Resistance to Targeted Anti-Cancer Therapeutics 21
Series Editor: Benjamin Bonavida

Ana C. Xavier
Mitchell S. Cairo *Editors*

Resistance to Targeted Therapies in Lymphomas

Springer

Resistance to Targeted Anti-Cancer Therapeutics

Volume 21

Series Editor

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Los Angeles, CA, USA

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ISSN 2196-5501 ISSN 2196-551X (electronic)
Resistance to Targeted Anti-Cancer Therapeutics
ISBN 978-3-030-24423-1 ISBN 978-3-030-24424-8 (eBook)
https://doi.org/10.1007/978-3-030-24424-8

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Lymphomas are a complex group of hematological malignancies that have distinctive etiology, epidemiology, clinical behavior, and response to therapy. For decades, multidrug chemotherapy and/or radiation therapy constituted sole backbones to treat those patients. However, the development of resistance to conventional therapy, due to a multitude of genetic, epigenetic, metabolic mechanisms among others, has contributed to hinder the therapeutic success in a significant proportion of patients.

More recently, remarkable advancements in the lymphoma field, with better understanding of lymphoma cell biology and its microenvironment, have contributed to the development of biologic or "targeted" agents and consequent rapid expansion of the therapeutic landscape. These agents are usually designed and developed based on specific target molecules present in key tumor or microenvironmental cells that once blocked or deregulated can lead to cell death, cell differentiation, or immune system recognition. Many clinical studies have focused on testing targeted agents as monotherapy or in combination with conventional chemotherapy with the goal of improving outcomes or reducing acute or long-term complications associated with therapy. Unfortunately, despite the well-thought rational behind each targeted agent development, transient or unsatisfactory responses to those new therapies are commonly described, suggesting the development of tumor-related or host-related treatment resistance as a culprit to treatment failure. In this book, we will review different classes of targeted drugs that have been developed, approved, or are under investigation in the field of lymphoma therapy. Our focus is to provide a comprehensive review of the mechanisms of action or clinical response of several targeted agents and to discuss mechanisms of tumor-related or host-related resistance and potentially how to overcome resistance. This understanding is crucial considering the dismal outcomes of patients with relapsed or refractory lymphomas. Collectively, the chapters offer a unique opportunity to review, understand, and reflect on the recent successes and pitfalls of the modern lymphoma therapy era.

Birmingham, AL, USA — Ana C. Xavier
Valhalla, NY, USA — Mitchell S. Cairo

Aims and Scope

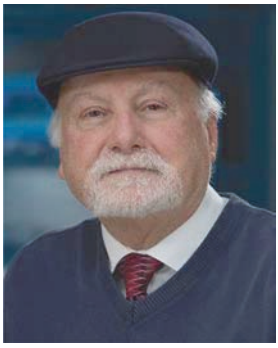
For several decades, treatment of cancer consisted of chemotherapeutic drugs, radiation, and hormonal therapies. Those were not tumor-specific and exhibited several toxicities. During the last several years, targeted cancer therapies (molecularly targeted drugs) have been developed, consisting of immunotherapies (cell-mediated and antibody) drugs or biologicals that can block the growth and spread of cancer by interfering with surface receptors and with specific dysregulated gene products that control tumor cell growth and progression. These include several FDA-approved drugs/antibodies/inhibitors that interfere with cell growth signaling or tumor blood vessel development, promote the cell death of cancer cells, stimulate the immune system to destroy specific cancer cells, and deliver toxic drugs to cancer cells. Targeted cancer therapies are being used alone or in combination with conventional drugs and other targeted therapies.

One of the major problems that arise following treatment with both conventional therapies and targeted cancer therapies is the development of resistance, preexisting in a subset of cancer cells or cancer stem cells and/or induced by the treatments. Tumor cell resistance to targeted therapies remains a major hurdle, and, therefore, several strategies are being considered in delineating the underlining molecular mechanisms of resistance and the development of novel drugs to reverse both the innate and acquired resistance to various targeted therapeutic regimens.

The new series "Resistance of Targeted Anti-cancer Therapeutics" was inaugurated and focuses on the clinical application of targeted cancer therapies (either approved by the FDA or in clinical trials) and the resistance observed by these therapies. Each book will consist of updated reviews on a specific target therapeutic and strategies to overcome resistance at the biochemical, molecular, and both genetic and epigenetic levels. This new series is timely and should be of significant interest to clinicians, scientists, trainees, students, and pharmaceutical companies.

Los Angeles, CA, USA — Benjamin Bonavida

Series Editor Biography



Dr. Benjamin Bonavida, Ph.D. (Series Editor), is currently Distinguished Research Professor at the University of California, Los Angeles (UCLA). His research career, thus far, has focused on basic immunochemistry and cancer immunobiology. His research investigations have ranged from the mechanisms of cell-mediated killing, sensitization of resistant tumor cells to chemo-/immunotherapy, characterization of resistant factors in cancer cells, cell-signaling pathways mediated by therapeutic anticancer antibodies, and characterization of a dysregulated NF-κB/Snail/YY1/RKIP/PTEN loop in many cancers that regulates cell survival, proliferation, invasion, metastasis, and resistance. He has also investigated the role of nitric oxide in cancer and its potential antitumor activity. Many of the above studies are centered on the clinical challenging features of cancer patients' failure to respond to both conventional and targeted therapies. The development and activity of various targeting agents, their modes of action, and resistance are highlighted in many refereed publications.

Acknowledgments

The Series Editor acknowledges the various assistants who have diligently worked in both the editing and formatting of the various manuscripts in each volume. They are Leah Moyal, Kevin Li, and Anne Arah Cho.

Acknowledgments

The editors would like to thank the dedication and valuable efforts of Erin Morris, RN, during the preparation of this volume and gratefully acknowledge the contributions of each author.

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About the Editors

Ana C. Xavier Dr. Ana C. Xavier is an Associate Professor at the Division of Hematology/Oncology, Department of Pediatrics, University of Alabama at Birmingham (Birmingham, Alabama). She received her medical degree from the University of Sao Paulo, Brazil, and completed her pediatric residency at the Medical University of South Carolina and Pediatric Hematology/Oncology Fellowship Training at the Wayne State University. She is Board Certified in Pediatrics and Pediatric Hematology/Oncology and currently serves as Associate Program Director of the Pediatric Hematology/Oncology Fellowship Program at the University of Alabama at Birmingham. She has authored numerous peer-reviewed manuscripts in highly reputed international journals and has presented several abstracts at various national and international conferences. She holds memberships with the American Academy of Pediatrics, American Society of Hematology, American Society of Pediatric Hematology/Oncology and Children's Oncology Group. Her clinical practice includes both pediatric oncology, and her research interest focuses on the treatment of pediatric patients with lymphoma.

Mitchell S. Cairo Dr. Cairo is currently the Associate Chairman and Professor (with tenure) in the Department of Pediatrics at New York Medical College (NYMC). His additional current leadership positions include being the Chief of the Division of Pediatric Hematology, Oncology, and Stem Cell Transplantation, Program Director of the Adult and Pediatric BMT Program, Director of the Childhood and Adolescent Cancer and Blood Disease Center, Medical and Scientific Director of the GMP Cellular and Tissue Engineering Laboratory at Westchester Medical Center (WMC), Medical Director of the WMC Hematotherapy Program, and Co-chair of the WMC Cancer Committee. His additional academic appointments include being a Professor of Medicine, Pathology, Microbiology and Immunology, Cell Biology and Anatomy, and Public Health at NYMC. Briefly, his past education includes his undergraduate studies at the University of Wisconsin, Madison, WI, graduating in 1972 with a BA and election to Phi Beta Kappa. He received his medical school training at the University of California, San Francisco (USCF), graduating in 1976 with an election to Alpha Omega Alpha (AOA). He trained as a Pediatric Resident at the UCLA Harbor General from 1976 to 1978 under the mentorship of Joseph St. Geme, MD, and then a Chief Residency in Pediatrics from 1978 to 1979 at the UCSF under the mentorship of Melvin Grumbach, MD. He completed a Pediatric Hematology-Oncology Fellowship as an American Cancer Society Fellow at Indiana University from 1979 to 1981 under the mentorship of Robert Baehner, MD. He joined the Faculty of Children's Hospital of Orange County (CHOC) in 1982 and established the BMT/Stem Cell Transplant Program there in 1985 as Director of Blood and Marrow Transplantation. Also, at CHOC, he was the Principal Investigator (PI) for Children's Cancer Group and PI of the Cord Blood Collection Center and Cord Blood Transplant Center under an NHLBI award. In 1997, he was recruited to Georgetown University where he became a Professor of Pediatrics, Medicine, and Pathology, Chief of the Division of Stem Cell Transplantation and Cellular and Gene Therapy, Director of the Adult and Pediatric Bone Marrow Transplantation Program at the Lombardi Cancer Center, and Medical Director of the NHLBI Cord

Blood Collection Center and Cord Blood Bank. In 2000, he was recruited to Columbia University and was a Professor of Pediatrics, Medicine, and Pathology, Director of the Division of Blood and Marrow Transplantation, Member of the Executive Committee of the Department of Pediatrics, Medical Director of the National Marrow Donor Unrelated Transplant Program, Chief of the Division of Pediatric Blood and Marrow Transplantation, and Member of the Executive Steering Committee of the Morgan Stanley Children's Hospital of New York-Presbyterian Hospital. In 2011, he was recruited to NYMC and WMC. He has over 410 peer-reviewed publications, over 1200 national and international abstract presentations, and over 50 book chapters and edited 2 textbooks. He is on the Editorial Board of *British Journal of Hematology*, *Blood Reviews*, and *Cell Transplantation* and Past Editorial Board Member of *Bone Marrow Transplantation* and *Experimental Hematology*. He is a regular NCI Reviewer for PPG and Spore applications. He has been a Member of the CCG/COG Bone Marrow Transplantation, now Cell Therapy Committee, for the last 20 years. He was the Chair of the ISCT Immuno-Gene Therapy Committee and currently is the ISCT North America Vice President Elect and Past Co-chair of the CIBMTR Cellular Therapy Committee. He is also a long-standing Member of the PBMTC Executive and Steering Committee and is an International Leader in the Biology and Treatment of Childhood and Adolescent Lymphomas and Leukemias, Stem Cell Transplantation, Developmental Therapeutics, Experimental Hematopoiesis and Immunology, Tumor Immunology and Biology, and Stem Cell Biology and Regenerative Therapy. He was a Pioneer in the use of cord blood stem cells for treating pediatric malignant and nonmalignant disease and the use of cord blood stem cells for potential regenerative therapy and haploidentical stem cell transplantation for patients with sickle cell disease. He is a Member of a number of national and international societies related to both Pediatrics and Hematology/Oncology/Stem Cell Transplantation, including elected to the Society of Pediatric Research (SPR) and the American Pediatrics Society (APS), and Member of AAP, ESPR, and hematology and oncology and stem

cell transplantation societies such as ASH, ASCO, ASBMT, CIBMTR, AAI, ISEH, AACR, ASPHO, SIOP, PBMTC, and COG. In summary, he has been an International Leader in basic, translational, and clinical research in childhood, adolescent, and young adults with emphasis in stem cell transplantation, stem cell biology, lymphoma, tumor immunology, and developmental therapeutics.

Chapter 1
Mechanisms of Glucocorticoid Response and Resistance in Lymphoid Malignancies

Lauren K. Meyer and Michelle L. Hermiston

Abstract Glucocorticoids (GC) are an integral component of multi-agent therapy regimens for a wide variety of lymphoid malignancies due to their potential effects to induce apoptosis in cells of the lymphoid lineage. Despite their clinical utility, *de novo* and acquired resistance to GC is a significant clinical problem that contributes to inferior outcomes for many of these diseases. This review summarizes what is currently known about mechanisms of GC resistance in lymphoid malignancies, with a particular focus on novel therapeutic strategies currently in preclinical or clinical development that are rationally-designed to overcome GC resistance and improve clinical outcomes.

Keywords Apoptosis · Glucocorticoid · Leukemia · Lymphoma · Metabolism MicroRNA · Drug resistance · Signal transduction

Abbreviations

2-DG	2-Deoxy-D-Glucose
3'UTR	3' Untranslated Region
B-CLL	B-Cell Chronic Lymphocytic Leukemia
BFM	Berlin-Frankfurt-Munster
cAMP	Cyclic Adenosine Monophosphate
CDK	Cyclin Dependent Kinase
ChIP-Seq	Chromatin Immunoprecipitation with Sequencing
CHOP	Cyclophosphamide, Adriamycin, Vincristine, and Prednisone
DBD	DNA Binding Domain
DEX	Dexamethasone

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A. C. Xavier, M. S. Cairo (eds.), *Resistance to Targeted Therapies in Lymphomas*, Resistance to Targeted Anti-Cancer Therapeutics 21,
https://doi.org/10.1007/978-3-030-24424-8_1

DLBCL	Diffuse Large B-Cell Lymphoma
EFS	Event-Free Survival
GC	Glucocorticoid(s)
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
GST	Glutathione *S*-Transferase
HDAC	Histone Deacetylase
HSD	Hydroxysteroid Dehydrogenase
LBD	Ligand Binding Domain
LOH	Loss of Heterozygosity
MAPK	Mitogen Activated Protein Kinase
miR	MicroRNA
MRD	Minimal Residual Disease
NCoR	Nuclear Co-Receptor
NHL	Non-Hodgkin Lymphoma
NTD	N-Terminal Transactivation Domain
PDE	Phosphodiesterase
PDX	Patient-Derived Xenograft
PGR	Prednisone Good Responder
PKA	Protein Kinase A
PPR	Prednisone Poor Responder
RT-PCR	Real-Time Polymerase Chain Reaction
T-ALL	T-Cell Acute Lymphoblastic Leukemia
TCR	T-Cell Receptor
WBC	White Blood Cell

Introduction

For decades, glucocorticoids (GCs) have been a key component of therapy for the treatment of lymphoid malignancies and are widely used in both frontline and salvage therapy regimens [1, 2]. In many of these cancers, the response to GC therapy is a strong prognostic indicator that is related to both overall and event-free survival (EFS) rates [1, 3, 4]. In particular, patients with acute lymphoblastic leukemia (ALL) treated on Berlin-Frankfurt-Munster (BFM) protocols can be classified as having a prednisone good response (PGR) or a prednisone poor response (PPR), defined based on the response to an upfront 1 week window of monotherapy consisting of the GC prednisone. In early ALL-BFM protocols, patients with a PPR had significantly inferior outcomes relative to patients with a PGR [1]. These data indicate that therapeutic strategies to overcome GC resistance may significantly improve patient outcomes. The objective of this review is to highlight key concepts regarding GC resistance in lymphoid malignancies, with a specific focus on therapeutic strategies designed to overcome GC resistance.

Mechanism of Glucocorticoid Action

GCs are a class of steroid hormones that bind to the GC receptor (GR). In the absence of endogenous or exogenous GC ligand, GR is largely retained in the cytoplasm through its association with a variety of molecular chaperone proteins, including HSP70 and HSP90 [5]. Upon ligand binding, GR undergoes a conformational change that promotes translocation of the GC-GR complex to the nucleus, where it associates with DNA sequences known as GC response elements (GREs). These GREs function as enhancer elements to modulate the activity of associated gene promoters, which in turn mediate the activation or repression of target gene expression (Fig. 1.1) [6]. These effects of GCs are highly tissue-specific due to differences in GRE binding patterns and transcriptional activities in different cell types. Importantly, while GCs exert pro-survival effects in many tissues, they potently induce cell death in cells of the lymphoid lineage [7], underlying their importance in the treatment of lymphoid malignancies.

While there is little overall consensus regarding the specific components of the GR-associated transcriptome that mediate the effects of GCs on lymphoid cells, many groups have demonstrated that activation of the intrinsic apoptotic pathway is required for GC-induced cell death [7]. Consistent with this idea, the concept of a "BCL2 rheostat" has been proposed whereby modulation of both the pro-and anti-apoptotic components of the intrinsic apoptotic pathway results in an altered

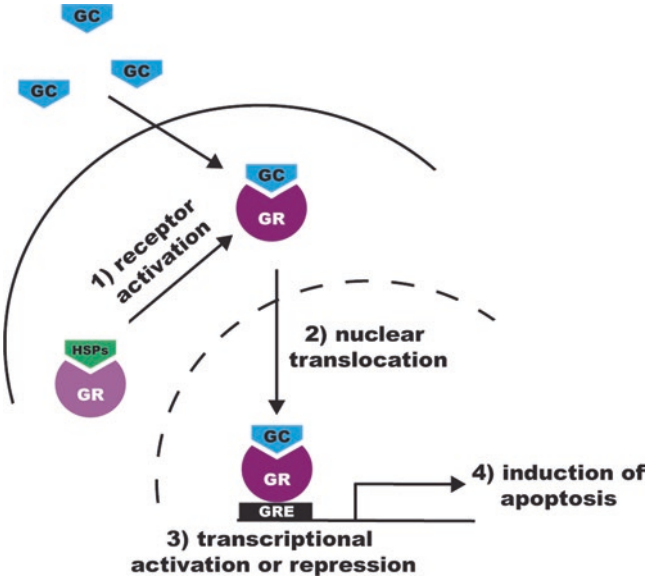

Fig. 1.1 Mechanisms of GC Action. GCs bind to a cytoplasmic GR (1), which induces translocation of the GC/GR complex to the nucleus (2). This complex binds to GREs to induce or repress transcription (3). In cells of the lymphoid lineage, this transcriptional activity alters the expression of components of the intrinsic apoptotic pathway, resulting in apoptosis (4)

apoptotic threshold that leads to cell death. Through an analysis of both basal and GC-induced expression of components of the intrinsic apoptotic pathway in primary ALL cells, Ploner et al. identified key expression patters that are associated with GC-induced apoptosis. Specifically, they noted potent induction of the pro-apoptotic family members *BIM* and *BMF*, and demonstrated that loss of expression of either of these proteins is sufficient to decrease GC sensitivity. Conversely, they demonstrated that overexpression of anti-apoptotic family members, including *BCL2*, *BCL-XL*, and *MCL1*, impairs GC-induced apoptosis, an effect that is reversed upon experimental silencing of these genes [8]. Taken together, these data suggest that coordinate modulation of both pro-and anti-apoptotic family members contribute to GC-induced apoptosis. Jing et al. further elucidated the importance of such a BCL2 rheostat through an analysis of ALL patient derived xenograft (PDXs). Using chromatin immunoprecipitation with sequencing (ChIP-seq), this group identified a novel GR binding site within an intronic region of the *BIM* gene. When this region was mutated to abolish GR binding, GC-induced upregulation of *BIM* expression was lost and GC sensitivity was significantly decreased, providing further evidence that GR-mediated upregulation of *BIM* is required for GC sensitivity. Furthermore, this study elucidated a series of GR-mediated transcriptional events that lead to downregulation of *BCL2* expression, and found that these events were also required for effective GC-induced apoptosis [9]. Given the importance of the intrinsic apoptotic pathway in mediating GC sensitivity in lymphoid cells, it is not surprising that while diverse mechanisms of GC resistance have been elucidated in lymphoid malignancies, these mechanisms largely converge on a failure to appropriately modulate the intrinsic apoptotic pathway.

Mechanisms of Glucocorticoid Resistance

GR Intrinsic Mechanisms of GC Resistance

NR3C1 Mutations

The GR protein, which is encoded by the *NR3C1* gene, is comprised of three major functional domains: the ligand binding domain (LBD), the DNA biding domain (DBD), and the N-terminal transactivation domain (NTD), which interacts with the transcriptional machinery and the transcriptional coregulators to mediate the effects of GR on gene expression [5]. Mutations in each of these domains have been identified in the context of familial and sporadic generalized GC resistance, where they lead to complete or partial insensitivity to target tissues to both endogenous and exogenous GCs [10]. In addition to generalized GC resistance, localized GC resistance that is attributable to *NR3C1* mutations has been reported in a number of disease contexts, including asthma and autoimmune diseases [11]. Given the precedent for GC resistance mediated by GR mutations, many groups have hypothesized that pre-existing mutations in the GR gene, or mutations acquired over the course of GC

therapy, may contribute to *de novo* or acquired GC resistance in lymphoid malignancies. Much of this work has focused on CCRF-CEM cells, a cell line model of human T-cell ALL (T-ALL) that has been studied extensively in the context of GC sensitivity and resistance. Early studies involving CCRF-CEM identified considerable heterogeneity in the clonal composition of the cell line, leading to the subsequent isolation and characterization of a number of subclonal cell lines with varying degrees of GC sensitivity [12]. In an analysis of the parental CCRF-CEM cell line, a heterozygous mutation has since been identified in the LBD, and functional studies demonstrated impaired functionality of this mutant allele [13–16]. However, this same heterozygous mutation has since been identified in both GC sensitive and GC resistant subclones derived from the parental CCRF-CEM cell line, suggesting that additional events are required to confer GC resistance. Consistent with this idea, it has been shown that the GC resistant subclones derived form the GC sensitive parental cell line express this mutant allele in the absence of a wild-type allele, resulting in complete impairment of GR activity [13]. Interestingly, this LBD point mutation was identified in biopsy tissue taken after the initiation of treatment from the patient from whom CCRF-CEM cells were derived, suggesting that it was acquired *in vivo* and was likely selected for over the course of GC treatment [17].

Similar to CCRF-CEM cells, Jurkat cells, another human T-ALL cell line, are heterozygous for a mutation that impairs GR transcriptional activity. Unlike CCRF-CEM cells however, Jurkat cells also express low basal levels of GR and fail to induce expression of GR upon GC exposure, resulting in profound GC resistance [18].

Based on this evidence supporting a role for *NR3C1* mutations as a cause of GC resistance in cultured cell lines, multiple groups have conducted studies to determine whether such mutations cause clinically relevant GC resistance in patients receiving GC therapy for the treatment of lymphoid malignancies. In an analysis of a panel of cell lines derived from paired diagnostic and relapsed samples taken from pediatric patients with ALL, Beesley et al. identified significant variability in GC sensitivity. Upon sequencing the *NR3C1* gene in these cell lines, this group identified a number of polymorphisms, all of which had previously been shown to have a negligible effect on GC sensitivity [11], but no deleterious mutations. This finding led them to conclude that *NR3C1* mutations are not a common mechanism of naturally-acquired GC resistance [19]. Consistent with these findings, sequencing of *NR3C1* in a larger cohort of diagnostic pediatric ALL samples revealed a similar distribution of polymorphisms, but these polymorphisms failed to correlate with the clinical response to prednisone therapy and did not occur at a significantly higher rate than previously reported in the general population [20], supporting the conclusion that *NR3C1* mutations are not a common cause of *de novo* GC resistance. However, there have been a number of reports demonstrating the presence of deleterious *NR3C1* mutations that are undetectable at diagnosis but are significantly enriched at the time of disease relapse, suggesting that the acquisition of such mutations may confer acquired GC resistance [21–23]. Taken together, the existing data suggest that *NR3C1* mutations are a relatively minor cause of GC resistance in human lymphoid malignancies, particularly at the time of diagnosis, but may be more important in the context of relapsed disease following the selective pressure of exposure to GC therapy.

Modulation of GR Expression and Function

In addition to GR mutations, expression levels of GR have been evaluated as a potential biomarker for GC sensitivity and resistance. Using large cohorts of diagnostic ALL samples, early clinical data suggested that the absolute number of GRs in lymphoblasts is positively correlated with the clinical response to GC monotherapy [24], the likelihood of disease remission [25], and with 5-year EFS rates [26]. More recently, GR expression has been shown to carry prognostic significance specifically in the context of pediatric B-cell ALL (B-ALL) harboring the *ETV6*/*RUNX1* fusion oncogene. In these patients, deletions of *NR3C1* resulting in loss of GR protein expression are associated with increased minimal residual disease (MRD) and with risk of relapse [27]. Despite these findings, other studies have failed to identify a clinically meaningful relationship between basal GR expression at diagnosis and the clinical response to GC therapy. In an analysis of GR protein expression in diagnostic samples taken from patients treated on ALL-BFM protocols, there was no significant difference in GR expression between PPR and PGR patient groups [28], suggesting that basal GR expression may be an unsuitable biomarker for predicting GC sensitivity. However, it has been shown that in lymphoid cells, exposure to GCs results in autoinduction of GR expression mediated by a direct transcriptional effect of GR [29], and multiple studies have demonstrated that expression levels of GR after autoinduction, rather than basal expression levels, are required for a GC response and may be a better predictor of GC sensitivity. Using a titratable expression system in human T-ALL cell line, Ramdas et al. demonstrated that basal levels of GR may be insufficient to confer GC sensitivity, but that levels comparable to those achieved following GC exposure and subsequent autoinduction of GR expression are sufficient to mediate GC-induced apoptosis [30]. Consistent with these data, a failure to autoinduce GR expression upon GC exposure has been implicated in GC resistance in Jurkat T-ALL cells [18] and in multiple myeloma cell lines [31].

Several studies have also identified other genetic and epigenetic events that lead to altered GR expression levels, and may therefore contribute to GC resistance. For example, loss-of-function mutations in the E3 ubiquitin ligase *FBXW7* have been associated with a favorable prognosis and an early response to GC therapy in ALL [32, 33]. A later study demonstrated that *FBXW7* mediates the ubiquitination and subsequent proteasomal degradation of GR, leading to insufficient GR levels to mediate GC-induced apoptosis. This same study found that inactivation of *FBXW7* in an *in vitro* system was sufficient to restore GR expression and consequently, GC sensitivity [34]. The NALP3 inflammasome has also been implicated as a modulator of cellular GR levels. In an analysis of GC resistant primary ALL samples, it was found that decreased promoter methylation of *CASP1* and *NLRP3* resulted in increased expression of the NALP3 inflammasome, and that the associated increase in caspase 1 activity caused increased cleavage of GR protein, leading to an attenuated GC response mediated by a loss of GR protein expression [35]. Therefore, while basal GR expression has not proven to be a tractable biomarker with clinical utility for predicting GC sensitivity, altered levels of GR expression may nonetheless contribute to a poor GC response.

