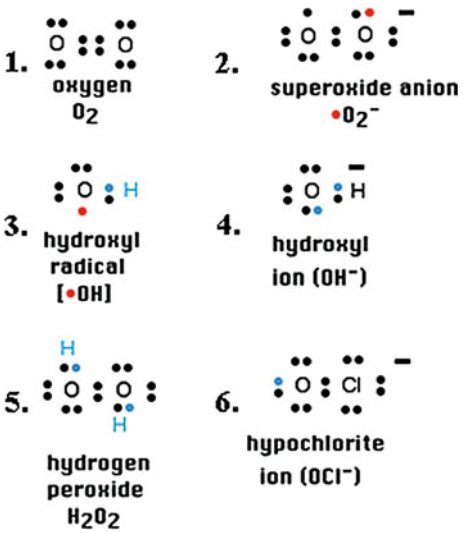

Fig. 1 Most common reactive oxygen species (ROS). *Red* is the unpaired electron which makes an extremely unstable configuration and reacts with other molecules or radicals to achieve stable configuration. The superoxide anion, which is both ion (*2*) and radical (*1*). Hydroxyl radical (*3*) is the most reactive of all radicals. It differs from the hydroxyl ion (*4*) and hydrogen peroxide (*5*). Ions like the hypochlorite ion (*6*) is also very reactive than other ions

How ROS Changes Cellular Pathways

Reactive oxygen species (ROS) with a highly reactive oxygen atom (Fig. 1) reacts with DNA, amino acids of proteins and unsaturated fatty acids leading to oxidation of these biomolecules. ROS can lead to oxidation of amino acid residues of side chains, formation of protein–protein cross-linkages and oxidation of the protein backbone resulting in protein fragmentation [5]. These oxidized proteins, in turn, modify normal protein functions, and have profound effects in cellular signaling and create oxidative stresses in the cell, thus, compelling cells to adopt altered molecular pathways.

How ROS is Generated or Maintained in Cancer Cells

ROS is generally produced at the electron transport chain of the mitochondria as a byproduct during ATP generation and at the plasma membrane to kill cellular microorganisms such as bacteria and viruses. Increasing evidence suggests that mitochondrial ROS generation is the principal contributor in the growth of cancer cells [6–8]. The Warburg effect states that cancer originates from a shift towards excess glycolysis due to defective mitochondrial oxidative phosphorylation that promotes differentiated cells to become undifferentiated cells [9]. Mutations in the TCA cycle enzymes, indeed, show mitochondrial dysfunction with excess glycolysis in cancer cells [10] and oncogene activation increases the chance of mitochondrial dysfunction [11].

External agents such as chemicals, radiation, and viruses induce mutations in cancer-causing genes and proteins that lead to cancer. Hypoxia (low glucose leading to low oxygen supply) increases HIF2-alpha expression leading to suppression of the DNA mismatch repair system that continually increases mitochondrial ROS generation and induces oxidative damage in the mitochondrial and

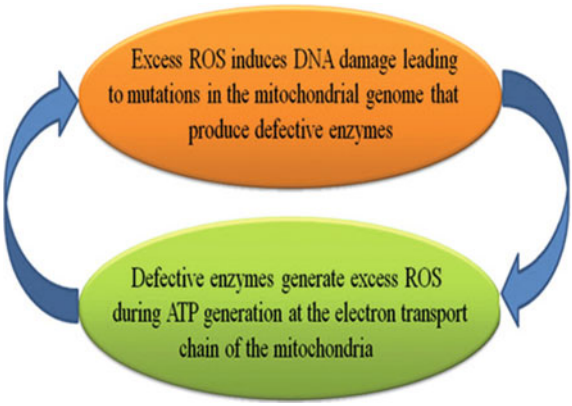

Fig. 2 *ROS generation in cancer cells*. Excess ROS in cancer cells induces mitochondrial DNA damage leading to secondary mutations that virtually produce nonfunctional enzymes and, in turn, generates more ROS through aberrant respiration. The excess ROS production at the mitochondria elevates the ROS level in cancer cells

nuclear genome. This view is widely supported as tumor cells have often found to harbor numerous mutations in the mitochondrial genome [12, 13] leading to abnormal metabolism and increased glycolysis [14] (Fig. 2). Thus, in cancer cells, a cycle is maintained when excess ROS induces mutations in the mitochondrial genome and excess mitochondrial genome mutations produce abnormal enzymes that induce ROS generation .

Apart from ROS generation, cancer cells have distinct mechanisms for ROS maintenance. Mitochondrial oxidative damage also triggers redox-sensitive transcription to regulate ROS levels by eliminating ROS-scavenging systems such as NFE2L2 (NRF2)-KEAP1, catalase, superoxide dismutases, glutathione peroxidases, peroxiredoxins, glutaredoxins and thioredoxins [15]. A general mechanism of ROS maintenance includes NF2EL2-KEAP1 systems (Fig. 3). NF2EL2 remains bound with KEAP1 in the cytoplasm. However, excess ROS in the cell releases KEAP1 from NF2EL2 and free NF2EL2 is phosphorylated by kinases and enters the nucleus. Phosphorylated NFE2L2 binds to Antioxidant Responsive Element (ARE) of the antioxidant genes such as catalase (*CAT*) or glutathione S-transferase (*GST*) [16] and facilitates transcription. The minimum sequence requirement for ARE is 5′-

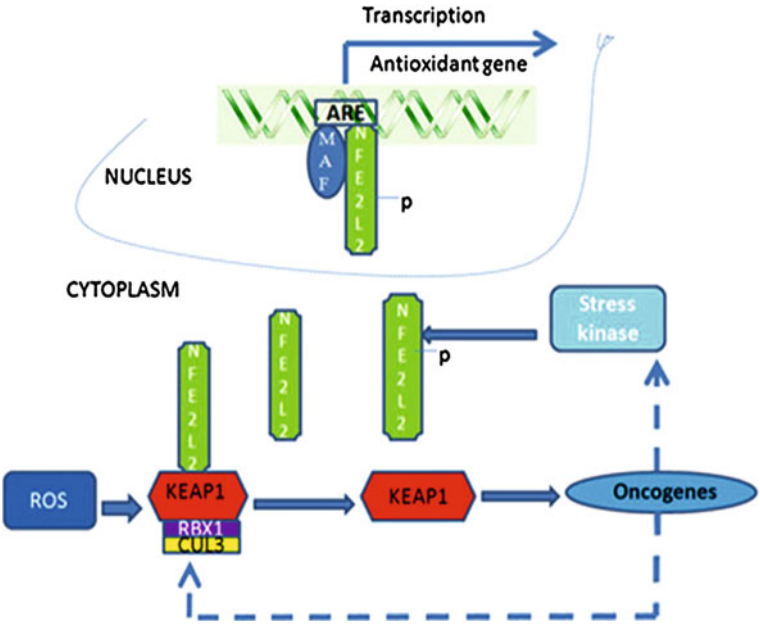

Fig. 3 NFE2L2—KEAP1 *mechanism for maintaining the ROS* ***level in cancer cells***. NFE2L2 remains bound with KEAP1, RBX1 and CUL3 at the cytoplasm. An excess ROS release KEAP1, RBX1 and CUL3 from NFE2L2 and free NFE2L2 is phosphorylated in the cytoplasm. Phosphorylated NFE2L2 travels to the nucleus and binds at the promoter of *ARE* sequence carrying antioxidant genes to facilitate transcription leading to increased antioxidant enzymes production that reduce excess ROS. P, the phosphate group, MAF-oncoprotein v-MAF family members

gagTcACaGTgAGtCggCAaaatt-3′ or TMAnnRTGAYnnnGCRwwww or TGA(C/T)nnnGCA [17, 18] and the presence of two or more copies of the ARE in close proximity to each other often serves as a bona fide ARE [19]. As a result of NFE2L2 binding at the promoter, increased transcription of antioxidant genes leads to increased protein synthesis that reduces the ROS level in the cell. However, all antioxidant genes do not possess ARE sequences at their promoters such as SOD1, SOD2, SOD3 etc. and must be activated through an unknown mechanism.

The Role of ROS in Tumor Development

ROS concentrations act as a double-edged sword for tumor progression as high concentrations of ROS are needed for cancer progression but are toxic to normal cells. The positive ROS signaling is a necessary prerequisite for the development of tumors [15, 20] and cancer cells have higher ROS content than normal cells [14, 21]. ROS acts as a second messenger and its signaling is necessary for cancer metastasis, cellular adhesion and spreading [22, 23]. Recent studies also strongly support the important relationship between the extracellular redox state and cancer cell aggressiveness [24]. ROS compartmentalization and distribution within cancer cells also plays a critical role in tumor progression [25]. ROS oxidizes and inhibits MAPK phosphatases resulting in enhanced cancer cell proliferation and survival. ROS also affects transcription through phosphorylation, activation, oxidation of transcription factors such as APEX1, NF-κB complex, p53, and HIF-2 alpha leading to changes in targeted gene expression [26–29].

An opposite view is also speculated by the observation of the fact that the increase of some of the oncogenes such as *K-Ras, B-Raf and Myc* induce *NFE2L2* expression that detoxify excess ROS in the cell and induce tumorigenesis in mice [30]. This hypothesis considers that low level of ROS promotes tumorigenesis. Several factors should be considered before considering this apparent conflict between the ROS reduction and generation-inducing tumorigenesis. The effect of only three oncogenes on an increase of *NFE2L2* expression may not be sufficient to assume that overall reduction of the ROS level induces tumor development. In human lung cancer, several somatic mutations in *KEAP1* are identified and although the functions of these individual mutations are not studied [31], it is believed that these mutations abolish KEAP1 binding with NFE2L2. Therefore, free NFE2L2 could activate the antioxidant system to decrease the ROS level in lung cancer but the direct evidence for such an assumption is not verified. Unfortunately, Keap1$^{-/-}$ mice expressed *NFE2L2* consistently but did not live more than 21 days, making it impossible to assess its direct role in tumorigenesis [32]. In contrast to oncogenes, tumor suppressor genes, such as *p53* down regulation induces ROS generation during tumorigenesis [33] and have also been shown to harbor numerous mutations in many cancers [34, 35]. Thus, *p53* mutation supports the view that positive ROS signaling is necessary for tumorigenesis.

In addition, no germline mutations in *NFE2L2* or *KEAP1* have been identified in any cancers that would justify the notion that an increase in antioxidants facilitates tumor development. It is believed that cancer stem cells or normal cells when becoming cancerous do need increased ROS signaling [20, 23], but mature tumor cells cannot sustain excessive ROS generation due to abnormal mitochondrial mutations in cancer cells [6, 14, 36]. Any cell, including a cancer cell is vulnerable to excess ROS, thus somatic mutations in *NFE2L2* or *KEAP1* genes observed in cancer cells could activate antioxidant systems to reduce the ROS level that actually helps tumor progression, but may not initiate tumor development. Importantly, several *NFE2L2* mutations are also observed in lung cancer patients (11/103 patients) and are believed to help tumor progression [31]. It has also been proposed that excess ROS, generated by cancer cells, itself drives cancer cells from their primary site towards the bloodstream and is a molecular basis for metastasis [37]. This assumes that the adherence properties of cancer cells are reduced in the presence of excessive ROS signaling and ROS-mediated physiological changes help to prepare them to move from primary sites. Although extensive research is needed to establish this, it appears that tumorigenesis depends on a critical level of ROS in a dose-dependent manner but not solely on a decrease or increase of ROS level in the cell [38, 39]. By combining both views it is also possible that before transformation of normal cells to tumor cells, the ROS level is low but it increases in the tumor cells due to secondary mutations in the mitochondrial genome and then reaches a tolerable level of cancer cells before metastasis.

Anticancer Drugs Induce the Generation of ROS to Kill Cancer Cells Through Apoptosis

Excessive ROS kills cancer cells through induction of apoptosis [40]. Most of the anticancer agents or drugs initially induce ROS generation to kill cancer cells through apoptosis [41, 42]. Numerous anticancer agents including rotenone, thermoquinone, sulforaphane, menadione, rosmarinic acid, tanshinone, chlorambucil, cisplatin, chrysophenol, dithiophene and others initially induce the generation of ROS in various cancer cells [43]. However, it is unknown how these anticancer agents influence cellular mechanisms to generate ROS. Direct relationships between anticancer drugs and antioxidant modulating genes or proteins are yet to be established. It is not known whether ROS generation is the only way to induce apoptosis, or if other mechanisms also play an important role in inducing apoptosis. It is evident that various anticancer agents induce ROS in various cancers by activating different genes and these genes activate the same set of master genes that could induce apoptosis. For example, in breast cancer, rotenone activates ERK1/2, JUN and MAPK8 [44], but sulforaphane is known to inhibit hTERT functions [45]. Dithiophene induces IL-24 in pancreatic cancer cells to activate apoptosis [46]. Similarly,

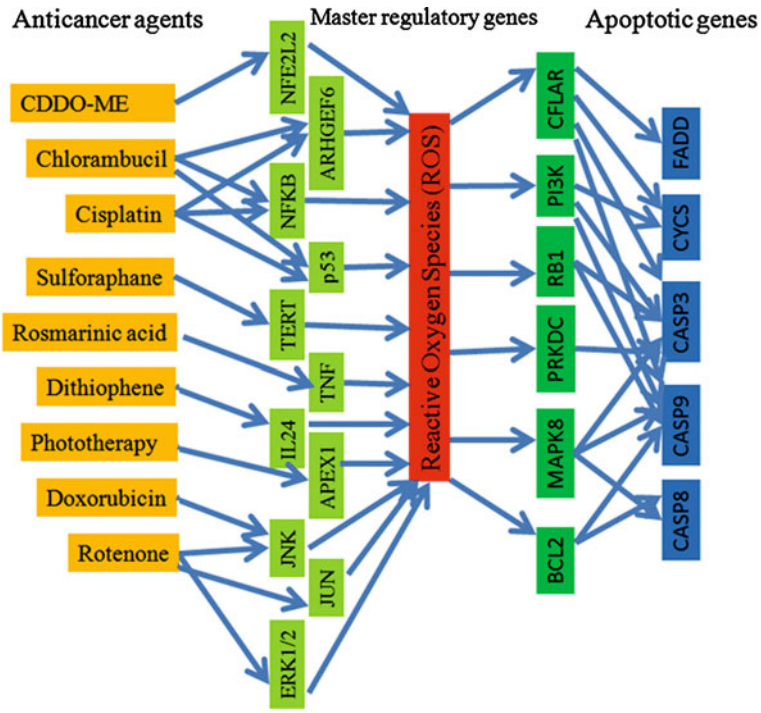

Fig. 4 *ROS-mediated apoptosis through common molecular pathways*. Various anticancer drugs activate different genes to induce ROS generation. However, these genes act on some master regulatory genes that act on apoptotic genes leading to the induction of apoptosis. Thus, anticancer agents share 'common molecular pathways' for ROS-mediated apoptosis

in ovarian cancer, chlorambucil and cisplatin activate NF-κB and p53 [47] whereas CDDO-ME (C-28 methyl ester derivative methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate) downregulates NFE2L2 [48]. *APEX1*, a DNA transcription factor and DNA repair gene, confers drug resistance through ROS generation and MDR activation [29]. Recently, Li et al. [49] showed that APEX1 regulates mitochondrial membrane potential and ROS production after photodynamic therapy of lung cancer cells and induces apoptosis. Again, ROS activates a master set of genes such as *CFLAR, MAPK8, PRKDC, p53*, and *RB1* that act on apoptosis-inducing genes leading to apoptosis [43, 50, 51]. IPA analysis (Ingenuity Pathway Analysis; www.ingenuity.com) has shown the interacting pathways of these genes (Fig. 4). These master regulatory genes could activate Caspases *(CASPs)*, *FADD/MORT* and cytochrome C to activate apoptosis. Thus, the ROS-apoptosis model demonstrates that various anticancer agents in most of the cancer cells induce apoptosis through 'common molecular pathways' [52].

ROS Reduction is the Key Underlying Mechanism of Drug Resistance

Although most of these anticancer drugs induce apoptosis through ROS generation, prolonged treatment with the same drug reduces the ROS level in ovarian cancer cells [47]. Thus, drug resistant cells have a lower ROS content than the drug sensitive cells. Addition of exogenous ROS in conjunction with the drug resensitizes drug resistant cells to drug sensitive cells. The specific mechanism for this observed phenomenon is unknown. Also, it is not clear whether a reduced ROS level makes the sensitive cancer cells become resistant or if resistant cells reduce ROS levels in the cell for their survival. More clearly, upon prolonged drug treatment, it is unclear whether ROS reduction is a primary mechanism for drug sensitive cancer cells to become drug resistant cells or a secondary consequence of resistant cells. Nevertheless, based on these observations, an ROS management cycle in cancer cells could be established, which demonstrates these events (Fig. 5).

In support of this view, evidence is accumulating that the reduced ROS level could be the primary reason for acquired drug resistance in various cancers. It is observed that drug resistant cells have a higher expression of catalase at the plasma membrane which could reduce some of the ROS level [53] in the cells derived from

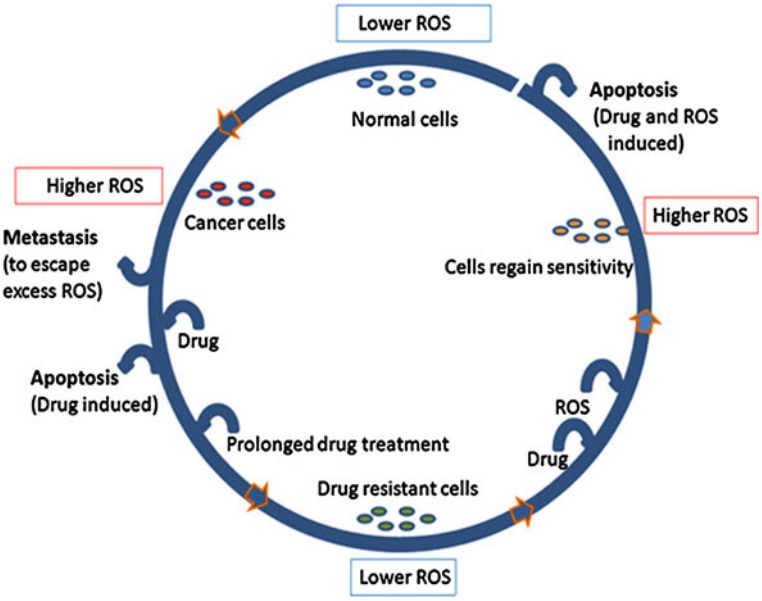

Fig. 5 *ROS cycle of drug resistance*. A flow cycle of ROS-mediated drug resistance is shown where anticancer drugs initially induce the generation of ROS that kills cancer cells by apoptosis. However, prolonged treatment of the same drug reduces the ROS level and transforms drug-sensitive cancer cells to drug-resistant cells. Exogenous ROS in combination with the drug sensitizes drug-resistance cells

human ovarian, gastric and cervical carcinomas. In fibrosarcoma cells, p21-mediated apoptosis could be blocked by overexpression of catalase at the mitochondria of these cells [54]. Overexpression of human catalase in transgenic mice breast cancer model shows resistance to H_2O_2 associated oxidative stress [55]. Recent observations also indicate that overexpression of *NFE2L2*, that mechanistically should reduce ROS, actually confers resistance to lung and ovarian epithelial cancers for platinum based drugs [56, 57]. Depletion of glutathione S transferase (GST), an antioxidant producing enzyme, induces apoptosis through Phenyl isothiocyanate (PEITC) in MCF7 breast cancer cells [58]. However, it remains to be seen whether prolong treated anticancer drug-mediated ROS reduction is a general mechanism of various cancer cells for most of the anticancer drugs.

Drug Resistance Could be Overcome Through Modulation of ROS Along with Drug Treatment as a 'Combinational Chemotherapy'

Elevation of the ROS level in combination with a drug is a useful strategy for 'combinational chemotherapy'. Along with drug treatment, ROS level in cancer cells could be elevated by modulating specific ROS level-elevating genes or proteins. It could be achieved by (1) inactivating ROS generating and maintenance genes or proteins, such as *CAT, SODs, GSTPs* or (2) modulating ROS regulatory master genes, such as, *ARHGEF6, NFE2L2, KEAP1, p53, APEX1* etc. or (3) direct delivery of ROS at the tumor site during chemotherapy.

(1) *Inactivating ROS generating and maintenance genes or proteins.*

Manipulation of drug-resistant mechanisms by redox modulation could have significant therapeutic implications by inhibiting the antioxidative enzyme systems of tumor cells [15, 53, 59]. These are superoxide dismutases (SOD1, SOD2 and SOD3), glutathione peroxidases, peroxiredoxins, glutaredoxins, thioredoxins and catalases. Using optimal concentrations of the catalase inhibitor [3-aminotriazole (3-AT)], or site-specific generation of hydroxyl radicals at the cell membrane of the tumor could prove useful for maintaining excess ROS levels in cancer cells [53]. The function of these target proteins could also be impaired by screening small molecules or siRNA or triplex oligo-mediated gene silencing.

Drugs such as mitocans are being developed that selectively target the mitochondria of malignant cells without adversely affecting mitochondria of normal cells. Mitocans selectively interfere with the bioenergetic functions of cancer cell mitochondria, causing major disruptions that lead to increased ROS production and induction of intrinsic apoptotic pathways [6].

(2) *Modulating ROS regulatory master genes.*

Modulation of ROS regulatory genes should have a significant impact to maintain the elevated level of ROS in the cell. As most cancers share common pathways for antioxidant regulation to induce apoptosis, these strategies could be useful for developing advanced chemotherapy for many cancers. siRNA silencing, triplex-mediated oligo or regulating gene specific microRNA (miRNA) could be used for elevation of ROS in cancer cells. Systematic knockdown of *NFE2L2* or *KEAP1* using siRNA confers resistance to carboplatin-treated epithelial ovarian cancer cells [57]. However, it is not known whether *KEAP1* downregulation overcomes complete resistance in these cells. Experiments with combined manipulation of several genes would be necessary for overcoming complete resistance in drug-treated cancer cells. Cancer cell specific delivery of inhibitors of these genes could be achieved using cancer specific biomarkers attached to suitable nanoparticles [60, 61]. In these cases, a tumor specific attached biomarker would guide the nanoparticles specifically to the tumor cells and the inhibitors directed to specific ROS-reducing genes, such as *NFE2L2 or ARHGEF6,* could be inhibited leading to excess ROS generation. Efficient delivery of oligo to cancer cells through lipoplexes and polyplexes has also been recently developed as a useful tool for efficient gene silencing [62].

(3) *Direct delivery of ROS at the tumor site during chemotherapy.*

Finally, the most important aspect is to explore the direct delivery of ROS into tumor cells along with drug treatment. Designing suitable vehicles to efficiently deliver or generate ROS in conjunction with specific drugs could help to overcome drug resistance in cancer cells in vivo. Among the emerging techniques, liposome-polycation-DNA (LPDI and LPDII) nanoparticles conjugated with an ROS-generating agent, is a promising discovery for treating cancer cells. LPD nanoparticles with a guanidinium containing cationic lipid such as DSAA [***N***,***N***-distearyl-***N***-methyl-***N***-2-(***N***′-arginyl) aminoethyl ammonium chloride] can induce ROS, down regulate MDR transporter expression (Pgp-glycoprotein mediated drug efflux), increase doxorubixin uptake and also shows a significant improvement in tumor growth inhibition [63]. Magnetically engineered nanoparticles also have advantages of inducing ROS in cancer cells [64]. In another approach, multicellular tumor spheroids (MCTS) offer an excellent in vitro system that mimics the endogenous oxidative stress often observed in tumors and could potentially be exploited for ROS generation in vivo [65].

Conclusion

Increased generation of ROS with an altered redox status might not only be exploited for treatment of primary tumors, but has the potential to be used for sensitizing drug-resistant cancer. Numerous antitumor agents induce ROS

production and activate ROS-dependent apoptotic pathways. As observed in ovarian carcinoma cells, anticancer drugs initially increase ROS levels, but prolonged treatment with the same drugs reduces ROS levels in cancer cells resulting in drug resistance. Thus, constant maintenance of higher ROS levels in cancer cells may be necessary for better drug efficacy. A rigorous investigation could be initiated to assess whether drug resistance due to ROS reduction is a general mechanism for most cancer types with most of the anticancer drugs. Nevertheless, using a 'combinational chemotherapy' with anticancer drug and elevation of ROS could be an efficient alternative to increase the efficacy of drug treatments, thereby precluding drug resistance.

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Conflicts of Interest No potential conflicts of interest were disclosed.

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Cancer Stem Cells in Resistance to Cytotoxic Drugs: Implications in Chemotherapy

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Abstract The efficacy of cytotoxic chemotherapy is limited by drug resistance presented by some cancer cells. Cancer stem cells (CSCs) are a sub-population of tumor cells that can initiate tumor formation. If chemotherapy kills bulk of cells within a tumor but not CSCs, the surviving CSCs can initiate the formation of recurrent tumors. This article discusses the inherent resistance of CSCs toward cytotoxic chemotherapy and some possible mechanisms involved. Approaches to target CSCs to improve the efficacy of chemotherapy will also be discussed.

Keywords Cancer stem cells · Drug resistance · Multidrug resistance · ATP binding cassette transporters · Quiescence · Cell survival · Nanog

Abbreviations

ABC	ATP binding cassette
ALDH	Aldehyde dehydrogenase
BCRP	Breast cancer resistance protein
CSCs	Cancer stem cells
EGFR	Epidermal growth factor receptor
ESC	Embryonic stem cells
LRCs	Label retaining cells
MDR	Multidrug resistance
SP	Side population

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Introduction

Cytotoxic chemotherapy is often used in the management of advanced cancer, especially when the disease becomes metastatic. But the efficacy of chemotherapy is often limited by drug resistant cancer cells [1–4]. The major target of cytotoxic chemotherapy is the rapidly dividing cells found within a tumor mass. This has the inherent consequence of collateral damages to other rapidly dividing cells found in the bone marrow, the intestinal and respiratory epithelial lining and cells of the hair bulb. Due to the limited therapeutic windows for most chemotherapeutic agents and steep toxicity curves, the drug-resistant tumor cells cannot be killed simply by dose escalations.