In addition to GR expression levels, post-transcriptional processing of the GR mRNA results in multiple GR isoforms, which may also play a role in dictating GC sensitivity. GRα is the most abundant GR isoform and has been shown to mediate the pro-apoptotic effects of GCs in lymphocytes. Exon 9 of NR3C1 encodes a portion of the LBD, and alternating splicing of this exon distinguishes the GRα isoform from the GRβ isoform [5]. The GRβ isoform does not bind GCs and does not have transcriptional activity, thereby impeding its pro-apoptotic activity [36]. Additionally, alternative splicing involving the intron between exons 3 and 4 gives rise to the GRγ isoform, which has an altered DBD. Therefore, GRγ retains ligand binding capacity but has limited transcriptional activity [5]. Finally, alternative splicing involving the LBD results in the production of the GR-A and GR-P isoforms, both of which fail to bind ligand [5]. As a result of the impaired activity of multiple GR isoforms, many groups have studied the relationship between GC sensitivity and the relative expression and distribution of these isoforms in a variety of lymphoid malignancies. One of the earliest such studies focused on a patient with chronic lymphocytic leukemia (CLL) who was found to have generalized GC resistance. An analysis of the expression pattern of GR isoforms in cells taken from this patient demonstrated decreased GRα expression and increased GRβ expression, resulting in an altered ratio between the two isoforms [37]. Given the dominant negative effect of GRβ on GRα, this group concluded that the altered ratio may contribute to the generalized GC resistance observed in this patient. Consistent with these findings, an analysis of 23 diagnostic ALL samples revealed an inverse correlation between the GRβ/GRα ratio and the number of apoptotic cells following *in vitro* exposure to prednisolone, further indicating that high expression of GRβ impairs GC sensitivity [38]. Relative to diagnostic samples, relapsed ALL samples have also been shown to have a decreased mRNA to protein ratio of GRα [39]. Similarly, GRγ expression has been shown to be increased in PPR patients relative to PGR patients, which is consistent with the idea that GRγ expression might impair the transcriptional activity of GRα, leading to an inferior GC response [40].

Further regulation of GR activity is mediated by the chaperone protein systems that interact with GR, the two most important of which are the HSP70 and HSP90 systems. These chaperones assist with maintaining GR in a conformation in which it is competent for ligand binding and they facilitate the subsequent nuclear translocation of ligand-bound GR [41]. Given the central role of chaperone proteins in modifying GR activity, several groups have hypothesized that aberrant expression or activity of these chaperone systems could contribute to GC resistance in lymphoid malignancies. However, in an analysis of PPR and PGHR patients treated on ALL–BFM trials, there was no correlation between *in vivo* GC sensitivity and HSP90 expression [42]. In a more in-depth analysis looking at mRNA expression of key chaperone proteins in GC sensitive versus GC resistant ALL cells, there were also no meaningful differences in transcript expression [43]. While this finding does not exclude the possibility that differences in protein expression of these chaperones may underlie differences in GC sensitivity, these studies suggest that chaperone proteins likely do not play a significant role in clinical GC resistance in lymphoid malignancies.

GR Extrinsic Mechanisms of GC Resistance

Epigenetic Regulation of GR Activity

Changes in GR target gene expression require the association of ligand-bound GR with a GRE [6]. Some of the cell-and tissue-specificity of GCs may be mediated by differences in chromatin accessibility, as GR binding has been shown to occur predominantly at accessible chromatin sites [44]. Given the requirement for pre-existing chromatin accessibility, a number of groups have assessed the role for an altered epigenetic landscape as a mediator of GC resistance. Chromatin accessibility is maintained in part through the activity of the SWI/SNF [44], and decreased expression of core components of this complex correlate with the occurrence of GC resistance in ALL cells [45]. In an analysis of gene expression and DNA methylation patterns in matched pairs of pediatric B-ALL samples obtained at the time of diagnosis and at relapse, Hogan et al. identified a distinct pattern of gene expression associated with relapse and found that this gene expression pattern co-occurred with increased promoter methylation [46]. With the addition of the DNA methyltransferase inhibitor decitabine, this relapse-specific gene expression pattern could be reverted, allowing for re-expression of hypermethylated genes. Exposure to decitabine, along with the histone deacetylase (HDAC) inhibitor vorinostat, resulted in significant potentiation of GC-induced apoptosis [47], suggesting that modification of the epigenetic landscape may facilitate GR-mediated changes in gene expression that lead to apoptosis. Similarly, it was shown that elevated expression of a number of HDAC genes is common in patients who have a PPR [48]. Consistent with these findings, Jones et al. reported a high frequency of deletions of *TBL1XR1,* a component of the nuclear receptor corepressor (NCoR) complex, in patients with B-ALL. These deletions stabilize NCoR, which represses GR activity by decreasing its recruitment to target gene loci and by recruiting HDAC3 to further promote inhibition of target gene expression. Treating these cells with an HDAC inhibitor was sufficient to restore GC sensitivity [49] Collectively, these data suggest that GC sensitivity is mediated in part by a permissive epigenetic landscape, and that the use of epigenetic modulators may represent a therapeutic strategy to enhance GC sensitivity in lymphoid malignancies that are associated with an altered epigenetic landscape.

Signal Transduction

Dysregulated signal transduction is a hallmark feature of many lymphoid malignancies including T-ALL [50], B-ALL [51], and non-Hodgkin lymphoma (NHL) [52]. Importantly, the downstream effectors of these signal transduction pathways exhibit known cross-talk with GR signaling and transcriptional activity [53]. As a result of these interactions, aberrant regulation of these signal transduction pathways is an important cause of GC resistance in lymphoid malignancies and significant

attention has been devoted to the use of targeted signal transduction inhibitors as a strategy to overcome GC resistance.

Cyclic Adenosine Monophosphate (cAMP) Signaling

cAMP is a second messenger molecule that initiates signaling cascades responsible for mediating a variety of immune cell functions. cAMP is generated through the catalytic activity of adenylate cyclases and is degraded by a family of enzymes called phosphodiesterases (PDEs) [54]. It has long been known that in addition to GCs, activation of cAMP decreases lymphoid cell proliferation and induces apoptosis [55]. Furthermore, it has been shown in T-cell lines that stimulation of cAMP signaling has a synergistic effect to induce cell death when combined with the GC dexamethasone (DEX) [56], and that cAMP and GCs likely converge to promote the upregulation of BIM expression [57, 58], thereby facilitating the induction of apoptosis. Given the pro-apoptotic effects of cAMP and the effects of PDEs to decrease the cellular pool of cAMP, significant attention has been devoted to the development of PDE inhibitors [59], and a number of groups have evaluated the efficacy of PDE inhibitors as a means of overcoming GC resistance in lymphoid malignancies. In the CCRF-CEM cell line, both a non-specific PDE inhibitor and rolipram, a PDE4-specific inhibitor, significantly potentiated DEX-induced apoptosis [60]. In primary CLL cells, rolipram synergized with GCs to induce apoptosis, and this effect was associated with increased GR-mediated transcriptional activity [61]. Furthermore, in these same cells, it was found that rolipram exposure resulted in an increase in both transcript and protein expression of GRα [62]. In patients, PDE4 overexpression has been observed in a cohort of primary diffuse large B-cell lymphoma (DLBCL) samples. Consistent with the data in leukemia cell lines, inhibition of PDE4 in DLBCL cells was sufficient to restore GC sensitivity [63]. Finally, in a large-scale gene expression analysis of primary DLBCL samples obtained from patients who received treatment with cyclophosphamide, adriamycin, vincristine, and prednisone (CHOP), elevation of PDE4 expression was enriched in patients with fatal or refractory disease relative to patients who were cured with CHOP therapy [64]. Taken together, these data suggest that alterations in cAMP pathway signaling may contribute to GC resistance in lymphoid malignancies and that therapeutic targeting of this pathway may have clinical utility.

Mitogen Activated Protein Kinase (MAPK) Signaling

The three best studied MAPKs are p38, ERK, and JNK, all of which become activated downstream of a signaling cascade induced by cellular exposure to mitogenic stimuli [65]. Each of these MAPKs has been shown to modulate GC sensitivity, resulting in a considerable number of studies devoted to investigating the therapeutic potential of MAPK pathway signaling modulators as a means of enhancing GC sensitivity. In GC resistant clones derived from the parental CCRF-CEM cells, inhibition of p38 MAPK decreased DEX sensitivity, while inhibition of ERK activity increased sensitivity. These data implicate p38 as a positive regulator of GC activity and ERK as a negative regulator of GC activity [66], suggesting that distinct arms of the MAPK signaling cascade interact differently with the GR pathway. Consistent with these findings, it has been shown that exposing

CCRF-CEM cells to DEX results in increased phosphorylation and activation of p38, one substrate of which is GR itself. Specifically, this study demonstrated that p38 mediates Ser-211 phosphorylation of GR [67], which has been shown to increase the transcriptional activity of GR [5], thereby providing a mechanistic explanation for the positive effect of p38 activity on GC sensitivity. Another study demonstrated that inhibition of p38 in CCRF-CEM cells resulted in decreased induction of BIM expression upon DEX exposure, leading to an attenuated apoptotic response and suggesting that p38 might further contribute to GC sensitivity by enabling the upregulation of BIM expression [68].

Other studies have focused on elucidating the molecular basis for the inhibitory effect of ERK signaling on GC sensitivity. Importantly, ERK has been shown to phosphorylate BIM, preventing it from interacting with other members of the intrinsic apoptotic pathway to induce apoptosis [69]. To determine whether this mechanism contributes to ERK-mediated GC resistance, Rambal et al. demonstrated in ALL cell lines and primary patient samples a synergistic interaction between a MEK inhibitor and DEX, with simultaneous exposure to both agents resulting in increased BIM expression due to a reduction in ERK-mediated BIM phosphorylation [70]. In addition to ERK, JNK activation has previously been implicated as a negative regulator of GC sensitivity. In contrast to p38, JNK is known to catalyze an inhibitory phosphorylation of GR, resulting in decreased transcriptional activity [71]. Jones et al. further established the role of ERK and JNK as negative regulators of GC sensitivity through an shRNA screen designed to identify genes that modify prednisolone sensitivity in B-ALL cell lines. Interestingly, this screen identified *MEK2*, which activates ERK, and *MEK4*, which activates JNK, as important candidate GC resistance genes. Through a variety of functional studies, the authors demonstrated that loss of MEK2 expression induced generalized chemosensitivity, including to GCs, through a p53-dependent mechanism and that loss of MEK4 increased expression of GR, leading to improved GC sensitivity. Furthermore, they demonstrated the clinical relevance of these findings by assessing ERK activity in paired diagnostic and relapse samples from patients with B-ALL and found increased levels of phosphorylated ERK in the relapsed samples [72], consistent with the idea that aberrant activation of ERK signaling may contribute to GC resistance. Given the large number of past and current clinical trials conducted in a wide variety of malignancies [73], the addition of small molecules that modulate MAPK pathway activity may be a feasible strategy for overcoming GC resistance in some lymphoid malignancies.

PI3K/AKT/mTOR Signaling

The PI3K/AKT/mTOR pathway is another signal transduction pathway that is commonly dysregulated in lymphoid malignancies and represents a potential therapeutic target for strategies aimed at overcoming GC resistance. In a recent study involving a large cohort of pediatric T-ALL samples, *AKT1* and *PTEN* mutations were two of only a handful of genetic lesions that had a univariable association with relapse [50], suggesting that mutational activation of this pathway may play a role in therapy resistance, including to GCs. In another analysis of primary B-ALL

samples, patients with increased phosphorylated AKT at diagnosis had a significantly inferior response to steroid-containing induction therapy and had decreased overall and relapse-free survival [74]. These studies provide correlative evidence for the role of aberrant PI3K/AKT/mTOR pathway activity in GC resistance. To more directly assess a mechanistic basis for this relationship, Piovan et al. demonstrated using co-immunoprecipitation that AKT1 binds to and phosphorylates GR on Ser-134, a phosphorylation event that impairs nuclear translocation of ligand-activated GR. Using the *PTEN*-null CCRF-CEM cell line, the authors demonstrated through both *in vitro* and *in vivo* studies that combined treatment with GCs and the AKT inhibitor MK2206 is sufficient to reverse GC resistance [75]. One class of proteins that has been found to cooperate with AKT to modulate GC activity is the 14–3–3 class of phospho-serine/threonine binding proteins, which regulate the subcellular localization of proteins with phosphorylated serine or threonine residues, including phosphorylated GR [76]. Consistent with this function, the 14–3–3σ protein interacts with GR upon AKT1-mediated Ser-134 phosphorylation, resulting in impaired nuclear translocation of ligand-bound GR and leading to reduced transcriptional activity in the presence of GCs [76, 77]. Similarly, it has been shown that more proximal inhibition of this pathway with a PI3K inhibitor results in synergy when combined with GCs, both *in vitro* and in an *in vivo* xenograft model [78]. In B-ALL cell lines and primary diagnostic patient samples, PI3K inhibition augmented nuclear translocation of ligand-activated GR through a reduction in Ser-134 phosphorylation [79], further confirming the effect of aberrant PI3K/AKT pathway inhibition to promote cytoplasmic retention of GR and prevent transcriptional activation.

One important downstream effector of PI3K/AKT pathway activation is mTOR [80], and many groups have studied the role of aberrant mTOR activation as a mediator of GC resistance in lymphoid malignancies. Using a chemical genomics approach, Wei et al. compared a large number of drug-associated gene expression profiles with the gene expression signature of GC sensitive and resistant ALL cells. Through this analysis, they determined that the changes in gene expression associated with exposure to the mTOR inhibitor rapamycin matched that associated with GC sensitive cells, suggesting that rapamycin may show efficacy by altering the gene expression pattern in GC resistant cells to better mimic that of GC sensitive cells. They further demonstrated that exposure to rapamycin sensitized cells to GCs through a mechanism involving downregulation of expression of the anti-apoptotic protein MCL1 [81]. Similarly, Gu et al. demonstrated a synergistic relationship between rapamycin and DEX in a panel of T-ALL cells, and further elucidated the mechanistic basis for this interaction by identifying a synergistic induction of expression of the pro-apoptotic BAX and BIM proteins in conjunction with downregulation of MCL1 [82]. In addition, it has been shown that simultaneous exposure to an mTOR inhibitor and GCs results in a synergistic induction of the cyclin-dependent kinase (CDK) inhibitor proteins p21 and p27 [82, 83], suggesting that mTOR inhibitors and GCs converge both to induce cell cycle arrest and activation of the intrinsic apoptotic pathway. This effect was further demonstrated *in vivo* using PDXs derived from primary patient T- and B-ALL samples, and the

combinatorial effect of mTOR inhibition and GCs was found to be particularly effective in T-ALL samples with loss of *PTEN* expression [84], providing further evidence that aberrant regulation of upstream PI3K/AKT pathway activity results in altered mTOR activity that can be targeted therapeutically to augment the GC response. Finally, given the direct effects of both AKT and mTOR on GC sensitivity, several groups have investigated the efficacy of the dual PI3K and mTOR inhibitor BEZ235, reasoning that dual inhibition at two critical points in this pathway may have a more profound effect to induce GC sensitivity. Indeed, in ALL cell lines and primary patient samples both *in vitro* and *in vivo*, synergy has been demonstrated between BEZ235 and DEX [85, 86], suggesting that multiple nodes within this pathway are viable therapeutic targets for augmenting GC sensitivity.

JAK/STAT Signaling

The JAK/STAT signaling pathway is the critical effector pathway of cytokine receptor signaling, which plays a crucial role in mediating survival, proliferation, and differentiation of lymphoid cells [87]. Not surprisingly, aberrant activation of this pathway is common in lymphoid malignancies [50, 88], and significant attention has been devoted to assessing the role of JAK/STAT pathway inhibition as a novel treatment modality. Activation of cytokine receptors recruits JAK proteins to intracellular domains of cytokine receptors, and these activated JAK proteins recruit and phosphorylate STAT proteins, which translocate to the nucleus and function as transcription factors [87]. Interestingly, GR and one of these STAT proteins, STAT5, have been shown to physically interact at certain genomic loci. Specifically, STAT5 is known to inhibit the action of GR on GR target genes [89]. Consistent with this inhibitory role of JAK/STAT signaling on GR activity, inhibition of this pathway has been shown to overcome GC resistance in a number of lymphoid malignancies. In Philadelphia chromosome-like B-ALL, which is associated with aberrant JAK/STAT pathway activation, the combination of a JAK2 specific inhibitor and DEX demonstrated *in vitro* synergy and showed improved survival in an *in vivo* xenograft model [90]. Similarly, in primary diagnostic T-ALL samples, exposure to the cytokine interleukin-7 resulted in increased JAK/STAT pathway activity that induced GC resistance and could be overcome with the addition of the JAK1/2 inhibitor ruxolitinib [91]. Finally, in CLL cells, GC resistance was found to be associated with autocrine activation of another STAT protein, STAT3, and inhibition of STAT3 activation with ruxolitinib resulted in increased sensitivity to DEX *in vitro* [92].

NOTCH Signaling

NOTCH receptors are transmembrane receptors that, upon ligand binding, undergo a series of cleavage events to release the activated intracellular component of NOTCH from the membrane, allowing it to translocate to the nucleus and function as a transcription factor. The γ-secretase complex mediates the final step in this processing [93]. Due to the important role of NOTCH signaling in the pathogenesis of T-cell malignancies, inhibitors of this γ-secretase complex have been evaluated as potential therapeutic agents for the treatment of these diseases in combination with GCs. Specifically, in T-ALL cell lines, γ-secretase inhibitors have been shown to sensitize cells to the cytotoxic effects of DEX [94]. Several groups have

demonstrated that the combination of γ-secretase inhibitors and GCs facilitates autoinduction of GR and potentiates the induction of BIM expression, leading to increased cell death in both *in vitro* and *in vivo* model systems [95, 96]. Despite these promising preclinical findings, the clinical utility of γ-secretase inhibitors has been limited by severe gastrointestinal toxicity [97]. However, in an elegant study conducted in a T-ALL xenograft model, it was shown that simultaneous exposure to a γ-secretase inhibitor and DEX not only overcame GC resistance, but also attenuated the toxicities associated with the γ-secretase inhibitor [95], suggesting that the combination of γ-secretase inhibitors and GCs may be a viable therapeutic strategy to enhance GC sensitivity. Finally, at least one study has evaluated the efficacy of an anti-NOTCH1 monoclonal antibody in a T-ALL PDX model and demonstrated potentiation of GC activity when given in combination [98].

Src Family Kinase Signaling

In T-cells, the Src family kinases Lck and Fyn mediate critical signal transduction events downstream of the T-cell receptor (TCR) [99]. Through the use of reverse-phase protein arrays applied to PPR and PGR T-ALL samples, Lck was found to be aberrantly activated in PPR patients relative to PGR patients [100]. Consistent with these findings, inhibition of Lck with the Src family kinase inhibitor dasatinib has demonstrated *in vitro* efficacy to enhance GC sensitivity [101] and has been shown to impair the engraftment of T-ALL cells *in vivo* relative to treatment with either agent alone [100].

Metabolism

In addition to studies demonstrating the importance of GR expression levels as a mediator of GC sensitivity, many groups have demonstrated that metabolic processes that limit the availability of GC ligand can similarly contribute to GC resistance. In normal physiology, the 11β-hydroxysteroid dehydrogenase (HSD) class of enzymes mediates the conversion between cortisol, the active endogenous hormone, and cortisone, the inert form of the hormone. Specifically, 11β-HSD1 regenerates cortisol from cortisone while 11β-HSD2 inactivates cortisol [102]. In an analysis of primary patient ALL samples, basal 11β-HSD1 expression was found to be higher in GC sensitive samples relative to GC resistant samples. Furthermore, 11β-HSD1 expression was upregulated in response to DEX exposure specifically in the GC sensitive samples but not in the GC resistant samples, suggesting that 11β-HSD1 may participate in a GC-regulated feedback loop to maintain the availability of ligand for GR binding [103]. The same group similarly analyzed 11β-HSD2 expression in the GC resistant T-ALL cell line MOLT4F and the GC sensitive CCRF-CEM cell line and demonstrated that 11β-HSD2 expression was higher in the setting of GC resistance. They further demonstrated that pharmacologic inhibition of 11β-HSD2 was sufficient to potentiate GC-induced apoptosis [104]. Consistent with these findings, 11β-HSD2 expression was compared between GC resistant T-ALL cell lines, GC sensitive NHL cell lines, and normal peripheral T-cells. In the GC

resistant cell lines, 11β-HSD2 expression was found to be significantly elevated relative to the GC sensitive cell lines or normal T-cells [105]. To determine how 11β-HSD2 is dynamically regulated in the presence of GCs, transcript and protein expression as well as enzymatic activity were assessed in the GC sensitive CEM-C7 cell line after exposure to DEX. This analysis demonstrated a reduction in expression and enzymatic activity upon DEX exposure, suggesting that, in contrast to 11β-HSD1, GC-induced downregulation of 11β-HSD2 may be important for maintaining GC sensitivity [106].

In addition to HSDs, glutathione *S*-transferases (GSTs) are a class of enzymes involved in the metabolism of a wide variety of drugs, including steroids [107]. In an analysis of PGR and PPR patient samples from children treated on an ALL-BFM protocol, deletion of the GST family member *GSTT1* was enriched in the PPR patient group and was associated with an increased risk of relapse [108]. These data suggest that genetic lesions involving GST genes might contribute to differences in clinical GC response, though further studies are needed to determine whether aberrant GST activity plays a significant role in altering the availability of GC ligand and whether this contributes to GC resistance.

While metabolism of GCs themselves may play a role in modulating GC sensitivity, GC resistance has also been attributed to the aberrant activity of key bioenergetic metabolic pathways. Specifically, it has been shown in both ALL cell lines and in primary patient ALL samples that GC resistance is associated with increased rates of glycolysis, oxidative phosphorylation, and cholesterol biosynthesis. In a gene expression profiling study using ALL cell lines, pathways involved in these metabolic processes emerged as the top biological pathways associated with GC resistance. Furthermore, when these gene sets were studied in the context of primary patient samples, enrichment for these gene sets was a strong predictor of relapse [109], suggesting that activation of these bioenergetic pathways may promote chemoresistance. The same group went on to demonstrate that inhibition of glycolysis, oxidative phosphorylation, or cholesterol biosynthesis was sufficient to sensitize GC resistant T-ALL cells to GCs [110], further supporting the idea that aberrant activation of cellular metabolic processes may confer GC resistance.

Based on these findings, significant attention has been devoted to studying the role of glucose metabolism as a modulator of GC sensitivity. In an analysis of a large cohort of primary B-ALL samples with varying degrees of *in vitro* prednisolone sensitivity, genes associated with carbohydrate metabolism were found to be differentially expressed between GC sensitive and GC resistant samples [111]. Furthermore, in ALL cell lines and primary patient samples, prednisolone resistance was found to correlate with increased glucose consumption, and inhibition of glycolysis with the metabolite 2-deoxy-D-glucose (2-DG) sensitized cells to GCs, supporting the idea that excessive metabolic activity may impair GC-induced apoptosis [112]. To further assess the relationship between glucose consumption and GC sensitivity, GC sensitive ALL cell lines and primary patient samples were exposed to DEX, which was found to inhibit glycolysis, leading to decreased glucose consumption that was mediated by a reduction in the expression of the glucose transporter GLUT1. This group went on to demonstrate that culturing cells in low glucose

conditions resulted in increased DEX-induced apoptosis [113]. Taken together, these data suggest that a reduction in glucose metabolism may be required for optimal GC-induced apoptosis. Consistent with this idea, it was shown that in prednisolone sensitive primary B-ALL samples, MCL1 expression decreased upon exposure to prednisolone, while it did not decrease in prednisolone resistant samples. Genetic silencing of MCL1 was found to be associated with an increase in glucose consumption, and simultaneous inhibition of glycolysis and silencing of MCL1 resulted in further sensitization to prednisolone [114]. The importance of excessive glucose metabolism as a mediator of GC resistance has also been studied in the context of NHL cell lines and primary patient samples. In these cells, inhibition of glycolysis was found to synergize with methylprednisolone to induce cell cycle arrest and apoptosis [115]. Providing a genetic explanation for the relationship between altered glucose metabolism and GC sensitivity, Chan et al. recently performed ChIP-seq to assess the binding pattern of transcription factors that are commonly inactivated in B-ALL, including PAX5 and IKZF1. They demonstrated that in B-ALL, these transcription factors are recruited to genetic loci that encode positive and negative regulators of glucose uptake. Re-expression of PAX5 and IKZF1 in B-ALL cells resulted in decreased glucose uptake and was sufficient to overcome prednisolone resistance [116]. The authors speculated that the hypermetabolic state associated with the deletion of these transcription factors facilitates leukemogenesis and simultaneously facilitates resistance to GC therapy.

In addition to glucose metabolism, aberrant lipid metabolism has also been shown to contribute to GC resistance. Specifically, lymphoid cells have been shown to have a unique dependency on exogenously synthesized cholesterol, and similar to glycolysis, GCs may exert their pro-apoptotic effects in part through inhibiting this cholesterol synthesis pathway. Indeed, in GC sensitive CEM-C7 cells, DEX was found to inhibit cholesterol synthesis, while this did not occur effectively in GC resistant CEM-C1 cells. Furthermore, exposure of CEM-C7 cells to exogenous cholesterol decreased DEX sensitivity, suggesting that DEX resistance may be mediated in part by increased cholesterol metabolism [117]. Further supporting these data, T-ALL PDXs treated with a GC-containing four-drug induction regimen that acquired *in vivo* drug resistance were found to have altered cholesterol metabolism. In these samples, exposure to DEX and simvastatin, an inhibitor of cholesterol biosynthesis, demonstrated *ex vivo* synergy [118]. These data suggest that additional preclinical studies may be warranted to evaluate the use of drugs that modulate bioenergetic pathways as a means of overcoming GC resistance.