Several possible mechanisms and molecular alterations associated with tumors have been implicated in resistance to chemotherapy, such as hypoxia associated with poor vascularization in tumors [5], activation of pro-surviving signals such as NF-κB [6, 7], overexpression of p-glycoprotein [8–10], defects in apoptosis [11–16], and presence of drug-resistant cancer stem cells (CSCs). These mechanisms are not necessarily mutually exclusive; in fact, they often overlap. In this review, we will evaluate the role of CSCs in resistance to cytotoxic drugs and its implications in chemotherapy.

The Conceptual Framework of Cancer Stem Cells

Tumors are quite heterogeneous. In a given tumor, there is considerable heterogeneity among tumor cells in terms of proliferation, differentiation, and their ability to form tumors when transplanted [4]. It is increasingly appreciated that cancer cells with stem cell-like properties are responsible for the initiation, maintenance and growth of the tumor [17]. Cancer stem cells (CSCs) are defined as a small subpopulation of cells within a tumor that possesses the capability to renew itself and gives rise to a tumor that can recapitulate all the heterogeneous cell lineages of tumor cells within the original tumors [18]. CSCs are also termed as “tumor initiating cells” based on their capacity to initiate tumors when transplanted into immuno-compromised mice. The number of CSCs varies from 5 to 30 % of the total malignant cell population [6]. Studies suggest that, although representing only a small percentage of cells within tumors, the CSCs isolated can reconstitute a new tumor with all the cell types represented in the tumor of origin when transplanted [19].

Identification and Characterization of Cancer Stem Cells

A study of acute myelogeneous leukemia provided the first experimental evidence on the existence of cancer stem cells. A subpopulation of leukemic cells, which expresses the CD34 surface maker but lacks the CD38 marker, was able to recapitulate leukemia in NOD/SCID mice [20]. The cells exhibited a cell surface immune-phenotype similar to the normal hematopoietic stem cells. In addition to leukemia, CSCs have been isolated from different types of cancers, with different but sometimes overlapping profiles of cell surface markers (Table 1). For example, in breast cancer, as few as 200 $CD44^+/CD24^-$/low cells were able to form tumors in immune deficient mice, whereas injections of 20,000 cells from the remaining population failed to form tumors [21, 22]. The tumorigenic population gave rise to additional $CD44^+/CD24^-$/low epithelial tumors; these cells were able to form tumors in other immune deficient mice when they were serially transplanted [21, 22].

Role of CSCs in Resistance to Cytotoxic Chemotherapy

A significant implication of the cancer stem cell theory is the intended target of cancer therapy such as chemo- or radio-therapy. If cancer treatment kills most of the cancer cells in the stage of transit amplifying and differentiation without killing the stem cells, the surviving CSCs will eventually lead to the recurrence of tumors. In fact, there are a number of studies demonstrating the increased resistance of CSCs toward chemotherapy [34]. For example, the CD133-positive cells isolated from a glioblastoma patient sample has exhibited significant resistance to the chemotherapeutic agents including temozolomide, carboplatin, and paclitaxel (Taxol) [35]. The increased resistance of CSCs to cytotoxic chemotherapy [34] raises an interesting question regarding how to kill CSCs in a more effective way.

Table 1 Various CSCs isolated on the basis of cell surface markers by cell sorting techniques

Cancer	CSC phenotype	Reference
AML	$CD34^+CD38^-CD90^-$	[20]
Breast cancer	$ESA^+CD44^+CD24^-$/lowLin^-, $ALDH1^+$	[21, 22]
Brain cancer	$CD133^+$	[23]
Colon cancer	$CD133^+$, $CD44^+$ $EpCam^+CD166^+$	[24, 25]
Osteosarcoma	$CD133^+$	[26]
Pancreatic cancer	$CD44^+CD24^+ESA^+$	[27]
Prostate cancer	$CD44^+\alpha2\beta1^+CD133^+$	[28]
CNS	$CD133^+$	[29]
Head and neck	$CD44^+$	[30]
Melanoma	$ABCB5^+$	[31]
Liver	$CD133^+CD13^+$, $ALDH^+$	[32, 33]

The mechanism of action of most chemotherapeutic drugs includes the impairment of cell division or mitosis, DNA damages, and induction of apoptosis and other types of cell death. Although the chemotherapeutic drugs kill most cells in a tumor, the key question is whether CSCs can survive and cause tumor recurrence. Indeed, CSCs have an inherent resistance to chemotherapy thorough various mechanisms, including increased functionalities of ATP-binding cassette efflux transporters, increased stress response and pro-surviving signals, and increased DNA repairs. Finally, but not lastly, the quiescence of CSCs can pose significant resistance to chemotherapy whose cytotoxic chemotherapy is more geared to target the rapidly proliferating tumor cells than to those quiescent tumor cells.

Role of ABC Efflux Transporters in CSC Resistance to Chemotherapy

Multi-drug resistance (MDR), a clinical phenomenon of decreased intracellular drug retention and changed tumor response, is one of the primary factors limiting effective therapy in cancer [36]. Many *in vitro* and *in vivo* models have been developed to study the development of MDR and to assess the potential clinical application of MDR modulators [7, 37]. The differential induction of adenosine triphosphate (ATP)-binding cassette (ABC) transporters has been associated with MDR in many cancers [38, 39]. These transporters transport solutes across membranes, and their roles in drug resistance have been extensively investigated. The ABC transporter gene products, such as ABCB1 (MDR1, P-gp), ABCG2 and BCRP, expressed in tumor cells allow cancer to resist the chemotherapies. For example, ABCB5, a super-family of ABC, has a significant role in chemo-resistance in human melanoma. When ABCB5 mRNA levels are intentionally reduced using siRNAs, sensitivity of melanoma tumor cells to various drugs, including 5-flourouracil, camptothecin, and mitoxantrone, improves considerably [40].

Both normal stem cells and CSCs commonly express drug pumps such as the ABC transporters, ABCB1 and BCRP. Historically, stem cells or CSCs have been isolated as the "side population" (SP) [37, 41], which express active efflux transporters [37, 41]. For example, CSCs enriched from leukemia SP fractions have an increased ability to pump daunorubicin and mitoxantrone out of the cell [42]. The CSCs have been found to be closely associated with the high expression of ABC transporters including ABCB1 and ABCG2. It has been experimentally shown that ABCG2 and other ABC transporters are responsible for the transporting of fluorescent dye out of normal murine and human cancer cells [43]. Among the common chemotherapeutics, vinblastine and paclitaxel are common substrate for ABCB1. Methotrexate, topotecan, and imatibib mesylate are substrates for BCRP. The enhanced expression and activities of the ABC transporters can enable CSCs to pump out chemotherapeutic agents, when they are the substrate for the ABC transporters, which can lead to an increased resistance to chemotherapy.

Role of Drug Modifying Enzymes in CSC Resistance to Chemotherapy

Aldehyde dehydrogenases (ALDH) (EC 1.2.1.3) are a group of enzymes that catalyze the oxidation (dehydrogenation) of aldehydes, leading to the detoxification of exogenously and endogenously generated aldehydes [44]. ALDH1 activities are frequently used to isolate breast cancer stem cells, and ALDH1 expression is associated with a poor prognosis [45]. Increased ALDH activities were also noted in leukemic CSCs [46–48]. In liver cancer, ALDH is associated with liver CSCs [33]. The increased ALDH expression/activity may confer CSCs with increased resistance to chemotherapeutics such as cyclophosphamide, as ALDH may oxidize and inactivate aldophosphamide/4-hydroxycyclophosphamide (4-HC), the bioactive metabolic byproduct of cyclophosphamide [49].

Role of Pro-Survival Signaling in CSC Resistance Toward Chemotherapy

Another mechanism of CSC resistance to chemotherapy is the preferential activation of pro-survival signaling. For example, hepatocellular carcinoma CSCs presented a preferential activation of the Akt signaling pathway in resistance to chemotherapy [50]. CD44, a receptor for hyaluronan (HA), is a major marker for CSCs in a variety of cancers. The binding of CD44 by HA can lead to the association of CD44 with the epidermal growth factor receptor (EGFR) [51, 52]. This association can cause the activation of MAP kinase and other cellular signaling pathways which promote cell survival in responses to chemotherapeutic drugs such as cisplatin, methotrexate, and adriamycin [51]. Further, the HA-CD44 interaction can activate the EGFR-elicited cellular signaling pathways without engagement of the ligand EGF [51, 52], which also leads to the resistance toward targeted anti-EGFR therapy. It is of interest to determine whether other CSC surface markers can initiate or potentiate pro-survival signaling in the resistance to chemotherapy.

Role of Increased DNA Repairs of CSC: Potential Role in Drug Resistance?

In a seminal study, it was found that CSCs isolated from glioblastomas presented an increased resistance toward radiotherapy via the preferential activation of DNA-damage repair mechanisms [23]. It was found that, while radiation caused equal levels of damage to all cancer cells, CSCs repaired the damage more rapidly than non-CSCs, through the ready activation of the DNA damage and replication

checkpoints that include the checkpoint kinases, Chk1 and Chk2 [23]. The increased ability of DNA repairs in CSCs can confer resistance toward chemotherapy, especially those that can cause DNA damages and checkpoint controls. For example, as a participant in the cytarabine-induced S-phase checkpoint activation, Chk1 can cause the resistance of AML cells toward cytarabine [53]. The inhibition of Chk1 sensitized AML cells toward cytarabine [53]. It is unknown, however, whether other mechanisms of DNA damage repairs are involved in the resistance of CSCs toward chemo- or radio-therapy. It is likely that the process is multi-factorial. Some unique features of stem cells or CSCs, such as slow cycling or quiescence, can make it possible for the cells to repair the damages.

Role of CSC Quiescence in Resistance to Chemotherapy

Normal adult stem cells are usually slow-cycling and quiescent. Under steady-state conditions, adult stem cells divide asymmetrically to maintain tissue homeostasis by keeping their number constant. Asymmetric division refers to the asymmetric partitioning of cell components and/or placement of daughter cells relative to external microenvironments. It is a defining strategy used by stem cells to self-renew and at the same time generate progeny cells for differentiation. Selective segregation of template DNA strands is one feature of stem cells that has been utilized to characterize stem cells known as long term label retaining cells (LRCs) [54]. Normal stem cells cycle less frequently than the more differentiated transit-amplifying cells, and therefore, adult stem cells are often LRCs.

Since most cytotoxic chemotherapeutic agents preferentially target the rapidly proliferative cells, another mechanism of CSC drug resistance is the quiescence. CSCs from acute and chronic myelogenous leukemias are relatively quiescent [55, 56], which may cause their resistance to chemotherapy. It is conceivable that the CSC can enter a state of quiescence under substantial selection pressure from chemotherapy. Therefore, there must exist a period of relative lack of proliferation, during which the CSCs can reduce their metabolic rate and enter into a quiescent state to adapt to the new selection pressure.

Role of Stem Cell Factors in Chemoresistance

The transcription factors Nanog, Oct3/4, and SOX2 constitute the core transcriptional regulatory circuitry in human embryonic stem cells (ESCs) [57, 58]. Ectopic expression of ESC-associated transcription factors can reprogram fibroblasts into pluripotent stem cells [59]. The Nanog expression at the mRNA level was detected in NT2 teratocarcinoma cells [60, 61], malignant cervical cancer [62], testicular carcinoma in site and germ tumors [63], oral carcinoma [64], Ewing's sarcoma [65], seminoma and breast carcinoma [66]. It was found that

Nanog functionally regulates tumor growth [67]. It has been demonstrated that NANOGP8, a pseudogene of NANOG, promotes cancer stem cell characteristics and enables prostate cancer to resist androgen deprivation therapy [68].

In addition to Nanog, Oct3/4 and SOX2 expressions in cancer have been reported. Oct3/4 expression promoted dedifferentiation of melanoma cells to CSC-like cells, with concurrent marked increase in resistance to chemotherapeutic agents [69]. Interestingly, Oct4-induced dedifferentiation was associated with the increased expression of endogenous Oct4, Nanog and Klf4 [69]. The stem cell factors also collaborate with CD44 to promote resistance of CSCs to cisplatin in head and neck squamous cell carcinoma [70]. In this study, it was found that HA stimulates the CD44v3 (an HA receptor) interaction with Oct4-Sox2-Nanog leading to both a complex formation and the nuclear translocation of three CSC transcription factors, stimulation of miR-302 expression in CSCs, cancer stem cell properties that include the resistance to chemotherapeutic agents such as cisplatin [70]. The studies suggest a critical role of stem cell factors in mediating CSC resistance to cytotoxic chemotherapy.

Can We Target CSCs to Eliminate Drug Resistance and Tumor Recurrence?

Given its role in tumor initiation, CSCs should be targeted to eliminate tumor recurrence after therapeutic interventions such as chemotherapy. However, this effort is made difficult by the inherent resistance of CSCs to chemotherapy. It is important to elucidate how CSCs acquire resistance to chemotherapy so that a strategy can be formulated to sensitize CSCs to chemotherapy. There are a number of promising approaches reported to sensitize CSCs to cytotoxic chemotherapies. For example, colon CSCs, which were resistant to fluorouracil or oxaliplatin, can be sensitized by an interleukin-4 blocking antibody [71]. It was shown that the autocrine stimulation of interleukin-4 receptors on CSCs may contribute to their stemness including the drug-resistant phenotype [71]. The drug resistance of CSCs is likely multi-factorial. By further understanding of the biology of CSC drug resistance and the factors/pathways involved, we can develop means to eliminate those drug resistant cells and, therefore, tumor recurrence will not occur.

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Two Birds with a Stone: Molecular Cancer Therapy Targeting Signal Transduction and DNA Repair Pathways

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Abstract The hallmarks of cancer cells are a higher proliferative activity and an aberrant genotype with respect to normal cells. These features can be exploited for the development of selective chemotherapeutic treatments against cancer. In particular, the connections among signal transduction pathways, cell cycle checkpoints and DNA replication and repair have the potential to provide new venues for the treatment of cancer. Here, we will review how the differences existing between normal and tumour cells, with respect to control of cell proliferation and maintenance of the genetic stability, can be exploited in cancer chemotherapy.

Keywords DNA repair · DNA polymerase · Cancer · Tyrosine kinase · Signal transduction · Anticancer drugsanticancer drugs

Abbreviations

BIR	Break induced replication
CK2	Casein kinase II
CML	Chronic myeloid leukemia
DDR	DNA damage response
DSBs	Double strand breaks
GISTs	Gastrointestinal stromal tumours
HER-2	Human epidermal growth factor receptor 2
MMEJ	Microhomology-mediated end joining
NRTKs	Non receptor tyrosine kinases
NSCLC	Non-small cell lung cancer
PDGFR	Platelet-derived growth factor receptor
PTEN	Phosphatase and tensin homology protein
SFK	Src family kinase

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SH2	Src homology-2
SH3	Src homology-3
SSA	Single-strand annealing
SSBs	Single strand-breaks
STKs	Serine-threonine kinases
TKs	Tyrosine kinases
VEGFR	Vascular endothelial growth factor receptor

Introduction

Tumour cells are characterized by a higher proliferative activity with respect to the surrounding cells. This hallmark of cancer cells has been regarded since the beginning as a feature to be exploited for the development of selective chemotherapeutic treatments against cancer. Indeed, anticancer chemotherapy can be regarded as the science of selective toxicity, since it is aimed at reducing the proliferation of cancer cells with minimal perturbation of the homeostasis of normal cells.

In order to achieve selectivity, however, a first requirement to be fulfilled is the identification of a suitable target. Ideally, such a molecular entity should play an essential role in cancer cells, while being dispensable for the normal life of healthy cells. Once a suitable target is identified, small molecules need to be developed which selectively suppress that particular molecular function, without interfering with other similar proteins eventually present in the cells.

In practice, neither of these two goals has been fully achieved in contemporary anticancer chemotherapy. Classical anticancer drugs, in fact, target proteins which are involved in the proliferation of both normal and diseased cells. Selectivity is achieved on the basis that in adult organisms only a small subset of cells have proliferative indexes similar to cancer ones, thus administering the correct doses of drugs for a limited period of time, may achieve suppression of cancer growth without making too much damage to the normal cells.

In recent years, thanks to the advancement of our understanding of the physiology of cancer cells, it was realized that tumourigenesis is almost invariably driven by aberrations in various signal transduction and DNA repair pathways. As a result, cancer cells are dependent, for their survival, on a narrower complement of molecular functions than normal cells. Fostered by these findings, novel targets have been identified, such as tyrosine kinases and DNA repair enzymes. Selective inhibitors of these new targets have been developed and have already entered the clinics. However, after a few years, also these new approaches did not completely fulfill the expectations of the researchers. In many instances, drug resistance readily developed and, thus, reducing the efficacy of the drugs. In addition, alternative pathways, sometimes tumour-specific, can be activated in cancer cells to surrogate for the functions inhibited by the drugs.

As a result, it is now clear that a shift of paradigm is required to develop a new generation of anticancer drugs. Genome-wide analyses of cancer cells proteomes and transcriptomes have provided the experimental evidence for the existence of truly different molecular phenotypes among different tumour types. Thus, cell proliferation cannot be considered anymore, according to the simplistic view of the past, as the hallmark of all cancers since the molecular pathways leading to uncontrolled proliferation are very diverse.

A more fruitful approach should be based on the understanding of the links connecting signal transduction pathways to cell cycle checkpoints and DNA replication and repair. Proliferative signals, which are transduced by tyrosine kinases, beside inducing DNA replication, also activate those pathways that are required to maintain a level of genomic stability compatible with cell survival, including DNA repair. These pathways are the same that also allow tumour cells to cope with the DNA damage induced by classic anticancer agents such as etoposide or cis-Pt. At the same time, tumours are very often defective in one or more DNA repair pathways, thus depending on the remaining ones for their survival. Thus, a strategy aimed to specifically target both signal transduction and DNA damage tolerance pathways may prove to be effective on a large fraction of tumours. An additional benefit of such a combination targeted chemotherapy could be to reduce the emergence of drug resistance. In fact, the genetic barrier required to develop at least one independent mutation for each target will be higher than in the case of monotherapy regimens. To date, several signal-transducing kinases are being regarded as attractive targets for selective cancer chemotherapy. In addition, the realization that in human cells there are at least 15 different DNA polymerases playing non-overlapping roles in DNA replication and repair, has provided entirely new venues for the development of novel cancer drugs.

Targeting Signal Transducing Pathways

Phosphorylation is a fundamental mechanism used in transduction pathways to propagate the signal to final effectors. The reaction consists in the transfer of the —phosphate from ATP to amino acidic residues in specific peptide substrates. The enzymes responsible for this reaction are tyrosine kinases (TKs) and serine-threonine kinases (STKs). These enzymes regulate multiple aspects of cellular metabolism, determining differentiation, adhesion, motility, genome stability, cell growth and death.

Receptor Tyrosine Kinases (RTKs) are single transmembrane domain receptors resident in the plasma membrane with high affinity for ligands like growth factors, cytokines or hormones. Unlike RTKs, non receptor tyrosine kinases (NRTKs) lack both extracellular and transmembrane domains and can be found free in the cytosol as well as in the nucleus, or linked to the inner cell membranes by myristoylation or palmitoylation modifications. These enzymes harbour protein–protein interaction domains like Src homology-2 (SH2), Src homology-3 (SH3)

and pleckstrin homology domains by which NRTKs interact with substrates or regulatory factors.

STKs include a large number of kinases whose activity can be regulated by numerous chemical signals, including DNA damage, cAMP/cGMP, diacylglycerol and Ca2+/calmodulin. Their activity is crucial in the genome stability and its maintenance. Given their roles within the cell, their overexpression or deregulation are linked to the onset, progression and malignancy in a wide range of cancers. Moreover, the deregulation of one or more specific kinases appears to have a positive effect on particular cancer cells survival. Therefore, the specific inhibition of such kinases leads to cancer cell death without impairing the survival of healthy cells. For these reasons, kinases represent ideal candidates for cancer targeted therapy.

BCR-Abl as the Prototype of Molecular Targeted Chemotherapy

The classic example of a successful therapy targeted to a protein kinase is the inhibition of the Abl kinase in leukemias. NRTKs Abl1 and Abl2 are members of the Abl family of kinases, involved in actin remodelling, cell adhesion and motility, DNA damage response and microbial pathogen response [1]. Equally to the Src Family Kinase (SFK) members, Abl proteins can exist either in an active (open), or inactive (closed) form. The shift from open to close conformation is regulated through self interaction between the regulatory domains SH2 and SH3 and the C-terminal part of the protein. *ABL* genes are constitutively activated by chromosome translocations in various haematopoietic malignancies. Chronic myeloid leukemia (CML) is characterized in almost all cases by a t(9q34;22q11) translocation that fuses the Bcr (breakpoint cluster region) and ABL1 genomic regions. The Bcr-Abl1 fusion gene product (p210) has a constitutive tyrosine kinase activity that leads to the activation of the downstream pathways of Abl, conferring to the haematopoietic cell a tumoral phenotype [2]. A similar translocation occurs in three to five percent of childhood [3] and 20–30 % of adult acute lymphoblastic leukemia (ALL) cases. Additional ABL1 fusion variants like NUP214-ABL1, EML1-ABL1 and ETV6-ABL1 [4–6] have been reported in some other leukemias, such as acute myeloid leukemias (AMLs) [7]. In all of these cases, the cell transformation activity by ABL fusion proteins is inextricably tied to their tyrosine kinase activity. In May 2001, the FDA approved Imatinib as the first-line treatment for CML. Imatinib is a selective TK inhibitor which competes with ATP in binding to the Bcr-Abl protein kinase. In particular, the drug occupies a part of the ATP-binding pocket of the enzyme and stabilizes the inactive, non-ATP-binding form of Bcr-Abl [8]. Imatinib has shown excellent activity against CML, inducing apoptosis in leukemia cells; its introduction in therapy greatly improved the outcome of CML patients with complete haematological remission

in more than 90 % of previously treated patients who are resistant to interferon treatment [9, 10].

Beyond Imatinib: New Generation TK Inhibitors

After Imatinib, many other small-molecule inhibitors specifically targeting different kinases have been approved for the treatment of different cancers or are in clinical trial (Table 1). Erlotinib and Gefitinib are small-molecule inhibitors targeting the Epidermal Growth Factor Receptor (EGFR) Family that are approved in breast and non–small cell lung cancers therapy. EGFR is the cell-surface receptor of extracellular protein ligands of the epidermal growth factor family (EGF-family) members. As for other RTKs, binding of the ligand stimulates the dimerization of EGFR resulting in autophosphorylation and, consequently, in the full activation of the kinase domain [11]. In non–small cell lung cancer (NSCLC), which accounts for approximately 85 % of lung cancer cases, EGFR is overexpressed or hyper activated through somatic gain-of-function mutations in exons encoding the EGFR tyrosine kinase domain (in-frame deletions in exon 19 or L858R substitution) [12, 13].

Table 1 Tyrosine kinase inhibitors in anticancer therapy

Inhibitor	Target
Small molecule inhibitors	
Axitinib	Multiple targets
Bosutinib	Bcr-Abl/SRC
Crizotinib	ALK/MET
Dastinib	Multiple targets
Erlotinib	EGFR
Gefitinib	EGFR
Imatinib	Bcr-Abl
Lapatinib	ErbB1/ErbB2
Nilotinib	Bcr-Abl
Pazopanib	VEGFR2/PDGFR/KIT
Pegaptanib	VEGFR
Ruxolitinib	JAX
Sorafenib	Multiple targets
Sunitinib	Multiple targets
Vandetanib	RET/VEGFR/EGFR
Vemurafenib	BRAF
Monoclonal antibodies	
Bevacizumab	VEGFR
Cetuximab	ErbB1
Panitumumab	EGFR
Ranibizumab	VEGFR
Trastuzumab	Erb2

Erlotinib and Gefitinib affect the EGFR kinase activity by acting as ATP competing molecules, binding in a reversible fashion to the ATP-binding site of the receptor. Like EGFR, Human Epidermal growth factor Receptor 2 (HER-2) is a member of the EGFR family. HER-2 is a RTK normally involved in signal transduction pathways leading to cell growth and differentiation, migration and apoptosis. It is considered an orphan receptor because none of the Epidermal Growth Factor (EGF) ligands are able to activate it. On the other hand, HER-2 is the preferential dimerization partner of all other members of the EGFR family. HER-2 amplification or overexpression in breast cancer was found to be correlated with aggressive tumour growth and poor clinical prognosis, rendering HER-2 an ideal candidate for chemotherapy [14]. Lapatinib is a selective, potent, small-molecule inhibitor of HER1 and HER-2 that is approved in combination with Capecitabine for the treatment of patients with advanced or metastatic breast tumour stages with overexpression of HER-2 [15, 16]. The binding mechanism resembles that one of Imatinib to Abl, hence, it binds to the inactive HER-2 form [17].