MicroRNAs

MicroRNAs (miRNAs) are short non-coding RNAs that are most commonly contained within introns. Once transcribed, they bind to complementary sequences within the 3' untranslated region (3'UTR) of target gene mRNAs. Through this activity, miRNAs function primarily as negative regulators of translation, though

they may have other repressive and activating roles [119]. Dysfunctional expression of miRNAs is a common feature of many cancers, including hematologic malignancies. In ALL samples, a miRNA microarray analysis of paired diagnostic and relapse samples identified a distinct miRNA profile in the relapse samples relative to the diagnostic samples [120]. Similarly, in a study involving miRNA sequencing of samples from patients with Burkitt lymphoma, DLBCL, and follicular lymphoma, many miRNAs were found to be aberrantly expressed in lymphoma cells relative to normal lymphoid cells. Functionally, these miRNAs were found to be associated with altered regulation of key signal transduction pathways, including the Ras/MAPK and PI3K/AKT signaling pathways, suggesting that these miRNAs may play a role both in lymphomagenesis and in chemoresistance, including GC resistance [121].

MiR-17

In B-ALL cell lines, DEX exposure downregulated expression of miR-17 in GC sensitive but not in GC resistant cells. ChIP-seq analysis demonstrated that this is mediated by direct GR binding to the miR-17 locus specifically in GC sensitive cells. Functionally, miR-17 was found to target the BIM transcript for silencing, and pharmacologic inhibition of miR-17 increased DEX sensitivity with an associated increase in BIM expression [122].

MiR-100/99a

The miRNA species miR-100/99a has also been implicated in GC resistance and is known to be downregulated in samples from ALL patients with clinically high risk features. Specifically, low expression has been associated with inferior leukemia-free and overall survival [123]. In cell lines, ectopic expression of miR-100/99a promoted DEX-induced apoptosis through a reduction in expression of the miR-100/99a target FKBP51. The reduction in FKBP51 expression was associated with increased nuclear localization of ligand-bound GR and decreased expression of mTOR, subsequently leading to a reduction in MCL1 expression which further potentiated apoptosis [123].

MiR-124

The role of MiR-124 as a mediator of GC resistance was first appreciated in the context of sepsis, where it was found that miR-24 represses the GRα transcript [124], suggesting that its overexpression might mediate GC resistance by decreasing the availability of GR protein for ligand binding. Indeed, miR-124 expression was found to be increased in prednisolone resistant ALL cell lines and in PPR patient samples and overexpression of miR-124 in ALL cells was associated with a reduction in GR protein expression [125]. However, at least one study has suggested the opposite effect of miR-124 in GC sensitivity. In DLBCL cells, miR-124 expression was found to decrease expression of PDE4B, thereby relieving the inhibitory

effect on cAMP signaling and increasing GC sensitivity [126]. Further studies are therefore needed to elucidate the role of miR-124 in modulating GC sensitivity in distinct lymphoid malignancies.

MiR-128b and miR-221

In MLL-AF4 ALL, miR-128b and miR-221 were found to be downregulated relative to other types of ALL. Overexpression of these miRNAs in MLL-AF4 ALL cell lines resulted in increased sensitivity to GCs, which was accompanied by downregulation of MLL, AF4, and their associated fusion genes [127]. Further implicating low miR-128b as a mediator of GC resistance in MLL-AF4 ALL, miR-128b mutations found in both cell lines and primary patient samples were shown to impair the appropriate processing of miR-128b. This resulted in GC resistance mediated by a failure to downregulate the expression of fusion oncogenes involving MLL and AF4, though the mechanisms by which MLL and AF4 themselves contribute to GC resistance are currently unknown [128].

MiR-142-3p

MiR-142-3p was initially shown in T-regulatory cells to target adenylyl cyclase 9 mRNA for silencing, resulting in a reduction in the cellular pool of cAMP due to the loss of adenylyl cyclase enzymatic activity [129]. Elevated expression of miR-142-3p in primary T-ALL samples was found to be associated with an increased risk of relapse and decreased leukemia-free survival relative to patients with lower miR-142-3p expression. Consistent with its known effects on the adenylyl cyclase 9 transcript, high miR-142-3p expression was associated with increased cAMP pathway activity. Furthermore, miR-142-3p was found to target the GRα transcript for repression via direct binding to the 3'UTR. In this context, inhibition of miR-142-3p overcame GC resistance both by facilitating an increase in cAMP pathway activity and an increase in GRα expression [130].

MiR-182

In an analysis of a variety of murine and human malignant lymphoid cell lines, miR-182 expression was higher in GC resistant cells relative to GC sensitive cells, and high expression was associated with decreased FOXO3A expression. One important downstream target of FOXO3A is BIM, and high expression of miR-182 was also associated with a reduction in BIM expression. Consistent with this activity, overexpression of miR-182 restored BIM expression, thereby overcoming GC resistance [131].

MiR-185-5p

Finally, miR-185-5p was found to be overexpressed in GC sensitive ALL cell lines. One target of miR-185-5p is the mTORC2 mRNA. Forced overexpression of miR-185-5p in GC resistant ALL cells restored GC sensitivity with a concomitant reduction in mTORC2 activity [132].

Conclusion

Given the pleiotropic effects of GCs and the innumerable interactions between GR and a wide variety of cellular processes, it is not surprising that the mechanisms of GC resistance are complex and that our understanding of these mechanisms is constantly evolving (Fig. 1.2). However, despite a well-justified concern for GC resistance and its associated clinical implications, GCs are profoundly efficacious in the treatment of lymphoid malignancies and will undoubtedly remain an integral component of therapy. Therefore, there is an urgent need to translate the findings from the numerous preclinical and clinical studies highlighted in this review into standard clinical practice for the treatment of these diseases. With the application of large-scale sequencing and epigenetic profiling technologies, the development of small molecule and biologic therapeutics, and increasing access to patient-derived tissue samples, there is significant potential for the elucidation of additional causes of GC resistance and the identification and implementation of novel therapeutic strategies to overcome them.

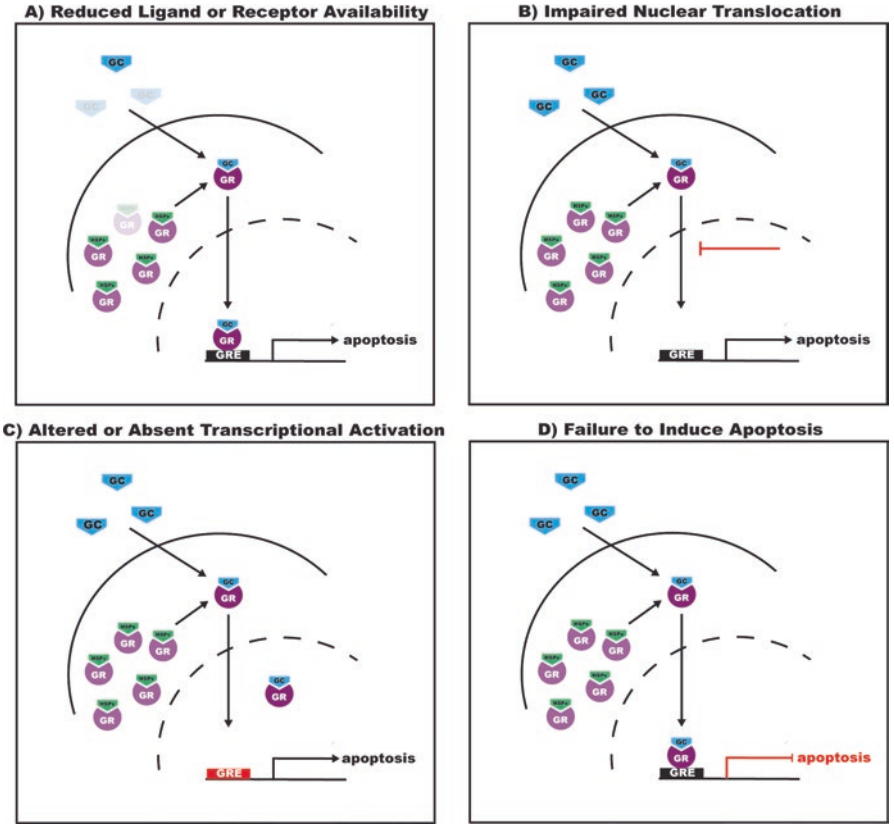

Fig. 1.2 Mechanisms of GC Resistance. GC resistance may arise due to processes that impact any component of normal GR signaling, including the availability of GC ligand or GR (**A**), nuclear translocation of the activated GC/GR complex (**B**), transcriptional activity of ligand-bound (**C**), or the induction of apoptosis (**D**)

Acknowledgements L.K.M. is supported by the UCSF Medical Scientist Training Program Grant T32 GM007618 and by a Genentech Foundation Award. M.L.H is supported by the National Cancer Institute Grant R01 CA193776, The Campini Foundation, The Buster Posey Family Foundation, and The Pepp Family Foundation. The authors thank Kevin Shannon and Anica Wandler for their critical reading of the manuscript.

Disclosure of Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 2
Resistance to Monoclonal Antibody Therapeutics in Lymphoma

Matthew J. Barth and Stanton C. Goldman

Abstract With the long history of rituximab use in CD20 positive lymphomas and the recent approval of brentuximab vedotin for the treatment of Hodgkin lymphoma and anaplastic large cell lymphoma, monoclonal antibody-based therapies are commonly utilized for the treatment of many lymphomas. Following decades of experience with rituximab, much has been learned about the mechanisms of action and potential mechanisms of resistance to monoclonal antibody therapies, but a thorough understanding of which mechanisms of action are most relevant to rituximab's efficacy and which resistance mechanisms are most clinically relevant is still elusive. Nonetheless, many approaches have been identified and continue to be investigated both pre-clinically and clinically to attempt to overcome or circumvent resistance to monoclonal antibody therapies in order to enhance treatment responses or improve survival at the time of relapse following monoclonal antibody based therapy.

Keywords Monoclonal antibody · Antibody drug conjugate · Non-Hodgkin lymphoma · Hodgkin lymphoma · Resistance

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A. C. Xavier, M. S. Cairo (eds.), *Resistance to Targeted Therapies in Lymphomas*, Resistance to Targeted Anti-Cancer Therapeutics 21,
https://doi.org/10.1007/978-3-030-24424-8_2

Abbreviations

ADCC	Antibody-Dependent Cellular Cytotoxicity
ADPC	Antibody-Dependent Phagocytic Cytotoxicity
AKT	Protein Kinase B
ALCL	Anaplastic Large Cell Lymphoma
ALL	Acute Lymphoblastic Leukemia
B-NHL	B-cell Non-Hodgkin Lymphoma
BiTE	Bispecific T-cell Engaging
CDC	Complement Dependent Cytotoxicity
CLL	Chronic Lymphocytic Leukemia
DLBCL	Diffuse Large B-Cell Lymphoma
EFS	Event-Free Survival
ERK1/2	Extracellular signal Related Kinase 1 and 2
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HACA	Human Anti-Chimera Antibodies
IFN-γ	Interferon Gamma
IL-2	Interleukin 2
IL-4	Interleukin 4
MAPK	Mitogen Activated Protein Kinase
MMAE	Monomethyl Aurostatin E
MS4A1	Membrane Spanning 4-Domain A1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NK-κB	Nuclear Factor Kappa B
NK-cell	Natural Killer Cells
PCD	Programmed Cell Death
PLCγ2	Phospholipase C Gamma 2
RIC	Radioimmunoconjugate
ROS	Reactive Oxygen Species
STAT3	Signal Transducer and Activator of Transcription 3
SYK	Spleen Associated Tyrosine Kinase
TNF-α	Tumor Necrosis Factor alpha

Introduction

The addition of monoclonal antibody therapy to the treatment of lymphoma has revolutionized its therapy over the past 2 decades. The proof of principle of monoclonal antibody therapies has been the addition of the anti-CD20 monoclonal antibody, rituximab, to therapy regimens for CD20 expressing mature B-cell lymphomas. The introduction of rituximab to the best backbone chemotherapy regimens for B-cell non-Hodgkin lymphoma (B-NHL) has improved event free survival (EFS) in high grade B-NHL. Well conducted randomized phase 3 studies have shown an approximately 15–20% absolute improvement in EFS (*vs*. chemotherapy alone) in

favor of rituximab in elderly patients with diffuse large B-cell lymphoma (DLBCL), younger patients with DLBCL and more recently adults with Burkitt lymphoma [1–3]. Until recently it was unknown whether the same would be true for pediatric mature B-NHL where the multiagent chemotherapy results alone were already greater than 80% survival. A recent international study in advanced pediatric Burkitt and DLBCL was halted early after the rituximab arm demonstrated a superior 1-year EFS (94%) compared to identical chemotherapy backbone alone (81%) [4]. Thus, rituximab (+ disease specific chemotherapy) is now considered standard of care in pediatric and adult patients with aggressive mature B-NHL.

While the success of rituximab is well documented, resistance to monoclonal antibody therapy has also been well described with multiple possible mechanisms of resistance reported. Numerous next generation monoclonal antibodies have been developed in an attempt to improve upon rituximab and circumvent mechanisms of resistance with varying degrees of success. Additionally, monoclonal antibodies modified to enhance interaction with host immune cells or conjugated to toxins or radiotherapeutic agents have been developed as an alternative approach to the use of naked monoclonal antibody therapies in the treatment of lymphoma. In this chapter, we will highlight resistance to monoclonal antibody therapies, focusing primarily on rituximab as the predominant monoclonal antibody utilized in the treatment of lymphoma, and the development of alternative approaches to overcome described mechanisms of resistance.

Resistance to Monoclonal Antibody Therapy in the Clinic

The efficacy of rituximab in treating B-NHL was first established in the setting of relapsed low-grade B-NHL where 4 weekly doses of rituximab single agent therapy led to responses in approximately 50% of patients in initial trials [5–8]. In patients with relapsed or refractory aggressive B-NHL variants, 8 weekly doses of rituximab led to responses in about 30% of patients [9]. In the setting of aggressive disease, patients with primary refractory disease, non-large cell variants and more bulky disease tended to be less likely to respond to single agent rituximab [9]. In low grade lymphoma patients having previously responded to rituximab, responses were noted in 40% of patients upon retreatment with single agent rituximab [10, 11]. These initial trials highlighted a failure to respond in more than half of relapsed patients treated with rituximab upon initial single agent treatment with more than half of initial responders developing resistance upon re-treatment. As an initial therapy for low grade B-NHL, rituximab induced a slightly higher response rate of greater than 60% as a single agent [12]. Rituximab also demonstrated the ability to sensitize lymphoma cells to the effects of cytotoxic chemotherapy and thus was subsequently combined with chemotherapy for treating both newly diagnosed and relapsed/refractory lymphoma patients. The combination of chemotherapy and immunotherapy with rituximab was initially investigated in the R-CHOP regimen (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) with 95% of patients

with low grade B-NHL achieving a response [13, 14]. In the setting of aggressive B-NHL, similar response rates were noted [15]. Rituximab has subsequently been combined with a variety of chemotherapy regimens in both indolent and aggressive B-NHL and has become standard of care in the treatment of CD20-positive B-NHL. However, with the introduction of rituximab to front-line therapy for B-NHL, a new phenomenon of resistance has been noted in the relapse setting. A large Phase 3 study of relapsed DLBCL, the CORAL study, highlighted the development of resistance following treatment with rituximab containing regimens with patients having previously been treated with rituximab exhibiting an inferior survival upon treatment with rituximab containing salvage therapy compared to patients not having previously received a rituximab containing regimen [16]. The degree of contribution of rituximab to resistance is difficult to assess since current treatment essentially universally combines rituximab with chemotherapy. However, resistance has been noted both upon initial exposure and upon re-exposure to rituximab heightening interest in the mechanisms of resistance to monoclonal antibody therapies and the development of new immunotherapeutic agents able to overcome resistance.

Antibodies to Rituximab are Unlikely to Play a Role in Resistance

Monoclonal antibodies are large antigenic proteins and can theoretically be ineffective because of the formation of anti-antibodies, especially with repeated exposure. In addition, the less fully humanized antibodies are at higher risk of inducing an antibody response from the host. On the other hand, rituximab is a powerful humoral immunosuppressant with prolonged reduction of mature non-malignant B-cells and serum immunoglobulins. During our studies of the first trial of rituximab plus aggressive multi-agent chemotherapy in children and adolescents with *de novo* mature B-NHL, we could not demonstrate any formation of human anti-rituximab (HACA) antibodies [17]. In addition, by using a dose dense approach, we were able to demonstrate very high serum rituximab levels with t ½ of 26–29 days. Thus, reduced serum levels of antibody, through anti-antibody formation (or other mechanisms), is unlikely to play a role in resistance.

Mechanisms of Monoclonal Antibody Activity

To understand the mechanisms of resistance to monoclonal antibody therapy, one needs to initially understand the varying potential mechanisms of activity of monoclonal antibodies. Monoclonal antibodies can function to kill tumor cells through a variety of mechanisms. These primarily include antibody-dependent cellular

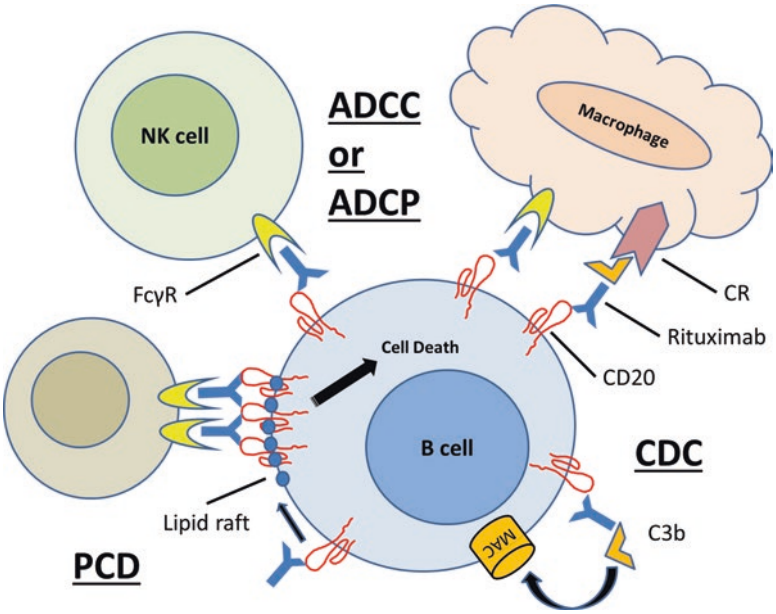

Fig. 2.1 Mechanisms of rituximab activity. Rituximab binding to surface CD20 leads to lymphoma cell death through several reported mechanisms. (1) Binding of rituximab leads to the activation of complement leading to the formation of the membrane attack complex (MAC) resulting in cell lysis. (2) Binding of the Fc portion of rituximab by Fcγ receptors (FcγR) or rituximab bound complement C3b by complement receptors (CR) on effector cells leads to cell killing by antibody dependent cellular cytotoxicity (ADCC) or phagocytosis (ADCP). (3) Binding of rituximab to CD20 leads to mobilization to lipid raft domains where crosslinking of CD20 bound rituximab leads to intracellular signaling and induction of apoptosis

cytotoxicity (ADCC) or phagocytic cytotoxicity (ADCP), complement dependent cytotoxicity (CDC) and direct induction of programmed cell death (PCD) (Fig. 2.1) [18–20]. Additionally, monoclonal antibodies can function to sensitize tumor cells to the effect of cytotoxic chemotherapy exhibiting synergistic activity in combination immunochemotherapy regimens [21]. The most relevant mechanism of action of an individual anti-CD20 antibody can largely be defined by whether the antibody is a type I antibody (e.g. rituximab) or a type II antibody (e.g. tositumomab, obinutuzumab). Type I anti-CD20 antibodies can localize CD20 into membrane lipid raft domains effectively activating complement and altering signal transduction through co-localization of receptors and effectors; while type II antibodies do not induce lipid raft localization and generally induce limited CDC, but more robust induction of PCD [22, 23].

ADCC/ADCP relies on the binding of the Fc fragment of the monoclonal antibody to receptors on surrounding immune effector cells [natural killer (NK) cells, monocyte/macrophages and neutrophils] inducing tumor cell death through triggering the immune effector cells to bind and kill the tumor. The role of ADCC on rituximab *in vivo* activity has been demonstrated by impaired activity in NK-cell

and neutrophil depleted mice and Fc receptor dependent activity [24, 25]. CDC relies on activation of the complement cascade through binding of the protein C1q to the Fc portion of the antibody leading to development of a membrane attack complex leading to cell lysis. The dependence on complement for activity has been demonstrated by a lack of rituximab activity in mice deficient in C1q or with complement depleted by exposure to cobra venom factor, though others have also demonstrated that deficiency of complement proteins had little impact on rituximab activity suggesting that Fc-receptor dependent ADCC activity was more critical to rituximab activity *in vivo* [25, 26]. Additionally, some have suggested that complement activation may impair other antibody mediated mechanisms of cell killing like ADCC [27]. This detrimental effect of complement activation has also been suggested in relation to an increase in progression free survival noted in follicular lymphoma and DLBCL patients with mutations in the gene encoding C1q that are known to cause lower C1q expression [28, 29].

While the direct induction of PCD by monoclonal antibodies has been demonstrated *in vitro*, the mechanism of such an effect *in vivo* has been difficult to demonstrate so that less is understood about the exact mechanism of antibody induction of cell death. The mechanism of induction of cell death also likely varies between antibody types with type I and type II anti-CD20 monoclonal antibodies demonstrating varying mechanisms. Rituximab binding to CD20 on the surface of malignant B-cells has been shown to induce a caspase-dependent apoptosis through activation of caspases 3 and 9 leading to PARP cleavage with these effects being inhibited by exposure to caspase inhibitors and enhanced by cross linking of CD20 bound rituximab molecules [30–32]. While this suggests a caspase-dependent mechanism of cell death induction, others have reported cell death associated with rituximab binding that is independent of caspase activation and resistant to caspase inhibition [33]. Apoptosis induction may also be dependent on altered calcium transport leading to increased intracellular calcium following rituximab exposure with calcium chelators inhibiting the apoptosis induced by rituximab [31, 32]. The cellular function of membrane bound CD20 is likely to be a calcium channel critical to B-cell signaling. This shift in intracellular calcium after rituximab binding to CD20 has been shown to be secondary to activation of Src-family protein tyrosine kinases leading to phosphorylation of phospholipase C gamma 2 (PLCγ2) [34]. Additional intracellular signaling effects reported following rituximab binding have been noted on the mitogen activated protein kinase (MAPK), extracellular signal related kinase 1 and 2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B (NF-κB) signaling pathways [35–38]. Rituximab binding has also been shown to alter expression of Bcl-2 family member proteins and other inhibitors of apoptosis proteins. Chemosensitization observed following rituximab exposure may in large part be due to the documented ability of rituximab to overcome Bcl-2 associated resistance following chemotherapy exposure [30, 39]. Some have also theorized that debris from apoptotic cells can have a "vaccination effect" leading to expansion of lymphoma specific cytotoxic T lymphocytes [40]. This effect has been demonstrated in mice where tumor re-challenge in mice previously treated with an anti-CD20 antibody led to impaired engraftment

Table 2.1 Mechanisms of resistance to monoclonal antibody therapy and approaches to overcoming resistance

Mechanisms of activity	Mechanisms of resistance	Approaches to circumventing resistance
CDC	Complement depletion [49, 71, 72]	Next generation mAbs with enhanced CDC activity [82–84]
	Complement variants [26]	Enhanced hexamer foundation [99]
	Complement inhibitory proteins [73–79]	Complement replacement [71, 72]
ADCC	FcγR polymorphisms [102, 103, 105, 108, 110, 111, 113, 118]	Next generation mAbs with enhanced FcR affinity [121–129]
	Inhibitory FcγR expression [100, 119, 120]	
PCD/ Apoptosis	Altered Bcl-2 protein expression/ intracellular signaling [146, 147]	Type II antibodies with enhanced cell death induction [21, 124, 128, 150, 151]
Antigen binding	Antigen variants [46, 52, 53]	Increase CD20 expression using epigenetic modulating agents or cytokines [19, 59–64, 67, 68]
	Antigen shedding [48, 49]	Type II monoclonal antibodies [51]
	Antigen internalization [50]	Dose dense dosing [17, 143–145]
	Circulating antigen [138–142]	
ADC	Drug transporter mediated efflux [158]	Alternative anti-neoplastic conjugates [160]

CDC, complement dependent cytotoxicity; ADCC, antibody dependent cellular cytotoxicity; PCD, programmed cell death; ADC, antibody-drug conjugate; mAB, monoclonal antibody

and the identification of macrophage-associated ADCC leading to dendritic cell uptake of immune complexes inducing anti-tumor adaptive responses [41–43].

The exact role and contribution of each mechanism of activity to the efficacy of monoclonal antibody therapies is still not clearly understood and is likely disease- and antibody-dependent. Each of these mechanisms of activity has also been associated with proposed mechanisms of resistance (Table 2.1).

Mechanisms of Monoclonal Antibody Resistance

Antigenic Alterations Leading to Resistance

For a monoclonal antibody to exert its effect, it needs to first bind to its target antigen. The level of expression or mutations in the surface antigen to which an antibody is targeted can impact the activity of the monoclonal antibody. One of the characteristics of CD20 that was believed to make it an ideal antigen for antibody targeting was a reported lack of internalization or shedding of the protein [18]. Despite this, early in the investigation of rituximab, reports began emerging

describing the loss of CD20 expression in patients with relapsed B-NHL following exposure to anti-CD20 monoclonal antibody therapy [44–47]. Though the relative incidence of CD20 loss after rituximab exposure in the clinic has generally been believed to be low, investigation of alterations of CD20 expression levels in rituximab resistant cells has indicated a possible role of this phenomenon in rituximab resistance.

In rituximab-resistant B-NHL cell lines developed by serial exposure of cell lines in culture to rituximab, decreased CD20 expression has been described in the resultant resistant cells reported to be due to transcriptional and post-transcriptional mechanisms [48]. Alternative splicing of CD20 mRNA may also impact rituximab response with an alternatively spliced, truncated version of the CD20 protein reported in B-lymphocytes that were either malignant or EBV transformed, but not present in non-transformed B-cells [49]. The variant CD20 was noted to increase in expression in rituximab-resistant cell lines developed by exposure to rituximab in vitro and also in primary patient cells following exposure to rituximab suggesting a role in development of rituximab resistance.