The Unmet Promises: Drug Resistance to TK Inhibitors

Despite the administration of these drugs that have greatly ameliorated the efficacy of the therapy, in many cases, the onset of drug resistance leads to treatment failure. Drug resistance can be achieved in different ways that are best described in ABL-related tumours. Indeed, the follow-up of CML patients receiving Imatinib showed that primary resistance (no response to Imatinib after the initial treatment) or secondary resistance (development of resistance after achieving an objective response) emerged in 31 % of the patients. In all cases, Imatinib resistance was characterized by the presence of active Bcr-Abl, rather than the activation by the cell of an alternative signaling pathway independent from Bcr-Abl [18] and, thus, indicating that the BCR-ABL signal transduction pathway is crucial to cancer cells survival. The mechanisms of acquired Imatinib resistance were due to BCR-ABL gene amplification or mutation events. In the first case, due to BCR-ABL gene amplification or overexpression, the intracellular Imatinib concentration is not high enough to inhibit all Bcr-Abl molecules in leukaemic cells [19, 20]. In the second case, BCR-ABL mutations emerged upon the selective treatment pressure. Up to now, nearly 100 ABL mutants have been described, some of these appearing with higher frequency than others: 15 single amino-acid substitutions account for more than 85 % of the reported mutations, and 66 % of reported cases occur specifically at seven sites only (G250, Y253, E255, T315, M351, F359, H396). Furthermore, different amino-acid substitutions can involve the same residue like F317C, F317L, and F317V, all showing reduced Imatinib sensitivity [21–23]. Since these mutations in the *BCR-ABL* fusion gene were observed only after Imatinib administration, it indicates that there must have been a low prevalence of mutant cells before the therapy. This idea is supported by the fact that these mutations do not provide any growth advantage in the absence of Imatinib, but are

selected specifically only upon drug exertion pressure [24]. Kinase activity is not abrogated by the mutations, although some mutants demonstrate lower enzymatic activity compared with the wild-type BCR-ABL. On the other hand, the kinase activity is enhanced by other type of mutation [25].

It has been shown that besides displaying inhibitory activity towards ABL, Imatinib is active against the RTKs KIT and PDGFRA [26]. KIT is a cytokine receptor expressed on the surface of hematopoietic stem cells and plays a role in cell survival, proliferation, and differentiation; PDGFRA is a member of the platelet-derived growth factor family implicated in mesenchymal cells proliferation. These RTKs are constitutively activated by gain-of-function mutations in most Gastrointestinal Stromal Tumours (GISTs) [27]. Experiments on human tumour cell lines, dependent on the KIT pathway, showed that Imatinib could block the activity of KIT in the GIST cells, arresting therefore proliferation and causing apoptosis. Currently, Imatinib is in phase II and III clinical trials for GIST treatment; the prognosis of GIST patients has dramatically improved after recruitment of Imatinib into the therapeutic arsenal (80 % clinical response rate). However, the mutation resistance issue arising during the Imatinib therapy significantly influences the clinical response also in the case of KIT. As for the BCR-ABL Imatinib resistance model, Imatinib efficacy is affected by the KIT mutations that directly block the drug binding or pose an energetic hindrance disfavouring the closed conformation of the kinase [28, 29].

Also in the case of Erlotinib and Gefitinib, targeting EGFR, after an initial response characterized by tumour regression and improvement in disease-related symptoms, most patients relapse. In response to Erlotinib or Gefitinib treatment, different resistance mechanisms can occur and one of the most frequent is the amplification of the *MET* proto-oncogene [30]. MET is another RTK (also known as Hepatocyte Growth Factor Receptor) and its amplification has also been observed in gastric and esophageal cancers [31, 32]. MET is involved in a pathway distinct from EGFR, and it is normally expressed by cells of epithelial origin where it promotes cell growth and motility [33]. In NSCLC cell lines whose Gefitinib resistance was obtained by continuous drug administration, a marked focal amplification within chromosome 7q31.1–7q33.3, containing the *MET* proto-oncogene, was observed. It is known that Gefitinib leads to the disruption of the signal cascade EGFR/ERBB3/PI3K/Akt [34, 35]. In Gefitinib resistant cell lines, MET amplification leads to PI3K/Akt signaling through ERBB3 activation, independently from EGFR; hence, MET inhibition restores sensitivity to Gefitinib. Resistance towards Erlotinib or Gefitinib can also occur as a consequence of mutations in the *K-RAS* gene, a GTPase of the Ras family, which is a downstream effector of EGFR, [36]. Similarly, treatment of HER-2 overexpressing cancers is not always effective because resistance to Lapatinib develops in some patients during prolonged exposure to the drug. It is known that resistance to Lapatinib in breast cancer arose through AXL overexpression. AXL is a RTK, closely related to MET, involved in cell proliferation and motility and, as the case of MET for NSCLC, its overexpression is associated with poor prognosis and increased invasiveness of several human cancers [37].

In order to avoid drug resistance, design of drugs with inhibitory properties against the mutant kinase forms is being actively pursued. It is the case of the ABL inhibitor Nilotinib, targeting Bcr-Abl with an increased potency (approximately 20-fold) when compared to Imatinib. Even if both drugs act in the same manner, hence, binding to the kinase domain of the inactive ABL form, Nilotinib binding is energetically more stable [38]. Nilotinib remains active towards all Imatinib resistant mutations lying in the activation domain (A-loop), as well as towards mutations spread across the entire Bcr-Abl kinase domain, including the P-loop. In fact, the clinical efficacy of Nilotinib was demonstrated in patients harbouring most Imatinib-resistant mutations, with the exception of Y253H, E255K/V, F359C/V and T315I BCR-ABL mutations. In particular, overcoming the Imatinib resistance mutation T315I, that confers resistance also towards Nilotinib with IC50 value 800-fold greater than against wild type Bcr-Abl, still constitutes a significant unmet medical need [39].

Overcoming Drug Resistance: The Dual Inhibitor Concept

A more innovative strategy to overcome drug resistance consists in the development of drugs able to inhibit not only the primary target kinase, but also other kinases that contribute to cancer survival. For instance, Src Family Kinases (SFKs) are downstream effectors of Bcr-Abl; Dasatinib, another second generation agent developed for the treatment of CML, besides inhibiting Bcr-Abl, has been shown to successfully inhibit also the SFKs Src, Lyn, Yes, and Lck [40]. In particular, Lyn and Hck are involved in signal transduction pathways downstream of Bcr-Abl. Thus, the activity of Dasatinib, especially towards LYN, contributes to the overall efficacy of this drug in the treatment of CML. Like Imatinib and Nilotinib, Dasatinib acts as a competitor of the ATP substrate, but differently from other drugs, it binds to ABL both in its active and inactive conformation [41]. Compared with Imatinib, Dasatinib has an approximately 300-fold increased potency in antiproliferative assays. Despite the fact that Dasatinib remains active towards the majority of Imatinib-resistance mutants, the mutations T315I/A and F317I still result in a completely resistant phenotype.

Many other drugs showing a dual activity against Abl and SFKs are already in different clinical trial phases and preliminary research investigations. For instance, Bafetinib and Bosutinib are other dual inhibitors of Abl and SFKs that have been tested in clinical trials for CML treatment. Bafetinib targets Abl and Lyn, showing limited inhibition against other SFKs [42]. Despite the fact that Bafetinib is effective in a heavily pretreated (Imatinib, Nilotinib, Dasatinib) population, it lacks any appreciable efficacy against T315I mutation and in blastic CML phases, or in Ph-positive ALL [43]. Another drug in phase III clinical trials for CML treatment, Bosutinib [44], shows similar limitations [39, 45].

The mutation T670I in KIT is selected in GIST tumours after prolonged Imatinib administration. T670 of KIT was identified as one of the key hydrogen

bonds for Imatinib binding [46], similarly to T315I in Bcr-Abl. In the same way, the EGFR T790M resistance mutation occurs after prolonged administration of Elrlotinib or Gefitinib in NSCLCs. Also, this mutation is in an analogous position to T315I in Abl and T670I in Kit [47].

In addition to small-molecule inhibitors, monoclonal antibodies (mAb) targeting RTK are also in use in clinical therapy (Table 1). Their use relies on the principle that the targeted receptors are expressed at higher levels on cancer cells than on healthy cells. Several mAbs are approved for cancer therapy or are in clinical trials. Targets of these mAbs are EGFR, HER2, VEGFR, MET, VEGFR2 and IGF1-R. The anti-cancer activity of a mAb is due to different mechanisms: the binding with the receptor can (1) prevent ligand-receptor interaction (2) promote receptor internalization (3) prevent receptor dimerization and activation and (4) induce apoptosis or immune response toward the target cells [48]. As for small-molecule inhibitors, primary and secondary resistance towards these drugs have emerged. Multiple studies showed that primary resistance can be conferred by activating mutations in KRAS, PIK3CA, BRAF or loss of PTEN expression. These mutations negatively correlate with the response to Cetuximab or Panitumumab, two EGFR-targeting mAbs [49]. Secondary resistance is due to (1) overexpression and aberrant phosphorylation of alternative RTK (2) expression of receptor variants (3) increased expression of the target receptor and (4) activation of alternative pathways [48]. To date, both point mutations in the target receptor or rearrangements in the corresponding genomic regions have been observed after mAb treatment.

Expanding the Dual-Inhibitor Concept: The Quest for Multi-Targeting TK Inhibitors

The drug development research field is still the problem of the unsatisfactory efficacy of to-date approved inhibitors towards the T315I substitution and analogue mutations in other kinases. Some encouraging data are now emerging in clinical trials with other kinase inhibitors. Sunitinib, approved as second line therapy of GIST and renal cell carcinoma (RCC), is a multi-targeted RTKs inhibitor active toward PDGFR, VEGFR, Kit, Ret, CSF-1R and Flt3 [50]. Contrary to Imatinib, it is also active towards several Imatinib-resistant KIT secondary mutations, including the T670I KIT mutant. In the same way, Sorafenib, a small-molecule inhibitor that targets the RTKs vascular endothelial growth factor receptor (VEGFR) and Platelet-derived growth factor receptor (PDGFR) and the STKs C-Raf and B-Raf [51], is also able to inhibit Kit mutation that provides resistance toward Imatinib and Dasatinib.

If the availability of inhibitors that target multiple kinases could result in a higher degree of transduction signal inhibition, and thus in higher efficacy on cancer treatment, it could also result in enhanced toxicity for healthy cells.

The identification of the hierarchical pattern of inhibition of a given compound against all the kinome is now emerging as an essential step in order to estimate the effect of a drug against a selected cancer. Recently, high-throughput screening approaches have been use by Anastassiadis et al. [52] and Davis et al. [53] with the aim to score the inhibition potency and selectivity of 72 and 128 known kinase inhibitors, respectively, against large panels of kinases (442 and 300, respectively). Traditionally, the discovery of kinase inhibitors starts from a high-throughput screening of small molecules with inhibitory properties targeting a kinase of interest. The selectivity of effective compounds is then evaluated against a panel of representative kinases. The same goal can be obtained by screening libraries of compounds against large panels of protein kinases, thus revealing the degree of selectivity of each compound. This method led to the identification of inhibitors that were unexpected for the kinase of interest, revealing multi-targeted inhibitors active on a number of kinase targets larger than predicted. For example, Sunitinib, known to target PDGFR, VEGFR, Kit, RET, CSF-1R and Flt3 [50], shows affinity for RET harboring gatekeeper mutations [RET (V804L/M)], which is resistant to the approved RET inhibitor Vandetanib. In the same way, PKC-412, a compound designed as a protein kinase C (PKC) inhibitor, was shown to be more active against the EGFR mutant T790M. These studies also revealed that compounds designed to target a specific kinase could show higher potency of inhibition against another, unrelated enzyme. For example, DMBI, designed as a Platelet-derived growth factor receptor (PDGFR) inhibitor, is an highly potent inhibitor of FLT3 and TrkC; SB202474, an inactive analog of the p38 MAP kinase inhibitor SB202190 [54], showed significant activity only against the haploid germ cell–specific nuclear protein kinase Haspin. These data open new opportunities for clinical use of drugs already tested for their pharmacological properties.

In order to improve the efficacy of target therapy and to fight/avoid the onset of resistance, a valid strategy should be the analysis of the signalling network surrounding a target kinase. The determination of a unique factor responsible for drug resistance is not possible in many tumours that show heterogeneous resistance due to partial contributions by multiple proteins. Network models paradigm conjecture that signalling pathways are made with no hierarchy and feedback loops and are redundant. This implicates that inhibiting a specific oncogene can lead to the rescue of the signaling by enrichment (gain of function, amplification, overexpression) of proteins that compose the web of interaction with the target. Astsaturov et al. [55] screened siRNA libraries targeting EGFR network in order to identify synthetic lethality with EGFR inhibitors. This approach consented to identify previously uncharacterized genes that can drive resistance modulating EGFR signaling or that can be considerate as concomitant target for the treatment of EGFR hyper activated cancers. For instance, in the case of the EGFR network, the inhibition of SFKs or Aurora A kinase enhances the effect on cancer viability of EGFR inhibitors. The application of this approach to other validated targets could greatly ameliorate the clinical strategy in other malignancies.

Why Targeting DNA Repair in Anticancer Chemotherapy?

RTKs and NRTKs are involved in transducing proliferative signals, hence, resulting in the activation of DNA replication and cell division. In turn, the high proliferative phenotype of tumour cells is very often accompanied by alterations in their genetic structure, leading to chromosomal aberrations and aneuploidy.

The genomic instability and the high mutation rate, typical features of human cancer cells, are mainly due to defects in DNA repair. In fact, very often at least one of the six major DNA repair pathways (mismatch repair MMR, base excision repair BER, nucleotide excision repair NER, homologous recombination HR, non-homologous end joining NHEJ and translesion synthesis TLS) is indeed defective in tumours. The mechanisms through which cancer cells respond to damaged DNA have important implications in the development of both the tumoral phenotype and the resistance to chemotherapy [56, 57].

Activation of the various DNA repair pathways during cell cycle progression depends on proper regulation of checkpoints, that are signalling cascades, often involving multiple kinases, leading to the activation of specific transcription factors. One of the main transcriptional effectors activated by DNA damage checkpoints is the protein p53. It is, thus, not surprising that *p53* is one of the most frequently mutated gene in human cancers due to its ability to halt cell growth and to modulate apoptosis after checkpoint activation when DNA damage and/or genotoxic stress occur [58].

Germline mutations of *p53* are associated with a disease called Li-Fraumeni Syndrome characterized by an increased risk of developing various cancers with an early age of onset [59]. Moreover, various clinical phenotypes in different types of cancer are associated with somatic *p53* mutations at specific residues [60]. The involvement of *p53* in DNA repair pathways has also a detrimental effect on chemosensitivity, helping cells to resist to DNA damage caused by therapy. An example is the well documented resistance against anthracyclines and mitomycin due to *p53* mutations in breast cancer as well as in haematological malignancies [61].

Polymorphism regarding *BER* genes such as *OGG1*, *APE1*, *MutYH* and *XRCC1* have been examined for their possible effect on cancer development [62]. As an example, a *DDR* gene strongly associated with cancer development is *MutYH*, whose missense mutations, insertions, deletions and duplications give rise to MutYH-associated polyposis, the most common colorectal cancer and polyposis syndrome. *MutYH* recognizes the mismatch 8-oxo-G:A and, through its action, restores the pair 8-oxo-G:C, that can be acted upon by OGG1, another BER glycosylase. The OGG1 polymorphism Ser326Cys is associated with the risk of lung cancer and increased risk of colorectal cancer; these findings confirm the roles of MutYH and OGG1 as essential players in the maintenance of genome stability against oxidative damage. In addition, the polymorphism Asp148Glu of APE1 seems to be associated with hypersensitivity to ionizing radiation and cancer risk and it can affect the prognosis of ovarian, gastro-oesophageal and pancreatico-biliary cancers [62–65]. Finally, polymorphisms of XRCC1, such as Arg399Gln

and Arg194Trp, are related to the risk of skin, upper aerodigestive and lung cancers; moreover, it is important to know that these genetic polymorphisms might be associated with overall survival and response to platinum-based chemotherapy in lung cancer patients [62, 66].

Another DNA repair pathway whose alterations are strongly related to tumour development is the MMR pathway. The Lynch syndrome, a tumour predisposition syndrome characterized by colorectal and endometrial cancer and other extracolonic malignancies, is indeed caused by monoallelic germline mutations in mismatch repair genes, such as *MLH1*, *MSH2*, *MSH6* and *PMS2*, while biallelic mutations lead to a more severe scenario called constitutional mismatch repair deficiency (CMMRD). Childhood onset of leukemia/lymphoma, brain tumours and other rare malignancies are all typical features of the CMMRD disease [67, 68].

Interestingly, distinct phenotypes and clinical manifestations are due to different *MMR* gene deficiencies; for example, an increased risk of colon cancer is particularly associated with *MLH1* mutations, whereas *MSH2* mutations have a higher incidence of extracolonic tumours. Also, as shown for clinical features, the chemotherapy resistance has different outcomes based on *MMR* gene loss; for example, the MSH2-deficient cells, but not MLH1-deficient, are sensitive to psoralen, a chemotherapic agent that induces DNA interstrand crosslinks [69].

These examples of DDR defective-associated cancers justify the strong interest in figuring out all the features related with the regulation of DDR to reach a deeper knowledge of cancer development and chemoresponse.

Targeting DNA Repair Enzymes in Cancer Therapy: The Concept of Synthetic Lethality

Somatic and hereditary mutations in DNA damage response (DDR) genes are thus associated with an increased cancer risk, but they can also offer new venues for cancer treatment. For example, the mutations of *BRCA1* and *BRCA2* genes, involved in double strand breaks repair via HR, are among the most studied, due to their strong correlation with breast and ovarian cancer development. Indeed, it was discovered that, due to their intrinsic deficiency in HR, these BRCA-deficient tumours are particularly sensitive to inhibitors of another DNA repair enzyme: poly(ADP-ribose) polymerase1 (PARP1).

PARP1 has the ability to bind single strand-breaks (SSBs) and facilitate their repair. Loss of PARP1 activity is supposed to cause formation of DNA-SSBs which are subsequently converted to double strand breaks (DSBs). In BRCA-positive cells, these DSBs are repaired by HR, but in BRCA1-or BRCA2- deficient cells they are presumed to accumulate leading to subsequent cells death [70, 71]. This discovery allowed the development of specific PARP1 inhibitors that, in the context of a BRCA1 or BRCA2 deficient genetic backgrounds, proved to be very effective in suppressing tumour growth in Phase I/II clinical trials. Such a synthetic

lethality approach, however, did not entirely meet the expectations and failed in Phase III clinical studies. An explanation for this behaviour could be the activation of an alternative DSB repair pathway, such as NHEJ, that allows the survival of HR-deficient cells. However, Patel et al. [71] turn this hypothesis upside down, demonstrating that disabling NHEJ diminished the genomic instability and lethality of PARP inhibition in HR-deficient cells rather than exacerbating it. In this context, an emerging role could be assumed by the X-family DNA polymerases; in fact, they are involved in many of the alternative pathways of the DSBs repair like the Microhomology-Mediated End Joining (MMEJ), Single-strand annealing (SSA), Break Induced Replication (BIR) and others. Recent results make polλ an ideal candidate for a new target therapy due to its ability to promote strand annealing and subsequent elongation between two DNA strands with limited homology (5–10 nt) [72].

New Candidates for Synthetic Lethality: Repair DNA Polymerases

The case of PARP1 inhibitors highlights a fundamental problem: namely, achieving a true synthetic lethality by suppressing a particular molecular function in the context of the high level of redundancy existing among the cellular metabolic pathways. A possibility to attain such a difficult goal might be to target enzymes common to different DNA repair pathways. The most obvious candidates are, of course, the DNA polymerases (pols). In fact, since DNA pols are essential to several repair pathways, their inhibition might potentially achieve a high level of tumour cell sensitization to chemotherapeutics.

Specialized DNA pols are required to bypass DNA damage lesions that would otherwise cause replication arrest and cell death. In recent years, a number of specialized DNA pols of the X and Y families have been identified. These are characterized by their ability to bypass different classes of lesions and to maintain a high degree of genetic fidelity by incorporating the nucleotide that would normally pair with the undamaged version of the base. Thus, under normal circumstances, specialized pols can be considered as agents that promote genomic stability. Trans-lesion synthesis (TLS), however, needs to be a highly regulated process because, when copying non-cognate lesions or undamaged DNA, the specialized pols have been shown to exhibit reduced fidelity. In addition to a role in mutagenesis, over-expression or increased activity of specialized pols could also result in enhanced TLS capability, allowing cancer cells to better cope with the high environmental stress that results from increased replication rates and higher level of oxidative damage. Moreover, increased TLS could provide cancer cells with an advantage in coping with the DNA damage resulting from the chemotherapeutic assault [73].

The Y-family members, in human cells, are DNA pol ι, DNA pol κ, DNA pol η and Rev1, each of them has a preference for catalysing DNA synthesis across certain kind of lesions. For example, the loss of DNA pol η reduces the efficiency to copy cis-syn cyclobutane dimer, one of the main lesion generated by sun exposure, and gives rise to the variant form of xeroderma pigmentosum (XP-V), a disease characterized by high susceptibility to sunlight-induced skin cancer. In addition, it has been shown that pol η has a key role in the cellular response to cisplatin and in the cellular resistance to this antitumour drug [74]. Another example is the role of pol ι in the induction of lung tumours, in fact pol ι knockout mice treated with urethane, a pulmonary adenoma inducer, did not develop cancer compared to wild type controls. Thus, it suggested that pol ι deficiency could lead to reduction of lung tumour [75]. On the other hand, Ziv et al. [76] discovered an important role of another Y-family enzyme, pol κ, in protecting cells from UV damages. Based on these findings, it is becoming clear that the mis-regulation of the Y-family members promotes genetic disorders and can be associated with a malignant phenotype [77].

Beside TLS, the BER and NHEJ DNA repair pathways seem to play a prominent role in promoting genetic instability in cancer cells. Central to these pathways are the X-family enzymes DNA pol β, λ, μ and TdT. TdT has the peculiar characteristic of elongating a single stranded DNA sequence without the need of a template strand. It normally acts during immunoglobulin and T cell receptor gene rearrangements, thus increasing the diversity of these molecules. A DNA pol with closely related amino acid sequence and functional domain organization to TdT is pol μ, whose roles in V(D)J recombination and NHEJ are well described. The over-expression of TdT and pol μ has been observed in several acute leukemia cells and in Non-Hodgkin's lymphomas, respectively, suggesting a possible role in tumourigenesis [78, 79].

Pol β is the smallest pol and is composed of a single 39 kDa polypeptide containing 335 amino acid residues. It is involved in BER pathways (short and long patch BER) and implicated in meiotic events associated with synapsis and recombination and SSB repair [80]. Mice carrying a target disruption of the pol gene show growth retardation and high perinatal lethality; histological examination of the embryos revealed defective neurogenesis, indicating that pol plays an essential role in neural development [81].

The over-expression of pol β has been found at both the mRNA and protein levels in many tumour types, in particular in uterus, ovary, prostate and stomach samples. In addition, its ectopic over-expression in cancer cells can increase mutagenesis and enhance resistance to chemotherapeutic agents, including cisplatin, while cancer cells deficient in pol are hypersensitive to oxaliplatin chemotherapy, indicating that BER impairment could affect the therapy outcome [73, 82]. Cells deficient in pol β converted into another kind of critical pro-apoptotic lesion during replication. These critical secondary DNA lesions are likely to be unrepaired DNA double strand breaks, which trigger apoptosis in a replication-dependent way by activating the mitochondrial death pathway, i.e. the decline of Bcl-2 level and activation of caspase-9 and caspase-3 [83].

Pol λ is a protein of 67–70 kDa which is expressed at highest level in the testis, ovary and fetal liver and it seems to be implicated in short patch BER repair, NHEJ, TLS over 8-oxo-G and V(D)J recombination [84, 85]. Despite the strong expression of pol λ seen in testis, POLL null mice were fertile and the only effect seemed to be a modification of heavy chain junctions during V(D)J recombination [86, 87]. Pol λ was found to be over-expressed at a significant extent in a range of different tumour types, albeit less frequently than pol β [73]. Recently, a new allelic variant of human pol λ has been described. It is the result of the amino acid change Arg 438 to Trp and it seems to have a reduced base substitution fidelity. Thus, the ectopic expression of R438W hPolλ variant in mammalian cells increases the mutation frequency, affects the DSB repair NHEJ pathway and generates chromosomal aberrations [88]. Another recent study reports that the pol λ protein level can be modulated in tumour cells, and in fact, NSCLC that express less protein amount are in a significantly more advanced stage [89]. Furthermore, it has been observed that more than 90 % of leukemic cells of acute lymphocytic leukemia and approximately 30 % of leukemic cells in chronic myelogenous leukemia crisis exhibit elevated TdT activity, which is associated with poor prognosis and response to chemotherapy and reduced survival time. Since leukemic cells also often over-express pol λ, which has been shown to possess a strong bona fide TdT-like activity, it is possible that both pol λ and TdT have an important role in tumourigenesis and progression of the acute leukemia [90]. In a recent study, pol λ seems to be involved in the incorporation of therapeutic nucleoside analogs into DNA during BER and NHEJ, thus it may have an impact on the cellular sensitivity to these compounds following DNA damage [91].

Recently, another DNA pol, member of the A-family, called DNA polymerase θ might be implicated in cancer development. It seems to be involved in tolerance of bulky adducts or in some DNA repair pathways such as BER, DNA interstrand crosslink and DNA break repair. Its overexpression is found in breast, colon and lung cancers and it is usually related to poor prognosis [92, 93]. In addition, a recent study demonstrated that DNA pol θ knockdown on a panel of tumour cell lines from different primary sites resulted in radiosensitization, whereas having little or no effects on normal tissue cell lines [94].

Development of Selective Inhibitors of Specialized DNA Polymerases

Currently, several classes of DNA pol inhibitors have been developed. Most of them are non-nucleosidic compounds of natural origin (polypeptides, fatty acid, triterpenoids, sulfolipids, polar lipids, secondary bile acids, phenalenone-derivates, anacardic acids, harbinatic acid, flavonoid derivates and pamoic acid), but only a few are enough specific and active to be potentially considered as drug candidates. Nucleoside analogs (NAs), on the other hand, mimicking the structure of the natural

nucleotides, can interact with the catalytic site of the pols and inhibit the DNA synthesis and/or repair by competing with the natural substrates. In addition, the lack of the 3′ hydroxyl group, typical feature of most NAs, prevents the elongation step, determining an abortive DNA replication and single strand breaks formation. Currently, eight NAs have been already approved by FDA for cancer treatment: mercaptopurine, thioguanine, fludarabine and cladribine (purines), cytarabine and gemcitabine (pyrimidines), fluorouracil and capecitabine (fluoropyrimidines); whereas clofarabine (CAFdA), nelarabine, immucillin H (BCX-1777, forodesine) and 8-chloroadenosine (8-Cl-Ado), all novel purine analogs are in advanced clinical phase. Unfortunately, NAs suffer from a number of drawbacks. First, they need to be subjected to three independent cellular phosphorylation steps to be converted to their active forms. Moreover, they can be easily degraded by other enzymes such as nucleosidases and phosphorylases and, finally, their inhibitory effects can affect also replicative polymerases, thus becoming toxic to normal cells [95, 96]. In this scenario, a novel diketo hexenoic acid (DKHA) analog was described as a selective non-nucleoside inhibitor of the template-independent activity of pol λ and TdT. Locatelli et al. [90] proved that this compound can selectively suppress cell proliferation of TdT+, but not Tdt^{-}, leukemic cells, holding the potential to be further developed as a novel antitumour agent.