CD20 expression may also be altered after rituximab exposure secondary to antigenic modulation or "shaving". Beum *et al*. described a so called "shaving effect" leading to loss of CD20 expression on malignant B-cells [50]. The described effect was reported both clinically, with reported rapid loss of CD20 and rituximab from B-cells without internalization, and in an experimental system where rituximab-CD20 complexes were noted to be removed from B-cells and taken up by monocytes in co-culture [51, 50]. Some reports have also suggested that, contrary to earlier data, CD20 may be internalized following rituximab binding. Beers *et al*. demonstrated using fluorescently labeled rituximab that internalization of the rituximab-CD20 complex occurred following rituximab exposure with trafficking of rituximab-CD20 complexes noted to endosomes and lysosomes in B-cells [52]. Variability in the internalization of CD20 was noted with different types of monoclonal antibodies with the Type I rituximab antibody leading to internalization while a Type II tositumumab-like antibody did not, highlighting potential differences in the mechanism of action and resistance to different antibody constructs [52]. Utilization of a Type II antibody, like the humanized, glycoengineered Type II anti-CD20 monoclonal antibody obinutuzumab may thus allow for activity without significant modulation from internalization, though recent studies have also reported on the "shaving phenomenon" occurring with obinutuzumab as an alternate mechanism for resistance in the absence of antigenic modulation [53].

In addition to antibody associated effects leading to altered expression of the CD20 antigen on the B-cell surface, others have reported on mutations in the gene encoding CD20 that may impair response to rituximab [54, 55]. For example, Turui *et al*. performed a mutation analysis of CD20 in 50 patients treated for a variety of NHL types including 9 patients with progressive disease [55]. They found that 11 patients (22%) had a mutation in CD20 and that those with a C-terminal deletion mutation had a significantly lower expression of CD20 compared to patients without a mutation or those with mutations defined as early termination or extracellular domain. Notably 4 of the 5 C-terminal deletions occurred in samples from patients

with progressive disease. Cells transfected with the C-terminal deletion mutated CD20 expressed similar CD20 RNA, but did not express CD20 on the cell surface with only weak cytoplasmic staining noted. These C-terminal mutations were subsequently reported to affect the extracellular large loop of the CD20 antigen also impacting the rituximab binding site [56]. Another relapse case was noted to have a homozygous deletion of the membrane spanning 4-domains A1 (MS4A1) gene, the gene encoding CD20, at relapse leading to loss of CD20 [57]. While these mutations have been reported, a larger analysis of DLBCL patients identified that such mutations occur at very low rates (0.4% of 264 newly diagnosed and 6% of 15 relapsed DLBCL patients analyzed) and may not significantly contribute to resistance except in a small percentage of cases [58].

Epigenetic regulation of the gene encoding CD20 has also been implicated in changes in CD20 surface antigen expression and thus possibly also related to rituximab resistance [59, 60]. Tomia *et al.* reported on a case of CD20 negative relapsed DLBCL after rituximab exposure with increased CD20 expression following exposure to the epigenetic modifier Trichostatin A [59]. A further analysis of mechanisms of epigenetic regulation of decreased CD20 expression identified the role of the Sin3A-HDAC1 co-repressor complex in downregulating transcription of MS4A1 with expression of CD20 increased following exposure to the histone deacetylase inhibitor Trichostatin A [60]. Similarly, numerous epigenetic modifying agents have been identified that can alter CD20 protein expression and augment the activity of rituximab through their effects on DNA methylation, DNA acetylation or the recruitment of transcription factors leading to altered CD20 expression [61–66]. The potential clinical impact of epigenetic modifiers has also been investigated in combination with rituximab containing regimens with some promising early findings [67, 68]. Alternative mechanisms of increasing CD20 expression have been reported using a variety of cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), IL-4 and IL-2 suggesting possible roles of combination therapies involving cytokine based therapies to increase CD20 expression prior to rituximab therapy [69, 70, 21]. An additional approach to enhance targeting of tumor cells using monoclonal antibodies is the use of multivalent antibodies targeting multiple antigens on a tumor cell or having multiple binding sites for a single antigen. Examples include an antiCD20/CD22 bivalent antibody which has demonstrated enhanced in vitro and in vivo cell killing compared to the individual antibodies or a combination of the two single antibodies [71]. A combination of a type 1 and a type 2 CD20 antibody into one bivalent antibody also exhibited enhanced CDC and direct killing [72]. In addition to approaches intended to enhance expression of CD20 to improve response to rituximab, antibodies targeting alternative lymphoma associated cell surface antigen targets continue to be developed for use in the setting of rituximab resistance or CD20 negative relapsed disease including, for example, monoclonal antibodies targeting CD19, CD22, CD79b, CD80 and CD40 with varying degrees of activity [73–78].

Complement Mediated Resistance

Binding of monoclonal antibodies to surface proteins can induce CDC via interactions of the Fc portion of the antibody with complement proteins. In chronic lymphocytic leukemia (CLL), a rapid depletion of complement proteins has been observed which may represent a limitation of rituximab activity [51]. This possible source of resistance was further supported by evidence that infusing rituximab with complement containing fresh frozen plasma may enhance rituximab activity [79, 80]. Polymorphisms in genes encoding C1q have also been reported to impact rituximab activity in patients with follicular lymphoma also highlighting the potential important role of CDC in rituximab activity, especially when given without chemotherapy [28].

Tumor cells can also inhibit CDC killing through the expression of complement inhibitory proteins CD46, CD55 and CD59 with altered expression of complement inhibitory proteins identified as a possible mechanism of resistance to monoclonal antibody therapies [81]. B-NHL cells resistant to rituximab, including tumor cell lines and primary patient cells, have been shown to exhibit increased expression of CD55 and CD59 leading to impaired CDC activity of anti-CD20 monoclonal antibodies [82–85]. The effect of complement inhibitory proteins on the CDC activity of rituximab has been demonstrated through increased rituximab associated CDC following inhibition of CD55 or CD59 [84, 81, 86, 87]. Despite this in vitro evidence of the detrimental effect of high complement inhibitory protein expression on rituximab activity, clinical investigation of the effect of high complement inhibitory protein expression on treatment response to rituximab has been conflicting with some analysis suggesting higher levels of CD55 and CD59 in non-responders while others suggest no impact of varying levels of CD46, CD55 or CD59 on likelihood of response to rituximab [88, 89].

Novel monoclonal antibodies have been developed which exhibit enhanced CDC activity in comparison to rituximab [90]. CDC activity has been linked to the proximity of antibody binding to the cell membrane and is dependent on redistribution of the antigen target into lipid soluble rafts within the cell membrane, an effect predominantly observed with Type I antibodies [91, 90]. The fully human type I anti-CD20 monoclonal antibody ofatumumab binds to a unique, more membrane-proximal epitope of the CD20 antigen compared to rituximab and has a slower off-rate while effectively inducing CD20 redistribution to lipid rafts [90, 92]. Likely secondary to these characteristics, ofatumumab has demonstrated enhanced CDC activity in comparison to rituximab including in the setting of rituximab resistance and high levels of CD55 and CD59 expression [90, 83, 93–95]. Clinically, ofatumumab has induced a high rate of responses, particularly in CLL alone or in combination with chemotherapy, and has received FDA approval for first line and refractory CLL alone and with various alkylator combinations. [96–100]. Ofatumumab demonstrated limited efficacy in aggressive B-NHL, where no significant benefit was observed over rituximab, though some responses have been noted

in rituximab resistant disease. This highlights that CDC may play a larger role in certain B-cell malignancies (like CLL) compared to others [101–105].

An alternative approach to enhancing monoclonal antibody associated CDC relates to the formation of antibody hexamers in order to activate complement effectively. This recently described hexamer formation of anti-CD20 antibodies increases C1q binding and enhances CDC activity [106]. Polymorphisms in the Fc portion of an antibody have been identified that enhance hexamer formation and thus increase CDC activity of the antibody. Introducing such polymorphisms into rituximab was shown to increase CDC in CLL samples and was also even shown to increase CDC induced by type II anti-CD20 monoclonal antibodies [107]. This represents another potential approach to overcoming resistance to CDC activity.

Fcγ Receptor Associated Resistance

Much of the function of rituximab and other monoclonal antibodies is dependent on the interaction of the Fc portion of the antibody with Fcγ receptors (FcγR), in particular FcγRIIIa and FcγRIIa receptors on myeloid effector cells [108, 25, 109]. FcγR deficiency in mice abrogates the activity of monoclonal antibody therapies providing evidence for their crucial role in monoclonal antibody activity [108]. Polymorphisms in FcγR leading to altered affinity for Fc binding have been noted to impact the efficacy of rituximab in vitro and in vivo. In particular, the 158F variant of FcγRIIIa has been noted to impair responsiveness to rituximab compared to the 158V variant which has a higher affinity for binding IgG1 antibodies [110]. In patients with previously untreated follicular lymphoma treated with rituximab, response rates were significantly higher in patients homozygous for the FcγRIIIa 158V variant compared to 158F carriers [111]. However, subsequent analysis in a variety of tumor types have provided conflicting results in particular in patients treated with rituximab in combination with chemotherapy [112–122]. There may also be an effect on toxicity associated with rituximab exposure as the high affinity FcγRIIIa 158V polymorphism has recently been associated with increased rates of late onset neutropenia following rituximab therapy [123–125]. Polymorphisms in FcγRIIa have also been implicated in response to rituximab, in particular the FcγRIIa H131R polymorphism which has been associated with improved response in tumors with a higher affinity H/H genotype, though similar to the FcγRIIIa polymorphisms, the impact on clinical outcome has been mixed with no impact noted in most recent studies of rituximab in combination with chemotherapy [121, 119, 118, 126, 116, 113].

Additionally, the expression of other inhibitory FcγR, such as FcγRIIb, may impair response upon binding of effector macrophages [108]. This has been demonstrated in transgenic mice lacking the FcγRII inhibitory receptor, in which tumors tend to be more responsive to monoclonal antibody therapies [108]. FcγRIIb has also been reported to interact with rituximab bound to CD20 to form a complex that promotes internalization of the rituximab-CD20 complex impairing Fc-dependent

functions and overall antibody efficacy [127, 52]. The clinical effect of high FcγRIIb has also been described in follicular lymphoma patients receiving rituximab monotherapy where patients with high FcγRIIb expression exhibited lower EFS [128].

With variability in FcγR binding affinity playing a potential role in response to rituximab, novel monoclonal antibodies have been developed with alterations aimed at enhancing Fc receptor affinity. Alterations to the Fc portion of monoclonal antibodies have improved affinity for lower affinity FcγRs leading to improved ADCC activity. Afucosylation (manipulation of the oligosaccharides to remove fucose) of the Fc portion of antibodies was shown to decrease steric hindrance that likely inhibited FcγR binding leading to enhanced receptor affinity and increased ADCC [129–131].

Obinutuzumab is the prime example of a third generation type II, humanized, glycoengineered anti-CD20 monoclonal antibody. Obinutuzumab has been shown to exhibit enhanced pre-clinical activity compared to rituximab both from enhanced ADCC and from enhanced direct cell killing typical of type II antibodies [132–137]. Despite this promise related to pre-clinical activity, the clinical development of obinutuzumab has led to variable results. In CLL, as a single agent, obinutuzumab induced a rapid decrease in circulating CD20 positive cells associated with a significant rate of infusion related reactions secondary to cytokine release [138]. In combination with chlorambucil in newly diagnosed CLL patients with coexisting conditions, obinutuzumab demonstrated high response rates and progression free survival compared to chlorambucil monotherapy or the combination with rituximab [139]. In patients with relapsed/refractory indolent NHL, obinutuzumab monotherapy induced responses in 62% of patients during a phase 1 dose escalation and 30% in the phase 2 portion of the trial including responses in patients having previously received rituximab [140]. The best overall response rate of all patients also seemed to be higher than that reported with ofatumumab as monotherapy in a similar population. A subsequent randomized study of obinutuzumab in comparison with rituximab reported a similar response rate of 44.6% in obinutuzumab treated patients compared to 26.7% with rituximab, as determined by a blinded review panel [138]. However, despite the apparent benefit in response, obinutuzumab did not lead to an improvement in progression free survival [138]. Due to an observed dose response effect with obinutuzumab, a randomized trial of 1000mg vs 2000mg was performed which seemed to confirm a higher response rate with increased dosing (67% vs 49%) in previously untreated CLL patients [141]. In patients with relapsed indolent NHL following prior rituximab containing therapy, obinutuzumab was randomly studied in combination with bendamustine compared to bendamustine alone with obinutuzumab maintenance given in patients responding to the combination. While the end of induction response rate was no different between the two arms, the obinutuzumab/bendamustine group experienced less events and had a prolonged progression free survival compared to bendamustine alone [142]. In DLBCL, obinutuzumab monotherapy resulted in responses in 32% of patients, a rate that is similar to responses to rituximab in rituximab naïve relapsed DLBCL patients, with 20% of 25 rituximab-refractory patients achieving a response and a suggestion of increased

responses in a higher dose group [143]. In newly diagnosed DLBCL patients, obinutuzumab/CHOP was compared to rituximab/CHOP with no difference in EFS noted [144]. Obinutuzumab has gained regulatory approval in the United States for treatment of newly diagnosed CLL in combination with chlorambucil and in combination with bendamustine followed by obinutuzumab monotherapy in patients with follicular lymphoma relapsed after a rituximab containing regimen. However, the results in aggressive NHL variants have been inconsistent and continue to be evaluated with no current indication for aggressive B-NHL to date.

Circulating Antigen

Many cell surface antigens can also be identified in circulation. These circulating CD20 (cCD20) antigens have been identified in patients with CLL, Hodgkin lymphoma and NHL in addition to healthy controls [145–147]. Patients with B-NHL had significantly higher levels of cCD20 compared to normal controls [146]. In CLL, high levels of cCD20 have been correlated with disease stage and inversely correlated with overall survival [148]. It has been suggested that high levels of cCD20 may complex with therapeutic monoclonal antibodies leading to enhanced clearance, a mechanism suggested to contribute to the impaired efficacy of rituximab in CLL [149]. A more recent report also suggested a role of cCD20 in clinical outcomes in B-NHL with patients with high cCD20 levels prior to receiving therapy and those with higher cCD20 after therapy having a significantly lower probability of survival [146]. Serum rituximab concentration has been correlated to response in some studies with patients achieving higher concentrations being more likely to respond and patients with higher disease burden generally attaining less ideal rituximab levels [150–152, 17]. Binding of rituximab to cCD20 may hinder it's binding to B-cell associated CD20 and possibly increase clearance leading to decreased rituximab concentrations which may be able to be overcome by increased rituximab dose intensity.

Resistance to Apoptosis

As previously discussed, binding of monoclonal antibodies to surface antigens can induce intracellular signals leading to induction of apoptosis without the need of third party effector cells or complement activation. Alterations in the signaling pathways leading to apoptosis can thus lead to impaired ability of antibody to induce this effect. Multiple groups have generated NHL cell lines resistant to rituximab following serial exposure to the antibody and have demonstrated that alterations in pro- and anti-apoptotic regulators of apoptosis likely contribute to the development of resistance [153, 154]. While rituximab has demonstrated the ability to induce apoptosis, the importance of this effect on lymphoma cell death is unclear.

Type II anti-CD20 monoclonal antibodies on the other hand have demonstrated a more significant induction of cell death when compared to rituximab. In experiments assessing the cell killing effect of Type I *vs.* Type II antibodies, $F(ab')_2$ fragments of Type II antibodies were able to induce significant cell death independent of Fc dependent mechanisms as opposed to the Type I antibody which required the Fc fragment to induce cell death primarily through complement activation [23]. Type II antibodies have been developed in order to improve on the cell death induction observed with rituximab. Obinutuzumab has also demonstrated significantly more induction of cell death than rituximab *in vitro* [132]. While as previously discussed, obinutuzumab has enhanced ADCC activity secondary to a glycoengineered Fc segment increasing FcR binding affinity, the same antibody without the glycoengineering still maintained superior cell killing compared to rituximab highlighting the increased induction of PCD by this type II antibody [155]. Obinutuzumab, similar to a previously developed Type II antibody tositumomab, induces a caspase independent cell death that correlates with high levels of homotypic adhesion not observed with rituximab, possibly indicating enhanced signaling effect [22, 133]. This caspase-independent cell death has also been identified to occur through the generation of reactive oxygen species (ROS) mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase independent of mitochondria and can be blocked by exposure to an ROS scavenger [156]. Though additional signaling effects of obinutuzumab compared to rituximab that may be related to an increased induction of cell death and possible chemosensitization continue to be investigated, there do appear to be differences in signaling effects compared to rituximab in cells that are either rituximab-sensitive or rituximab-resistant with more significant effects noted on activation of protein kinase B (AKT), spleen-associated tyrosine kinase (SYK) and PLCγ2 following obinutuzumab exposure *in vitro* [157, 158].

Alternative Antibody Mediated Therapeutics

In addition to the use of naked monoclonal antibodies, other immunotherapy approaches utilize antibody-based approaches to direct therapy. Antibody drug conjugates (ADCs) or radioimmunoconjugates (RIC) utilize an antigen targeting antibody to deliver a drug or radioactive molecule that is covalently bound to the antibody to tumor cells in a targeted fashion. RICs have been thoroughly investigated in B-NHL and have earned regulatory approval for some limited treatment indications including indolent lymphomas relapsed after rituximab therapy (I^{131}-tositumomab and ibritumomab tiuxetan) or newly diagnosed follicular lymphoma following a response to initial therapy (ibritumomab tiuxetan).

In addition to these radioimmunoconjugates, other antibody drug conjugates have been evaluated in lymphoma. The most established is Brentuximab vedotin, an ADC targeting CD30 conjugated with monomethyl auristatin E (MMAE), a potent microtubule stabilizing agent. Brentuximab vedotin demonstrated significant

responses in relapsed refractory Hodgkin lymphoma and anaplastic large cell lymphoma, two lymphoma types with high CD30 expression; and it is approved for use in adult classical Hodgkin lymphoma patients who have relapsed after stem cell transplant or 2 chemotherapy regimens, as consolidation after transplant or with newly diagnosed stage 3 or 4 disease and in relapsed systemic or anaplastic large cell lymphoma or mucosis fungoides [159, 160]. Despite low levels of CD30 expression, brentuximab vedotin has also exhibited activity in treatment of some B-NHLs, in particular DLBCL and primary mediastinal large cell lymphoma [161].

Since these agents rely on the antibody primarily for targeting purposes, resistance mechanisms relating to Fc associated mechanisms of activity previously discussed are generally less relevant. However, changes in antigen expression can have a role in resistance to ADCs which continue to rely on antigen expression for appropriate delivery of their cargo. A single case of CD20 negative relapse following treatment with I^{131}-tositumomab has been reported though this was a very early progression raising the question of monoclonal antibody blocking binding of anti-CD20 antibody used for immunohistochemistry analysis [162]. Additionally, CD30 negative relapse of ALCL has been reported following treatment with brentuximab vedotin [163, 164].

Similarly, since the efficacy of ADCs is dependent on the anti-neoplastic agent conjugated to the antibody, additional mechanisms of resistance common to other chemotherapeutic agents can contribute to resistance. Chen *et al.* described mechanisms of resistance to brentuximab vedotin in ALCL and Hodgkin lymphoma cell lines generated to be resistant following serial exposure [165]. In addition to down-regulation of CD30 expression affecting ADC targeting, resistance to MMAE was observed. MMAE intracellular accumulation was lower in resistant cells following exposure to the ADC or to free MMAE suggesting possible impaired delivery related to decreased antigen expression, but also resistance to MMAE itself. Investigation of mechanisms of resistance to the MMAE identified an increased expression of the multi-drug resistance gene MDR1, with resistance to MMAE partially reversed following inhibition of p-glycoproteins. Similar increase in positivity for drug transporters was observed in patient samples from relapses following brentuximab vedotin therapy.

Additionally, altered induction of target cell apoptosis may contribute to resistance to the conjugated molecule. For example, in pre-clinical investigation of a novel ADC targeting CD79b and conjugated with MMAE, increased expression of the anti-apoptotic Bcl-2 family protein Bcl-xL was demonstrated to be associated with resistance to this investigational ADC with enhanced responses noted following inhibition of Bcl-2 family proteins using ABT-263 [166]. Alternative antibodies with enhanced antigen targeting or targeting alternative surface antigens, conjugates with more efficient conjugation of anti-neoplastic compounds and alternative anti-neoplastic agents not known to be substrates for drug transporter mediated efflux represent potential options for alternative ADCs to circumvent these identified mechanisms of resistance.

Another use of monoclonal antibody therapy is the ability to target tumor cells to cytotoxic effector cells using bi-specific antibodies that can bind the target cell and an effector cell. The prime example is the bispecific T-cell engaging (BiTE) antibody blinatumomab. Blinatumomab is a bivalent antibody targeting CD19 present on B-cells and CD3 on T-cells leading to enhanced immune-mediated clearance of tumor cells. It has been approved for use in acute lymphoblastic leukemia (ALL) and is under investigation in B-NHL with responses in 69% of 76 relapsed/refractory B-NHL patients including 55% of patients with DLBCL [75]. With data primarily in ALL, resistance to blinatumomab has been noted with CD19-negative relapses and primary resistance possibly due to high expression of the checkpoint inhibitor ligand PD-L1 on tumor cells [167–169]. Alternative bispecific antibodies constructed to enhance immune surveillance of malignant cells continue to be developed and evaluated in B-NHL including a CD20-CD3 bispecific antibody [170].

Summary

Monoclonal antibodies have been a cornerstone of therapy for lymphomas for decades following the first ever approval of a monoclonal antibody therapy, rituximab, for the treatment of cancer. Despite the overwhelming success of rituximab in treating NHLs, resistance exists both in primary refractory cases and on relapse following treatment with rituximab. Mechanisms of resistance have been identified that target all described mechanisms of monoclonal antibody activity including altered antigen expression or binding, impaired CDC or ADCC, altered intracellular signaling effects and inhibition of direct induction of cell death. Multiple next generation anti-CD20 monoclonal antibody therapies developed to overcome described resistance mechanisms continue to be investigated with two, ofatumumab and obinutuzumab, already approved for the treatment of B-cell malignancies albeit with narrow indications. Alternative monoclonal antibody based immunotherapeutic approaches more recently developed include the use of ADCs and bispecific or multivalent antibody constructs. The ADC brentuximab vedotin has approvals for indications in Hodgkin lymphoma and relapsed ALCL, while the BiTE antibody blinatumomab is approved for use in B-ALL. Understanding of these newer monoclonal antibody based therapeutic approaches and mechanisms of resistance to them continue to be studied, with alternative agents from each class already in development to try to improve on the significant activity already observed with each agent.

Acknowledgements S.C.G. is supported by Hyundai Hope on Wheels.

Disclosure of Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 3
Resistance to Antibody-Drug Conjugate

Jessica Hochberg and Sarah Alexander

Abstract Immune therapies have shown significant efficacy in the treatment of pediatric lymphomas. Monoclonal antibodies, whether naked or conjugated have emerged as an attractive option for targeted therapy while minimizing toxicities. Monoclonal antibodies conjugated to small molecule drugs were developed as a way to combine highly potent agents with tumor specificity. Current challenges include careful selection of tumor targets, the management of potential toxicities, identification of ideal patient selection and therapy regimens, and a better understanding of antibody-drug conjugates (ADC) mechanisms of action and resistance. The right combination is critical for a successful ADC. Mechanisms of resistance to ADCs can be inherited to the ADC or acquired by the host environment and can developed against each of the individual components of the ADC. Given the rational design of ADCs, there is the ability to modify each of the components to develop improved agents that can overcome resistance.

Keywords Pediatrics · Lymphoma · Antibody · Conjugates · Resistance Immunotherapy

Abbreviations

ABVD	Doxorubicin, Bleomycin, Vinblastine, Dacarbazine
ADC	Antibody-Drug Conjugate
ADCC	Antibody Dependent Cellular Cytotoxicity

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A. C. Xavier, M. S. Cairo (eds.), *Resistance to Targeted Therapies in Lymphomas*, Resistance to Targeted Anti-Cancer Therapeutics 21,
https://doi.org/10.1007/978-3-030-24424-8_3

Ag-Ab	Antigen-Antibody
AKT	Activated Tyrosine Kinase
ALCL	Anaplastic Large Cell Lymphoma
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
AVD	Doxorubicin, Vinblastine, Dacarbazine
Bv	Brentuximab Vedotin
CI	Confidence Interval
COG	Children's Oncology Group
CR	Complete Response
DLBCL	Diffuse Large B-Cell Lymphoma
FDA	Food and Drug Administration
GO	Gemtuzumab Ozogamicin
HL	Hodgkin Lymphoma
HR	Hazard Ratio
IO	Inotuzumab Ozogamicin
mAB	Monoclonal Antibody
MF	Mycosis Fungoides
MMAE	Monomethylauristatin E
NHL	Non-Hodgkin Lymphoma
OR	Objective Response
pcALCL	Primary cutaneous Anaplastic Large Cell Lymphoma
PFS	Progression-Free Survival
RR	Response Rate

Introduction

Immune therapies play an increasing role in the treatment of cancer overall and have shown significant efficacy in the treatment of Hodgkin (HL) and Non-Hodgkin Lymphoma (NHL). Monoclonal antibodies, such as the CD20 targeted agent rituximab, emerged as an attractive option for targeted therapy while minimizing toxicities. Lessons learned from the use of rituximab in NHL have shown that monoclonal antibodies can be used alone and in combination with multi-agent chemotherapy to improve outcomes in both pediatric and adult lymphoma. Furthermore, the use of novel immune therapy agents allows for the potential reduction in cytotoxic therapies and minimization of long term side effects. However, the clinical efficacy of naked antibodies as single agents remains limited. Thus, monoclonal antibodies conjugated to small molecule drugs were developed as a way to combine highly potent agents with tumor specificity. Over the past 30 years of antibody-drug conjugate (ADC) research, several new linkers, and conjugation strategies have been discovered. Designing an effective ADC is a complex process, requiring thoughtful combination of optimal antibody target, linker, and tolerable drug. Lessons learned from the first-generation of ADC's have guided the design of improved compounds which are now in clinical trials. Current challenges include

careful selection of tumor targets, the management of potential toxicities, identification of ideal patient selection and therapy regimens, and a better understanding of ADC mechanisms of action and resistance. Here we will review the structure and mechanisms of resistance to ADCs, specifically as they relate to lymphoma.