Tying the Ends Together: Targeting Proliferation and Repair in Cancer Cells

The inhibition of different targets in order to obtain synthetic lethality within cancer cells could greatly ameliorate the efficacy of target therapy. In this way, it is possible to hypothesize the concomitant inhibition of targets that belong to different cellular process. A fascinating idea regards the targeting of factors that link signal transduction and DNA repair. In fact, oncogenic kinases activity is linked to DNA repair [97]. For example, it is known that tumours dependent on fusion kinases, like Bcr-Abl, present an elevated number of DNA double-strand breaks (DSBs). These are caused by high ROS levels generated by the altered cell metabolism and also by chemotherapy [98]. The DSBs repair processes require the activity of Werner Helicase/Exonuclease (WRN) which plays a critical role in optimizing DSB repair mechanisms due to its DNA end-processing activities. Slupianek et al. [99] showed that Bcr-Abl is able to enhance WRN expression via c-Myc–induced transactivation and Bcl-xL–dependent inhibition of caspase-mediated cleavage. Moreover, the Bcr-Abl kinase forms a complex with WRN protein which results in constitutive phosphorylation and activation of WRN itself. As result, WRN promotes the survival of Bcr-Abl positive leukemia cells under oxidative and genotoxic stress. Furthermore, in these cells, WRN promote alternative DSBs repair mechanisms such as Homologous Recombination (HR) and Single Strand Annealing (SSA). Additionally, in Bcr-Abl positive leukemia cells,

WRN caused aberrant Non Homologous End Joining (NHEJ) repair products [100]. Altogether, these effects can promote survival of cancer cells, inducing at the same time the accumulation of genetic aberrations in CML, a mechanism by which cancer cells could acquire resistance mutation to the therapy.

Other studies underline a strict interplay between signal transduction proteins and DNA repair factors. For example, inhibition of Chk1, a STK that is activated in response to DNA damage, was found to be synthetically lethal with Src or ERK inhibitors in myeloma and leukemia cells, respectively [101–103]. Also, EGFR was found to interact with BRCA1 in highly aggressive breast cancers. Concomitant inhibition of EGFR with Lapatinib and PARP1 with ABT-888 led to transient DSBs repair deficiency which resulted in the activation of the intrinsic pathway of apoptosis [104].

Casein Kinase II (CK2) is an ubiquitously expressed STK, whose activity is implicated in cell growth and proliferation. Its overexpression is linked to cancerogenesis and attenuated apoptosis induced by chemotherapeutic drugs [105], posing CK2 as an attractive target for cancer treatment. In particular, CK2 inhibits the tumour suppressor activity of promyelocytic leukemia protein (PML) and phosphatase and tensin homology protein (PTEN) [106]. CK2 phosphorylates

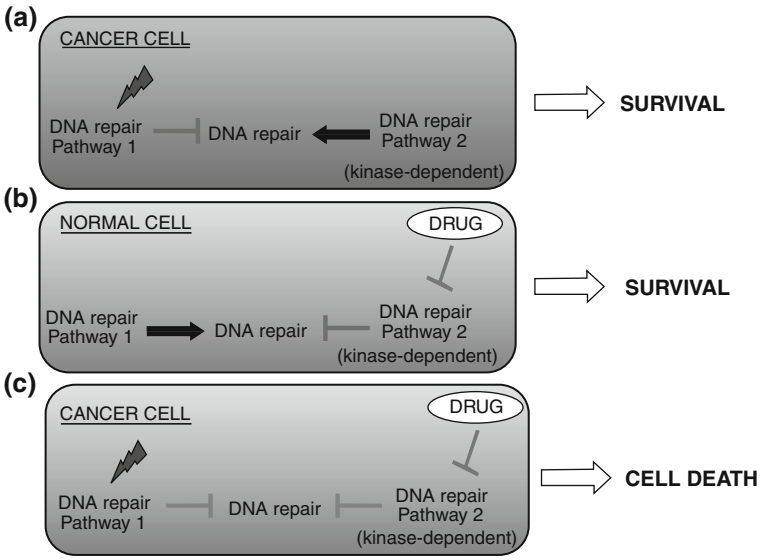

Fig. 1 *The synthetic lethality approach through targeting DNA repair and signal transduction*. **a** In a cancer cell, a defect in a DNA repair pathway can be overcome through the induction of an alternative pathway, often under the control of signal-transducing kinases. **b** In a normal cell, where all the DNA repair pathways are effective, inhibition of one of them, either by targeting the apical kinase or directly the repair DNA polymerase, will not affect its survival. **c** In a cancer cell, already defective for a given DNA repair mechanism, inhibition of the existing alternative pathways, either by targeting the apical kinase or directly the repair DNA polymerase, will result in apoptosis

XRCC1, a scaffold protein that plays a critical role in DNA base excision repair (BER) by interacting with Ligase III (LigIII). Phosphorylation of XRCC1 in human cell extracts is required for XRCC1-Lig III complex stability and phosphorylation reduction leads to DSBs accumulation [107]. Thus, inhibition of CK2 would lead not only to apoptosis in response to chemotherapeutic drugs, but also in accumulation of DSBs that will enhance the apoptotic effects of the therapy.

Conclusions

The above-discussed examples highlight the intimate connections between the proliferative signal transduction pathways and the DNA repair. In such a context, inhibition of specialized DNA pols, that act in different repair mechanisms, will reduce the ability of cancer cells to cope with the genotoxic stress imposed by proliferation and/or cancer treatment. Thus, simultaneously targeting signal transduction and DNA repair pathways will drive very efficiently cancer cells to apoptosis (Fig. 1). The investigation of the interplay between signal transduction pathways and DNA repair pathways in cancer cells can lead to the identification of novel therapeutical targets and to the design of cancer treatment strategies that avoid the onset of resistance to the therapy.

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Conflicts of Interest No potential conflicts of interest were disclosed.

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Collateral Sensitivity in Drug-Resistant Tumor Cells

Mohamed Saeed, Henry Johannes Greten and Thomas Efferth

Abstract Collateral sensitivity is a term for the hypersensitivity of otherwise drug-resistant cells. The selective killing of tumor cells by drugs exerting collateral sensitivity might be used as a novel treatment strategy. In this chapter, we give an overview on drug resistance phenotypes with known collateral sensitivities; furthermore, their molecular and cellular mechanisms were discussed to explain mediation of these hypersensitivities.

Keywords ABC transporter · Cancer · Chemotherapy · Collateral sensitivity · Cross resistance · Multidrug resistance · P-glycoprotein

Abbreviations

ABC transporter	ATP-binding cassette transporter
P-gp	P-glycoprotein

Cross-Resistance and P-glycoprotein

Chemotherapy still belongs to the main options to treat cancer. A major problem with drug treatment is that tumors frequently develop resistance. Concentrations high enough to kill tumor cell subpopulations frequently cannot be applied because of severe side effects of most classical anti-cancer drugs, e.g. myelosuppression, gastrointestinal mucositis, alopecia, sterility, etc. Clinically two major types of resistance are observed: (1) primary or inherent drug resistance, whereby tumors

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do not respond to chemotherapy from the beginning of therapy and (2) secondary or acquired resistance, whereby tumors initially respond to drugs but, during the course of chemotherapy, drug-resistant subpopulations overgrow the entire tumor and ultimately lead to refractoriness and treatment failure.

During the past three decades, the concept of multidrug resistance has been developed. The basic idea of combination treatment is to kill tumor subpopulations resistant to one drug by another drug in the combination. While this concept undoubtedly is a mainstay of cancer therapy leading to improving survival times of patients during the past decades by constantly optimizing combination regimens, nevertheless, cancers can develop resistance to many drugs at the same time. While cross-resistance of tumors to many different anticancer drugs was a well-known phenomenon already in the early days of cancer chemotherapy [1], a specific profile of cross-resistance was described as multidrug resistance. This profile comprises cross-resistance between anthracyclines, *Vinca* alkaloids, taxanes, epipodophyllotoxins, but not antimetabolites, alkylating agents or platinum compounds. The reason for this classical type of multidrug resistance is a membrane-located efflux transporter which extrudes drugs out of tumor cells. This drug transporter has been termed P-glycoprotein and is encoded by the *MDR1* (*ABCB1*) gene and belongs to the ATP-binding cassette (ABC) transporter family.

A typical, but unusual feature of the P-glycoprotein is that it translocates a wide variety of chemically diverse compounds. While many speculations on the mechanism of action of P-glycoprotein have been made, the only common property of P-glycoprotein substrates is their relative hydrophobic, amphiphilic nature [2, 3]. While initially one binding domain and subsequently two binding sites have been proposed for the P-glycoprotein, more recent investigations suggested multiple different binding sites [4, 5]. An alternative model hypothesized that the P-glycoprotein extrudes diverse drugs by an induced-fit mechanism [6].

Recently, homology models for the P-glycoprotein based on the crystal structure of the bacterial ABC transporter from *Staphylococcus aureus* Sav1866 have been described [7]. Three main membrane-related binding regions were outlined in the P-glycoprotein. The binding region 1 is located at the interface between the membrane and the cytosol and two other binding regions are located in the transmembrane parts of the protein. The regions contain multiple binding pockets. Hence, it is possible that drugs, depending on their structural properties, may bind to either more hydrophobic or more hydrophilic pockets, or even to more than one pocket simultaneously. Additionally, a large binding pocket resides in the protein cavity, which may represent an "escaping" site, where the compounds that bind to any of these regions are released from the protein. Site-directed mutagenesis experiments fit to these putative binding sites of the homology models [8], but a final proof can only be delivered by drug-protein crystal structures. Considering these complex and unresolved situations, the substrate specificity of P-glycoprotein may be much broader than estimated thus far. The binding of established compounds to the P-glycoprotein as a first step in the drug efflux process is illustrated in Figs. 1, 2, 3, 4. By means of a molecular docking approach, we have shown that compounds of diverse drug classes

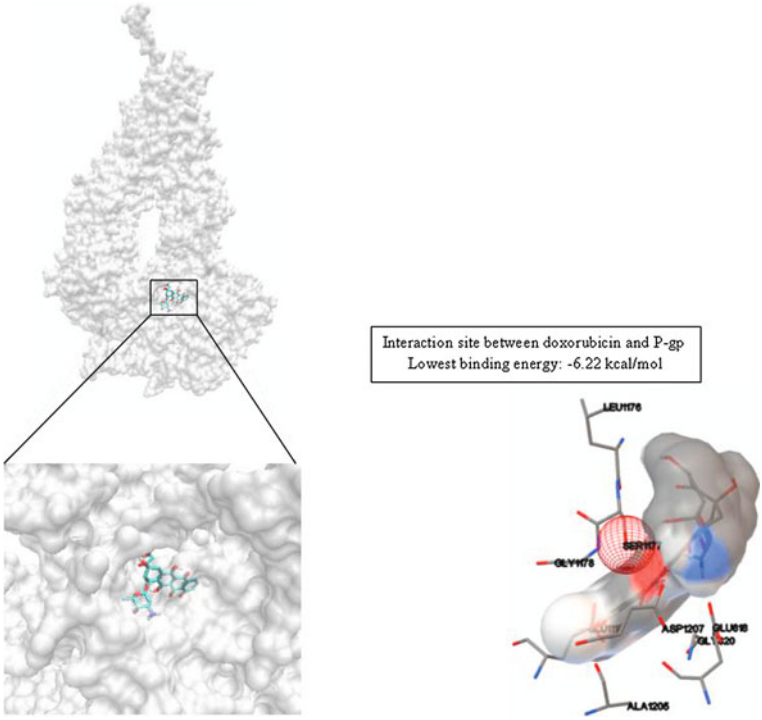

Fig. 1 Graphical representation of the binding of doxorubicin to the human P-glycoprotein (P-gp). Human P-gp was homology-modeled using MODELLER software [111] with the mouse P-gp crystal structure (PDB-ID 3G5U at www.pdb.org) as template. The docking was performed using AutoDock version 4.2 (http://autodock.scripps.edu/). The best docked position of doxorubicin was illustrated in different styles. *Top* The P-gp surface was calculated and drawn as a *black shadow* (ghost mode) for better contrast with the drug. *Bottom left* For a clearer view of the cavity where the drug was docked, the *box* shows a close-up view of the drug in the cavity. *Bottom right*: Amino acids in close proximity to the docked drug molecule, which constitute the binding cavity

and mode of action bind to different binding sites on the human P-glycoprotein (doxorubicin, Fig. 1; vinblastine, Fig. 2; paclitaxel, Fig. 3; etoposide, Fig. 4).

The human genome contains 49 ABC transporter genes encoding for proteins with diverse functions [9, 10]. After characterization of the classical multidrug resistance phenotypes and the P-glycoprotein, atypical forms have been described with other cross-resistance profiles [11, 12], Atypical multidrug resistance can be caused by other ABC transporters, e.g. multidrug-resistance related proteins (MRPs, ABCCs) or breast cancer resistance protein (BCRP, ABCG2) or proteins not related to the ABC transporters such as DNA topoisomerases, enzymes related to the glutathione redox cycle or DNA repair enzymes [13, 14].

After the description of the calcium channel blocker verapamil as an inhibitor of the P-glycoprotein a plethora of synthetic drugs from diverse pharmacological classes [15] and natural products [16] have been identified. The concept was to

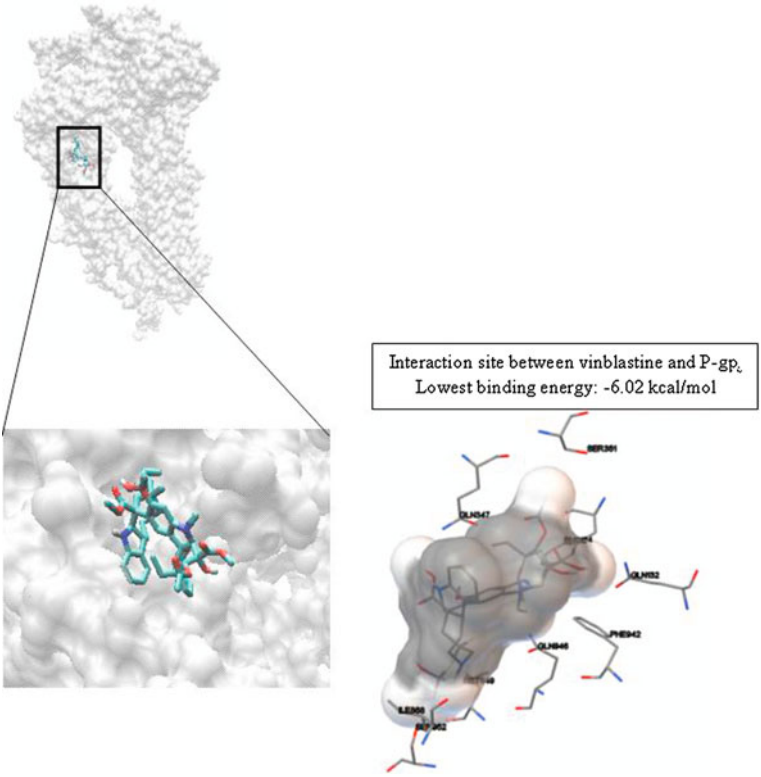

Fig. 2 Graphical representation of the binding of vinblastine to the human P-glycoprotein. For details see Fig. 1 legend

inhibit the efflux function of the P-glycoprotein and, thereby, to increase anti-cancer drug concentrations by the combination regimen of cytostatic drugs and P-glycoprotein inhibitors.

Unfortunately, clinical trials with P-glycoprotein inhibitors were not successful to reverse multidrug resistance [17], although the relevance of the P-glycoprotein for treatment failure and worse survival time prognosis of cancer patients has been unambiguously shown [18–20]. Among the reasons for the unfavorable outcome of these clinical trials were: (1) P-glycoprotein inhibitors have been initially developed for the treatment of other diseases than cancer. For instance, verapamil blocks calcium channels in the heart and is an established drug to treat heart rhythm disturbances. These main drug activities may appear as non-tolerable side effects in cancer therapy, (2) The P-glycoprotein is not only expressed in tumors, but also in normal tissues to detoxify potentially harmful xenobiotic compounds taken up with food. High P-glycoprotein expression can be found in the gastro-intestinal tract, liver, kidney, blood brain barrier, blood placenta barrier etc. Systemic treatment with P-glycoprotein inhibitors may, therefore, not only affect

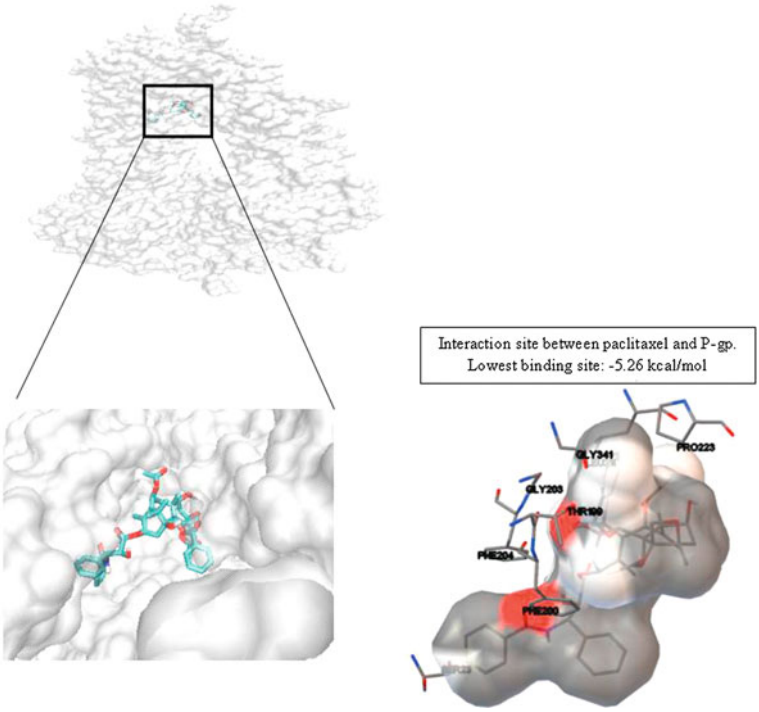

Fig. 3 Graphical representation of the binding of paclitaxel to the human P-glycoprotein. For details see Fig. 1 legend

P-glycoprotein's functions in multidrug-resistant tumors, but also in healthy tissues and (3) some P-glycoprotein inhibitors are strong inducers of cytochrome P450 monooxygenase (CYP) enzymes in the liver. Up-regulated CYP enzymes metabolize anticancer drugs more rapidly and efficiently leading rather to treatment failure than to reversal of multidrug resistance.

The fact that it was not possible as of yet to establish novel treatment protocols to combat multidrug resistance by the addition of P-glycoprotein inhibitors to standard treatment -protocols resulted with much disappointment in the scientific community.

Hypersensitivity in Resistant Cells

In the hype to search for P-glycoprotein inhibitors, it was sometimes overseen that multidrug resistance comprises not all classes of anticancer drugs and that P-glycoprotein-expressing tumor cells retain sensitivity e.g. to antimetabolites, alkylating agents or platinum compounds. This finding opened the opportunity to bypass multidrug resistance by treatment with non-cross-resistant drugs.

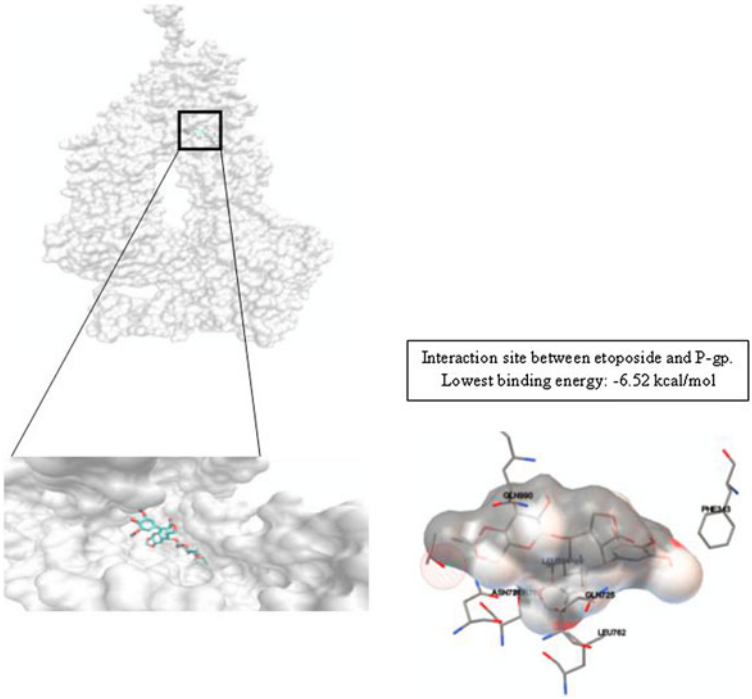

Fig. 4 Graphical representation of the binding of etoposide to the human P-glycoprotein. For details see Fig. 1 legend

Even more, some of these non-cross-resistant drugs are hypersensitive in multidrug-resistant cells, i.e. they kill multidrug-resistant cells at lower concentrations than their drug-sensitive counterparts. This phenomenon has been termed collateral sensitivity. Hypersensitivity or collateral sensitivity is not a unique feature of multidrug-resistant tumor cells as it has first been described in drug-resistant *Escherichia coli* [21]. Collateral sensitivity was also observed in an otherwise pleiotropic cross-resistant mutant of *Saccharomyces cerevisiae* [22]. These data indicate that collateral sensitivity is a general biological and pharmacological phenomenon, by which organisms react to toxic insults. Collateral sensitive drugs have also been recognized in tumor cell lines, long before the P-glycoprotein was discovered [1]. Bech-Hansen et al. [23] were the first to describe collateral sensitivity in colchicine-selected P-glycoprotein expressing, multidrug-resistant Chinese hamster ovary cells.

The selective killing of P-glycoprotein-expressing tumor cells by taking advantage of collateral sensitivity has been compared to the concept of synthetic lethality [24, 25]. Synthetic lethality is a novel approach that specifically targets cancer cells with specific gene mutations that are not found in normal cells. If two parallel pathways both contribute to an essential cellular process and one pathway is inhibited by a specific mutation, the second pathway may be inhibited by small

molecules leading to the death of cancer cells. Normal cells will be spared from the cytotoxic effects of the small molecule, since they escape using the intact first pathway without gene mutation [26, 27]. One example for the concept of synthetic lethality is the inhibition of PARP-1 to specifically kill cancer with mutations in the *BRCA1* or *BRCA2* genes. The idea in the context of multidrug resistance is to find a drug that specifically kills P-glycoprotein-over-expressing multidrug-resistant tumor cells, but not other cells.

Although drugs exerting collateral sensitivity in multidrug-resistant cells do not differentiate between normal and cancerous tissue (because the P-glycoprotein is also expressed in normal organs), it is an intriguing idea to take advantage of collateral sensitivity as "Achilles' heel" to specifically target otherwise refractory tumors.

In the past three to four decades, a plethora of compounds have been identified to exert collateral sensitivity in P-glycoprotein expressing otherwise drug-resistant tumors, including local anesthetics, detergents, antimetabolites, alkylating agents, platinum compounds as well as natural products such as saponins or flavonoids (Table 1). Collateral sensitivity is a phenomenon which is not restricted to the expression of P-glycoprotein, although some studies provided strong evidence by analyzing revertant cell lines or applying siRNA targeting the *MDR1* gene that collateral sensitivity was linked to the presence of the P-glycoprotein (Table 1). It can be speculated that different forms of collateral sensitivity may exist depending on the drug used to select for resistance and the molecular mechanisms of drug resistance in resistant cell lines.

Mechanisms of Collateral Sensitivity

Increased ATP Hydrolysis and ROS Generation by Futile Cycling

Intriguingly, many P-glycoprotein inhibitors are also substrates of this efflux pump. This means that these substances are exported out of the cell under ATP consumption, diffuse back into the cell, and are exported again and so on. These futile cycles cause increased ATP hydrolysis [28, 29]. The process of multidrug-resistant cells to replenish ATP generates oxidative stress leading both to increased levels of reactive oxygen species (ROS) provided from oxidative phosphorylation and to oxidation of glutathione as an antioxidant stress response. Non-detoxified ROS may lead to induction of apoptosis causing collateral sensitivity of multidrug-resistant cells. These effects have been shown for verapamil as a model compound for P-glycoprotein inhibition [30–32]. Whether this mechanism is also valid for other classes of P-glycoprotein inhibitors should be investigated in the future. Interestingly, verapamil causes collateral sensitivity not only in P-glycoprotein-expressing cells, but also in MRP1-expressing ones, although verapamil is a much weaker substrate for MRP1 than for the P-glycoprotein [33–35]. It has been

Table 1 Collateral sensitivity in drug-resistant tumors

Cell line	Tumor type	Collateral sensitivity	Resistance	P-gp expression	Reference
CH^RC5	Chinese hamster ovary	Local anaesthetics (procaine, xylocaine, propanolal), steroid hormones (1-dehydrotestosterone, corticosterone, 5-beta-pregnan-3,20-dione), triton-x	Colchicine	Yes	Bech-Hansen et al. [23], Ling et al. [53]
CH^RC5 CH^RB30 RPMI 8226	Human breast cancer	Dexamethasone	Doxorubicin	Yes	Dalton et al. [54]
H69AR	Human small cell lung cancer	1-dehydro-testosterone, lidocaine	Doxorubicin	No	Mirski and Gerlach [55]
S180/ADR	Mouse sarcoma	Methotrexate, 5-fluorouracil	Doxorubicin	Yes	Volm et al. [56]
CHO-K1	Chinese hamster ovary	Prednisolone	Actinomycin D	N.A.	Diddens et al. [57]
HN-1/DAR-II	Squamous cell carcinoma of the tongue	Cisplatin	Etoposide	N.A.	Lock and Hill [58]
HT1080/DR4	Human fibrosarcoma	Nicardipin	Doxorubicin	No	Cole et al. [59]
HL60/MX2	Human promyelocytic leukemia	Melphalan, mitomycin	Mitoxantrone	No	Harker et al. [60]
G3361/CP G3361/4HC G3361/PAM	Human melanoma	Carmustine carmustine carmustine	Cisplatin, hydroxyper-oxycyclophosphamide, melphalan	N.A.	Wang et al. [61]

(continued)

Table 1 (continued)

Cell line	Tumor type	Collateral sensitivity	Resistance	P-gp expression	Reference
RPMI-8402 CPT-K	Human leukemia	Daunorubicin, doxorubicin, mitoxanthrone, m-AMSA, etoposide, THP-adriamycin, vincritine, vindesine, cyclophosphamide, cisplatin	CPT-11	N.A.	Oguro et al. [62]
RPMI-8402 CPT-T	Human leukemia	Vindesine, cyclophosphamide	CPT-11	N.A.	Oguro et al. [62]
H69AR	Human small cell lung cancer	Buthionine sulfoximine	Doxorubicin	No	Cole et al. [63]
DU 145/EMR	Human prostate carcinoma	Cytochalasin B	Estramustine	No	Speicher et al. [64]
9L/ACNU	Rat glioma	L-asparaginase	ACNU	No	Saito et al. [65]
SH-SY5Y/VCR	Human neurobastic	Progesterone megestrol acetate	Vincristine	Yes	Fleming et al. [66]
NCI-H69/DAU4	Human small cell lung cancer	Cytarabine, cisplatin, carmustine	Daunorubicin	Yes	Jensen et al. [67]
GLC4/ADR	Human small cell lung cancer	Doxycycline oligomycin	Doxorubicin	No	de Jong et al. [68]
GM3639/L100	T cell acute lymphoblastic leukemia	Cyclosporine A	Vincristine	Yes	Larssson et al. [69]
CHO VRT 5/15/25	Chinese hamster ovary	Verapamil, nicardipine	Vincristine	Yes	Stow and Warr [70]
RPMI 8226/DOX	Human myeloma	Streptozotocin carmustine (BCNu)	Doxorubicin	Yes	Futscher et al. [42]

(continued)

Table 1 (continued)

Cell line	Tumor type	Collateral sensitivity	Resistance	P-gp expression	Reference
CHO/ACTD	Chinese hamster ovary	Dexniguldipine	Actinomycin D	Yes	Neumann et al. [71]
LI210/VCR	Murine leukemia	11-deoxycorticosterone, dexamethasone	Vincristine	Yes	Barancik et al. [72]
H69/DAU4	Human small cell lung cancer	5-fluorouracil, cytarabine, carmustine, bleomycin, camptothecin, cisplatin, hydroxyurea	Danuorubicin teniposide vincristine	Yes No No	Jensen et al. [73]
NYH/VM	Human small cell lung cancer	5-fluorouracil, cytarabine, carmustine, bleomycin, hydroxyurea, methotrexate, mitomycin C	Danuorubicin teniposide vincristine	Yes No No	Jensen et al. [73]
H69/VP	Human small cell lung cancer	5-fluorouracil, mrmustine, cytarabine, carmustine, bleomycin, camptothecin, cisplatin, hydroxyurea, iododoxorubicin	Danuorubicin teniposide vincristine	Yes No No	Jensen et al. [73]
AA8-Mtp^{R10}	Chinese hamster ovary	Etoposide, vincristine	Metoprine	No	Assaraf and Slotky [74]
AA8-Pyr^{R10}	Chinese hamster ovary	Actinomycin D, colchicine, doxorubicin, etoposide, gramicidin D, vinblastine, vincristine	Pyrimethamine	No	Assaraf and Slotky [74]
AA8-Tmp^{R500}	Chinese hamster ovary	Actinomycin D, colchicine, doxorubicin, etoposide, vinblastine	Trimethoprim	No	Assaraf and Slotky[74]

(continued)

Table 1 (continued)

Cell line	Tumor type	Collateral sensitivity	Resistance	P-gp expression	Reference
CH^RA3 CH^R110 CH^RC5 CH^RB30	Chinese hamster ovary	Polyoxyethaylene amphiphiles (triton X-100 nonidet P-40). dibutylphthalate	Colchicine	Yes	Loe and Sharom [75]
LYC5	Human colon carcinoma	Rotenone, anti-mycin, oligomycin, vincristine, actinomycinD, rhalamine-123, melphelan, doxorubicin	N-(5-indanylsulfonyl)-N'-(4-chlorophenyl) urea (ISCU)	Decreased P-gp	Sosinski et al. [43]
KB8-5	Human epidermoid carcinoma	Rotenone, antimycin, oligomycin, diarylsulfonylurea derivatives	Colchicine	Yes	Sosinski et al. [43]
TE-671 MR	Human rhabdomyo-sarcoma xenogrraft	Etoposide	Melphalane	N.A.	Friedman et al. [44]
P388/MITOX	Murine leukemia	Cisplatin, bleomycin	Mitoxantrone	Yes	Fichtner et al. [76]
P388/ADR	Murine leukemia	Cyclophosphamide	Doxorubicin	Yes	Fichtner et al. [76]
P388/MTX	Murine leukemia	Cyclophosphamide, daunorubicin, etoposide, cisplatin	Methotrexate	Yes	Fichtner et al. [76]
P388/VCR	Murine leukemia	Cyclophosphamide	Vincristine	Yes	Fichtner et al. [76]
RPMI 8226/DOX 40	Human myeloma	Lovastatin	Doxorubicin	Yes	Holmberg et al. [77]
RPMI-7932 VCR4.5/4, VBL-4, BMCOL-1	Human melanoma	N1, NU-diethylnorspermine	Vincristine, vinblastine, colchicine	Yes	Porter et al. [78]

(continued)

Table 1 (continued)

Cell line	Tumor type	Collateral sensitivity	Resistance	P-gp expression	Reference
L100	Human T cell acute lymphoblastic leukemia	Gamma-irradiation, cisplatin	Vincristine	Yes	Cho et al. [79]
P388	Murine leukemia	Cisplatin, alkylating agents	Colchicine	Yes	Demidova et al. [80]
CH B30 MCF 7/ AdR	Chinese hamster ovary human breast cancer	Opiates (pentazocine, naloxone, pethidine, morphine) opiates (pentazocine, naloxone, pethidine, morphine)	Colchicine doxorubicin	Yes Yes	Callaghan et al. [40] Callaghan et al. [40]
2007/C13*	Human ovarian carcinoma	Doxorubicin, colcemide, vincristine, vinblastine, paclitaxel	Cisplatin	No	Parekh and Simpkins [81]
KB-8-5 KB-8-5-11 KB-C1	Human epidermoid carcinoma	2-deoxy-D-glucose	Colchicine	Yes	Bentley et al. [82]
M5/CDDP M5/ CDDPc	Murine ovarian reticulosarcoma	Etoposide doxorubicin	Cisplatin	N.A.	Belvedere et al. [83]
SUSA/VPC2	Human testicular teratoma	ICRF-159	Etoposide	N.A.	Davies et al. [45]
H69/BCNU	Human small cell lung cancer	Vindesine, vincristine, taxol, taxotere, doxorubicin, mitoxantrone m-AMSA, etoposide, teniposide, mitomycin cytarabine, gemicitabine	Carmustine	N.A.	Jensen et al. [77]
NYH/CIS	Human small cell lung cancer	Teniposide, cytarabine	Cisplatin	N.A.	Jensen et al. [84]

(continued)

Table 1 (continued)

Cell line	Tumor type	Collateral sensitivity	Resistance	P-gp expression	Reference
NYH/CAM	Human small cell lung cancer	Doxorubicin, etoposide, teniposide, hydroxyurea	Camptothecin	N.A.	Jensen et al. [84]
NYH/TPT	Human small cell lung cancer	Teniposide	Topotecan	N.A.	Jensen et al. [84]
H69/DAU	Human small cell lung cancer	Cisplatin, cytarabine, gemicitabine	Daunorubicin	Yes	Jensen et al. [84]
NYH/VM	Human small cell lung cancer	Cytarabine	Teniposide	N.A.	Jensen et al. [84]
H69/VP	Human small cell lung cancer	Mitomycin, cisplatin, cytarabine, hydroxyurea	Etoposide	Yes	Jensen et al. [84]
MCF-7/OAP	Human breast cancer	E09	Oxazaphosphorine	N.A.	Rekha and Sladek [85]
CHO/OAR2-3	Chinese hamster ovary	Cisplatin, nitrogen mustard	Okadaic acid	Yes	Tohda et al. [86]
SBC-3/OADM 100	Human small cell lung cancer	Methotrexate, TNP-351 (antifolate)	Doxorubicin	Yes	Matsuo et al. [46]
SW-1573/2R160 2R120	Human lung carcinoma	No 5-fluorouracil, AG337	Doxorubicin doxorubicin	Yes No	Van Triest et al. [87]
C6/VP	Rat glioma in vivo	Lomustine, cisplatin, cytarabine	Etoposide	No	Taki et al. [88]
IGROV-1/Pt0.5 IGROV-1/Pt-1	Human ovarian carcinoma	Taxol	Cisplatin	N.A.	Perego et al. [89]
A2780/m248 A2780/m273	Human ovarian carcinoma	Tumor necrosis factor	Transfected mutated p53	N.A.	Sleijfer et al. [90]
CEM/ara-C	T cell acute lymphoblastic leukemia	Idarubicin 6-thioguanine, vincristine, 6-mercaptoguanosine	AraC	Yes	Martin-Aragon et al. [91]

(continued)

Table 1 (continued)

Cell line	Tumor type	Collateral sensitivity	Resistance	P-gp expression	Reference
PC-6/Tax 1-1	Human oat cell carcinoma	Cisplatin, 5-FU, DX-8951f camptothecin	Taxol	Yes	Ishii et al. [92]
PC-6/ADM 2-1	Human oat cell carcinoma	Cisplatin, camptothecin	Doxorubicin	Yes	Ishii et al. [92]
PC-6/VCR29-9	Human oat cell carcinoma	Cisplatin, 5-fluorouracil, DX-8951f, SN-38, camptothecin	Vincristine	Yes	Ishii et al. [92]
PC-6/VP1-1	Human oat cell carcinoma		Etoposide	Yes	Ishii et al. [92]
PC-14 KDDP PC-9/CDDP	Human non-small cell lung cancer	Aragusterol	Cisplatin	N.A.	Fuhuoka et al. [93]
H69/DAU NYH/VM	Human small cell lung cancer	Gemicitabine, cytarabine gemicitabine, cytarabine	Daunorubicin teniposide	Yes No	Bergman et al. [48, 49]
EBG-2/R	Human lung squamous cell carcinoma	Vindesine	Cisplatin/ifosfamide/vindesine	No	Kawai et al. [94]
SR-2	Human small cell lung cancer	Azidothymidine	Cisplatin	N.A.	Savaraj et al. [95]
PyrR100	Chinese hamster ovary	Cholate, etoposide, doxorubicin, vincristine	Pyrimethamine	N.A.	Stark et al. [96]
CCRF-CEM	Human T cell acute lymphoblastic leukemia	Cyclosporine A chloroquine dexamethasone	Antirheumatic drug (sulfa-salazine)	N.A.	van der Heijden et al. [97]
P388/F11782	Murine leukemia in vivo	Cisplatin, topotecan, colchicine, *Vinca* alkaloids	F11782 (topoisomerase inhibitor)	N.A.	Kruczyuski et al. [98]

(continued)

Table 1 (continued)

Cell line	Tumor type	Collateral sensitivity	Resistance	P-gp expression	Reference
K562/D1-9	Human chronic myeloic leukemia	Arsenic trioxide	Daunorubicin	Yes	Seo et al. [99]
	Human renal cell carcinoma	2-chlorodeoxyadenosine, fludarabine	2-deoxy-tubercidin	N.A.	Pan and Nelson[100]
H69/AR	Small cell lung cancer	Verapamil, buthionine, sulfoximine, apigenin	Doxorubicin	No	Laberge et al. [35]
CH^RC5	Chinese hamster ovary	Verapamil	Colchicine	Yes	Laberge et al. [28]
CEM/ADR 9000	Human T cell acute lymphoblastic leukemia	Artesunic acid homodimer	Doxorubicin	Yes	Horwedel et al. [101]
KB-VIN	Human epidermoid carcinoma	6,6,8-triethyl-desmosku-montin B	Vincristine	Yes	Nakagawa-Goto et al. [102]
KB-V1	Human epidermoid carcinoma	Tiopronin	Vinblastine	Yes	Goldsborough et al. [52]
L1210R	Mouse leukemia	Cold stress	Daunorubicin	Yes	Cerezo et al. [103]
MCF-7TaxR	Human breast cancer	Platinum	Paclitaxel	N.A.	Ajabnoor et al. [104]
KB-V1, KB-C1, KB-A1	Human epidermoid carcinoma	2-deoxy-D-glucose	Vinblastine, colchicine, doxorubicin	Yes	Bell et al. [36]
Diverse	Diverse	NSC73306 and derivatives	Vinblastine, colchicine, docorubicin	Yes	Hall et al. [24, 105]
MCF-7/ADR	Breast cancer	2-deoxyglucose	Doxorubicin	N.A.	Kaplan et al. [106]

(continued)

Table 1 (continued)

Cell line	Tumor type	Collateral sensitivity	Resistance	P-gp expression	Reference
KB-VIN	Human epidermoid carcinoma	6,8,8-triethyl analogues of desmosdumotin B	Vinblastine	Yes	Nakagawa-Goto et al. [107], Nakagawa-Goto et al. [102]
KB-V1	Human epidermoid carcinoma	20 NSC compounds	Vinblastine	Yes	Türk et al. [108]
MES-Sa Dx 5	Uterine sarcoma	20 NSC compounds	Doxorubicin	Yes	Türk et al. [108]
CHO-VCR/A CHO-VCR/T	Chinese hamster ovary	Verapamil	Vincristine		Warr et al. [109, 110]

hypothesized that verapamil increases MRP1-mediated glutathione efflux rather than being effluxed itself by MRP1 [33]. Increased glutathione efflux reduces the detoxification capacity for ROS, hence, leading to increased apoptosis and collateral sensitivity.

Inhibition of ATP Production

The fact that ATP depletion plays a role for preferential killing by verapamil of multidrug-resistant tumor cells led to the hypothesis that ATP depletion by metabolic inhibitors may also cause collateral sensitivity of multidrug-resistant cells. Indeed, the glycolysis inhibitor 2-deoxy-D-glucose, the electron transport chain inhibitors rotenone and antimycin A are hypersensitive in P-glycoprotein-expressing cells [25, 36, 37]. Tunicamycin reduced GLUT1-mediated glucose transport and potentiated the effects of 2-deoxy-D-gluxose in P-glycoprotein-expressing cells [38]. This indicates that tunicamycin decreased intracellular glucose levels, thus, leading to decreased ATP production and enhancement of collateral sensitivity.

Effects on Membrane Fluidity

Collateral sensitivity also occurs in the absence of P-glycoprotein as shown in Table 1. Membrane-active compounds may account for P-glycoprotein-independent effects. Membrane fluidity has been described as a P-glycoprotein-independent mechanism of doxorubicin resistance affecting the uptake rates in sensitive and multidrug-resistant tumor cells [39].

Membrane-active compounds such as detergents (Triton-X) or local anesthetics perturb the biophysical properties of cell membranes [23]. Thereby, they may not only reverse multidrug-resistance by increasing uptake of co-applied anticancer drugs, but also cause collateral sensitivity if applied alone. As membrane perturbation by these compounds is more pronounced in multidrug-resistant cells than in parental, drug-sensitive cells reversal of resistance and collateral sensitivity seem to be specifically linked to membrane-active drugs such as detergents and local anesthetics [40]. Differences of membrane fluidity occur independently of P-glycoprotein in multidrug-resistant cells, since P-glycoprotein-transfected cells did not show such alterations [41]. Hence, biophysical membrane alterations are rather a consequence of the drug selection process during resistance development than a direct functional linkage to the presence of P-glycoprotein.

Alterations of Target Proteins for Collateral Sensitive Drugs

Tumor cell lines resistant to typical drugs involved in multidrug resistance and drug-resistant cells with other phenotypes may show specific alterations in activity

and expression of target proteins for other drugs which then cause collateral sensitivity. It is not known whether these changes occur as specific adaptation during selection pressure by the corresponding resistance-including agent or whether these alterations occur more or less by chance as secondary events.

Double selection of tumor cells with doxorubicin and verapamil led to collateral sensitivity to streptozotocin and carmustine due to functional loss of DNA repair enzyme O^6-methyl guanine DNA methyltransferase (AGMT) [42]. Diarylsulfonylurea resistant cells show collateral sensitivity to vincristine and actinomycin D [43]. The hypersensitivity to these two drugs can be explained by decreased expression of P-glycoprotein. Melphalan-resistant xenograft tumors were collateral sensitive to etoposide [44], which could be explained by an increase in protein expression and activity of DNA-topoisomerase II, which is the target protein of etoposide. Davies et al. [45] observed that collateral sensitivity to the DNA topoisomerase II alpha-inhibitor, ICRF-159, was associated with down-regulation of this protein. The collateral sensitivity to methotrexate and the antifolate TNP-351 in doxorubicin-resistant cells was associated by a faster uptake and intracellular accumulation, possibly pointing to increased activity of an uptake transporter [46]. A multidrug-resistant P-glycoprotein expressing cell line revealed collateral sensitivity to 5-fluorouracil due to increased thymidylate synthase protein and mRNA expression and activity. The marine steroid aragusterol provoked collateral sensitivity in cisplatin-resistant cells [47]. Treatment with the compound caused G_1 phase arrest in the cell cycle, decreased R6 phosphorylation and expression of cyclins and cyclin-dependent kinases as well as decreased p53 expression. Collateral sensitivities to gemcitabine and cytarabine in daunorubicin- or teniposide-resistant cells may be explained by specific alterations in metabolic enzymes, e.g. increased activity of the gemcitabine and cytarabine-activating deoxycytidine kinase. Furthermore, deoxycytidine deaminase was decreased, which inactivates both drugs [48, 49].

Conclusion

A synopsis of the literature on collateral sensitivity in drug-resistant tumor cells clearly shows that multiple mechanisms account for this phenotype. As collateral sensitivity has not been investigated in the same detail as the role of P-glycoprotein for multidrug resistance, it is possible that our knowledge on collateral sensitivity is still very incomplete and that we know only the tip of the iceberg as yet. More systematic analyses are required to unravel the full biological and pharmacological relevance.

The molecular mechanisms of some compounds have been significantly investigated (e.g. verapamil) but the modes of action of collateral sensitivity of many other drugs are still sparely known. This is true for both drug resistance-related and -unrelated with ABC transporters. Collateral sensitivity of P-glycoprotein- or MRP-1-expressing cells has been analyzed, but less is known about

collateral sensitivity in resistant cells expressing other ABC transporters. For instance, compounds causing collateral sensitivity in P-glycoprotein-expressing cells do not show hypersensitivity in MRP4-, MRP5-, or BCRP-expressing cells [50]. It is also largely unknown whether collateral sensitivity plays a role in the clinical context. In vitro tests of patient samples of chronic lymphocytic leukemia have been performed by Bosanquet and Bell [51]. Comparisons of untreated and treated patients revealed that chlorambucil treatment induced sensitivity to steroids. It still has to be clarified, whether collateral sensitivity is apparent in refractory tumors and whether it can be used to improve survival times of cancer patients. From a pharmacological point of view, the identification of drugs with high degrees of collateral sensitivity is desirable. Most drugs described to exert collateral sensitivity have not been identified in a targeted search for such compounds, but frequently appeared as side products in test panels to characterize cross-resistance profiles. As a consequence, most collateral sensitive drugs only exert modest degrees of collateral sensitivity. As exemplarily shown by Goldsborough et al. [52]. more potent drugs causing hypersensitivity are needed, if collateral sensitivity should be further developed as a novel treatment strategy of otherwise drug-resistant tumors.

Conflicts of Interest No potential conflicts of interest were disclosed.

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Human Cancer Resistance to Trail-Apoptotic Pathway-Targeted Therapies

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Abstract Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) mediates innate and adaptive immunity against the tumorigenesis and tumor progression. TRAIL binds its two death receptors, DR4 and DR5, which activate intracellular pathway of apoptosis for self destruction of tumor cells. To target this apoptotic pathway, recombinant human TRAIL and monoclonal antibodies to DR4 and DR5 have been generated as TRAIL agonists for clinical cancer therapies. A number of TRAIL agonists have passed drug safety evaluation in phase I trials; however, the data from phase II trials thus far are disappointing: TRAIL agonists either in monotherapy or combination have failed to show clinical antitumor activity. In this chapter, we will provide a historic review of the advances and the challenges in the development of TRAIL agonists for clinical treatment of human cancers.

Keywords Apoptosis · Cancer · Caspase · Death receptor · Therapies · TNF · TRAIL

Abbreviations

Apaf1	Apoptotic protease activating factor 1
Bid	Bcl-2 inhibitory BH3-domain-containing protein
tBid	truncated Bid
cFLIP	cellular FADD-like interleukin-1β-converting enzyme-like inhibitory protein
CYLD	cylindromatosis
CUL3	Cullin 3
CDDO	Cyano-3,12-dioxooleana-1,9-dien-28-oic acid

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CDDO-Me	CDDO-methyl ester
CDDO-IM	CDDO-imidazolide
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
DcR	Decoy receptor
DED	death effector domain
DD	death domain
DISC	death-inducing signaling complex
DR	death receptor
DIABLO	direct inhibitor of apoptosis binding protein with low pI
DFF45	DNA fragmentation factor 45
DUB	deubiquitinating
ERK 1/2	extracellular signal-regulated kinase 1/2
FADD	Fas-associated death domain
FasL	Fas ligand
FLICE	FADD-like interleukin-1β-converting enzyme
GPI	glycosyl phosphatidylinositol
IAP	inhibitors of apoptosis proteins
cIAP	cellular inhibitors of apoptosis proteins
IKKγ	inhibitor of κB (IκB) kinase γ
mAb	monoclonal antibody
mTOR	mammalian target of rapamycin
NHL	non-Hodgkin lymphoma
NSCLC	non-small cell lung carcinoma
NF-κB	nuclear factor-κB
OTU	N-terminal ovarian tumor domain
PEA-15	phosphopritein enriched in astrocytes-Mr 15,000
PED	phosphoprotein enriched in diabetes
PLAC	preligand assembly complex
PI3K	phosphatidylinositide-3-kinase
rhTRAIL	recombinant human TRAIL
RIP	receptor interacting protein
Smac	second mitochondria-derived activator of caspase
TNF	tumor necrosis factor
TNFSF	TNF ligand superfamily member
TNFR	TNF receptor
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
TRAILR	TRAIL receptor
TRADD	TNFR1-associated death domain
TRAF2	TNFR-associated factor 2
TNFAIP3	TNFα-induced protein 3
TNFSF15	TNF ligand superfamily member 15
UB	ubiquitin
XIAP	X-linked inhibitor of apoptosis

Introduction

Apoptosis is a genetically programmed cell death that eliminates unwanted cells and maintains tissue homeostasis under physiological and pathological conditions. More than 230,000 articles have been published on apoptosis in the last four decades since Kerr, Wyllie and Currie first used the term of apoptosis for programmed cell death in 1972 [1]. More than 70 % of these publications occurred in the last decade after Brenner, Horvitz and Sulston were awarded with the 2002 Nobel Prize in Medicine for their work on programmed cell death. These efforts have contributed to our understanding of programmed cell death in the aspects of biology, biochemistry, genetics, physiology, pathology and pharmacology, ultimately leading to the genesis of a new class of apoptotic pathway-targeted therapeutic agents. To date, thousands of patients have received apoptotic pathway-targeted therapies in treating various human diseases.

Human cancers are genetic diseases in which genomic alterations result in the deregulation of cell growth and death pathways and thus lead to the relentless cancer cell growth at expense of cancer cell death [2]. The ultimate goal of apoptotic pathway-targeted therapies is to restore the endogenous programmed cell death and drive cancer cells into self destruction [3, 4]. To date, two classic pathways of apoptosis have been well characterized for therapeutic targeting: the death receptors-mediated extrinsic pathway [5] and the mitochondrial intrinsic pathway [6]. Therapeutic agents targeting these two cell death pathways have been developed and entered clinical trials for cancer treatments [7–9].

Death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily that can trigger apoptosis upon binding of ligands of the tumor necrosis factor (TNF) family. TRAIL is a TNF family ligand that binds death receptor-4 (DR4) and DR5 on the cell surface and activates intracellular pathway of programmed cell death [7, 10]. Recombinant human TRAIL (rhTRAIL) and DR4 and DR5 agonistic antibodies have been developed as TRAIL agonists for clinical trials [11, 12]. Despite the excitement from phase I trials, however, the data from phase II trials have clearly established the resistance of human cancers to the treatment of TRAIL agonists [13]. In this chapter, we will provide a historic review of the development of death receptors-targeted cancer therapies and discuss the challenges that we currently face in cancer resistance to the targeted therapies.