ADC Structure and Components

ADCs are typically comprised of a fully humanized monoclonal antibody (mAb) targeting an antigen specifically/preferentially expressed on tumor cells, a cytotoxic drug, or payload, and a suitable linker (Fig. 3.1) [1]. The right combination is

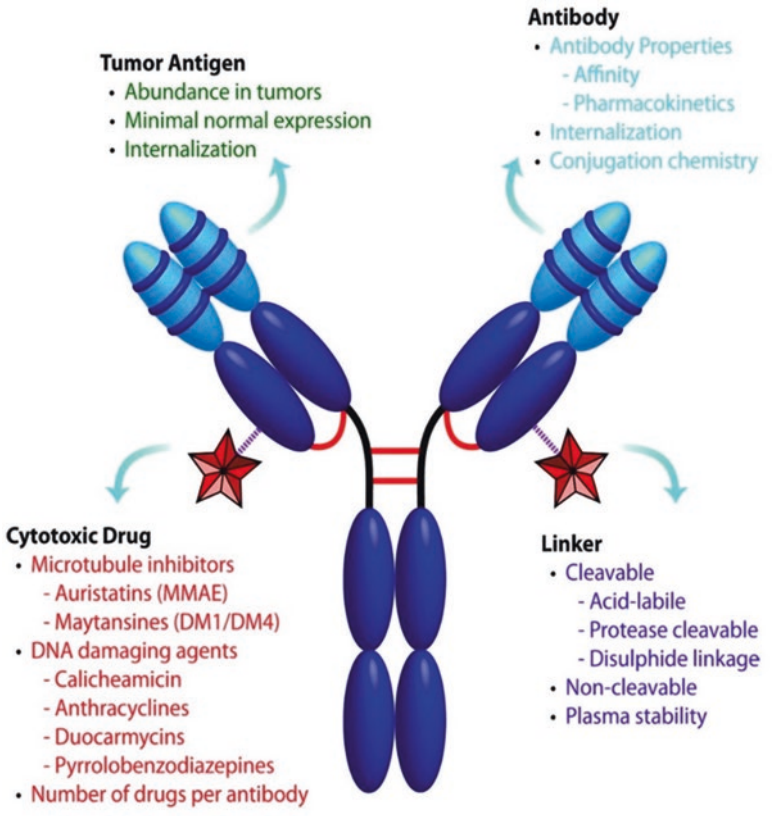

Fig. 3.1 Critical factors that influence ADC therapeutics. ADCs consist of a cytotoxic drug conjugated to a monoclonal antibody by means of a select linker. These components all affect ADC performance and their optimization is essential for development of successful conjugates. (from Siler Panowski, Sunil Bhakta, Helga Raab, Paul Polakis & Jagath R Junutula (2014) Site-specific antibody drug conjugates for cancer therapy, mAbs, 6:1, 34–45, DOI: https://doi.org/10.4161/mabs.27022)

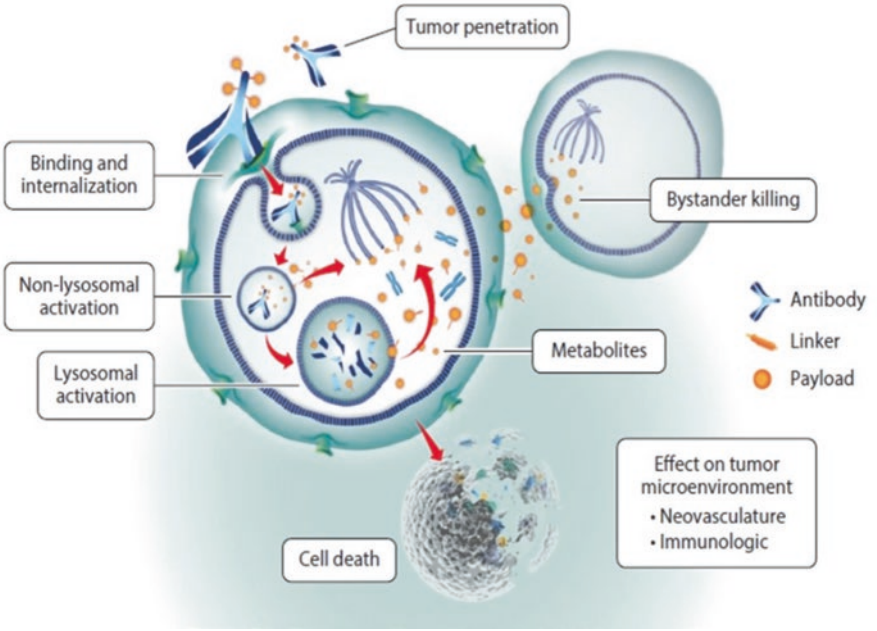

Fig. 3.2 The ADC must enter tumor tissue from the vasculature, bind to its cell surface target, and then be internalized via the endosome–lysosome pathway, where the linker is cleaved and/or the antibody is degraded to release the payload, which ultimately diffuses into the cytoplasm to reach its target. (*from* John M. Lambert and Anna Berkenblit, Antibody–Drug Conjugates for Cancer Treatment. Annu. Rev. Med. 2018. 69:191–207)

critical for a successful ADC. The target antigen must be highly expressed on the surface of the tumor cells with relatively little expression on normal cells, thus minimizing the chances of off target cytotoxicity. Upon binding, the antibody must be absorbed through rapid internalization followed by lysosomal degradation, and finally release of the cytotoxic drug inside the cell (Fig. 3.2) [1–3].

Antibody Selection

The selection of mAbs for specific tumor targeting leads to ADCs precision acting only on cancer cells, increasing the therapeutic index while minimizing off-target side effects. Therefore, determining which antigen to target is the first and most important step in ADC development [2]. Overexpression on tumor cells with minimal to no expression on healthy cells ensures specific targeting delivery of cytotoxic agents. Monoclonal antibodies also must have the ability to bind strongly and then penetrate tumor. If the target antigen is shed easily from tumor tissues or if the antibody does not strongly bind, systemic clearance from

circulation can alter the potency and pharmacokinetics of the ADC [1]. There must remain a balance between internalization and disassociation rates of the antigen-antibody (Ag-Ab) complexes in order to ensure effective drug utilization. Isotype selection of the mAb influences the ability of the ADC to stimulate immune system mediated actions with IgG1 able to support antibody-dependent cellular cytotoxicity (ADCC), whereas IgG2 and IgG4 are limited in this function [4]. Minimizing immune system actions can help limit unwanted side effects [1].

Cytotoxic Agents

The most frequently used cytotoxic agents are DNA damaging (such as calicheamicin analogs) or anti-microtubule compounds (such as auristatin analogs) [5]. Other drugs used in clinical-stage ADCs are topoisomerase inhibitors, DNA-alkylators, and RNA polymerase II inhibitors [1]. Drugs are selected for high cytotoxicity, so that they can destroy tumor cells at intracellular concentrations achieved after ADC delivery.

Linker Design

One of the main challenges in developing ADCs is to incorporate a linker that has high stability in systemic circulation for a prolonged period over several days and efficient lysosomal release of the drug only after internalization into the tumor. Premature release of drugs in the circulation can lead to systemic toxicity and a lower overall efficacy. Linkers are generally divided as cleavable and non-cleavable [2]. Cleavable linkers can be acid sensitive, only hydrolyzed in the lower pH environment of the lysosome; glutathione-sensitive disulfide linkers, where tumor cells express elevated levels of thiols; or lysosomal protease-sensitive peptide linkers. Non-cleavable linkers have better plasma stability which is beneficial in decreasing plasma drug release. Stability is achieved by attaching the linker to amino acid residues of the mAb through a non-reducible bond [2, 3, 6].

ADC in Lymphoma

At the time of writing of this review there is one ADC, brentuximab vedotin (Bv), which is Food and Drug Administration (FDA) approved for the treatment of patients with HL and NHL. In addition, three ADCs are approved for other diseases including gemtuzumab ozogamicin for the treatment of newly-diagnosed adult

patients with CD33-positive acute myeloid leukemia (AML) and for children and adults with relapsed or refractory CD33-positive AML, inotuzumab ozogamicin (IO) for adults with relapsed or refractory B-cell precursor acute lymphoblastic leukemia (ALL) and trastuzumab emtansine for patients with HER2 positive metastatic breast cancer. There are dozens of ADCs in development, including more than 20 being investigated for the treatment of patients with HL and NHL [7].

Brentuximab Vedotin

Bv is a chimeric anti-CD30 monoclonal antibody linked by a cathepsin B sensitive cleavable dipeptide valine-citruline linker to monomethylauristatin E (MMAE). Bv was the first ADC approved by the FDA, originally in 2011 for the treatment of patients with relapsed anaplastic large cell lymphoma (ALCL) following systemic chemotherapy and for those with relapsed HL following autologous stem cell transplant. Approval for the treatment of patients with relapsed and refractory ALCL was based on a phase 2 study in adults in which Bv was given at a dose of 1.8 mg per kilogram every 3 weeks. In this trial, 50 out of 58 (86%) patients achieved an objective response (OR), with 57% achieving complete response (CR) and 97% showing tumor reduction [8]. Subsequently, indications for Bv expanded to include treatment of adults with primary cutaneous ALCL (pcALCL) or CD30 negative expressing mycosis fungoides (MF) who have received prior systemic therapy. In addition the ECHELON-1 study in adults with newly diagnosed advanced stage HL demonstrated that Bv in combination with conventional AVD (doxorubicin, vinblastine, dacarbazine) chemotherapy (A + AVD) demonstrated 2-year modified progression-free survival (PFS) rates in the A + AVD and ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) groups of 82.1% [95% confidence interval (CI_{95}), 78.8–85.0] and 77.2% (CI_{95}, 73.7–80.4), respectively, with a hazard ratio (HR) for an event of progression, death, or modified progression of 0.77; CI_{95}, 0.60–0.98; P = 0.04) [9, 10].

Bv has been shown to be active in adult patients with relapsed and refractory diffuse large B cell lymphoma (DLBCL) with CD30 expression [11]. Safety of single agent Bv has been studied in children with relapsed and refractory HL and ALCL as well [12]. The safety and efficacy of Bv in combination with conventional chemotherapy for children with ALCL and for those with HL is being investigated through two Children's Oncology Group (COG) trials (www.clinicaltrials.gov identifiers NCT01979536 and NCT02166463).

Inotuzumab Ozogamicin

Inotuzumab ozogamicin (IO) is a humanized anti-CD22 monoclonal antibody with a cleavable hydrazine linker attached to N-acetyl-gamma-calicheamicin. It was FDA approved in 2017 for the treatment of adult patients with relapsed or

refractory precursor-B ALL. IO has been studied in multiple trials in adults with relapsed and refractory CD 22 positive indolent and aggressive B cell lymphomas as a single agent, in combination with rituximab and in combination with conventional chemotherapy with most studies describing favorable response rates while others showed no clear advantage when compared to alternate regimens [13–15].

ADC for Lymphoma in Development

There are numerous ADCs in all stages of pre-clinical and clinical trial development. Anti-CD19 agents that have undergone phase 1/2 studies in patients with B-lineage NHL include denintuzumab mafotin, coltuximab ravtansine and loncastuximab tesirine [16–19]. Pinatuzumab vedotin is a CD22-targeted ADC linked to MMAE that has and is being studies as a single agent and in combination with rituximab and obinutuzumab [20]. Polatuzumab vedotin is an anti-CD79b antibody conjugated to MMAE that has and is being investigated as a single agent and in combination with rituximab, obinutuzumab, conventional chemotherapy regimens in patents with DLBCL [21–23]. Additional ADC directed at CD19, 22 and 79b are in earlier phase clinical trials in addition to those directed at CD37, 70 and 25 [7].

General Mechanisms of Resistance

Mechanisms of resistance to ADCs can be inherent to the ADC or acquired by the host environment and can develop against each of the individual components of the ADC, namely the mAb, linker mechanism or the cytotoxic drug (Fig. 3.3) [6].

Antigen-Related Resistance

Changes in the levels of the antigen recognized by the mAb can occur through down regulation of cell surface protein expression, shedding of antigen or high level of expression of antigen in other tissues [6]. If the mAb is unable to bind sufficiently to the target cell, potency will be limited. Other mechanisms of antigen-related resistance may include masking or truncation of the epitope, or the presence of additional ligands to the antigen although these have not yet been reported in preclinical models of ADCs for lymphoma.

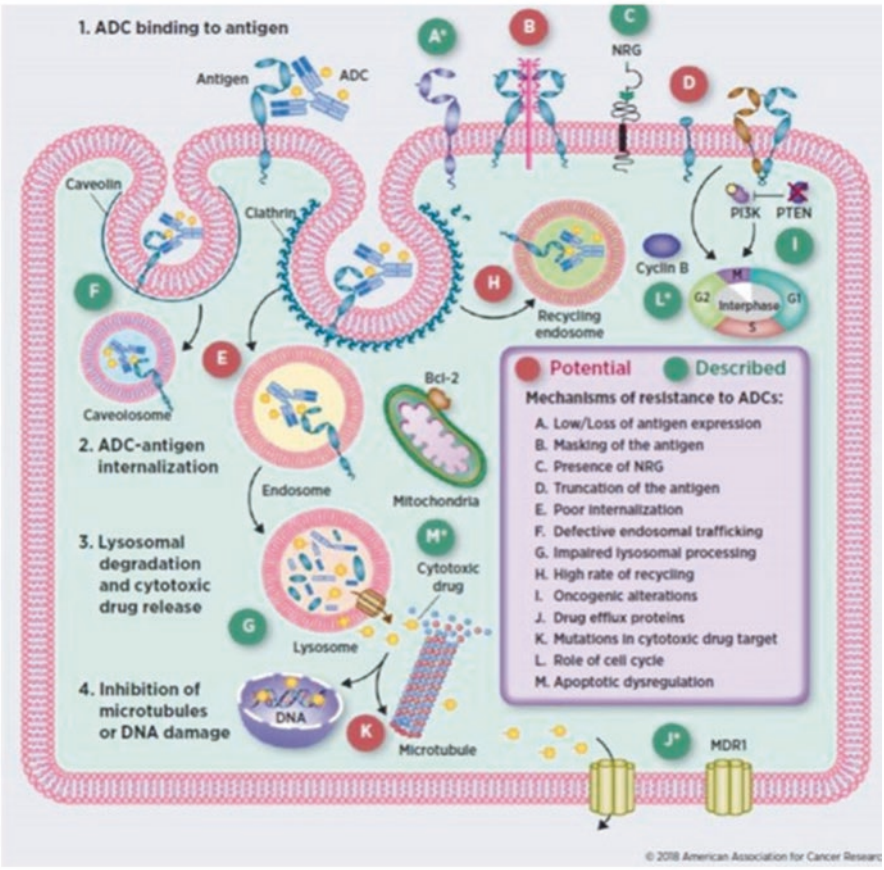

Fig. 3.3 The antibody binds to its target on the plasma membrane (1); then, ADC-target complexes enter cells via receptor-mediated endocytosis (2). The internalized complexes are initially contained within endocytic vesicles that fuse to become early endosomes and eventually mature to lysosomes (3). The ADC undergoes catabolism to release the cytotoxic agent, which can then be transported from the lumen of lysosomes to the cytosol. The intracellular cytotoxic agent exerts its action, generally damaging DNA or inhibiting microtubule polymerization (4), which ultimately leads to cell death. Alterations in any of these events may lead to resistance acquisition. Circled letters indicate potential (red) or already described in the literature (green) mechanisms of resistance to ADCs. The asterisks indicate mechanisms of resistance verified using patient-derived material. (*from* Sara García-Alonso, Alberto Ocana, and Atanasio Pandiella, Resistance to Antibody–Drug Conjugates. Cancer Res; 78 (9) May 1, 2018)

Defects in Internalization and Lysosomal Function

ADC efficacy requires endocytosis of the antibody into the cell (Fig. 3.3) [6]. Endocytosis can occur by different internalization routes which have all been implicated in possible resistance mechanisms [24]. Once delivered, impaired lysosomal

function can occur as a result of low proteolytic activity due to elevations in lysosomal pH [25].

Drug Efflux Pumps

A common mechanism of resistance for chemotherapies is the elimination of the agent from the cellular cytoplasm by drug efflux pumps. Commonly used cytotoxic agents, such as calicheamicin, auristatins, maytansines, taxanes, and doxorubicin are well-known substrates of efflux transporters [26–28].

Signaling Pathways and Apoptosis

Activation of downstream signaling pathways may contribute to the acquisition of resistance to ADCs. Activated PI3K/AKT signaling has been associated with gemtuzumab ozogamicin (GO) resistance *in vitro* in primary AML cells and it has been shown that the AKT inhibitor MK-2206 significantly sensitized resistant cells to GO or free calicheamicin [29]. A role for the pro-apoptotic proteins BAX and BAK in the regulation of GO sensitivity in AML has also been described previously as well as the overexpression of the anti-apoptotic proteins BCL-2 and BCL-X [30].

Mechanisms of Resistance to ADC in Lymphoma

In the pivotal phase 2 trial of Bv in adults with relapsed and refractory ALCL, the overall response rate (RR) was 86% and, of those, 57% obtained a CR [8]. However, despite ongoing therapy, all of the patients who did not achieve a CR eventually developed progressive disease. Understanding the mechanisms for those who had no response or loss of response is currently a focus on intense research.

Loss of Cell Surface Antigen

In vitro data using ALCL-derived cell lines selected for resistance to Bv has demonstrated that a mechanism for the evolution of resistance is the downregulation of CD30 expression [31]. This mechanism of ADC resistance has been suggested in case reports of individuals treated with Bv for CD30 positive lymphomas who had initially responsive disease and were found to have had loss of CD30 expression on pathologic samples at the time of disease progression [32–34].

Drug Efflux Pumps

In HL cell lines resistant to Bv, increased MDR1 drug exporter expression have been identified as possible mechanism of drug resistance [35]. Additionally, in a mouse xenograft model of Bv resistant cell lines with MDR1 upregulation, resistance was overcome by the addition of the MDR1 inhibitor cyclosporine [31]. MDR1 activity has been demonstrated *in vitro* to be associated with IO resistance in NHL and cell lines [28]. Similarly in NHL derived cells lines resistant to pinatuzumab vedotin and polatuzumab vedotin, MDR1 expression was identified as being the major driver of drug resistance. In this model, resistance was able to be overcome by replacing the MMAE moiety with an anthracycline derivative, NMS249, in the ADC complex leading the authors to hypothesize that the return of drug sensitivity was based on NMS249 being a poor substrate for MDR1.

Changes in Apoptotic Regulation

BCL-XL is a member of the BCL-2 family and is a mitochondrial transmembrane anti-apoptotic protein. It is associated with chemotherapy resistance and poor prognosis in adults with follicular lymphoma [36]. *In vitro* studies of an anti-CD79b-vc-MMAE ADC demonstrated that the expression level of BCL-XL was associated with less sensitivity to the drug [37]. In a subsequent xenograft model, the authors were able to demonstrate that the BCL-2 inhibitor ABT-263 (known currently as navitoclax) was able to restore tumor responsiveness to treatment with anti–CD79b-vc-MMAE.

Strategies for ADC Optimization

Given the rational design of ADCs, there is the ability to modify each of the components to develop improved agents that can overcome resistance. Low levels of antigen on tumor cells can be overcome by designing ADCs that have a significant bystander effect or by utilizing bispecific mAbs to recruit additional cytolytic immune cells [6]. Drug efflux pumps can be avoided by utilizing agents that are poor substrates for MDR1 or modifying the linker used for delivery [6]. Effective linker designs using hydrophobic compounds with homogenous drug-antibody conjugation were found to have enhanced potency [2]. Drug-to-antibody ratios also become very important in determining ADC efficacy. Drug-to-antibody ratio is defined as the number of drug molecules per mAb. This determines the dose needed to produce the desired efficacy. There is a limited number of drug molecules that can be efficiently delivered to the target site which significantly contributes to the pharmacokinetics of the ADC. If fewer drug molecules are conjugated per mAb, the

ADC system will not be effective clinically. However, too many drug molecules per mAb will make the ADC unstable, toxic and may lead to aggregation and increased immunogenic reactions [2, 38]. Most ADCs in current clinical development utilize conjugation to either lysine or cysteine residues of the antibody, which has led to an average drug-to-antibody ratio in the range of 3.5–4.0. This ratio was initially thought to minimize the amount of non-conjugated antibody and avoid too high of a ratio which can cause issues with manufacturing and stability [3]. Newer methods of ADC production have been developed for site-specific conjugation, which can enable lower drug-to-antibody ratios while avoiding excessive modification of the antibody. This approach is especially useful for highly potent or hydrophobic drugs, for which drug-to-antibody ratios greater than 2 are undesirable [3]. Likely, the best strategy for overcoming ADC resistance will be to combine with other cytotoxic and immune therapies such as multidrug chemotherapy regimens or with checkpoint blockade [39].

Conclusion

Drug resistance, either inherent or acquired, remains an obstacle to efficacy in oncology treatment. The need to develop improved treatment paradigms which utilize the power of the immune system with the potential to minimize short and long term side effects of therapy make ADCs attractive novel agents. However, many of the same resistance concerns are emerging and novel mechanisms of ADC resistance are being described with increased use. Further identification and characterization of these mechanisms of resistance will lead to more optimal ADC design with improved efficacy for all.

Disclosure of Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 4
Resistance to Proteasome Inhibitor Therapy in Non-Hodgkin Lymphoma

Rodney R. Miles and Paul J. Galardy

Abstract The proteasome is a cytosolic proteolytic system that not only degrades damaged proteins but also has a critical role in cellular function through highly-regulated, targeted degradation of proteins. Inhibition of the proteasome system has been shown to have therapeutic potential in certain hematological malignancies. In this chapter, we will provide an overview of the ubiquitin proteasome system, focusing our discussion in the mechanisms of action and resistance to small molecule proteasome inhibitors currently approved or in development for therapeutic use in cancer.

Keywords Proteasome inhibitors · Non-Hodgkin lymphoma · Ubiquitin-activating enzymes · Ubiquitin-conjugating enzymes · Bortezomib

Abbreviations

ABC	Activated B-Cell
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia

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A. C. Xavier, M. S. Cairo (eds.), *Resistance to Targeted Therapies in Lymphomas*, Resistance to Targeted Anti-Cancer Therapeutics 21,
https://doi.org/10.1007/978-3-030-24424-8_4

CHOP	Cyclophosphamide, Doxorubicin, Vincristine, Prednisone
COG	Children's Oncology Group
CNS	Central Nervous System
CR	Complete Response
DLBCL	Diffuse Large B-Cell Lymphoma
DUB	Deubiquitinating Enzymes
E1	Ubiquitin-Activating Enzyme
E2	Ubiquitin-Conjugating Enzyme
E3	Ubiquitin-Protein Ligases
ER	Endoplasmic Reticulum
FDA	Food and Drug Administration
GCB	Germinal Center B-Cell Type
HL	Hodgkin Lymphoma
IC50	Half Maximum Inhibitory Concentration
IHC	Immunohistochemical
LL	Lymphoblastic Lymphoma
MCL	Mantle Cell Lymphoma
MHC	Major Histocompatibility Complex
MM	Multiple Myeloma
NHL	Non-Hodgkin Lymphoma
OS	Overall Survival
ORR	Overall Response Rate
PFS	Progression-Free Survival
PMBCL	Primary Mediastinal large B-Cell Lymphoma
R-CHOP	Rituximab, Cyclophosphamide, Doxorubicin, Vincristine, Prednisone
R/R	Relapsed/Refractory
VR-CAP	Bortezomib, Rituximab, Cyclophosphamide, Doxorubicin, and Prednisone

Introduction

Since the early 2000's, proteasome inhibition has taken center stage for the therapy of the plasma cell cancer multiple myeloma (MM). Although efficacy has not been as robust in other cancers, proteasome inhibitors have found utility in hematologic cancers, and recent clinical trials through the Children's Oncology Group (COG) have included bortezomib – the first in class proteasome inhibitor – in the initial therapy for patients with acute myeloid leukemia (AML) and T-cell acute lymphoblastic leukemia (ALL)/lymphoma. While ongoing work continues to push the boundaries of where bortezomib and newer next-generation proteasome inhibitors fit into the therapy for these diseases, there has been tremendous insight into the mechanisms by which cancers may evade these novel therapeutics. Here we will provide an overview of the ubiquitin proteasome system, the small molecule inhibitors of the proteasome that are approved and in development, and delve into the mechanisms of resistance with insight into potential avenues to preserve efficacy in non-Hodgkin lymphoma (NHL).