Historic Review of TNF Family in Cancer therapies

In 1975, TNFα, the prototype of TNF family ligands was isolated from the serum of mice treated with endotoxin and named as such because it caused tumor necrosis [14]. Nineteen TNF family ligands have been identified as type II transmembrane proteins [15] and seventeen of them have C-terminal TNF homology domains that bind the cysteine repeat domains of receptors of the TNF receptor (TNFR)

Table 1 The TNF Family Ligands and Death Receptors and Toxicities

Ligands	Death Receptors	Therapeutic Agent	Toxicity
TNFα	TNFR1	rh TNFα	Hypotension and hepatotoxicity
FasL	Fas	Fas antibody	Hepatotoxicity
TNFSF15	DR3		
TRAIL	DR4, DR5	tagged rhTRAIL	Toxicity to hepatocytes and neural cells

superfamily. The TNFR family receptors are expressed by various cells in different organ systems; in contrast, however, almost all of the TNF family ligands are expressed by cells of the immune system, including B and T lymphocytes, natural killer and dendritic cells, and monocytes [16]. Ligand-receptor interactions initiate multiple signaling pathways promoting cell survival, death, differentiation, and inflammation in the receptor-expressing cells, depending on the activation state of the cells and the expression levels of the TNFR superfamily receptors.

Twenty-eight receptors of the TNFR family have been reported [7] and classified into three groups: death receptors, decoy receptors and TNFR-associated factor binding receptors [17]. The death receptors have cysteine rich extracellular domains that recognize their death ligands and homologous cytoplasmic domains that activate intracellular apoptosis pathway [18]. The following four sets of death ligands and death receptors have been reported (Table 1): TNFα [14, 19, 20] and TNFR1 (CD120a, DR1, p55, p60) [21]; Fas ligand (FasL, CD95L) [22] and Fas (APO-1, CD95, DR2) [23]; TNF ligand superfamily member 15 (TNFSF15, TL1A) [24–26] and DR3 (Apo3, LARD, TR3, TRAMP, WSL-1) [27–31]; TRAIL [32, 33] and TRAIL receptor-1 (TRAILR1, APO-2, DR4) [34, 35] or TRAILR2 (DR5, KILLER, TRICK2) [35–38]. Since their discovery, preclinical and clinical efforts have been made for development of these pathways-targeted therapeutic agents for clinical treatments of cancers. The first major roadblock in translation of these therapeutic agents into successful clinical therapies was the hepatotoxicity that these agents caused in mice and human.

TNFR1 and Fas Targeted Cancer Therapies and Toxicities

In 1984, *TNFα* gene was cloned from human [19] and recombinant human TNFα (rhTNFα) was soon generated and studies showed its antitumor activity but also toxicity to normal cells in mice [39]. Despite the toxicity of rhTNFα observed in mice, phase I and II trials of rhTNFα were launched in the 1990s and dose-limiting side effects of hypotension and hepatotoxicity were observed in patients [40–44]. The systemic use of rhTNFα as a therapeutic agent has therefore been abandoned since. In 1989, an agonistic antibody against Fas was shown to have the ability to activate Fas-mediated apoptosis [45]. Systemic injection of the Fas agonistic antibody in mice, however, caused profound hepatotoxicity [46] so that clinical trials of

Fas agonistic antibody have never been considered. Currently, clinical use of TNFα and FasL is limited to local delivery to avoid the systemic side effects [47, 48].

Tagged rhTRAIL-Induced Hepatotoxicity

TRAIL was identified in 1995 by Immunex [32] and 1996 by Genentech [33] and rhTRAIL was soon generated as a TRAIL agonist for cancer therapies [49, 50]. Studies of these recombinant proteins, however, revealed the toxicities of tagged forms of rhTRAIL to normal cells: a leucine zipper-fused rhTRAIL killed isolated human astrocytes in culture [49]; a polyhistidine-tagged rhTRAIL was toxic to isolated human hepatocytes [51]; and a Flag-tagged rhTRAIL induced apoptosis of neural cells in human brain slice culture [52]. These in vitro studies raised the same safety concern as those expressed with rhTNFα and Fas agonistic antibody and triggered a call for "steering anti-cancer drugs away from the TRAIL" [53]. In contrast, however, studies of non-tagged native sequence soluble forms of rhTRAIL showed that non-tagged rhTRAIL was not toxic to normal cells in non-human primates [50] and isolated human astrocytes in culture [54]. A mouse monoclonal antibody (mAb) against DR5 (TRA-8) was generated as a TRAIL agonist for having antitumor activity without causing liver injury in mice [55]. Systemic injection of non-tagged rhTRAIL inhibited the growth of cancer xenografts but caused no injury to human hepatocytes in chimeric mice reconstituted with human livers [56]. It appears that non-tagged rhTRAIL contains sufficient bound zinc ions and forms the homotrimers whereas polyhistidine-tagged rhTRAIL has poor zinc ion coordination and forms disulfide-liked dimmers that cause hepatotoxicity [57]. Moreover, normal human cells express the cell surface DR4 and/or DR5 not sufficiently enough to engage intracellular death pathway [56, 58].

Phase I Trials of the Safety of TRAIL Agonists

Preclinical findings that non-tagged rhTRAIL and its agonistic antibody are non-toxic to normal cells have provided renewed impetus for the launch of clinical trials of these TRAIL-apoptotic pathway-targeted therapeutic agents. In 2005, phase I trials of full human mAb against DR4 (HGS-ETR1, Human Genome Sciences) and DR5 (HGS-ETR2, Human Genome Sciences) were reported at the 96th Annual Meeting of the American Association for Cancer Research (AACR), revealing that these DR4 and DR5 agonistic antibodies were safe and well tolerated in patients. At the 42nd Annual Meeting of the American Society of Clinical Oncology (ASCO) in 2006, a phase I trial of rhTRAIL (Apo2L/TRAIL, Genentech/Amgen) showed that monotherapy with this no-tagged rhTRAIL was well tolerated in patients with no dose-limiting toxicity. In addition, this phase I trial showed the monotherapy of rhTRAIL was associated with partial response and

stable disease in patients with advanced cancer. These preclinical and clinical studies have finally put TRAIL toxicity issue behind and, as a result, a number of DR4/DR5 agonistic antibodies have been generated and quickly entered clinical trials as TRAIL agonist [11].

TRAIL-Induced Apoptosis in Cancer Immunity

Since its discovery in the middle 1990s [32, 33], TRAIL has been well studied for its physiological functions. The studies of mice treated with TRAIL-neutralizing antibody revealed for the first time that TRAIL is required for natural killer cells-mediated innate immunity against cancer progression and metastasis in mice [59, 60]. Studies of TRAIL-deficient mice further showed that TRAIL plays a critical role in immunosurveillance against tumorigenesis [61]. In addition, TRAIL contributes to T lymphocyte-mediated adaptive and dendritic cell-mediated innate immunity against cancers [62, 63]. Therefore, these studies have established TRAIL as a natural cancer killer of immune system in immunosurveillance and it can drive cancer cells into self destruction through activation of the endogenous pathway of programmed cell death [16].

TRAIL-Induced Extrinsic Pathway of Apoptosis

TRAIL is a type II transmembrane protein with a short intracellular amino-terminal and a long extracellular carboxy-terminal [32, 33]. The extracellular carboxy-terminal containing the receptor-binding domain [64] can be cleaved to yield a biologically active soluble protein of the amino acids 114–281 of the full TRAIL protein [65]. TRAIL has two death receptors, DR4 [34, 35] and DR5 [36–38]; both are type I transmembrane proteins with two extracellular cysteine-rich domains that can bind TRAIL and a cytoplasmic death domain (DD) that can engage intracellular machinery of apoptosis. DR4 and DR5 exist either as homotrimers or heterotrimers linked through an interaction between the pre-ligand assembly domains in the extracellular termini [66]. Upon TRAIL ligation, DR4 and DR5 recruit the intracellular adaptor, Fas-associated death domain (FADD) [36, 67]. FADD contains a carboxy-terminal DD and an amino-terminal death effector domain (DED) [68], through which, FADD recruits DED-containing apoptosis-initiating protease caspase-8 [69–71] and caspase-10 [71–74] to the receptors for the formation of a death-inducing signaling complex (DISC), similar to the one reported in Fas apoptotic pathway [75, 76]. In the DISC (Fig. 1), caspase-8 and caspase-10 are dimerized and cleaved, become enzymatically active, and cleave apoptosis effector caspase-3 and caspase-7 in the execution of programmed cell death [77].

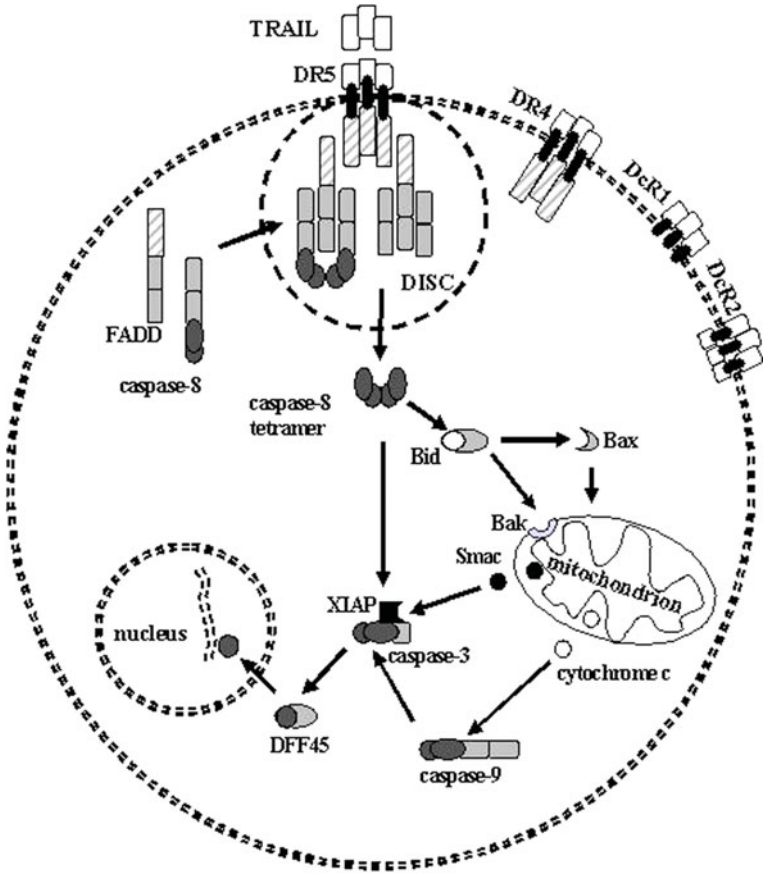

Fig. 1 TRAIL-induced apoptosis through the extrinsic and intrinsic pathways

TRAIL-Induced Intrinsic Pathway of Apoptosis

The intrinsic mitochondrial pathway of apoptosis is required for TRAIL-induced apoptosis (Fig. 1). Once cleaved and enzymatically activated in the DISC, caspase-8 active enzyme was released from the DISC and cleaves Bcl-2 inhibitory BH3-domain-containing protein (Bid) in cytoplasm [78]. The truncated Bid (tBid) in turn interacts with Bax and Bak and induces the protein oligomerization in mitochondrial membrane, leading to the change of the membrane potential and subsequent release from the mitochondria of cytochrome *c* [79] and second mitochondria-derived activator of caspase (Smac) or direct inhibitor of apoptosis binding protein with low pI (DIABLO) [80, 81]. In the cytosol, cytochrome *c* binds to Apoptotic protease activating factor 1 (Apaf1) and recruits dATP and caspase-9 for the formation of apoptosome (Fig. 1). Herein caspase-9 is cleaved through autoproteolysis and becomes active and cleaves downstream effector caspase-3 [82]. The caspase-3

cleavage, however, is inhibited in cancers due to the expression of X-linked inhibitor of apoptosis (XIAP) and interaction of XIAP and caspase-3 [83]. Therefore, TRAIL-induced extrinsic pathway can be rescued by the release of mitochondrial Smac that binds XIAP, removes it from caspase-3 and thus releases its inhibition of caspase-3 cleavage [84].

Caspase-8 in TRAIL-Induced Apoptosis

Caspase-8 (also termed FADD-like interleukin-1β-converting enzyme, FLICE) is an apoptosis-initiating caspase that is synthesized as a zymogen and exists in two isoforms (p55, p53); each consists of two DED domains and a protease domain made of two subunits, p18 and p12 [85]. Through its DEDs, caspase-8 zymogens are recruited to the DISC where they become cleaved through two-step autoproteolytic processes: the first intra-molecular cleavage generates p12 subunit [86, 87] and the second cleavage results in the release of the p18 subunit from the DED and p10 from the p12 subunit [88, 89]. The caspase-8 p18 and p10 subunits become enzymatically active and cleave downstream caspase-3 and caspase-7 [77]. Caspase-3 and caspase-7 are effector caspases and each of them consists of a protease domain with a large and small subunit. Once cleaved and activated, caspase-3 cleaves downstream DNA fragmentation factor 45 (DFF45) in the execution of programmed cell death [90].

It remains debated about how a caspase-8 zymogen becomes cleaved and activated when there is no protease above it. An induced proximity model initially suggested that caspase-8 zymogens were brought into close proximity in the DISC for the autoproteolytic cleavage [91, 92]. Further in vitro biochemical studies proposed the dimerization model that caspase-8 zymogens first form dimers through an interaction of alanine 397 residues of their protease domains and become proteolytically active and cleaved to form the enzymatically active p18 and p10 tetramer [93–95]. However, this model of caspase-8 activation remains to be established in vivo in normal or cancerous cells. In addition, the question remains why there are two apoptosis initiators, caspase-8 and caspase-10 in the DISC.

TRAIL's Antitumor Activity

The cytotoxicity of rhTRAIL was first reported in cancer cell lines originated from various organs and its antitumor activity was shown in the cell lines-derived xenografts in mice [49, 50, 96]. The expression of DR4 and/or DR5 on the surface of cancer cell lines and in some cancer tissues were reported [54, 97–100] and the cytotoxicity of TRAIL agonists were shown due to the induction of apoptosis in cancer cell lines [54, 55, 101–103]. TRAIL-induced apoptosis was further shown to involve the DISC assembly through DR4 and/or DR5-mediated recruitment of

FADD, caspase-8 and caspase-10 [71] in the lipid rafts [104]. In the DISC, caspase-8 and caspase-10 are cleaved through autoproteolysis and once released from the DISC, caspase-8 and caspase-10 enzymatically active subunits cleave Bid and thus induce mitochondrial release of cytochrome *c* and Smac, leading to the cleavage of XIAP and caspase-3 in the execution of TRAIL-induced apoptosis [99].

The preclinical studies have developed therapeutic modalities for TRAIL distribution in tumors. Systemic administration by intraperitoneal and intravenous injection of rhTRAIL and DR4/DR5 agonistic antibody was shown to inhibit the growth of tumor xenografts in mice [55, 96]. A local convection-enhanced delivery effectively distributed rhTRAIL throughout xenografts in rat brains [105] and intratumor injection of TRAIL-secreting neural stem cells inhibited the growth of brain tumor xenografts in mice [106]. Furthermore, intratumor injection of an adenovirus vector specifically replicating in tumor cells and expressing TRAIL significantly inhibited the growth of tumor xenografts in mice [107, 108]. These in vitro and in vivo preclinical studies suggest that TRAIL-apoptotic pathway exists in cancer cells and thus can be targeted for clinical cancer therapies.

Trail Resistance in Human Cancers

The understanding of TRAIL physiology in tumor immunosurveillance boosts enthusiasms in the development of TRAIL agonists for cancer therapies [16]. On the other hand, however, the notion that TRAIL mediates innate and adaptive immunity against cancer predicts that cancers occur in patients by evasion of TRAIL-mediated immunosurveillance and are resistant to the treatment of TRAIL agonists. This seems to be true since studies of human cancer cell lines from the earlier stage showed that only a fraction of the cell lines undergo apoptosis under TRAIL treatment [54] and most if not all in vivo studies utilized TRAIL sensitive cancer cell lines in generation of xenografts [50, 56, 109]. While it remains under intensive investigation about how cancers invade the immunosurveillance, studies of cancer human cancer cell lines and derived xenografts have revealed the multiple checkpoints of TRAIL resistance from the upstream death receptors down to the cleavage of effector caspases.

Genomic Defects in TRAIL Apoptotic Pathway

Genetic mutations, epigenetic silencing and chromosomal alterations have been identified in human cancers, resulting in the loss or silencing of apoptotic genes of TRAIL apoptotic pathway. Somatic inactive mutations of *Bax* gene were first identified [110], leading to TRAIL resistance in colon cancer cells [111]. Somatic inactive mutations of *DR4* and *DR5* were reported in non-Hodgkin lymphoma (NHL) and lung, head, neck, gastric and breast cancers [112–116]. The mutations

occur in the DD of DR4/DR5 and block DR4/DR5-mediated intracellular signal transduction [117, 118]. *DR4* and *DR5* genes map to human chromosome 8p12-23 and cytogenetic analysis revealed loss of chromosome 8p12-23 in TRAIL-resistant glioblastoma cell lines [119]. Caspase-8 is silenced in neuroblastoma by DNA methylation, leading to the cancer resistance to the treatments of TRAIL agonists [120, 121]. Somatic inactive mutations of *caspase-8* were reported in colorectal and gastric carcinomas [122, 123] and loss of the *caspase-8* loci on chromosome 2q33-34 was identified in glioblastoma cell lines [119]. Inactive mutations and genomic loss of TRAIL apoptotic genes in human cancers lead to the cancer resistance to the treatments of TRAIL agonists.

Inhibition by Decoy Receptors

In addition to DR4 and DR5, TRAIL has two membrane-associated decoy receptors, DcR1 (TRAIL-R3) [124–126] and DcR2 (TRAIL-R4) [127–129]. DcR1 is a glycosyl phosphatidylinositol (GPI)-anchored membrane protein with cysteine-rich extracellular domains but no cytoplasmic DD. DcR2 is a type I transmembrane protein with extracellular domains and a cytoplasmic DD. Through their extracellular domains, DcR1 and DcR2 interact with rhTRAIL and inhibit TRAIL-induced apoptosis either by competing with death receptors for TRAIL binding [130, 131] or by interrupting the homotrimeric formation of death receptors [132]. In addition, the interaction of TRAIL and DcR2 activates the nuclear factor-κB (NF-κB) pathway for cancer cell survival [129]. These studies have suggested DcR1/DcR2 act as TRAIL inhibitors and therefore raised concerns about the usefulness of rhTRAIL as compared to DR4/DR5 agonistic antibodies. This concern, however, is mainly based on the studies in transfectants and analysis of human cancer cell lines and tissues has detected neither DcR1 nor DcR1 and thus rule them out in TRAIL resistance [133].

DISC Modifications and Inhibition of Caspase-8 Cleavage

The DISC can be modified by intracellular adaptors that either inhibit caspase-8 or activate cell survival pathways (Fig. 2). Receptor interacting protein (RIP) and TNFR1-associated death domain (TRADD), first identified as DD adaptor proteins in TNFR1 and Fas pathway [134–136], were found in the DR4/DR5-mediated DISC in transfectants [38], but analysis of human cancer cell lines detected RIP, but not TRADD in the DISC and showed that RIP recruits an inhibitor of κB (IκB) kinase γ (IKKγ), through which IKKα and IKKβ are subsequently recruited to the DISC for the activation of IKKα/β kinases, the phosphorylation of IκB, and the activation of nuclear factor-κB (NF-κB) [133, 137–139].

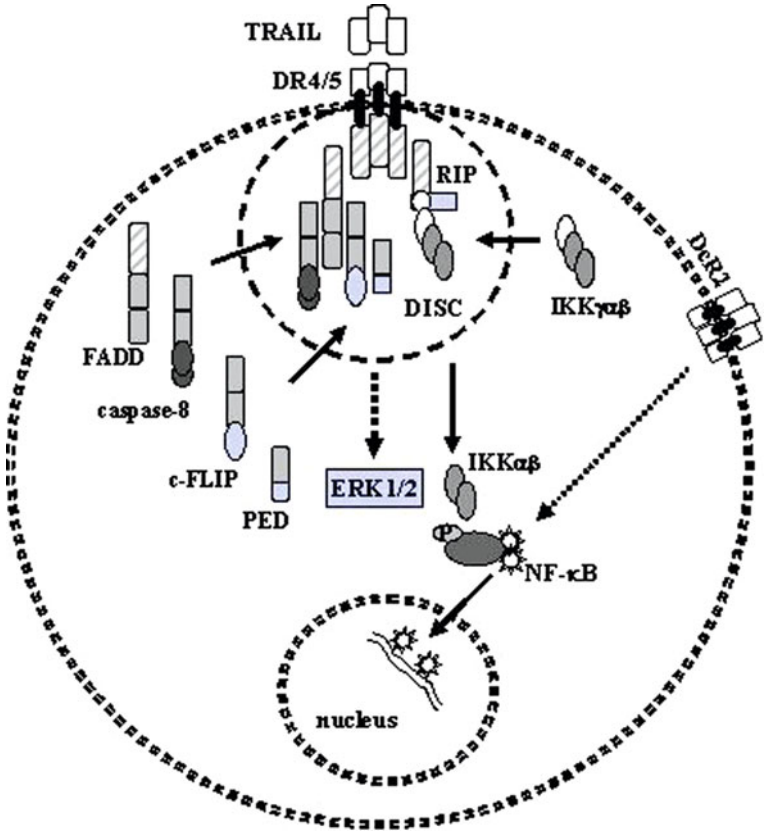

Fig. 2 The DISC modification in TRAIL-induced non-apoptotic signals

FADD is an adaptor protein that interacts with DR4/DR5 through its DD and caspase-8 through its DED. Through the DED interaction, FADD can also recruit two DED adaptors to the DISC: one is cellular FADD-like interleukin-1β-converting enzyme [FLICE]-inhibitory protein (c-FLIP) [140] and another is phosphoprotein enriched in diabetes or phosphoprotein enriched in astrocytes-15 kDa (PED/PEA-15) [141]. The c-FLIP exists in two isoforms: a short form (c-FLIP$_S$) consisting of two DEDs, and a long form (c-FLIP$_L$) containing two DEDs and a caspase-like domain that lacks catalytic activity [140, 142]. PED/PEA-15 possess a DED but not a caspase-like domain [141, 143]. Through the DED, c-FLIP and PED/PEA-15 interact with caspase-8 and inhibit caspase-8 cleavage in the DISC [71, 144]. In addition, c-FLIP and PED/PEA-15 may interact with RIP and TNFR-associated factor 2 (TRAF2) and thus link the DISC to NF-κB and extracellular signal-regulated kinase 1/2 (ERK1/2) pathway [145–147].

NF-κB Pathway

Treatment of human cancer cell lines with rhTRAIL can activate NF-κB and ERK1/2 signaling pathways through RIP, c-FLIP and PED/PEA-15; however, the question then is whether NF-κB and ERK1/2 activity in cancer cells lead to the cell resistance to TRAIL. Some have reported that NF-κB activity inhibits TRAIL-induced apoptosis in lymphoid cells [148] and promotes the growth of ductal carcinoma cells [149], thus raising concern about the therapeutic use of rhTRAIL and its agonists [150]. These effects might be cell type-dependent as no such effects were observed in other cancer cells such as non-small cell lung carcinoma (NSCLC) [104]. NF-κB activity has been shown to upregulate anti-apoptotic genes *c-FLIP* and *Mcl-1* and *cIAP2* [151–153] as well as apoptotic genes *TRAIL* and *DR5* [154, 155]. TRAIL-induced NF-κB activity requires IκB phosphorylation and NF-κB nuclear translocation; however, targeting these steps affects neither c-FLIP expression nor TRAIL resistance [156, 157]. In contrast, knockdown of RIP, c-FLIP and PED releases the inhibition of caspase-8 cleavage and converts TRAIL- resistant cells to the sensitive phenotype [104, 133, 158, 159]. These studies suggest that the DISC is the molecular switch that controls caspase-8 cleavage and thereby the intracellular cell death and survival machinery.

Inhibition of Mitochondrial Pathway and Effector Caspases

TRAIL-induced apoptosis requires the activation of the intrinsic mitochondrial pathway that is regulated by the Bcl-2 family proteins. The Bcl-2 family can be divided into three subfamilies: Bcl-2, Bax and BH3-only proteins [160]. Bid is a BH3-only protein and once cleaved by caspase-8, tBid interacts with Bax and Bak of the Bax subfamily, resulting in the change of mitochondrial membrane potential whereas Bcl-2 and Bcl-X_L of the Bcl-2 subfamily interact with Bax and Bak to maintain the mitochondrial membrane potential [161, 162]. Overexpression of either Bcl-2 or Bcl-X_L blocks TRAIL-induced apoptosis [163–165] and inactivation of Bax leads to the cell resistance to TRAIL [111]. However, it is largely unknown how these Bcl-2 family proteins are expressed in human cancers and consequently how they interact with each other and control the mitochondrial pathway.

The family of inhibitors of apoptosis proteins (IAP) consists of XIAP, cellular IAP1 (cIAP1), cIAP2, survivin, and livin. IAPs interact with caspase-3 and caspase-7 to prevent their cleavage and enzymatic activation [166]. XIAP, cIAP1, and cIAP2 have been shown to be highly expressed in various human cancer cell lines and tissues in correlation with poor clinical prognosis [83, 167–169]. Inhibition of the expression of XIAP and survivin enhances TRAIL-induced apoptosis in cancer cells [170, 171]. Targeting of XIAP by Smac peptide [172], Smac mimic [173] and XIAP antisense RNA [174] has been shown to enhance TRAIL-induced apoptosis in already sensitive cancer cell lines; thus, the ability of these XIAP-targeted

agents to overcome TRAIL resistance in resistant cell lines remains to be seen. In addition, it is unclear whether Bcl-2 and/or IAP family proteins constitute the checkpoints that block TRAIL apoptotic pathway in any given human cancers.