The Ubiquitin Proteasome System

The proteasome is the major cytosolic proteolytic system in eukaryotic cells. Comprised of a large multi-subunit complex, the proteasome recognizes and unfolds proteins marked for degradation, threads the polypeptide into the proteolytic chamber, and releases peptide fragments following proteolysis (Fig. 4.1) [1]. These peptides may be further degraded to recycle amino acids, or they may be incorporated into major histocompatibility complex proteins and trafficked to the cell surface for surveillance by T-lymphocytes [2]. While often envisioned as the garbage basket of

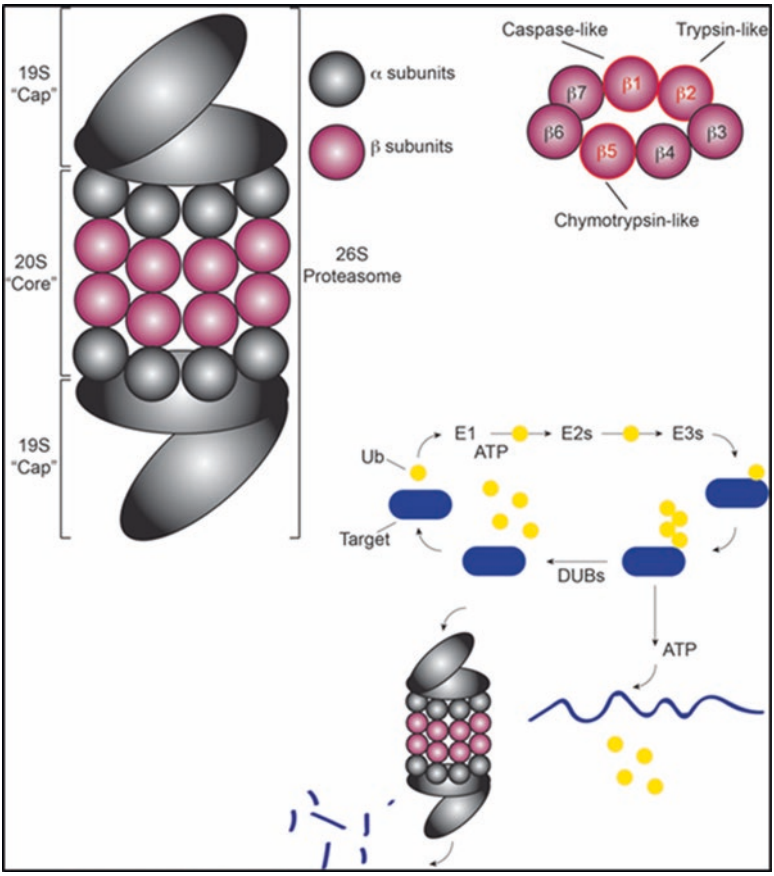

Fig. 4.1 The 26S proteasome is composed of the 20s core particle with a 19S regulatory particle at each end. The core 20S proteasome particle is comprised of a series of four stacked rings with seven subunits each that include the three catalytic subunits β1, β2, and β5. The enzymatic ubiquitination cascade involves an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s) for specific protein substrate ubiquitination. Once ubiquitinated, the protein is degraded by the proteasome or possibly targeted by deubiquitinating enzymes (DUBs) that remove ubiquitin

the cell that degrades proteins that have become damaged or otherwise have reached the end of their usefulness, the targeted degradation of many proteins is essential for events including receptor tyrosine kinase signaling and signal transduction from these receptors to the nucleus [3], cell cycle transitions [4], DNA damage repair [5], gene transcription [6], and others [7]. As such, activity of the proteasome is regulated at multiple levels, and inhibition of its activity has a broad array of cellular effects.

Ubiquitination

A major mechanism that regulates the activity of the proteasome is the selective marking of proteins with the modifier ubiquitin. Itself a 76 amino acid protein, the addition of ubiquitin to proteins is regulated by a large family of enzymes that attach or remove ubiquitin, often in a substrate and signal-specific manner [7]. The attachment of a single ubiquitin molecule, known as mono-ubiquitination, is affected by an enzymatic cascade that involves an ubiquitin-activating enzyme (E1) that expends ATP to install an energetic thioester on the C-terminus of ubiquitin. This enables its transfer to one of dozens of ubiquitin-conjugating enzymes (E2s) that work together with one of hundreds of ubiquitin-protein ligases (E3s) that bind to specific protein substrates to either directly transfer ubiquitin to the target (HECT-class E3s) or to simply act as a scaffold that brings the ubiquitin-loaded E2 in proximity with the targeted substrate [8]. In nearly all cases, the ubiquitin is attached to the ε-amino group on lysine side chains of the substrate [8]. Once a single ubiquitin has been attached, the process may be repeated with subsequent cycles of ubiquitination onto one of six potential lysine side chains on the initial ubiquitin molecule. Most proteasome substrates are marked by the presence of poly-ubiquitin chains comprised of at least four ubiquitin molecules linked sequentially through lysine 48 on each ubiquitin moiety [9]. Degradation of chains linked through lysine 11 generated by the anaphase promoting complex working with the Ube2c and Ube2s E2 enzymes has also been described [10]. Once marked by ubiquitination, modified proteins may either be recognized and degraded by the proteasome or they may be acted upon by one of nearly 100 ubiquitin isopeptidases (deubiquitinating enzymes, DUBs) that remove ubiquitin – possibly providing a reprise for the targeted protein [11, 12]. Although proteins may be degraded due to damage, the regulated ubiquitination of highly cancer relevant substrates such as cell cycle regulators is a highly orchestrated to occur at specific instances to promote cell cycle progression. As such, the ubiquitin proteasome system is less a garbage dump and more like a molecular sniper that is poised to eliminate select proteins to allow cellular events to occur in a highly ordered fashion.

Proteasome Structures

The proteasome exists in several forms, with the most predominant being the 20S and 26S proteasome [13]. The core 20S proteasome particle is comprised of a series of four stacked rings with seven subunits each that assemble to form a barrel shaped structure [14, 15]. Within the core 20S particle are three catalytic subunits β1, β2, and β5 that are largely responsible for three catalytic activities of the particle known as the chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolyzing respectively [14, 15]. These catalytic subunits may be replaced by subunits β1i, β2i, and β5i the expression of which is induced by interferon-γ to form what is termed the immunoproteasome [16]. The catalytic activity of this alternative form is altered compared with the constitutive proteasome to favor the production of peptides that associate with the major histocompatibility complex (MHC) proteins that are involved in presenting antigens to T lymphocytes [16, 17]. Proteasome inhibitors have variable inhibitory activities towards the various catalytic subunits, though most available compounds target both the constitutive and immunoproteasome subunits equally. Selective immunoproteasome inhibitors are under development with potential application in hematologic cancers and inflammatory diseases [18].

Each end of this cylindrical particle may be capped with one two protein complexes known as the 11S or 19S regulatory particles [13]. The most common is the 19S regulatory particle that contains receptors for polyubiquitin and ATPases that assist in the unfolding of protein substrates and deubiquitinating enzymes that remove ubiquitin from the substrates so that it may be recycled [19, 20]. The combined structure that includes the 20S core particle with the 19S regulatory particle at each end is termed the 26S proteasome [5]. There is good evidence also that the activity of these deubiquitinating enzymes, counteracted by a proteasome-associated ubiquitin-protein ligase known as E4, will affect the rate at which relatively poorly ubiquitinated substrates are degraded [20, 21].

Feedback Induction of Proteasome Assembly

While there has been substantial excitement about the use of proteasome inhibitors in lymphoid malignancies due to the success in myeloma, other cancers seem more resistant to inhibition of this enzyme complex that is thought to be central to all cellular functions. While myeloma and other lymphoid cells may have enhanced sensitivity due to the increased reliance on protein synthesis and therefore to stress induced by the unfolded protein response [1, 22], it has been unclear as to why other cancers seem able to bypass the fundamental need for targeted proteolysis. Studies that examine resistance mechanisms have noted that some cells increase the synthesis of proteasomes in response to reduced proteasome activity [1, 23]. Through elegant work by the Deschais, Hay, and Goldberg laboratories, the mechanism that couples proteasome activity with the synthesis of its subunits was elucidated

[24–26]. The Nrf1 protein is synthesized as an endoplasmic reticulum (ER) resident protein and immediately engages with the ER-associated degradation pathway where it is retro-translocated to the cytosol with the assistance of p97. When proteasomes are abundant and active, the Nrf1 protein is degraded coincidentally with its translocation from the ER to the cytosol. When proteasome activity is limiting, the Nrf1 protein is processed by an ER membrane protease to release a transcriptionally active domain that translocates to the nucleus and promotes the transcription of proteasome subunit genes. This mechanism therefore results in increased proteasome assembly which then suppresses Nrf1 and resumes the baseline state. When combined with inhibitors that bind irreversibly to the proteasome, the effect is to enhance proteasome activity leading to clinical resistance. While irreversible proteasome inhibitors may be more effective, strategies that also incorporate inhibition of the p97 assisted retro-translocation pathway are being explored as ways to circumvent this resistance mechanism [27, 28].

Proteasome Inhibitors

Bortezomib

Proteasome inhibitors were first developed as tools to aid in biochemistry studies seeking to understand the role of the proteasome in cell biology (Table 4.1). Because of the central role of the proteasome as a critical regulator in all cells and tissues, it was initially assumed that systemic inhibition of the proteasome would lead to unacceptable toxicity. The potential for interfering with the proteasome as a cancer therapy was hinted at in initial studies where blocking ubiquitination – or proteasome function – was found to cause cell cycle arrest *in vitro*. Developed by Millennium Pharmaceuticals (Cambridge, MA) and initially referred to as PS-341, bortezomib (Velcade®) was the first-in-class proteasome inhibitor and rapidly generated excitement given its profound inhibitory effects on the NCI-60 cell line panel with a low nanomolar half maximum inhibitory concentration (IC50) for the entire panel [29]. A dipeptide boronic acid compound, bortezomib shows greater selectivity towards the proteasome compared with commonly used biochemical tools such

Table 4.1 Proteasome inhibitors in use or under development

Agent	Binding site(s)	Mode	Route
Bortezomib	β1, β5	Reversible	IV
Carfilzomib	β5	Irreversible	IV
Ixazomib	β5	Reversible	PO
Oprozomib[a]	β5	Irreversible	PO
Delanzomib	β1, β5	Reversible	IV, PO
Marizomib	β1, β2, β5	Irreversible	IV, PO

[a]in development
IV, intravenous; PO, oral

as MG-132 due to the reactivity of boronic acid with threonine residues at the proteolytic active sites [29]. With mechanisms that included inhibition of NF-κB activation and suppression of IL-6 release, the drug rapidly caught the attention of MM investigators where it showed potent activity in both drug sensitive and resistant cell lines [30]. After it showed remarkable clinical activity in heavily pre-treated patients with advanced MM [31, 32], bortezomib earned US Food and Drug Administration (FDA) approval in 2003 (www.fda.gov) for the treatment of relapsed/refractory (R/R) MM and opened a new therapeutic avenue in cancer. The use of bortezomib in myeloma progressed rapidly due to its efficacy as an agent reserved for R/R disease and by 2007 had been incorporated into the up-front treatment as part of the total-therapy 3 regimen [33]. With this success of course came interest in next generation proteasome inhibitors, particularly those that may have lower levels of toxicity (neuropathy due to off-target inhibition of HtrA2/Omi) [34], improved ease of administration, and the potential to circumvent bortezomib resistance due to differing chemical structures. The efficacy of proteasome inhibitors *in vitro* showed a strong correlation with the degree of proteasome inhibition, though concern persisted relating to the extent to which patients would tolerate more lengthy and profound inhibition of this central enzymatic complex [1].

Bortezomib binds to and inhibits the β1 and β5 proteasome subunits, though its effect on β5 substantially outweighs that towards β1 [35–37]. Though potent and selective, the partial inhibition of chymotrypsin-like activity that maps to the β5 subunit leaves substantial activity remaining that ultimately limits therapeutic efficacy. This is compounded by the relatively rapid clearance of the drug from plasma, and the reversible nature by which bortezomib binds to β5 [1, 38]. The net effect of these characteristics is that the proteasome is inhibited for a relatively short time in target tissues, particularly those that may be sub-optimally perfused such as solid tumors that are outstripping blood supply. The search was then launched to develop better inhibitors that were more potent with slower recovery rates that may result in clinically efficacy in diseases beyond that seen in MM.

Carlfizomib

With its ancestry based in the biochemical proteasome inhibitor epoximycin, carfilzomib is an irreversible inhibitor of the β5 subunit [39, 40]. Developed by Proteolyx (a partnership born out of the laboratories of Craig Crews and Ray Deschais), carfilzomib initially was designed to address the kinetic problems associated with bortezomib. Unfortunately, the drug is rapidly cleared from the plasma due to distribution and rapid metabolism [40]. Furthermore, despite the irreversible nature of its binding with the proteasome, the recovery time of proteasome activity that was measured in tissues was only slightly improved over bortezomib [39]. Nonetheless, carfilzomib has strong activity in R/R MM patients due its differing structure and mechanism, including in cells that are resistant to bortezomib, and has a reduced incidence of neuropathy compared with bortezomib. In 2012, the FDA approved the use of carfilzomib for R/R MM.

Ixazomib and Delanzomib

As with bortezomib, ixazomib and delanzomib are boronic acid-based reversible proteasome inhibitors – but with the advantage of oral bioavailability [41, 42]. Both compounds primarily bind to and inhibit the β5/chymotrypsin-like activity with much lower activity towards the β1/caspase-like activity. Ixazomib is more selective compared with bortezomib with little of the off-target inhibition of HtrA2/Omi that is thought to produce the neuropathy seen in the latter [41]. With a weekly dosage frequency when combined with lenalidomide, and a favorable safety profile, ixazomib has gained rapid approval in Europe and the US for the treatment of MM. The development of delanzomib is lagging behind that of ixazomib, and currently there are no open trials including this drug registered with Clinicaltrials.gov. This may be due to the occurrence of substantial skin toxicity compared with other agents and the lack of apparent clinical benefit compared to the other drugs under development [38, 43].

Oprozomib

As ixazomib is an orally administered descendant obortezomib, oprozomib was developed as an orally bioavailable tripeptide epoxyketone related to carfilzomib [44]. As with carfilzomib, it is a selective inhibitor of the β5/chymotrypsin-like activity. Thus far oprozomib has shown encouraging pre-clinical activity *in vitro* and in xenograft studies with MM models [45]. There are currently several early phase trials examining the use of oprozomib alone or in combination with other conventional or targeted agents in refractory myeloma, hematologic malignancies, and solid tumors.

Marizomib

In a complete departure from the other agents, marizomib is a naturally occurring compound derived from the marine actinomycete *Salinospora tropica* [46]. Structurally it also is a departure from prior inhibitors in that it lacks a peptide backbone structure having instead a bicyclic ring structure. It also differs in at least partially, though irreversibly, inhibiting all three subunits and activities of the proteasome, though as with other agents its most potent activity is towards the β5 contained chymotripsin-like activity [47]. Unique to this agent is the appearance of some central nervous system (CNS) toxicities and possible activity towards glioma cells. As such, there are three open studies actively investigating its use in patients with gliomas – particularly glioblastoma multiforme [48]. As other proteasome inhibitors have little penetration of the blood brain barrier, marizomib may be uniquely situated to use in patients with brain tumors or hematologic malignancies involving the CNS.

Use of Proteasome Inhibitors in Lymphoma

The impact of proteasome inhibitors in MM is in part related to the dependency of myeloma cells on protein processing with high level immunoglobulin production [49]. However, proteasome inhibition also impacts signaling pathways as described earlier. This section will address the emerging therapeutic role of targeting signaling pathways via proteasome inhibition in B-cell non-Hodgkin lymphoma (NHL) (Table 4.2). Specifically, the discussion will center on mantle cell lymphoma (MCL) and subtypes of diffuse large B-cell lymphoma (DLBCL).

Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is a lymphoma of mature B-cells that primarily affects older adults and accounts for around 4% of lymphomas in the US and 7–9% in Europe [50]. Mantle cell lymphoma responds well to therapy initially, but remissions tend to be short with an overall survival (OS) of 3–6 years [51]. The clinical course is quite variable, however, with some patients showing highly aggressive disease and others an indolent course with long-term survival [51]. Standard therapy for MCL patients has often included cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), with more recently the inclusion of the anti-CD20 monoclonal antibody rituximab (R-CHOP).

Table 4.2 Representative clinical trials using bortezomib in B-cell non-Hodgkin lymphoma

Disease	Setting	Therapy	Results	Reference
B-NHL	R/R	BTZ monotherapy	ORR 41%	52
Indolent B-NHL. MCL	R/R	BTZ monotherapy	ORR 58%	53
			ORR 50% for MCL	
Indolent B-NHL. MCL	R/R	BTZ, R, bendamustine	ORR 83%	54
			ORR 71% for MCL	
MCL	Frontline	VR-CAP	PFS 24.7 months *vs.* 14.4 months for R-CHOP	55
DLBCL	R/R	BTZ + DA-EPOCH-B	ORR 85% in ABC DLBCL	61
			ORR 13% in GCB DLBCL	
Waldenström macroglobulinemia	Frontline	BTZ, R, dexamethasone	ORR 90–95%	64

ABC, activated B-cell; B-NHL, B-cell non-Hodgkin lymphoma; BTZ, bortezomib; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; DA-EPOCH, dose-adjusted etoposide, vincristine, doxorubicin, cyclophosphamide, prednisone; GCB, germinal center B-cell; MCL, mantle cell lymphoma; ORR, overall response rate; PFS, progression-free survival; R/R, relapsed/refractory; R, rituximab; VR-CAP, bortezomib, rituximab, cyclophosphamide, doxorubicin, prednisone

Bortezomib was first used in B-cell NHL in the R/R setting, where monotherapy achieved an overall response rate (ORR) of 41% [52, 53]. A similar phase 2 trial of R/R B-cell NHL including MCL found an ORR of 58%, with 50% for MCL patients [53]. When combined with bendamustine and rituximab, the ORR improved to 71% in pretreated patients including those refractory to rituximab [54]. The FDA approved bortezomib for the treatment of MCL in patients who have received at least one prior therapy in 2006 and for frontline therapy in 2014. A phase 3 study showed significant improvement in progression-free survival (PFS) with the replacement of vincristine by bortezomib in R-CHOP (bortezomib, rituximab, cyclophosphamide, doxorubicin, and prednisone, VR-CAP) [55]. PFS in the VR-CAP group was 24.7 months *vs*. 14.4 months for R-CHOP ($p < 0.001$). Overall response rates and the durability of response were both superior with VR-CAP.

Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) is one of the most common types of NHL. Although it is curable for a subset of patients, biologic heterogeneity of DLBCL has increasingly been linked to variable responses to therapy and survival. In an early such study, two molecular subtypes of DLBCL were identified by gene expression profiling: the germinal center B-cell type (GCB) and the activated B-cell type (ABC) [56]. Highly expressed genes that defined the GCB subtype were also highly expressed in normal germinal center B-cells, while most of the genes that defined ABC DLBCL were expressed in activated peripheral blood B-cells. This study also demonstrated that the ABC subtype DLBCL patients had worse outcomes, and identifying targeted therapies to improve outcomes in this subtype has been an ongoing objective of multiple studies since that time.

Several studies subsequently demonstrated the activation of and survival dependence on NF-kB pathway activation in ABC DLBCL [57–60]. Because proteasome inhibition was known to negatively regulate NF-kB pathway activation, these studies led to a clinical trial using bortezomib in DLBCL. Bortezomib alone had no activity in DLBCL, but when combined with chemotherapy in relapsed DLBCL patients, it demonstrated a significantly higher response (83% *vs.* 13%; $P < .001$) and median OS (10.8 vs 3.4 months; $P = .003$) in ABC compared with GCB DLBCL. Further, 41.5% of ABC DLBCL patients achieved complete remission (CR) compared to only 6.5% of GCB DLBCL. These results suggested that bortezomib enhances the activity of chemotherapy in ABC but not GCB DLBCL [61].

The finding that proteasome inhibitor therapy is subtype-dependent in DLBCL has important implications for the diagnostic approach to this lymphoma. The 2017 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues recommends that GCB/ABC subtyping be a part of routine initial diagnosis in DLBCL [62]. However, subtyping by gene expression profiling is still not widely available in clinical laboratories, and the upfront therapy remains the same for both subtypes. Although immunohistochemical (IHC) staining can be used as a surrogate assay to

attempt to subclassify DLBCL, the approach is less specific in well-controlled studies let alone among varied pathology practices utilizing different laboratories and IHC. For now, further clinical studies are necessary to better define the therapeutic role for proteasome inhibition in DLBCL. If subtyping of DLBCL to guide therapy becomes the standard of care, better assays will need to be deployed in clinical laboratories.

Other Non-Hodgkin Lymphomas

Data supporting the use of proteasome inhibitors in other subtypes of B-cell NHL are quite limited. Primary mediastinal large B-cell lymphoma (PMBCL) is a much less common subtype of DLBCL that is distinct from GCB and ABC types by gene expression profiling [63]. This subtype shows more similarities to classical Hodgkin lymphoma (HL) and is characterized in part by NF-kB pathway activation [63]. This suggests a potential role for proteasome inhibitors in PMBCL therapy, but this has not yet been demonstrated in clinical trials. Proteasome inhibitors are also used to treat Waldenström macroglobulinemia, where bortezomib combined with dexamethasone and rituximab leads to overall response rates of 90–95%, although CRs are uncommon [64].

The potential role of bortezomib in T-lymphoblastic lymphoma (LL) therapy is currently under investigation by the COG. Due to the similarities between LL and ALL, most pediatric patients with LL are treated on regimens developed to treat ALL. The distinction between lymphoma and leukemia in lymphoblastic disease is based on the somewhat arbitrary designation of leukemia for those cases with ≥25% bone marrow blasts [62]. T-ALL blasts often have considerable activation of the NF-kB pathway, frequently as a consequence of activated Notch signaling [65]. Although similar data are lacking for T-LL, these patients were enrolled on an arm of this study and randomized to standard ALL therapy or standard ALL therapy plus bortezomib. The results of this trial are not yet available, but a positive signal may establish a role for bortezomib in T-LL therapy.

Therapy Resistance

Studies of bortezomib resistance in lymphoma cell lines have implicated apoptotic pathways and the unfolded protein response. Roue et al. demonstrated a high correlation between proteasome inhibitor resistance and up-regulation of the pro-survival chaperone BiP/Grp78 in MCL cells [66]. The stabilization of BiP/Grp78 was mediated by an increase in activity of heat shock protein of 90 kDa (Hsp90). With targeted inhibition of this pathway, bortezomib cytotoxicity was restored in MCL cell lines. Mechanisms of lymphoma resistance to bortezomib were explored by comparative analyses of two DLBCL cell lines, SUDHL-4 and SUDHL-6, which

are resistant and sensitive to bortezomib, respectively [67]. This study demonstrated bortezomib-induced apoptosis in sensitive SUDHL-6 cells, but not in resistant SUDHL-4 cells. Inhibition of the proteasome was similar in the two cell lines. Gene expression profiling was performed to compare up- and down-regulated transcripts in response to bortezomib in SUDHL-4 and SUDHL-6 cells. The resistant SUDHL-4 cells showed induction of heat shock proteins and other chaperone proteins, which have been associated with bortezomib resistance. In the sensitive SUDHL-6 cell line, bortezomib induced ATF3 and ATF4, which induced apoptosis and the unfolded protein response, respectively [67]. Similar mechanisms of resistance have been described in MM, where bortezomib induces expression of ATF4 that in turn drives increased expression of the BCL2 family anti-apoptotic protein MCL-1 leading to increased cell survival [68].

Conclusions

The proteasome is a cytosolic proteolytic system that not only degrades damaged proteins but also has a critical role in cellular function through highly-regulated, targeted degradation of proteins. Drugs that specifically inhibit the proteasome first showed clinical utility in myeloma, but the therapeutic realm for these compounds has expanded to include NHL. As with many targeted inhibitors in cancer therapy, resistance to proteasome inhibitor therapy often emerges. Ongoing and future studies seek to optimize combination therapies including proteasome inhibitors as well as to overcome resistance mechanisms.

Disclosure of Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 5
Resistance to Histone Deacetylase Inhibitors in the Treatment of Lymphoma

Allyson Flower and Oussama Abla

Abstract Outcomes for patients with lymphoma have improved through the use of chemo/immunotherapy. However, therapy for patients with advanced disease and relapsed/refractory disease remains inadequate. In addition, off target side effects result in significant short and long-term toxicity. The use of targeted molecular therapy introduces an opportunity for improvement in efficacy and reduction in undesirable off target effects. Histone Deacetylase (HDAC) inhibitors are a class of targeted molecular therapies that have been extensively evaluated for the treatment of refractory malignancies including subtypes of lymphoma. However, critical resistance mechanisms are well described. Optimal efficacy of HDAC inhibitors in the treatment of lymphoma is dependent upon successful strategies to overcome drug resistance.

Keywords Lymphoma · Histone deacetylase inhibitor · Resistance · Epigenetics

Abbreviations

AITL	Angioimmunoblastic T-Cell Lymphoma
AML	Acute Myeloid Leukemia
CHOP	Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone
CR	Complete Response
CTCL	Cutaneous T-Cell Lymphoma
DLBCL	Diffuse Large B-Cell Lymphoma
ER	Endoplasmic Reticulum

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A. C. Xavier, M. S. Cairo (eds.), *Resistance to Targeted Therapies in Lymphomas*, Resistance to Targeted Anti-Cancer Therapeutics 21,
https://doi.org/10.1007/978-3-030-24424-8_5

FDA	Food and Drug Administration
FL	Follicular Lymphoma
HAT	Histone acetyltransferase
HDAC	Histone Deacetylase
HDACi	HDAC inhibitors
HL	Hodgkin Lymphoma
MCL	Mantle Cell Lymphoma
MM	Multiple Myeloma
MZL	Marginal Zone Lymphoma
NAD	Nicotinamide Adenine Dinucleotide
NHL	Non-Hodgkin Lymphoma
OR	Overall Response
ORR	Overall Response Rate
PCTCL	Primary Cutaneous T-Cell Lymphoma
PFS	Progression-Free Survival
PR	Partial Response
PTCL	Peripheral T-Cell Lymphoma
r/r	relapsed/refractory
SLL	Small Lymphocytic Lymphoma

Introduction

Survival for patients with lymphoma has improved over time through the use of multi-agent chemotherapy and immunotherapy (Fig. 5.1) [1, 2]. However, outcomes for patients with advanced disease or relapsed/refractory (r/r) disease remain poor. Also, importantly, current therapies for the treatment of lymphoma can lead to harmful short and long-term toxicities including cardiac dysfunction, endocrine dysfunction and secondary malignancies. Histone deacetylases (HDACs) are a set of naturally occurring proteins that contribute to gene expression through epigenetic modification. Dysfunctional HDAC activity leads to aberrant gene expression, which ultimately results in the generation of malignancy [3–7]. HDAC inhibitors (HDACi) are a class of targeted molecular therapies that, in addition to extensive alternative mechanisms of action, reverse the activity of dysfunctional HDACs. HDACi have been extensively evaluated for the treatment of refractory malignancies including subtypes of lymphoma, leading to United States Food and Drug Administration (FDA) approval of vorinostat for the treatment of primary cutaneous T-cell lymphoma (PCTCL), romidepsin for PCTCL and peripheral T-cell lymphoma (PTCL), and belinostat for PTCL. Panobinostat is approved by the FDA for use in combination with bortezomib for the treatment of multiple myeloma (MM), but also has activity against PCTLC and other lymphoma subtypes. Chidamide is approved by the China FDA for the treatment of r/r PTCL. However, the efficacy of HDACi in the treatment of lymphoma is limited by several well-described resistance mechanisms including DNA damage repair, reactive oxygen species redox pathways, drug

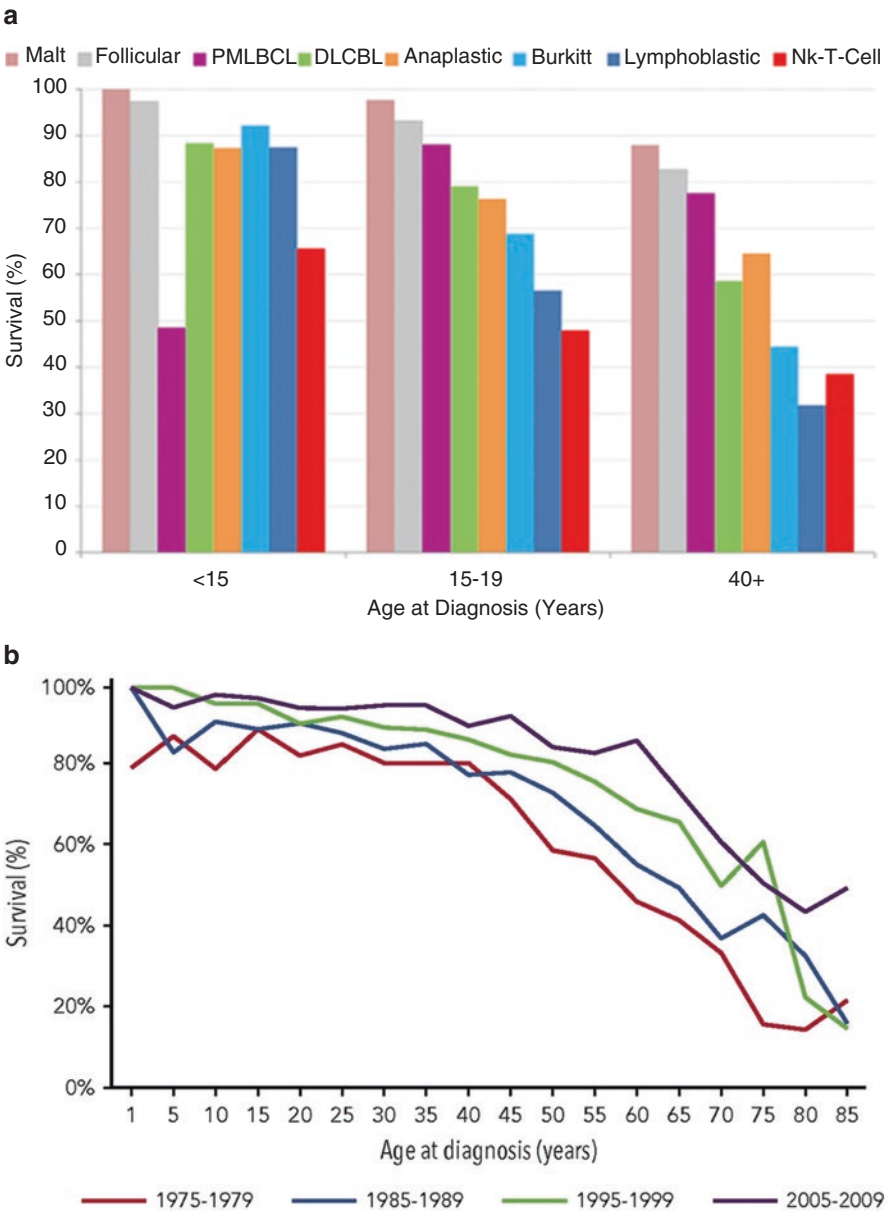

Fig. 5.1 (**a**) NHL 5 year lymphoma specific survival by histologic type and age, 2000–2011. United States SEER data. Source: National Cancer Institute: SEER 18 [1]; (**b**) Hodgkin lymphoma survival by age, 1973–2014. United States SEER data. Source: National Cancer Institute: SEER 18 [2]

efflux, cellular signaling, autophagy, endoplasmic stress pathway signaling, acquired resistance and target HDAC expression. This chapter will focus on HDACs, HDACi, drug resistance mechanisms, and strategies to overcome HDAC resistance in the setting of lymphoma.

Histone Deacetylases

Within the cell nucleus, DNA is wrapped around 4 key histone proteins (H2A, H2B, H3, and H4) to form nucleosomes (Fig. 5.2). When compacted, nucleosomes form the structure of condensed chromatin. Each histone protein is attached to a lysine tail, which extends out from the nucleosome and is accessible for modification. DNA within the nucleosome is controlled, in part, by modification of the lysine tail. Acetylation is one mechanism by which the lysine tail is modified. This occurs through the action of histone acetyltransferases (HAT) and HDACs [8]. HATs add acetyl groups to the histone lysine tail. This action neutralizes the lysine tail and reduces the attraction of negatively charged DNA. As a result, chromatin forms an open structure, and DNA is accessible by transcription factors and RNA polymerase. HDACs are enzymes that control acetylation of histones and other essential

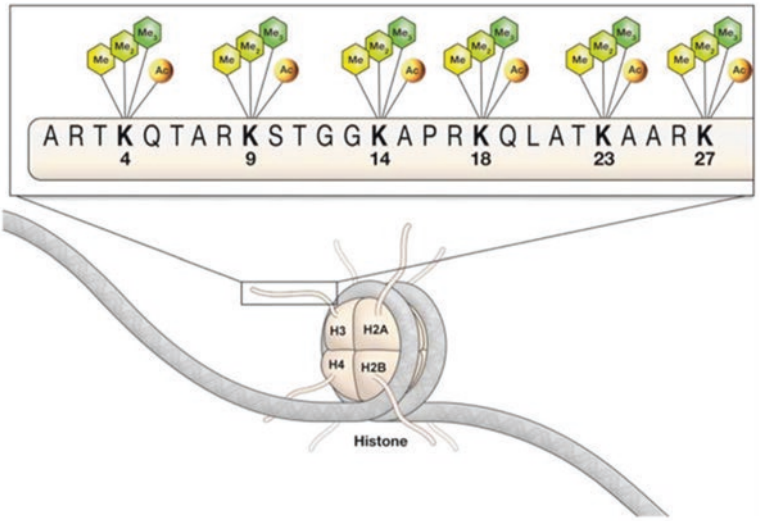

Fig. 5.2 Modification of lysines in histone tails. DNA is wound around four core histone proteins: H2A, H2B, H3, and H4. Each of the histones possess lysine-rich tails and accessibility of the DNA is controlled by modifications to the tail. Lysines can either be multiply methylated, or acetylated. Methylation and deacetylation of lysines both contribute to a more condensed chromatin structure, preventing transcription of genes. Demethylation and acetylation promote a more open chromatin structure allowing for increased gene transcription [8]

proteins by catalyzing deacetylation. Hypoacetylation results in condensed chromatin structure and decreased gene transcription [9].

Acetylation is a key epigenetic mechanism by which gene expression is modified [10]. HATs and HDACs affect cellular function essential to survival and proliferation through their regulation of gene expression [9]. Acetylation of proteins is not limited to histones. Non-histone proteins p53, c-Myc, BCL-2, BCL-6, E2F, HIF1α, hsp90, Ku70, NFκB, pRb, and STAT3 are critical to cellular function and are also affected by HAT and HDAC regulated acetylation [11–13]. The effects of deregulated acetylation have been implicated in the oncogenesis of some malignancies including diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and PTCL [3–7].

HDACs are classified as either zinc dependent or nicotinamide adenine dinucleotide (NAD) dependent [14, 15]. HDACs are further grouped into 4 classes based on their similarity to yeast HDAC proteins. Tissue specificity of HDACs and distribution within the intracellular compartment varies. Class I HDACs (1, 2, 3 and 8) are ubiquitously expressed in human tissues. Class IIA HDACs (4, 5, 7 and 9) and Class IIB HDACs (6 and 10) are differentially expressed in human tissues. Class III HDACs are known as sirtuins. Activity of these HDACs is NAD dependent. HDAC 11 is the only known Class IV HDAC [8]. Class I, IIA, IIB and IV HDACs are zinc dependent.

Expression of HDACs in lymphomas is not fully defined. Class I HDACs are ubiquitously expressed in lymphoid cell lines and primary lymphoid tumors whereas class II HDACs are differentially expressed (Table 5.1) [16]. The expression of HDACs in lymphoma subtypes, and among tumor tissue in individual patients is essential to the rationale for treatment of lymphoma with HDAC inhibitors. However, the specific targets of HDAC inhibitors in the treatment of lymphoma have not yet been identified.

Table 5.1 Immunoactivity of HDAC activity in reactive lymph nodes [16]

Cell type	HDAC 1	HDAC 2	HDAC 3	HDAC 6	HDAC 10
Germinal center B-cells	+	+	+	–	+
Mantle zone B-cells	+	+	+	–	+
Plasma cells	+	+	+	+	+
T-cells	+	+	+	–	+ strong
Follicular dendritic reticulum cells	+ weak	+ strong	+	–	+
Interdigitating reticulum cells	+ weak	+ strong	+	–	+ weak
Fibroblastic reticulum cells	+ weak	+ strong	+	–	+
Macrophages	+ weak	+ strong	+	–	+
Sinus histiocytes	+	+ strong	+	–	+
Endothelial cells	+	+ strong	+	–	+

Histone Deacetylase Inhibitors

HDACis limit the deacetylation activity of HDACs. This results in unrestricted HAT mediated acetylation of histones and promotes gene transcription. In addition to histone proteins, HDACis regulate gene expression of non-histone proteins critical to cell proliferation and survival by multiple mechanisms.

HDACis induce cell cycle arrest in both healthy and malignant cells [17–20]. The cell cycle phase affected by HDAC inhibition is dose dependent. The same cell is affected in the G0/G1 phase at low doses or the G2/M phase at higher doses. Cell Cycle arrest is mediated by p21 or p15 [21]. In addition, cell cycle arrest is induced by HDACi mediated transcriptional repression of genes involved in DNA synthesis (CTP synthase, thymidylate synthetase) and reduced expression of cyclins [22]. HDAC inhibition upregulates proapoptotic proteins and downregulates anti-apoptotic proteins in the Bcl-2 family and XIAP [23]. Extrinsic apoptosis is stimulated by HDAC inhibition via increased expression of Fas, TNFα and TRAIL death receptors [24].

Other mechanisms by which HDACis induce cell death include inhibition of DNA repair, post translational modification of proteins, decreased angiogenesis, generation of reactive oxygen species, and promotion of inflammation and immunity [12, 25–27]. HDAC inhibition results in decreased angiogenesis via enhanced degradation of HIF1α and decreased production of VEGF [28, 29]. The targeted anti-cancer activity of HDAC inhibitors is based on DNA repair mechanisms functioning in healthy tissues, but not in malignant cells.

HDACis are classified by their chemical structure (hydroxamic acid, cyclic peptide, electrophilic ketone, short chain fatty acid, or benzamide) (Fig. 5.3) [30–32]. Each HDACi differs in potency, pharmacodynamics and off-target effects. Selectivity also varies between HDACis. Vorinostat, panobinostat and balinostat inhibit both Class I and Class II HDACs. Mocetinostat and entinostat inhibit Class I HDACs only, while romidepsin preferentially inhibits Class II HDACs [33–35].

Histone Deacetylase Inhibitor Use in Lymphoma

Three HDAC inhibitors are FDA approved for treatment of patients with r/r lymphoma in the United States. Ongoing clinical trials will further define their role as well as the role of emerging clinical grade HDAC inhibitors.

Vorinostat (suberoylanilide hydroxamic acid)

Vorinostat belongs to the hydroxamic acid class of HDAC inhibitors. Vorinostat inhibits Class I and Class II HDACs. Single agent vorinostat demonstrated a response rate of 24–30% in patients with r/r PCTCL, which led to FDA approval in

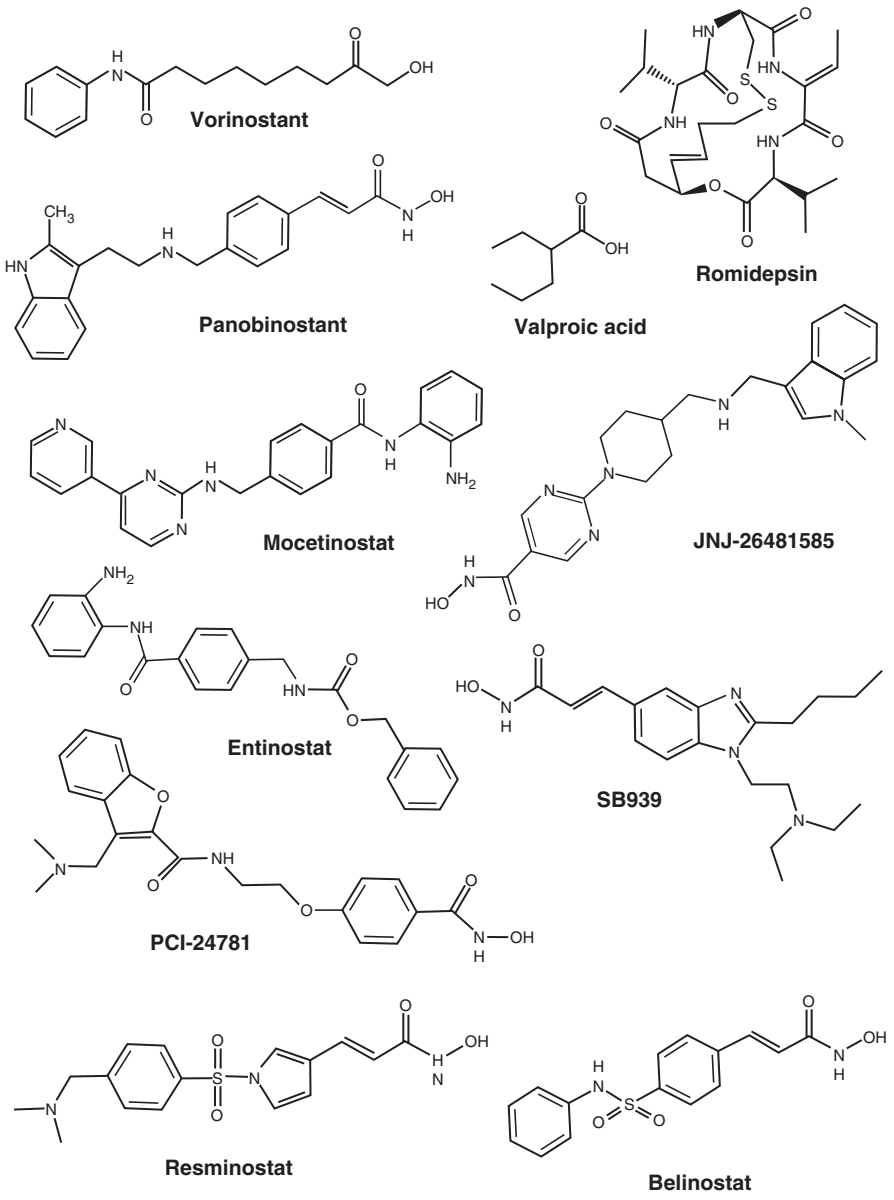

Fig. 5.3 Structure of some of the histone deacetylase inhibitors currently in clinical trials [8]

2006 [36–38]. Safety of administration and activity of vorinostat against Hodgkin lymphoma (HL) and small lymphocytic lymphoma (SLL) were demonstrated in a small number of patients in a preliminary phase 1 clinical trial [39]. However, the objective response rate for a small number of patients with DLBCL was only 5.6% in an early phase 2 clinical trial [40].

Although outcomes for patients with r/r HL treated with single agent vorinostat in a phase 2 clinical trial were dismal, an overall response rate (ORR) of 29% was demonstrated for patients with r/r indolent non-Hodgkin lymphoma (NHL). Though complete or partial response (CR/PR) was demonstrated for patients with FL or marginal zone lymphoma (MZL), no patient with mantle cell lymphoma (MCL) achieved a response [41]. Single agent vorinostat demonstrated a 49% ORR for patients with r/r FL [42]. Vorinostat is administered intravenously or orally. Common side effects associated with vorinostat include fatigue, diarrhea and nausea. Vorinostat has also been associated with thrombocytopenia, dehydration, and rare cases of pulmonary embolism, squamous cell carcinoma, severe anemia and QTc-interval prolongation [43].

Romidepsin (depsipeptide)

Romidepsin is a prodrug that is reduced to an active form intracellularly and has demonstrated activity against Class I HDACs and HDAC 6 [44, 45]. It has been shown to induce malignant cell differentiation, cell cycle arrest and apoptosis, inhibit angiogenesis and deplete hsp90 dependent proteins [46]. Romidepsin was approved by the FDA in 2009 for the treatment of r/r PCTCL and in 2011 for the treatment of r/r PTCL after demonstrating ORRs of 34–38% in affected patients [47–52]. Durable response of up to 48 months was demonstrated for patients with r/r PTCL after treatment with single agent romidepsin [53]. Patients with angioimmunoblastic T-cell lymphoma (AITL) experienced a 33% overall response rate after treatment with single agent romidepsin [54]. Romidepsin is administered intravenously. Adverse events associated with romidepsin include nausea, fatigue, infection, vomiting, anorexia, anemia and neutropenia. It has also been associated with hypomagnesemia and hypokalemia, which can lead to arrhythmia including prolonged QT.

Panobinostat

Panobinostat inhibits Class I, II and IV HDACs. It has been shown to mediate acetylation of H3 and H4, increase p21 levels, disrupt the chaperone function of hsp90, induce cell cycle arrest, and induce apoptosis [55]. Panobinostat was FDA approved for use in combination with bortezoimb for the treatment of MM in 2015. Panobinostat also has activity against PCTCL, HL and DLBCL in clinical trials. Single agent panobinostat therapy resulted in disease response in a small number of patients with PCTCL [56]. An objective response rate of 27% was demonstrated for patients with r/r HL treated with panobinostat [57–59]. Patients with r/r DLBCL achieved response rates of 28% after treatment with panobinostat [60]. Panobinostat

is administered orally. The most common adverse events associated with panobinostat are nausea, vomiting, diarrhea and fatigue. Common adverse grade 3 and 4 adverse events associated with panobinostat are thrombocytopenia, anemia and neutropenia.

Belinostat

Belinostat is a pan-HDACi. It has been shown to inhibit cell proliferation, induce apoptosis, inhibit angiogenesis, and induce differentiation of malignant cells [61, 62]. Belinostat was FDA approved for the treatment of r/r PTCL in 2014. The ORR for patients with r/r PTCL treated with single agent belinostat was 26% [63]. Belinostat is available for intravenous or oral administration. Adverse events associated with belinostat include nausea, vomiting, fatigue, pyrexia, and anemia.

Mocetinostat

Mocetinostat inhibits HDAC 1, 2, 3, and 11 [64]. It has been shown to inhibit the expression of thymus and activation-regulated chemokine/chemokine ligand 17 [65]. Single agent mocetinostat demonstrated an ORR of 18% and 11% for the treatment of patients with r/r DLBCL and FL, respectively [66]. Mocetinostat has also induced disease stability in patients with r/r HL [67]. Mocetinostat is administered in oral form. Common adverse events associated with mocetinostat include fatigue, anemia, nausea, anorexia, hyponatremia, neutropenia, and thrombocytopenia.

Entinostat

Entinostat inhibits HDAC 1 and 3. Entinostat inhibits cell proliferation by down-regulation of XIAP and induction of apoptosis [68]. Etinostat has been shown to increase IL2, p40–70, IP10, and RANTES and to decrease I13 and IL4. As a result, TH1 cytokines are increased. Entinostat also induces expression of tumor associated antigens SSX2 and MAGE-A [68]. Entinostat has activity against HL. The ORR for patients with r/r HL treated with single agent entinostat was 11% [69]. Entinostat is administered orally. The most common side effects associated with entinostat are thrombocytopenia, anemia, neutropenia, leukopenia, hypokalemia and hypophosphatemia.

HDACi Inhibitor Resistance Mechanisms

DNA Damage and Repair Mechanisms

Through hyperacetylation of histones, HDACis alter DNA stability and increase exposure to cytotoxic agents, radiation and reactive oxidative species. In addition, HDACis decrease expression of genes encoding homologous recombination DNA repair proteins (RAD51, RAD52, BRCA1/2, CtIP, Bloom Syndrome Gene, Nijmegan Breakage Syndrome1, XRCC2) and non-homologous recombination end-joining DNA repair proteins (Ku70, Ku86, DNA-PKCs, XRCC4, DNA ligase 4) [70–78]. The mechanism by which normal cells escape HDACi induced DNA damage has been demonstrated through the use of vorinostat [79]. Vorinostat induces accumulation of DNA DSBs in both normal and malignant cells. In malignant cells, vorinostat decreases levels of DNA repair proteins resulting in accumulation of DNA damage leading to cell death. Checkpoint kinase 1 (Chk1) has been proposed as the key factor for resistance to HDACis in normal cells [80]. Exposure of vorinostat, romidepsin or entinostat in combination with Chk1 inhibitor results accumulation of DNA DSBs and induces cell death in normal cells. HDACis induce cell cycle arrest in G1 and G2/M and cell depletion in S phase [81–84]. The effect of HDAC inhibition on mitotic chromosome breakage occurs in malignant but not in normal cells and persists after withdrawal of exposure [79, 80, 85].

Reactive Oxidative Species and Redox Pathways

Accumulation of reactive oxidative species induced by HDAC inhibition results in cell death. Thioredoxin is a thio reductase, which protects healthy cells from oxidative damage caused by reactive oxidative species. There is a negative correlation between sensitivity to HDACis and thioredoxin level [39]. However, vorinostat selectively increases thioredoxin levels in healthy cells. This mechanism plays a role in healthy cell resistance to damage caused by HDACis. In contrast, exposure of vorinostat to transformed cells induces upregulation of thioredoxin binding protein, a negative regulator of thioredoxin. As a result, reactive oxidative species accumulate and ultimately cause cell death. Increased expression of antioxidant genes including thioredoxin, super oxide dismutase 2, and glutathione reductase have been associated with HDACi resistance in acute myeloid leukemia (AML) cells [86, 87]. Lethality of leukemia cells after exposure to HDAC inhibition was significantly diminished by reactive oxidative species scavengers [88, 89]. This evidence suggests that modulation of reactive oxidative species after exposure to HDAC inhibition contributes to resistance in malignant cells.

Drug Efflux Mechanisms

P-glycoprotein-mediated transport of drugs across the cell membrane is a known entity contributing to multidrug resistance in cancer therapy. Exposure to HDAC inhibition including valproic acid, apicidin, romidepsin and sodium butyrate has been associated with increased levels of MDR1 mRNA and p-glycoprotein expression in cancer cells [90–94]. Romidepsin has been identified as a substrate of p-glycoprotein and romidepsin resistant cell lines express increased levels of p-gycoprotein [95, 96]. The increase in p-glycoprotein level is correlated with exposure to romidepsin and is reversible upon withdrawal of exposure [94]. As a result, HDAC activity is not affected [93]. Verapamil mediated inhibition of p-glycoprotein or MDR1 inhibition results in reversal of resistance of romidepsin and apicidin [97]. MDR1 expression is increased in circulating peripheral blood mononuclear cells from patients with romidepsin resistant malignancies [98]. While romidepsin induces cell death in non-p-glycoprotein expressing cells, vorinostat and oxamflatin are active in both non-p-glycoprotein expressing and p-glycoprotein expressing cells [99].

Cell Signaling-Related Mechanisms

Malignant cells may overcome HDAC-mediated cytotoxicity through the counteractive mechanism of specific cell signaling pathways. The BCL-2 protein family consists of both pro-apoptotic and anti-apoptotic groups; antiapoptotic BCL-2 proteins, proapoptotic BAX-like proteins, and proapoptotic BH3-only proteins [100]. The antiapoptotic activity of BCL-2 and BCL-XL limits HDACi-induced cell death. Cytotoxicity of transformed cells treated with vorinostat, sodium butyrate, valproic acid and entinostat is reduced in the presence of BCL-2 or BCL-XL [101–104]. The Eμ-myc mouse model provides a mechanism for evaluation of B-cell lymphoma with defined alterations in apoptotic pathways. Using this model, tumor cell apoptosis was correlated with therapeutic efficacy after exposure to vorinostat. This activity was found to be dependent upon the intrinsic apoptotic pathway, specifically proapoptotic BH3-only proteins Bid and Bim [100]. In contrast, overexpression of pro-apoptotic protein BCL-2 was associated with T-cell lymphoma resistance to vorinostat, romidepsin, and panobinostat [14, 105]. The apoptotic activity of panobinostat, romidepsin and m-carboxycinnamic acid bis-hydroxamide can be blocked by suppression of pro-apoptotic proteins BMV, BAX, or MCL-1 [105–107].

Activation of nuclear factor kB is a contributor to drug resistance in solid tumors and hematologic malignancies [108]. Apoptosis is indirectly downregulated by nuclear factor kB mediated transcription of anti-apoptotic genes including TNF receptor associated factors, Mn-SOD, and BCL-XL [109]. Vorinostat, trichostatin A, entinostat and pabinostat activate nuclear factor kB in malignant cells. Through

this mechanism, the cytotoxicity of HDACis is reduced [110–113]. Exposure to HDAC inhibition in addition to nuclear factor kB inhibition eliminates nuclear factor kB-mediated resistance [110, 111, 113, 114].

The JAK/STAT pathway promotes oncogenesis through interaction with antiapoptotic target genes and plays a key role in drug resistance in solid tumors and hematologic malignancies [115]. HDACis downregulate transcription of STAT target genes [116]. In cutaneous T-cell lymphoma (CTCL) cell lines and peripheral blood lymphocytes, HDACis reduce expression of STAT protein expression [117]. However, overexpression of antiapoptotic STAT proteins is associated with resistance to HDACis [118]. Increased expression of STAT proteins has also been associated with therapy resistance in lymphoma cell lines. In addition, nuclear accumulation of STAT1 and pSTAT3 was associated with lack of clinical response in CTCL patients [119]. Vorinostat in combination with JAK2 inhibitor demonstrated synergy in antiproliferative effect and downregulation of antiapoptotic genes [119]. This synergistic effect was also demonstrated through the use of panobinostat in combination with JAK inhibitor [120].