Ubiquitin Regulation of TRAIL Pathway

The caspase-8 dimerization and cleavage are the critical upstream events in TNF family-induced apoptosis [93, 94, 175, 176] and post-translational modification of proteins by ubiquitin regulates these biochemical processes [177]. Ubiquitin (UB) is covalently attached to lysine residues of the substrate proteins through the catalytic reactions mediated by UB-activating (E1), conjugating (E2) and ligase (E3) and removed by deubiquitinating (DUB) enzymes [178]. UB has seven lysine (K) residues; each of them and its N-terminal methionine (M1) can be linked to the C-terminal glycine residue of another UB to form polyUB chains [179], which regulate TNFα-induced signal [177]. Upon TNFα binding, TNF receptor 1 (TNFR1) recruits receptor-interacting protein 1 (RIP1), cellular inhibitor of apoptosis protein 1 and 2 (cIAP1 and cIAP2) and TNFR-associated factor 2 (TRAF2) for the assembly of TNFR1-associated complex I [173]. TRAF2, cIAP1 and cIAP2 E3 ligases that activate nuclear factor-κB (NF-κB) through attaching polyUB chains to RIP1 [180] and binding of the polyUB chain to IκB kinase γ (IKKγ) [181]. TRAF2 and RIP1 then detach from TNFR1 and recruit FADD and caspase-8 for the assembly of the cytoplasm complex II [182], where cylindromatosis (CYLD), a DUB removes the polyUB chains from RIP1 and promotes caspase-8 cleavage for TNFα-induced apoptosis [183].

Unlike TNFR1, however, DR4 and DR5 recruit FADD and caspase-8 in the assembly of a plasma membrane bound DISC where caspase-8 becomes dimerized and cleaved, initiating apoptosis [69, 70]. Cullin 3 (CUL3), an E3 ligase adds K48 and K63-linked polyUB chains to caspase-8 and facilitates its dimerization and cleavage in the DISC, where A20 DUB removes the polyUB chains from caspase-8 [184]. A20 (TNFα-induced protein 3; TNFAIP3) is well known for its anti-inflammatory activities [185] through its N-terminal ovarian tumor domain (OTU) that acts as a DUB and removes K63-linked polyUB chains from RIP1, TRAF6 and RIP2, thus restricting TNFR1, Toll-like receptor and nucleotide-binding oligomerization domain-induced NF-κB signal [186–188]. In contrast to the role of A20 in the TNFR1 pathway, the A20 C-terminal Zinc finger (Znf) domain of an E3 ligase [189] can mediate RIP1 polyubiquitination through K63-linked polyUB chains in TRAIL-induced DISC [190]. In TRAIL-resistant glioblastoma cell lines, A20 and DR5 form a plasma membrane-bound preligand assembly complex (PLAC) under physiologic conditions and TRAIL treatment leads to the recruitment of caspase-8 to the PLAC for the assembly of the DISC, in which the polyUB chains attached to RIP1 bind to the caspase-8 protease domain and inhibit caspse-8 cleavage and the initiation of TRAIL-induced apoptosis. It remains to be seen whether this revised TRAIL model can be applied in other cancers and whether

A20 E3 ligase inhibitors, which are currently under development, can overcome TRAIL resistance for combination cancer therapy with TRAIL agonists.

TRAIL-Based Combination Therapies

The genetic defects in TRAIL apoptotic genes have been detected only in a small fraction of human cancers [119]. The vast majority of human cancers appears to have intact TRAIL apoptotic pathway and thus can be therapeutically targeted by TRAIL agonists. Unfortunately, the pathway is often inhibited in human cancers at multiple points in the apoptotic pathway through numerous mechanisms [11, 191]. Therapeutic agents targeting these resistance points have been developed for combination therapies with TRAIL agonists. Moreover, cancer-addicted oncogenes can inhibit cancer cell death pathway and thus drive cancer growth; thus, oncogene-targeted therapeutic agents may have potential for synergistic antitumor activity with TRAIL agonists. Finally, chemotherapy and radiation therapy remain the standard clinical treatment of human cancers and have shown synergistic antitumor activity with TRAIL agonists.

Targeting of the DISC Modification and Mitochondrial Pathway

A number of therapeutic agents that target the TRAIL inhibitors, c-FLIP, Bcl-2 and XIAP have been developed as single agents or in combination with TRAIL agonists for cancer therapies. 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) is a synthetic oleanane triterpenoid [192] and its derivatives, CDDO-methyl ester (CDDO-Me) and CDDO-imidazolide (CDDO-Im) have been shown to be able to inhibit c-FLIP expression and promote TRAIL-induced apoptosis in cancer cell lines [193, 194]. In a phase I trial of CDDO, sponsored through the collaboration between the National Cancer Institute and Reata Pharmaceuticals, thromboembolic side effect was revealed and considered as dose-limiting toxicity [195]. Antisense oligodeoxynucleotides against Bcl-2 (Genasense; Genta Inc., Berkeley Heights, JN) [196], XIAP (AEG35156; Aegera Therapeutics, Montreal, Quebec, Canada) [197] and survivin (LY2181308; Eli Lilly and Company, IN) [198] have passed phase I trials of drug safety and pharmacokinetic analysis. In addition, a number of Smac mimetics with a high affinity for XIAP including synthetic Smac N-terminal peptide [172], small molecule Smac mimic [173], Smac peptidomimetic (LBW242, Novartis, Cambridge, MA) [199] and SM-164 [200] have been generated for clinical treatment of cancers. It remains to be seen whether the combination of these agents with TRAIL agonists may lead to effective cancer therapies.

Targeting Oncogenes-Driven Signaling Pathways

Mutations and/or deletions of tumor suppressors such as *TP53* and *PTEN* are commonly seen in human cancers [201], but it is unclear whether the genomic status of each of the genes correlates to TRAIL sensitivity. Studies of glioblastoma cell lines failed to show this correlation [54, 99], but other studies suggest that DR5 is p53 target gene and p53 rescue compound can induce the expression of DR5 [37] and thus enhance TRAIL killing of cancer cells [202]. Oncogene-driven pathways such as mammalian target of rapamycin (mTOR) have been reported to contribute to TRAIL resistance through induction of c-FLIP$_S$ expression [203]. Other mechanisms in TRAIL resistance include DNA methyltransferases through the up-regulation of PED/PEA-15 [204], heat shock protein 90α facilitating c-FLIP$_S$ recruitment to the DISC [205], microRNA-21 inhibiting TRAIL killing of tumor cells [206] and the ubiquitin–proteasome pathway blocking c-FLIP$_S$ degradation [207].

Therapeutic agents targeting oncogene-driven signaling pathways have been developed for clinical treatments of cancers. These therapeutic agents have also been evaluated in preclinical studies for the synergistic antitumor activity in combination with TRAIL agonists, including proteasome inhibitor bortezomib [208, 209], epidermal growth factor receptor inhibitors [210], histone deacetylase inhibitor [211, 212], DNA methylation inhibitor [204], cyclooxygenase 2 inhibitors [213, 214], mTOR inhibitor [203], phosphatidylinositide-3-kinase (PI3K) inhibitor [215], kinase inhibitor sorafenib [153], and CD20 antibody Rituximab [216, 217]. The molecular mechanisms by which these therapeutic agents can overcome the cancer resistance to TRAIL and the clinical value of the combination with TRAIL agonists for cancer therapies remain to be investigated.

Combination of TRAIL Agonists with Chemotherapy and Radiation

Chemotherapeutic agents can interact with DNA and form intra-strand cross-links and thus affect intracellular signal pathways [218]. Unfortunately, human cancers eventually become resistant to chemotherapy. In addition, chemotherapy non-specifically targets both cancer and normal cells and causes a broad range of unwanted side effects at therapeutic doses. On the other hand, studies have shown that chemotherapeutic agents enhance TRAIL-induced apoptosis in cancer cells [98, 219–229]; although the molecular mechanisms in the synergistic effects remain controversial. Some studies showed that the treatment with cisplatin and etoposide up-regulate DR4/DR5 mRNA [219], but others observed that cisplatin has no effect on the expression of DR4 and DR5 protein [222]. In addition, studies showed that camptothecin up-regulates Bax [98]; cisplatin activates JNK/p38 [227]; cisplatin down-regulates c-FLIP$_S$ [98] and 1-(2-chloroethyl)-3-cyclohexyl-

1-nitrosourea (CCNU), temozolomide and topotecan have the synergistic cytotoxicity with TRAIL [229] perhaps through down-regulation of c-FLIP$_S$ [99]. While the mechanisms remain controversial, these studies suggest that chemotherapy can enhance TRAIL apoptotic effects on human cancer cell lines and derived xenografts.

Radiation therapy is the standard adjuvant treatment of human cancers. Studies have shown the synergistic cytotoxic effects of radiation with TRAIL agonists. Combination treatment with rhTRAIL and ionizing radiation can kill human glioblastoma cells through activation of the extrinsic and intrinsic apoptotic pathways [230]. Radiation treatment can up-regulate caspase-8 [231] and DR5 [232] and combination treatment of radiation and TRA-8 antibody increases the survival of mice with intracranial glioblastoma xenografts [232]. Clearly, the combination treatment of TRAIL agonists with radiation requires the presence of TRAIL death machinery in human cancers.

Clinical Trials of TRAIL Agonists

The unraveling of TRAIL apoptotic pathway in cancer cells has resulted in a rapid development of the pathway-targeted cancer therapies. A number of therapeutic agents and modalities have been developed, of which rhTRAIL and DR4/DR5 agonistic antibodies have been under clinical investigation [11, 12]. The advantage in clinical use of rhTRAIL is that the recombinant ligand can target DR4 and DR5. On the other hand, antibodies have been proven to be effective clinical therapeutics because they selectively target specific antigens and have a much longer half-life than recombinant proteins. Two major categories of DR4 and DR5 mAbs have been developed: humanized mouse and fully human monoclonal antibodies (Table 2). Several of DR4 and DR5 agonistic antibodies and rhTRAIL have been evaluated in phase I-II trials for their safety, pharmacokinetics and therapeutic efficacy [9, 11].

Clinical Trials of rhTRAIL

A soluble form of non-tagged rhTRAIL (Apo2L/TRAIL, dulanermin) is currently under clinical development jointly by Genentech and Amgen for cancer therapy [233]. The data from phase I trial were first reported at the 42nd ASCO Annual Meeting in 2006 and published in 2010 [234]: 41 patients with advanced cancers and non-Hodgkin lymphoma were enrolled; the monotherapy was well-tolerated with partial response in 3 % and stable disease in 53 % of patients. The data of phase Ib trial of 24 patients with advanced non-small-cell lung carcinoma (NSCLC) was then published, showing that the addition of paclitaxel, carboplatin and bevacizumab (PCB) to rhApo2L/TRAIL (dulanermin) improved the response

Table 2 Clinical Trails of TRAIL Agonists

Target	Agonist	Molecular type	Sponsoring company	Clinical trial
DR4/5	Apo2L/TRAIL (dulanermin)	rhTRAIL	Genentech/Amgen	Phase I/II
DR4	HGS-ETR1 (mapatumumab)	fully human mAb	HGS	Phase I/II
DR5	HGS-ETR2 (lexatumumab)	fully human mAb	HGS	Phase I/II
DR5	HGS-TR2J (KMTR2)	fully human mAb	HGS and Kirin Pharma	discontinued
DR5	CS-1008 (tigatuzumab)	humanized mouse mAb	Daiichi Sankyo	Phase I
DR5	AMG 655 (conatumumab)	fully human mAb	Amgen	Phase I/II
DR5	PRO95780 (drozitumab)	fully human mAb	Genentech	Phase I/II
DR5	LBY135	chimeric mouse/human mAb	Novartis	Phase I

rates such as progression-free survival of patients [235]. It was therefore a surprise when the data from the phase II trial of 213 patients with advanced NSCLC were published in 2011, showing that the addition of PCB to rhApo2L/TRAIL (dulanermin) did not improve the response rates [236]. The small sample size and lack of control in the phase Ib may explain the difference in the outcome between the phase Ib and II trials. The phase II trial, however, has clearly established the resistance of advanced NSCLC to the TRAIL treatment, either in monotherapy or in combination with chemotherapy.

Humanized Monoclonal Antibodies against DR5

In 2001, a mouse mAb against human DR5 (TRA-8) was generated by immunizing mice with a fusion protein of DR5 extracellular domain and IgG [55]. This mouse mAb was then humanized for clinical treatment of cancers by Daiichi Sankyo [237]. The data of phase I trial of the humanized IgG_1 mAb (CS-1008, tigatuzumab) was published in 2010: 17 patients were enrolled with 16 of them diagnosed with carcinoma and 1 patient with lymphoma; all these patients tolerated well of the monotherapy of tigatuzumab and 7 of the 17 patients presented with stable disease [238]. At the 2008 ASCO Annual Meeting, Novartis presented the data from a phase I trial of a chimeric (mouse/human) mAb, LYB135, either in monotherapy or combination with capecitabine in treating patients with advanced solid cancers: LBY135 was well tolerated in 56 patients enrolled and showed signs of clinical activity.

At the 2012 ASCO Annual Meeting, the preliminary data from the phase II trial of tigatuzumab (CS-1008) in combination with carboplatin and paclitaxel were

reported: 100 patients with naïve metastatic and unrespectable NSCLC were enrolled and the treatments did not improve the efficacy of carboplatin and paclitaxel. A phase II trial of tigatuzumab (CS-1008) in combination with the nanoparticle albumin-bound paclitaxel is currently ongoing in treating patients with metastatic, triple-negative breast cancers.

Fully Human Monoclonal Antibodies Against DR4

At the 93rd AACR Annual Meeting in 2002, Human Genome Sciences in collaboration with Cambridge Antibody Technology reported for the first time the generation of fully human monoclonal antibodies against human DR4 and DR5 through phage display: the DR4 agonistic human mAb HGS-ETR1 (mapatumumab) and the DR5 human mAb HGS-ETR2 (lexatumumab) and HGS-TR2J (KMTR2). The antibodies bind DR4/DR5 directly and trigger apoptotic cell death in human cancer cell lines [239]. The data from the phase I trials of HGS-ETR1 showed that the monotherapy of HGS-ETR was well tolerated in patients [240, 241]. Phase II trials of HGS-ETR1 in treating patients with NSCLC [242] and colorectal cancer [243] showed no clinical activity of a single agent. At the 2010 ASCO Annual Meeting, the data from phase II trial showed that the combination of HGS-ERT1 with paclitaxel and carboplatin did not improve the response rates in the patients with advanced cancer.

Fully Human Monoclonal Antibodies Against DR5

The data of a phase I trial of the first fully human mAb HGS-ETR2 (lexatumumab) in treating patients with advanced solid cancer were published in 2007 and 2010: the 10 mg/kg dose was identified as the maximum tolerated dose; dose-limiting toxicity was reached at 20 mg/kg; and twelve of the patients had stable disease [244, 245]. At the 2007 ASCO Annual Meeting, the data from a phase Ib trial showed that patients tolerated well with HGS-ETR2 in combination with gemcitabine, pemetrexed, doxorubicin or FOLFIRI and tumor shrinkage was observed in the FOLFIRI and the doxorubicin combination. HGS-ETR2 is currently in phase I and II trials as a single agent or in combination with chemotherapeutic agents. HGS-TR2J (KMTR2) is a fully human mAb derived from trans-chromosomal mice expressing a human Ig locus immunized with the DR5 extracellular domain under the co-development of Human Genome Sciences and Kirin Pharma [246]. Recently, however, the companies announced to discontinue clinical development of this DR5 mAb.

At the 2007 ASCO Annual Meeting, Genentech reported the generation of a fully human DR5 mAb, Apomab (PRO95780, drozitumab) through phage display [247] and the preliminary data of phase I trial: this mAb was well tolerated in

patients with advanced cancers. At the 2010 ASCO Annual Meeting, the data from two phase II trials were released, showing that PRO95780 (drozitumab) plus rituximab in treating patients with relapsed NHL and in combination of PCB in treating patients with NSCLC did not improve the response rates in the patients.

At the 2007 AACR Annual Meeting, Amgen reported its pre-clinical study of a fully human DR5 agonistic mAb, AMG655 (conatumumab). The preliminary data from phase I trial were then reported at the 2007 ASCO Annual Meeting and the phase I trial data were published: showing that this mAb was well tolerated in Japanese patients [248] and the patients in US [249]. The subsequent data from a phase I/II trial showed that the addition of AMG655 to doxorubicin was safe but did not improved cancer control in patients with soft tissue sarcoma [250].

Conclusions and Future Directions

Since the discovery of TNFα in 1975 [14], a tremendous amount of efforts have been made with the hope that these efforts will lead to an effective clinical treatment of human cancers. 30 years later in 2005, TRAIL agonists of the TNF family finally passed the phase I trials of drug safety test and became available for clinical treatment of cancers. Unfortunately, the data from phase II trials of therapeutic efficacy evaluation have been disappointing: TRAIL agonists, alone or in combination, have failed to show antitumor activities. More data are expected to be released from ongoing clinical trials of TRAIL agonists in treating various human cancers. The hope is that these phase II trials of efficacy test may identify the types of cancers and/or the fraction of the type cancer that can be effectively treated with TRAIL agonists. Clearly, however, the vast majority of human cancers are resistant to the treatment of TRAIL agonists, alone or in combination.

TRAIL mediates the innate and adaptive immunity against cancer. This physiological role of TRAIL suggests that cancers occur in patients by escape from TRAIL-mediated immunity and thus predicts that cancers are resistant to the treatment of TRAIL agonists. In consideration of this possibility, most phase II trials started with a combination of TRAIL agonists with various cancer therapeutic agents, but still failed to show antitumor activity of TRAIL agonists. This is truly a surprise and a disappointment, but it does bring us back to the fundamental question: how cancers evade the immunosurveillance. Understanding how TRAIL-mediated immunity works against cancer will certainly lead to the smart design of TRAIL-based cancer therapies.

The current molecular models of TRAIL modulations in human cancer have been established mainly through studies of cancer cell lines and cell lines-derived xenografts. There is no doubt about the value of these cancer research models in establishment of the fundamental principles. At this stage of the development of TRAIL agonists, it may become more important that we analyze human cancer tissues and tissues-derived stem cell models genetically and functionally. This may allow us to determine whether TRAIL apoptotic pathway exists and can be

therapeutically targeted, in particular in each individual patient. If TRAIL resistance occurs, this approach may allow us to identify the roadblock for targeted therapy to remove the resistance. It seems that we know much more about molecules, cell lines, and animal xenografts, but very little about human cancers when it comes to how TRAIL pathway works. It has been a 35-year journey from the discovery of TNFα to the clinical trials of TRAIL agonists. It may take the same amount of time and efforts to develop this new class of cancer therapeutics as effective clinical therapeutics; but this will only occur, if we finally understand the therapeutic targets – patient's cancers.

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Conflict of Interest No potential conflicts of interest were disclosed.

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The Dark Side of Apoptosis

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Abstract Apoptosis is conventionally regarded as an anti-cancer mechanism that eliminates or prevents mutant cell expansion necessary for tumor development and progression. However, evidence for the paradoxical role of apoptosis in tumor progression is accumulating. In this chapter, we describe the mechanisms by which apoptosis serves as a vehicle for accumulating genomic instability to promote malignant progression of tumors, and show a direct association between apoptosis and tumor progression in clinical settings. The negative therapeutic implications of increased apoptosis on clinical outcome and the need to inhibit apoptosis or disable proliferation in apoptotic tumors are discussed.

Keywords Genomic instability · Horizontal transfer · Cell–cell fusion · Cancer stem cell · Comedo-ductal carcinoma in situ

Abbreviations

CAD gene	Trifunctional protein with carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase activities
DCIS	Ductal carcinoma in situ
EBV	Epstein-Barr virus
REF	Rat embryo fibroblast
MEF	Mouse embryo fibroblast
IAP	Inhibitors of apoptosis
PUMA	P53 upregulated modulator of apoptosis
FLIP	FADD-like IL-1β-converting enzyme)-inhibitory protein
TRAIL	TNF-related apoptosis-inducing ligand
DR4, DR5	Death receptors

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Introduction

Apoptosis represents a major mechanism by which tissue maintains homeostasis. Apoptosis also plays an important role in protecting tissues against tumorigenesis and malignant conversion. Cell loss occurs once tumors have grown more than a few million cells [1, 2]. Intracellular stresses such as hypoxia, nutrient deprivation, telomere shortening, and cell elimination by the host immune system are thought to contribute to steady attrition of a portion of cancer cells in solid tumors [3–5]. A hypothetical link between apoptosis and tumorigenesis has been described by Hanahan and Weinberg [6]. According to their model, the process of tumorigenesis and malignant conversion forces expression of proapoptotic factors that assist with elimination of 'mutant' cells and protection from transformation. Thus, mechanisms contributing to acquired resistance to apoptosis serve as a potential mechanism for cell survival and neoplastic conversion.

For cells to acquire the ability for limitless replicative potential or resistance to apoptosis, cells need to uncouple their growth/proliferation programs from environmental signals. It is, however, becoming abundantly clear that there are situations in which tumor cells undergo programmed cell death under conditions of optimal growth stimulation and in the absence of environmental stress. In these tumors, the apoptosis program is wired independently of cell–cell and cell-environmental communications. Interestingly, despite the proficiency for spontaneous cell death, the surviving tumor cells exhibit increased ability for malignant progression. This suggests that the very same process used for protecting cells against malignant conversion can contribute to greater vulnerability to malignancy. In metastatic melanoma, spontaneous regression of individual metastatic deposits is seen throughout the life history of the disease, yet the final outcome is usually death [7]. Similarly, spontaneous regression has been reported in metastatic renal carcinoma, neuroblastoma, colorectal carcinoma, and a wide range of solid tumors [8], suggesting that spontaneous cell loss is not an infrequent event. Yet, despite the loss of tumor cells, the final outcome is not often favorable. The fact that cell loss can occur in vitro under optimal growth and normoxic conditions suggests that cell loss seen in vivo is not necessarily due to nutrient deprivation or hypoxia but rather these tumor cells are intrinsically programmed to undergo apoptosis.

Causes of spontaneous death. Spontaneous loss of cells occurring in the absence of extracellular factors suggests that the fully transformed cells may be intrinsically unstable due to specific genetic abnormalities, and that in the process of achieving stability they undergo apoptotic cell death. Factors responsible for spontaneous apoptosis in tumors are diverse. In some tumors apoptotic cells are found near foci of confluent necrosis suggesting that mild ischemia may contribute to the initiation of apoptosis. The release of cytokines such as TNF-α by infiltrating macrophages is regarded as one of the contributing factors. The extrinsic or death receptor pathway of apoptosis involves stimulation of external surface receptors by FasL and TRAIL leading to activation of caspase-8. In the intrinsic or mitochondrial pathway, proapoptotic signals meet at the mitochondria with resultant

loss of the mitochondrial membrane potential, release of cytochrome c and activation of the caspase-9 signaling cascade [9, 10]. The intrinsic pathway is regulated by members of the Bcl-2 family that have either antiapoptotic (Bcl-2, Bcl-xL, Bcl-W, Mcl-1) or proapoptotic (Bax, Bim, Bak, Bid) function. The p53 tumor suppressor protein functions in apoptosis by transcriptional upregulation of genes (e.g., *Bax*, *PUMA*) that are directly involved in apoptosis [11, 12]. Both apoptotic pathways converge on the same execution pathway resulting in the activation of caspase-3, DNA fragmentation and formation of apoptotic bodies. Tumor cells exhibit resistance to apoptosis through overexpression of FLIP, reduced expression of CD95, TRAIL DR4 or DR5 receptors, or by overexpression of IAPs or altered expression of the Bcl-2 family members [10].

Paradoxical role of Bcl-2 and Bax in tumor progression. Upregulation of Bcl-2, Bcl-xL, and Mcl-1 are associated with inhibition of apoptosis; however, their expressions do not often correlate strictly with poor clinical prognosis [13, 14]. In breast cancer, Bcl-2 overexpression is associated with normal ploidy, estrogen receptor positivity, and absence of metastasis; all characteristics associated with better clinical outcome and a more favorable prognosis that is contradictory to its predicted role in apoptosis resistance [15, 16]. In colorectal adenoma, Bcl-2 levels are decreased compared to the adjacent normal tissue [17]. Similar data are reported in cervical, prostate, and endometrial cancers [18]. Overexpression of Mcl-1 is associated with poor prognosis for lung, and head and neck cancers [17–20]. Thus, there are malignancies in which Bcl-2 family member overexpression correlates with favorable prognosis and those in which it is correlative of high tumor grade. Paradoxically, elevated expressions of the proapoptotic proteins Bax and Bak have been associated with poor prognosis in esophageal carcinoma and bladder cancer, respectively [21–23]. In breast tumors, an increase in the proportion of apoptotic cells was observed in recurrent tumors as compared with primary lesions, and patients with tumors with higher apoptotic indices were associated with shorter survival [24]. Several examples of a positive correlation between the apoptotic index and the tumor grade have been reported [25–27]. The role of apoptosis suppression as a vehicle for enhancing genomic instability was tested by measuring the effects of Bcl-2 overexpression on the frequency of CAD gene amplification in cells exposed to the CAD inhibitor PALA. Bcl-2 overexpression failed to increase the frequency of CAD gene amplification. However, similar analysis on cells expressing mutant *p53* showed enhanced frequency of CAD gene amplification. These data suggest that although both p53 inactivation and Bcl-2 overexpression suppress apoptosis, apoptosis inhibition by Bcl-2 overexpression does not make the cells genetically unstable [28]. These data are consistent with low grade tumors produced by Bcl-2 overexpressing cells, further confirming that apoptosis inhibition by Bcl-2 overexpression does not favor selection of tumor cell variants to increase genomic instability [28].

Apoptosis as a mechanism for driving genomic instability. Reduced rates of apoptosis correlate with fewer cell divisions and, hence, the possibility of generating fewer mutant clones. On the other hand, higher rates of apoptosis would

require increased rates of cell division to compensate for cell loss, and consequently lay the foundation for accruing genomic instability [29, 30]. As discussed above, Bcl-2 overexpression and p53 loss both act by blocking apoptosis; however, only the latter enhances accumulation of genomic instability. In this context, it is interesting to note that Bcl-2 overexpression and mutant p53 are rarely coexpressed in a tumor. Bcl-2 overexpression was found in breast and head and neck cancers expressing wild type p53 [13], [31–34], whereas breast tumors with p53 accumulation (indicative of mutant p53 expression) showed low Bcl-2 expression [31, 35]. p53 is inactivated late in most cancers, suggesting that loss of p53 function in later stages of tumorigenesis may translate into higher rates of apoptosis and consequently higher rates of proliferation during the course of tumor development that would enable expansion and accumulation of mutant clones favoring malignant progression. Clinical data show that noncomedo-ductal carcinoma in situ (DCIS) breast cancers express normal levels of Bcl-2 compared to comedo-DCIS that express weak or negligible Bcl-2, strong Bax and mutant p53. Accordingly, comedo-DCIS lesions are characterized by high apoptotic and mitotic indices [36], and are at greater risk for recurrence and malignant progression compared to noncomedo-DCIS. Evidence for a direct link between increased apoptosis and tumorigenesis was recently demonstrated with a mouse model harboring the loxP-targeted allele of Mcl-1 and albumin promoter-driven Cre-recombinase. Hepatocytes of Mcl-1 fl/fl-AlbCre mice lacking Mcl-1 exhibited increased apoptosis and spontaneously developed hepatocellular carcinoma-like lesions at >50 % higher incidence [37]. These data suggest that apoptosis and proliferation work in concert to drive malignant progression.

Mechanisms contributing to tumor progression in apoptotic tumors. Apoptosis allows elimination of unwanted or unstable cells by processing them into an array of smaller bodies called apoptotic bodies by cellular blebbing and fragmentation. Apoptotic bodies have intact plasma membrane and adequate energy supply to maintain their membrane integrity. Cells undergoing apoptosis and apoptotic bodies expose phosphatidylserine on their surface that promote recognition by professional (macrophages, dendritic cells, B-lymphocytes) and nonprofessional (epithelial cells and fibroblasts) phagocytes, resulting in quick ingestion and lysosomal degradation [38]. It is generally believed that apoptosis does not induce inflammation because the apoptotic bodies are rapidly cleared to prevent release of harmful immunogenic materials from the dying cells [39]. We posit that the clearance rates of apoptotic bodies and the presence of associated inflammation will depend upon the extent and rate of apoptosis. Tumor tissues undergoing massive apoptotic cell loss such as comedo-DCIS breast cancers are generally associated with a strong inflammatory response. It is possible that these cells are recruited to mop-up the overwhelming amounts of released apoptotic bodies, or that the slow clearing of apoptotic bodies induces the inflammatory response. Either way, a tumor microenvironment rich in inflammatory cells would promote tumor progression. Additionally, slow or incomplete removal of apoptotic bodies would result in the accumulation of released genetic material into the

cellular milieu and, thus, trigger the horizontal transfer or uptake of the DNA by neighboring tumor or stromal cells.

Horizontal transfer of genetic material via cell–cell fusion. Horizontal transfer of genes has been reported in bacteria and fungi and plays an important role in the generation of antibiotic resistance and the adaptation to new environments [40]. Thus, horizontal transfer of genes represents a powerful mechanism for bacterial diversification. Cell–cell fusion is considered to play an important role in horizontal transmission of genes and malignant transformation [41–43]. Transfer of genetic information could occur via fusions between tumor and stromal cells or between tumor cells. Human glioblastoma grafted to hamster cheek pouches produced hybrid human/hamster tumors in which human chromosome segregation occurred within the first transplant generation and showed widespread metastasis. At least seven genes from the six human chromosomes were retained of which three genes implicated in oncogenesis (*CD7*, *CXCR4* and *PLAGL2*) showed continued expression [44]. CXCR4 (also called fusin), a G-protein coupled chemokine receptor for SDF-1 has been implicated in proliferation, motility, homing and metastasis of cancer cells and is associated with regions of cell death and angiogenesis. These data suggest that in vivo stability of the resulting hybrids depends upon the selective growth advantage provided by the DNA taken up.

Horizontal transfer of DNA via apoptotic conversion. There is increasing evidence that even cell-free cancer DNA can be transferred to induce malignancy. Holmgren et al. [45] demonstrated that DNA can be transferred from apoptotic cells to recipient neighboring cells by phagocytosis. Cocultivation of cell lines containing integrated copies of the Epstein-Barr virus (EBV) resulted in rapid a uptake of EBV DNA to the nucleus of the phagocytosing cell. Once transferred, the expression of EBV encoded genes was detected at both the mRNA and protein levels. Similarly, apoptotic bodies derived from *c-Ha-ras*^{Val} [12] and *c-myc* transformed rat embryo fibroblasts (REFs) were able to transform p53-/- or p21-/- mouse embryo fibroblasts (MEFs) but not wild type MEFs [46, 47]. FISH analysis confirmed that entire chromosomes are transferred from the apoptotic bodies of REFs and become integrated into the mouse host genome. However, the stability of the integrated DNA can be maintained only if it confers a selective growth advantage to the recipient cell. Following uptake of the apoptotic genetic material, normal recipient cells with the activated Chk2/p53/p21 DNA damage response pathway block replication of the transferred DNA, thereby protecting them from the potentially harmful effects of the apoptotic DNA [48]. Similar uptake of apoptotic bodies with resultant acquisition of and propagation of drug resistance genes has been demonstrated in prostate cancer cells [49]. Since p53 is lost in most cancers, horizontal transfer of genetic material from the dying tumor cells to recipient tumor cells may serve as a driving force for accumulation of genomic instability and high mutability of tumor cells. Apoptotic DNA from the dying tumor cells can also be transferred to recipient stromal cells (fibroblasts, endothelial cells, macrophages). Such transfers could actively modify the structure and behavior of the surrounding microenvironment, and potentially provide an explanation for pro-tumorigenic and pro-metastatic properties of the tumor

microenvironment. Apoptotic DNA was detected in the nuclei of ~15 % of the phagocytosing cells [45], suggesting that horizontal DNA transfer is an efficient mode of enhancing genomic diversity of tumor cells that is dictated by the rate and extent of apoptosis in the tumor. In contrast, mutation of specific genes is an inefficient process that requires amplification of the mutated cells and is limited by activities of surveillance mechanisms that monitor and maintain the genomic integrity. It is possible that the fluidity of the cell membrane is increased in cells sensitive to apoptosis, making it more receptive for cell fusion and DNA transfer. Given the manner in which even highly differentiated epithelial cells are stimulated to become phagocytic by the proximity of an apoptotic body, it is one of the less studied but more remarkable components of apoptosis [48, 50]. Horizontal DNA transfer or apoptotic conversion may be clinically important particularly in tumors characterized by high spontaneous apoptosis such as comedo-DCIS breast cancer since these tumor cells also express mutant p53.

Genetic exchanges occurring either via cell–cell fusions or by apoptotic conversion can provide the residual tumor with new attributes for survival, growth, progression and metastasis. It is possible that the phenotypic and genotypic diversity (or heterogeneity) observed in cells within a tumor, between primary and recurrent tumors, primary and metastatic tumors, and/or between metastases of the same tumor arise at least in part by horizontal transmission of genes and gene products by cell–cell fusions or direct incorporation of apoptotic DNA into cancer or stromal cells.

Cancer stem cell activation. Recent studies have invoked the role of cancer stem cells to explain the relationship between enhanced apoptosis and tumor progression. It has been proposed that in tumors with high apoptotic rates, the dying cells may free up space for cancer stem cells to proliferate into and populate the tumor [51, 52]. To determine the association between apoptosis and increased tumor growth/progression, Enderling et al. [53] simulated tumor development for different spontaneous cell death rates, and initialized each simulation with one cancer stem cell, stopping the simulation after 35 months or when the tumor reached confluence. When random cell death among tumor cells was increased, an increase in the number of stem cells was observed [53]. While spontaneous cell death can reduce the number of tumor cells in the short run, they can facilitate sufficient symmetric stem cell divisions to enrich the stem cell pool and ultimately promote malignant expansion [54]. These findings have therapeutic implications since conventional anti-cancer therapies are directed towards eradicating apoptosis-sensitive tumor bulk populations while sparing the therapy of resistant cancer stem cells. Thus, the accelerated tumor recurrence following therapy may be explained by the opportunistic proliferation of quiescent tumor cells into the space made available by the initial killing. This treatment recovery cycle could favor the creation of new stem cells by symmetrical division of previously quiescent stem cells as the latter are considered to be more resistant to radiation [55] and chemotherapy [56] compared to their nonstem counterparts [53]. As the tumor returns to its pretreatment size, the tumor could become more refractory to treatment. This notion is supported by observations that high rates of apoptosis in cancer cells

correlate with tumor progression [30], whereas upregulation of anti-apoptotic factors suppresses tumor progression and improves prognosis [18, 28]. Alternatively, apoptosis may accelerate tumor generation or progression by preferentially eliminating cells that retain normal apoptosis sensitivity while sparing apoptosis-resistant mutant cells for expansion of mutant clones [57, 58].

A prototype clinical disease depicting pertinence of apoptosis adverse effects. The relevance of detrimental effects of apoptosis to clinical settings is best illustrated with comedo-type ductal carcinoma in situ. Among the several ductal carcinoma in situ (DCIS) subtypes of preinvasive breast cancer, comedo type DCIS or comedo-DCIS accounts for $\sim$10 % of all DCIS and confers the greatest risk for progression and post-operative recurrence [59, 60]. Comedo-DCIS tumors are easily distinguished from other DCIS by the characteristic central comedo-necrosis [61] that results from extensive spontaneous apoptosis [62]. Yet despite abundant cell loss, comedo-DCIS often demonstrates microinvasion, chromosome aneuploidy, and higher proliferation and recurrence rates compared to non-comedo DCIS tumors [63–65]. MCF10DCIS.com human breast cancer cells produce tumors that resemble clinical comedo-DCIS and recapitulate the temporal sequence of progression from in situ to invasive cancer [62, 66, 67]. Using the MCF10DCIS.com model, we have demonstrated that spontaneous apoptosis contributes to the etiology and progression of comedo-DCIS [62] (Fig. 1). Spontaneous MCF10DCIS.com cell loss is activated by the mitochondrial pathway with upregulation of Bax, decreases in Bcl-2 and loss of p53 [62]. Clinical comedo-DCIS, like MCF10DCIS.com cells, show a significant drop in Bcl-2 expression combined with an increase in mutant p53 levels [36]. MCF10DCIS.com cells undergo spontaneous apoptosis in vitro under optimal growth conditions indicating that cell loss in vivo is not due to extraneous factors, but rather they are preprogrammed to undergo apoptosis [62]. The high rates of apoptosis in comedo-DCIS are accompanied by compensatory increases in PCNA-positive cells that are enriched for the $CD44^+/CD24^-$ phenotype (Fig. 2). $CD44^+/CD24^-$ cells have tumor-initiating properties in breast cancer [68]. The $CD44^+/CD24^-$ phenotype is associated with stem cell-like characteristics [69], enhanced potential for invasion [70], radiation resistance [71] and with distinct genetic profiles that correlate with adverse prognosis [72]. These data corroborate that abundant cell loss in comedo-DCIS provides increased opportunities for selecting cell variants with greater malignancy potential. The presence of an intact myoepithelial layer is indicative of DCIS lacking invasive potential. Interestingly, apoptosis is also implicated in the initiation of progression of clinical and MCF10DCIS.com-derived comedo-DCIS tumors as both luminal epithelial and myoepithelial cells are concurrently eliminated by apoptosis [62] (Fig. 2). These data provide clinical support for the adverse effects of apoptosis in neoplastic progression and show that apoptosis is tightly linked with the coordinated and concerted events that lead up to malignant progression (summarized in Fig. 3).

Therapeutic implications. The majority of anti-cancer therapies work by inducing apoptosis in tumors. In tumors undergoing high rates of natural (spontaneous) or therapy-induced apoptosis, increased horizontal transfer of genetic

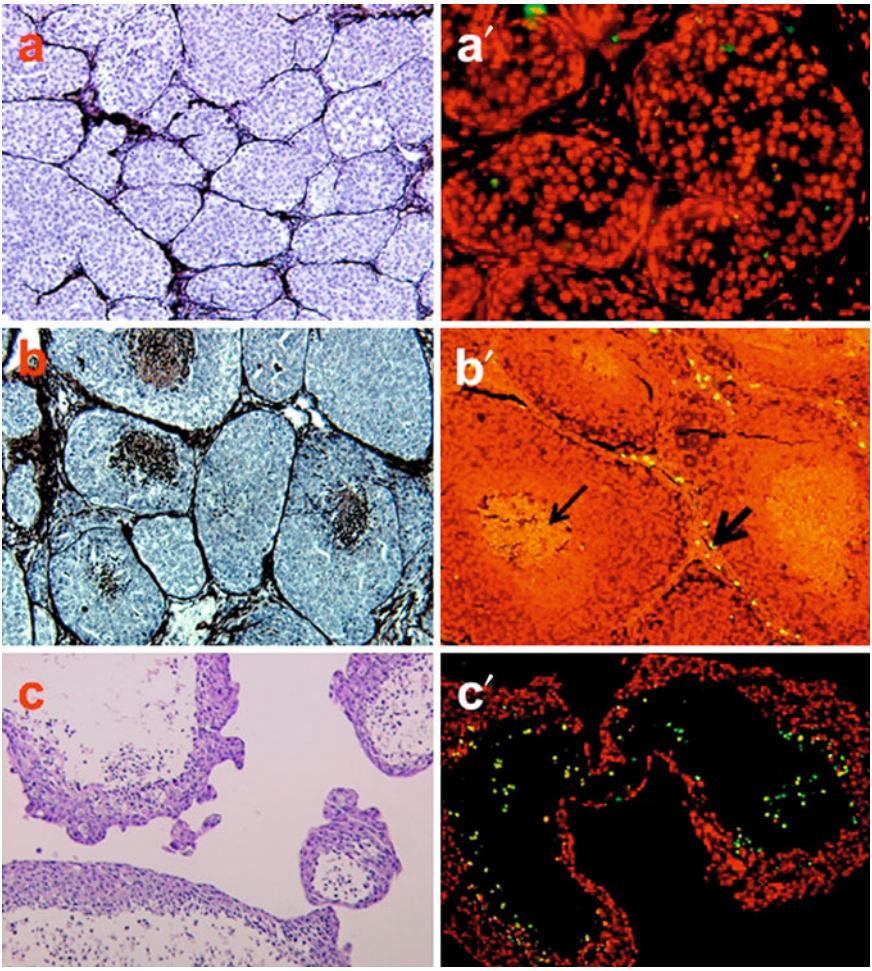

Fig. 1 Progression of MCF10DCIS.com to comedo-DCIS occurs by high rates of spontaneous apoptosis of luminal epithelial and myoepithelial cells. Panels **a**, **a′**: early DCIS lesions; **b**, **b′**: comedo-DCIS lesions; **c**, **c′**: MCF10DCIS.com multicellular tumor spheroids undergoing spontaneous apoptosis in vitro. Panels **a** and **c**, H&E staining; **b**, silver staining; **a′**–**c′**, TUNEL staining. *Thin* and *thick arrows* in **b′** show apoptotic luminal epithelial and myoepithelial cells, respectively

material by cell–cell fusions or apoptotic conversion, cancer stem cell activation, and/or associated inflammatory response may all contribute to the adverse effects of chemo- and radio-therapy, viz., initial tumor regression that is accompanied by quicker relapse, a greater tumor burden and therapy resistance [73]. Thus, it would seem that using therapies that induce apoptosis in tumors experiencing high rates of spontaneous apoptosis is counterintuitive. Inhibiting apoptosis in these tumor tissues may be beneficial as it could decrease compensatory proliferation of

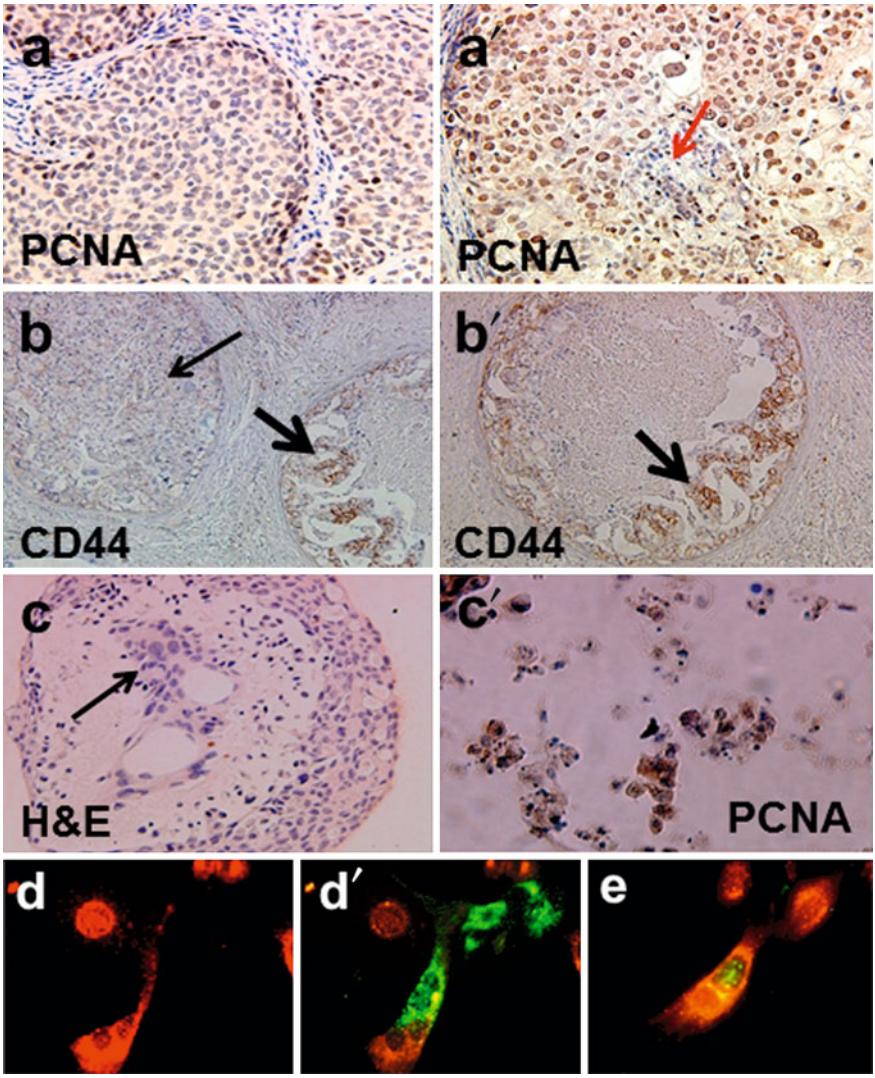

Fig. 2 Progression of MCF10DCIS.com-derived comedo-DCIS lesions is accompanied by increase in PCNA-positive and $CD44^{+}$ ($CD24^{-}$negative, not shown) cells. Panels **a**, **a′** PCNA expression in early and comedo-DCIS lesions, respectively. Note the presence of cells with uniform nuclei in early lesions (*panel* **a**) compared to cells with larger or pleiomorphic nuclei in the vicinity of the apoptotic core (indicated by *arrow*) of advanced lesions (*panel* **a′**). Panels **b**, **b′** CD44 expression. Note low CD44 expression in early lesion (*thin arrow* in **b**) and strong CD44 expression in advanced comedo-DCIS (*thick arrow* in **b** and **b′**). Panels **c**, **c′** H&E and PCNA staining, respectively, of MCF10DCIS.com multicellular tumor spheroids generated in vitro. Note the appearance of large nucleated cells in the apoptotic core (*arrow* in **c**) with PCNA expression (**c′**). Panels **d–e** cell–cell fusion propensity. MCF10DCIS.com cells were prelabeled with DiI (*red*) or DiO (*green*) and cocultured. Note the formation of fused cells in panel **d′** and **e**

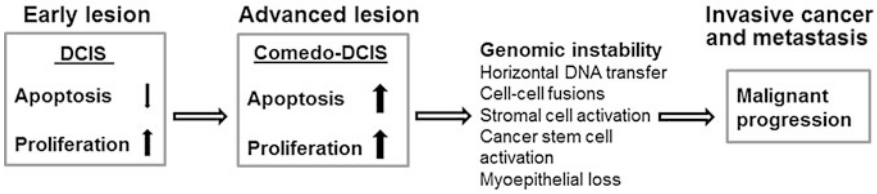

Fig. 3 Schematic model depicting the role of high apoptosis in malignant progression using the comedo-DCIS model of breast cancer

surviving tumor cells, decrease activation and expansion of initiated or cancer stem cells, decrease acquisition of genetic instability by inhibiting horizontal DNA transfer and reduce inflammation. Taking into account the adverse effects of apoptosis in malignant transformation and progression, it would seem that disabling proliferation of stem and nonstem cells in "apoptotic tumors" may be necessary to achieve a longer lasting clinical response.

Conclusion

Contrary to the popular belief that apoptosis acts to safeguard cells from neoplastic conversion and progression, apoptosis must also be viewed as a "not so innocent" participant that actively promotes tumor progression. The latter paradoxical role becomes relevant to tumors experiencing high rates of natural (spontaneous) apoptosis or therapy-induced apoptosis, and must be taken into account when therapy decisions are made.

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Erratum to: Overcoming Drug Resistance Through Elevation of ROS in Cancer

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Erratum to:
Chapter "Overcoming Drug Resistance Through Elevation of ROS in Cancer" in: B. Bonavida (ed.), *Molecular Mechanisms of Tumor Cell Resistance to Chemotherapy*, DOI 10.1007/978-1-4614-7070-0_7

In Chap. 7, Figs. 1, 2, 3 are replaced with high resolution artwork for readability.

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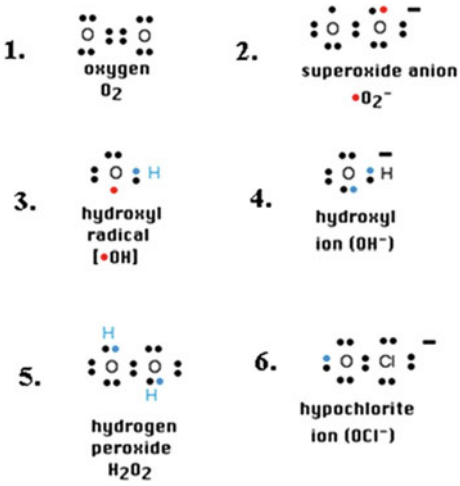

Fig. 1 Most common reactive oxygen species (ROS). *Red* is the unpaired electron which makes an extremely unstable configuration and reacts with other molecules or radicals to achieve stable configuration. The superoxide anion, which is both ion (*2*) and radical (*1*). Hydroxyl radical (*3*) is the most reactive of all radicals. It differs from the hydroxyl ion (*4*) and hydrogen peroxide (*5*). Ions like the hypochlorite ion (*6*) is also very reactive than other ions

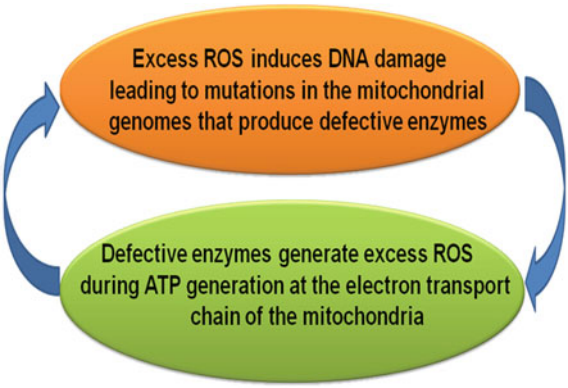

Fig. 2 *ROS generation in cancer cells*. Excess ROS in cancer cells induces mitochondrial DNA damage leading to secondary mutations that virtually produce nonfunctional enzymes and, in turn, generates more ROS through aberrant respiration. The excess ROS production at the mitochondria elevates the ROS level in cancer cells

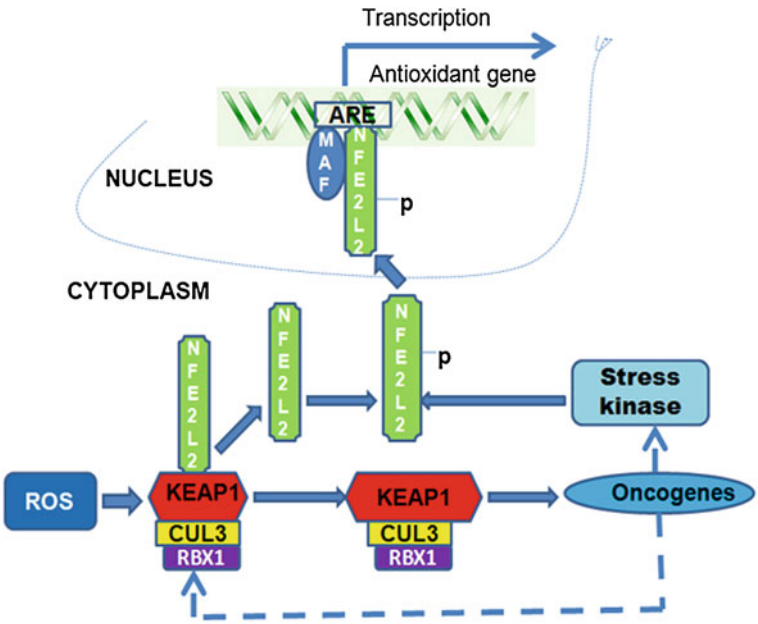

Fig. 3 NFE2L2—KEAP1 *mechanism for maintaining the ROS* ***level in cancer cells***. NFE2L2 remains bound with KEAP1, RBX1 and CUL3 at the cytoplasm. An excess ROS release KEAP1, RBX1 and CUL3 from NFE2L2 and free NFE2L2 is phosphorylated in the cytoplasm. Phosphorylated NFE2L2 travels to the nucleus and binds at the promoter of *ARE* sequence carrying antioxidant genes to facilitate transcription leading to increased antioxidant enzymes production that reduce excess ROS. P, the phosphate group, MAF-oncoprotein v-MAF family members

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