Retinoids modulate growth, differentiation, and apoptosis of cancer cells. Induction of TRAIL-mediated death signaling contributes to the therapeutic value of retinoids [121]. Inhibition of retinoic acid signaling is mediated by retinoic acid receptor α and preferentially expressed antigen of melanoma, and contributes to HDAC resistance [122]. Elimination of retinoic acid receptor α and preferentially expressed antigen of melanoma promotes HDAC inhibitor induced cell death [123].

Autophagy

Autophagy is the process by which cells maintain homeostasis and is mediated by lysosome dependent degradation of cellular components. Autophagosomes engulf and deliver non-essential cellular components to the lysosome for processing [124]. Accumulation of autophagosomes has been associated with resistance to HDAC inhibition and autophagy genes are upregulated by HDACis [125]. Chloroquine or bafilomycin interfere with autophagy and increase cell death in HDAC inhibitor resistant cells [125, 126].

Endoplasmic Reticulum Stress-Related Signaling

The endoplasmic reticulum (ER) is a cellular organelle that maintains homeostasis through control of protein synthesis, folding, delivery, and degradation [127]. Excessive unfolded proteins within the endoplasmic reticulum induce stress and promote the unfolded protein response. The unfolded protein response triggers glucose regulated protein 78 to bind to unfolded proteins and chaperone them to the

proteasome for degradation [128]. HDAC inhibitors induce overexpression of GRP78, which leads to HDAC inhibitor resistance [129–132].

Acetylation of GRP78 by panobinostat or vorinostat leads to cellular apoptosis [133, 134]. This proapoptotic signaling is mediated by protein kinase RNA-like EF kinase (PERK), which activates CAAT/enahancer binding protein homologous protein (CHOP) [127, 133]. Depletion of PERK or inhibition of CHOP lead to HDAC inhibitor resistance [135].

HDAC Expression in Lymphoma

The pattern of HDAC enzyme expression in lymphoma subtypes in incompletely understood. Amongst lymphoma tissue samples, expressions of class I and class II HDACs is variable (Table 5.1). Absent or low expression of HDAC targets in lymphoma is critical to the efficacy of selected HDACi therapy and is a mechanism of HDACi resistance. Class I HDACs are expressed in lymphoma cell lines and primary tumors including the non-malignant HL cellular microenvironment. The most variably expressed HDAC in lymphoma is HDAC 6, which is undetected or only weakly expressed in 64% of lymphoid cell lines and 93% of primary lymphoma tissue samples. Cells expressing HDAC 6 are more sensitive to Class I HDACi MGCD0103 [16]. Absent expression of HDAC 3 has been associated with vorinostat resistance and panobinostat cross resistance in CTCL T-cell leukemia/lymphoma [136].

Acquired Resistance to HDAC Inhibitors

Exposure to therapeutic agents can lead to drug resistance in cancer cells. Due to their heterogeneity, malignant cells have the capacity to induce mutations to promote cell survival and support resistant stem cell-like cells lines [137, 138]. Cancer cells with acquired resistance to HDACis exhibit decreased acetylation of histones and loss of G2 checkpoint inhibition [139]. Acquired resistance to HDACis can cause cross resistance. Resistance to vorinostat causes cross resistance to hydroxamic acid and the aliphatic acid-based HDACis [139]. Cancer cells with resistance to dacinostat show cross resistance not only to other HDACis vorinostat and panobinostat, but also to other chemotherapeutic agents. Hsp90 inhibitor reverses acquired resistance to dacinostat [140]. Belinostat resistant T-cell lymphoma cell lines show cross resistance with vorinostat, romidepsin, panobinostat, and ricolinostat [141].

Acquired resistance to HDAC inhibitors in malignant cells can be reversed. Reversible drug resistance is mediated by epigenetic modification. Restoration of HDAC3 expression by hypomethylation in cutaneous T-cell lymphoma and T-cell leukemia/lymphoma cell lines results in sensitization of cells previously proven to be vorinostat resistant [142].

Strategies to Overcome HDAC Inhibitor Resistance in Lymphoma Therapy

Chemotherapy Sensitization

Open chromatin structure after HDACi therapy increases DNA exposure to chemotherapeutic agents. The use of panobinostat in combination with ifosfamide, carboplatin and etoposide resulted in significantly improved CR rates for patients with r/r HL in comparison to ifosfamide, carboplatin and etoposide alone. However, the addition of panobinostat resulted in significantly increased incidence of neutropenia and thrombocytopenia [143].

Belinostat, cyclophosphamide, doxorubicin, vincristine and prednisone each target cell cycle arrest with different mechanisms of action, and their antineoplastic effects are potentially additive. Belinostat used in combination with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) resulted in an overall response rate of 89% for patients with peripheral T-cell lymphoma. The most frequent grade 3 or 4 adverse events included neutropenia and anemia [144].

Chidamide monotherapy for patients with r/r PTCL resulted in an ORR of 39%. However, when used in combination with CHOP-like, cisplatin based, or other chemotherapy regimens, overall response (OR) and progression-free survival (PFS) were significantly improved. Grade 3 and 4 adverse events included neutropenia and thrombocytopenia [145, 146].

Epigenetic Therapy

HDACs are one of many contributors to modification of DNA expression. In addition to acetylation, other epigenetic alternations including methylation, phosphorylation, ubiquitylation, and sumolyation influence gene expression without changing the DNA sequence. Alternative epigenetic factors also influence oncogenesis, and are potential targets for combination therapy.

The mTOR pathway influences cellular metabolism, growth and metabolism. Sirolimus is an mTOR inhibitor which has synergistic activity when used in combination with vorinostat. Patients with r/r HL experienced a response rate of 44% after treatment with sirolimus plus vorinostat with limited toxicity. The mTOR inhibitor everolimus used in combination with panobinostat resulted in objective responses in patients with indolent lymphoma, T-cell lymphoma, MCL and an ORR of 43% for patients with r/r HL. The toxicity profile included thrombocytopenia, neutropenia, anemia, infection, fatigue and dyspnea [147].

Bortezomib is a proteasome inhibitor. When used in combination with vorinostat, patients with r/r MCL achieved and ORR of 31.8%. However, limited activity was seen using these agents for the treatment of r/r DLBCL [148]. Patients with r/r PTCL achieved an ORR of 43% after treatment with combination borezominb plus panobinostat [149].

Immunotherapy

Rituximab is an anti-CD20 monoclonal antibody. Rituximab used in combination with panobinostat resulted in ORR of only 11% for patients with r/r DLBCL. The toxicity profile was similar to that seen with single agent HDAC inhibition and included thrombocytopenia, fatigue, anemia, diarrhea, nausea, lymphopenia, anorexia and hypophosphatemia [150].

Belinostat-resistant T-cell lymphoma cell lines exhibit decreased JAK/STAT activity and increased levels of reovirus receptor junctional adhesion molecule-A (JAM-A). After exposure to reovirus formulation, belinostat resistant T-cell lymphoma cell lines are sensitized both *in vivo* and *in vitro.* Enhanced sensitivity is associated with increased viral load and is mediated through endoplasmic reticulum stress mediated apoptosis. This preclinical data suggests that oncolytic reovirus is a promising therapeutic strategy for the treatment of HDAC resistant lymphomas. An early phase clinical trial is planned [141]

Toxicity of HDAC Inhibitors

HDAC inhibitors are associated with a range of adverse effects including myelsupression, diarrhea and cardiac arrythmias, specifically ST segment abnormalities and QTc interval prolongation, atrial fibrillation, and rarely ventricular tachyarrhythmias [151]. Monitoring of these and other side effects will be critical to the safety evaluation of HDAC inhibitors used alone or in combination with other agents in early phase clinical trials.

Conclusion

The use of HDAC inhibitors for treatment of subtypes of lymphoma is promising. However, the efficacy of single agent HDAC inhibition for patients with r/r CTCL, PTCL and HL is limited. This may, in part, be due to the aggressive nature of these diseases. However, multiple mechanisms of drug resistance also contribute to refractoriness. A more comprehensive understanding of resistance mechanisms through the identification of key pathways is critical to the development of strategies to overcome this limitation for the treatment of patients with lymphoma. The use of HDACi in combination with chemotherapy, alternative epigenetic modifiers or immunotherapy has demonstrated efficacy in lymphoma subtypes with limited toxicity. Evaluation of strategies to improve HDAC inhibitor efficacy in the treatment of lymphoma is ongoing.

Acknowledgements AF reviewed the literatures, developed the design of the paper and wrote the manuscript. OA critically revised the manuscript and have approved the final version for publication. The authors would like to thank Erin Morris, RN for her excellent assistance with the preparation of this manuscript.

Disclosure of Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 6
Resistance to Bruton's Tyrosine Kinase Signaling Pathway Targeted Therapies

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Abstract Activation of B-cell receptor (BCR) signaling is an important mechanism of the development and growth of B-cell lymphomas. Bruton's tyrosine kinase (BTK) is a key component of BCR signaling and functions as an important regulator of cell proliferation and cell survival in various B-cell lymphomas. BTK inhibitors, especially ibrutinib, have shown promising anti-tumor activity in preclinical and clinical studies. High response rates of ibrutinib were reported in patients with a variety of B-cell non-Hodgkin lymphoma (B-NHL) such as chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL). However, clinical evidence shows primary and acquired resistance to BTK inhibitors in patients. Understanding the molecular mechanisms underlying BTK inhibitors' resistance is of paramount importance. In this review, we highlight the potential resistant mechanisms, which include mutational resistance in BTK, mutational resistance in other proteins than in BTK, chromosomal abnormalities, activation of prosurvival pathways, B-cell lymphoma 2 (BCL-2) family members mediated resistance, and tumor microenvironment mediated resistance. We also discuss the strategies that are utilized to overcome BTK inhibitors' resistance: non-covalent inhibitors of BTK, alternate kinase inhibitors, combination therapies with other oncogenic inhibitors, BCL-2 inhibitors, anti-CD20 antibodies, anti-CD19 chimeric antigen receptor (CAR) T cells, CD19/CD3 bispecific antibody, or with inhibitors targeting other cellular processes.

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A. C. Xavier, M. S. Cairo (eds.), *Resistance to Targeted Therapies in Lymphomas*, Resistance to Targeted Anti-Cancer Therapeutics 21,
https://doi.org/10.1007/978-3-030-24424-8_6

Keywords Targeted therapies · Bruton's tyrosine kinase · Ibrutinib · Drug resistance · B-cell lymphoma · Activation of B-cell receptor

Abbreviations

ABC-DLBCL	Activated B-Cell- Diffuse Large B-cell Lymphoma
AKT	Protein Kinase B
AS-PCR	Allele-Specific Polymerase Chain Reaction
BCR	Activation of B Cell Receptor
BCL-2	B-Cell Lymphoma 2
BL	Burkitt Lymphoma
B-NHL	B cell Non-Hodgkin Lymphoma
BLNK	B-cell Linker Protein
BTK	Bruton's Tyrosine Kinase
CAR	Chimeric Antigen Receptor
CCND1	Cell Cycle Regulator Cycline D1
CLL	Chronic Lymphocytic Leukemia
CARD11	Caspase Recruitment Domain Family, Member 11
CDK4	Cyclin-Dependent Kinase 4
CR	Complete Response
CRM1/XPO1	Chromosome Region Maintenance1/Exportin-1 Protein
CXCR4	C-X-C Chemokine Receptor type 4
DPPYs	Diphenylpyrimidine Derivatives
DLBCL	Diffuse Large B-cell Lymphoma
DLT	Dose-Limited Toxicities
EFS	Event Free Survival
EGFR	Epidermal Growth Factor Receptor
EIF2A	Eukaryotic Translation Initiation Factor 2A
ERK	Extracellular Signal-Regulated Kinase
FDA	Food and Drug Administration
FL	Follicular Lymphoma
FLIPI	Follicular Lymphoma International Prognostic Index
GBC	Germinal Center B cell
HCL	Hairy cell Lymphoma
HDAC	Histone Deacetylase
HL	Hodgkin Lymphoma
IC50	Half Maximal Inhibitory Concentration
IκB	Inhibitor of Kappa B
IKKb	Inhibitor of Kappa Light Polypeptide Gene Enhancer in B-cells
ITAM	Immunoreceptor Tyrosine-Based Activation Motifs
Itk	Interleukin-2-Inducible T-Cell Kinase
LCK	Lymphocyte-Specific Protein Tyrosine Kinase

LNA	Locked Nucleic Acid
MALT1	Mucosa Associated Lymphoid Tissue Lymphoma Translocation Protein 1
MAPK	Mitogen-Activated Protein Kinase
MCL	Mantle Cell Lymphoma
MLL2	Mixed Lineage Leukemia 2
MOMP	Mitochondrial Outer Membrane Permeability
MPFS	Median Progression-Free Survival
MRD	Minimal Residual Disease
mTOR	Mechanistic Target of Rapamycin
MYD88	Myeloid Differentiation Primary Response Gene (88)
MZL	Marginal zone Lymphoma
NHL	Non-Hodgkin's Lymphoma
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NGS	Next-Generation Sequencing
NIK	NF-Kappa-B-Inducing Kinase
NSG	NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ
OS	Overall Survival
ORR	Overall Response Rate
P	Phosphorylation
PARP-1	Poly [ADP-ribose] Polymerase 1
PFS	Progression-Free Survival
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-Kinase
PIM1	Serine/threonine Kinase pim-1
PIP3	Phosphatidylinositol (3,4,5)-Trisphosphate
PLCγ2	1-phosphatidylinositol-4,5-Bisphosphate Phosphodiesterase Gamma-2
PMBCL	Primary Mediastinal B-cell Lymphoma
PR	Partial Response
RPS15	40S Ribosomal Protein S15
R/R	Relapsed/Refractory
scFv	Single Chain Fragment of Variable Region
SFK	Src Family Tyrosine Kinases
SH2	Src Homology 2
SH3	Src Homology 3
SNPs	Single Nucleotide Polymorphisms
SLL	Small Lymphocytic Lymphoma
SYK	Spleen Tyrosine Kinase
Tec	Tyrosine Kinase Expressed in Hepatocellular Carcinoma
TLR	Toll-Like Receptor
TME	Tumor Microenvironment
TRAIL	Tumor Necrosis Factor Related Apoptosis Inducing Ligand

TRAIL-R	Tumor Necrosis Factor Related Apoptosis Inducing Ligand Receptors
Txk	Tyrosine-Protein Kinase TXK
WES	Whole-Exome Sequencing
WM	Waldenström's Macroglobulinemia
XLA	X-Linked Agammaglobulinemia
2p+	Gain of the Short Arm of Chromosome 2

Introduction

B-cell lymphoma represents a heterogeneous group of B-cell malignancies with distinct pathological characteristics, clinical features and prognoses [1]. The most common types of B-cell lymphoma include chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), Waldenström's macroglobulinemia (WM), and Burkitt lymphoma (BL). In children, the vast majority of B-cell lymphomas are BL and DLBCL, rarely primary mediastinal B-cell lymphoma (PMBCL) and FL are found. Cairo et al. previously demonstrated that short but intensive chemotherapy is associated with an 80% 5-year event free survival (EFS) in patients with advanced mature B-cell non-Hodgkin lymphoma (B-NHL) [2–4]. Further, an international multi-cooperative group study showed a 90% 5-year overall survival (OS) in patients with newly diagnosed mature B-NHL [5–8]. Unfortunately, the outcome is dismal in patients with aggressive B-NHL, who relapse or progress due to chemoradiotherapy resistance [5, 8]. Therefore, facilitating the development of alternative novel therapeutic strategies is required to improve the outcome in patients with relapsed/refractory (R/R) B-cell lymphoma.

Activation of the BCR signaling pathway (Fig. 6.1) is critical to the development and maturation of B cells [9, 10] and the viability of a variety of B-cell lymphomas such as DLBCL [11], marginal zone lymphoma (MZL) [12], MCL, FL [13] and BL [14]. The BCR consists of the antigen-binding immunoglobulin heavy (IgH) and light (IgL) chains coupled to the heterodimeric CD79a and CD79b proteins, which contain tyrosine-based activation motifs [15]. Crosslinking of BCR by antigen triggers the phosphorylation of tyrosines within the immunoreceptor tyrosine-based activation motifs (ITAMs) of CD79A and CD79B by Src family tyrosine kinases (SFKs) [15]. The phosphorylated ITAMs serve as a scaffolding platform for engaging and activating Src homology 2 (SH2) domains containing kinases, including spleen tyrosine kinase (SYK). Activated SYK phosphorylates the B-cell linker protein (BLNK) to further recruit both Bruton's Tyrosine Kinase (BTK) and phospholipase C- γ2 (PLC- γ2) through their SH2 domains. BTK is then phosphorylated and activated by SYK to drive the activation of downstream signaling pathways such as phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [16]. The complex of

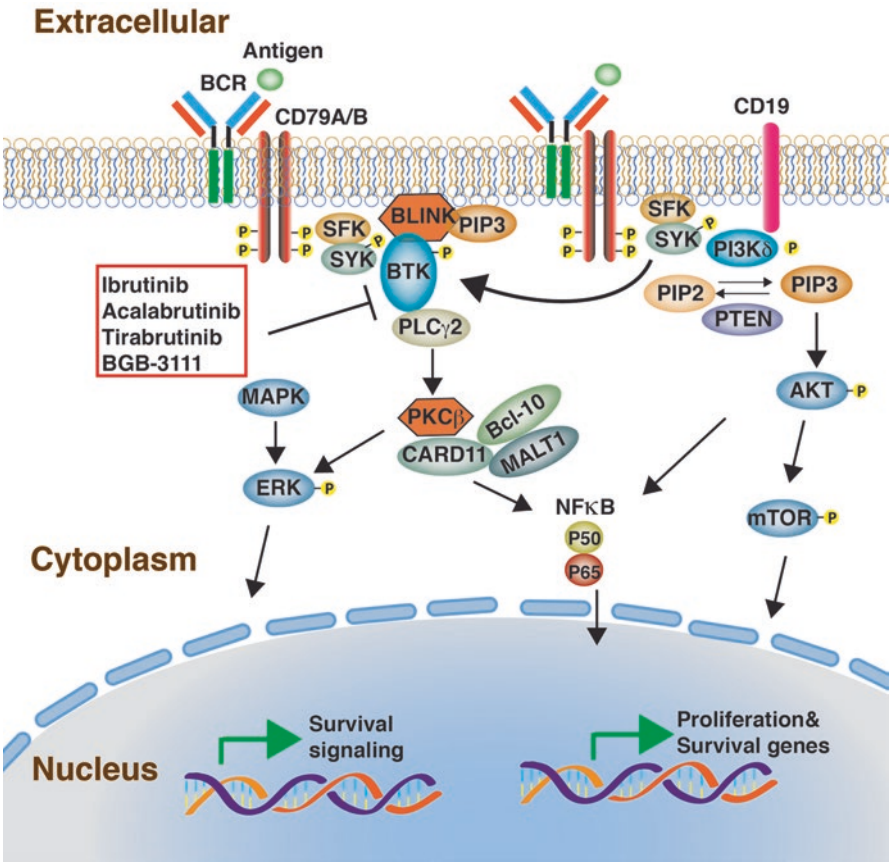

Fig. 6.1 Simplified B-cell receptor signaling. The BCR consists of the antigen-binding immunoglobulin heavy (IgH) and light (IgL) chains coupled to the heterodimeric CD79a and CD79b proteins. Antigen binding triggers the phosphorylation of tyrosines within the ITAMs of CD79A and CD79B by SFKs. And the phosphorylated ITAMs recruit SYK, which is followed by the activation of BLNK, BTK and PLCγ2. BTK is then phosphorylated and activated by Syk to drive the activation of PKCβ, PI3K/Akt/mTOR and NF-κB. PKCβ phosphorylates and activates ERK and NF-κB transcription factors. Ibrutinib, acalabrutinib, tirabrutinib, spebrutinib, and BGB-3111 inhibit BTK activities. The complex of CARD11, MALT1, and BCL10 is an important part of the pathway activating NF-κB. Additionally, the BCR co-receptor CD19 phosphorylation is also involved in BTK recruitment and activation by recruiting PI3K to generate PIP3 and activate the PI3K–AKT pathway. The activated ERK, PI3K/Akt/mTOR and NF-κB pathways upregulate the genes that are involved in cell proliferation and survival in B cell lymphoma

caspase recruitment domain family member 11 (CARD11), mucosa-associated lymphoid tissue lymphoma translocation protein 1(MALT1), and B-cell lymphoma/leukemia 10 (BCL10) is an important part of the pathway activating NF-κB. Additionally, the BCR co-receptor CD19 phosphorylation is also involved in BTK recruitment and activation by recruiting PI3K to generate phosphoinositide

phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) [17]. The BCR signaling pathway offers a wealth of therapeutic targets such as SYK, BTK and PI3K, and drugs targeting these kinases are in development and clinical trials to evaluate their efficacy against a variety of B-cell lymphomas.

BTK is a member of the tyrosine kinase expressed in hepatocellular carcinoma (Tec) family of the non-receptor tyrosine kinases and was discovered during cloning the genes that were associated with X-linked agammaglobulinemia (XLA) in 1993 [18, 19]. The gene encodes a 659 amino acid protein that consists of several putative domains: an N-terminal Pleckstrin homology (PH) domain that binds membrane PIP3, is followed by SH2, Src homology 3 (SH3), and proline rich domains that regulate binding to other cellular signaling molecules [20]. Activation of BTK correlates with an increase in the phosphorylation of two regulatory BTK tyrosine residues: Y551 and Y223 [21]. Y551 within the Src kinase domain is transphosphorylated by the kinases Syk or Lyn during BCR signaling and promotes the catalytic activity of BTK. Y223 is an autophosphorylation site within the BTK SH3 domain and the phosphorylation of this site has little discernible influence on BTK catalytic activity *in-vitro* or *in vivo* but may be a mechanism to modify protein–protein interactions [21]. BTK has been widely characterized as a critical mediator in signaling through BCR and the Fcγ receptor (FcγR) and is important for B cell development, differentiation, proliferation and survival [22, 23]. Mutations in BTK gene lead to inactivating the BTK gene through an in-frame insertion of a lacZ reporter in mouse embryonic stem cells resulting in defects of B cell development from pre-B cells to immature B cells in the bone marrow and B-cell differentiation arrest during the maturation from IgD(low)IgM(high) to IgD(high)IgM(low) stages in the periphery [24]. In humans a wide spectrum of BTK loss-of-function mutations such as a PH domain mutation in the BTK gene lead to an almost complete absence of peripheral B cells and antibodies in XLA [25, 26].

In this review, we summarize the clinical results of BTK inhibitors, discuss the resistant mechanisms of BTK inhibitors, especially ibrutinib, based on the clinical and preclinical studies in B-cell lymphoma. In the end, we describe current and future novel therapeutic strategies to overcome the resistance.

Overview of BTK Inhibitors and Clinical Response

BTK is a regulator of normal B-cell development and is activated upon BCR stimulation. Activation of the BCR signaling pathway has now emerged as a central oncogenic pathway that promotes growth and survival in both normal and malignant B-cells. Antigenic activation of the dimeric membrane immunoglobulin B-cell receptor, which induces phosphorylation of BTK and PLCγ2, results in the activation of a number of signaling pathways including mitogen-activated protein kinase (MAPK), NF-κB and Akt [27] (Fig. 6.1). Selective and covalent BTK inhibitors such as ibrutinib can inhibit BTK activation to further block chronic active BCR signaling [28, 29].

Ibrutinib

Ibrutinib (PCI-32765, Imbruvica®) is the first-in-class, selective and irreversible small molecule inhibitor of BTK and covalently binds to cysteine residue 481 on the BTK kinase domain, thereby inhibiting the autophosphorylation of tyrosine 223 on exon 8 and resulting in irreversible inhibition of BTK enzymatic activity [30]. Ibrutinib has been demonstrated to be an active agent in activated B-cell-like diffuse large B cell lymphoma (ABC-DLBCL), a NHL subtype that is characterized by constitutively activated NF-κB signaling [31]. Preclinical studies of ibrutinib in CLL and MCL suggested that ibrutinib inhibits cell proliferation *in-vitro* in the range of 1.0–25.0 μM [32, 33].

Ibrutinib's unique biochemistry and *in vivo* activities in mice and dogs paved the way for not only human clinical phase 1 trials but also phase 2 and 3 (Table 6.1) trials in patients with mature B-cell lymphomas [30]. In a phase 1 study, ibrutinib was well tolerated in 50 evaluable adults with R/R B-cell lymphomas including MCL, FL, DLBCL, MZL and CLL [34]. It was associated with an overall response rate (ORR) of 60%, including complete response (CR) 16% [34]. The safety and efficacy of ibrutinib in MCL and CLL patients who had received at least one prior therapy were evaluated in single-arm, open-label, multicenter trials (NCT01236391, NCT01105247) in 2013 [35, 36]. The drug demonstrated substantial improvement on a clinically significant endpoint over available therapies. In the MCL trial, a response rate of 68% (75 patients) was observed, with a CR of 21% and a partial response rate (PR) of 47% [35]. In the CLL trial, the ORR was 71% and the PR ranges from 15–20% based on the doses [36]. Based on the highly effective treatment of refractory and relapsed adult patients with CLL and MCL, ibrutinib was granted breakthrough therapy designation and has been approved for the treatment in patients with R/R CLL or MCL with at least one prior therapy in 2013 [35–38].

To evaluate the efficacy and tolerability of ibrutinib in relapsed or refractory WM, and to examine the impact of myeloid differentiation primary response 88 (MYD88) (L265P) and WHIM-like C-X-C chemokine receptor type 4 (CXCR4) mutations on ibrutinib response, 63 patients with R/R WM were enrolled for a phase 2 study with an ORR of 90.5% and a major response rate (PR or better) of 73% with a median time to response of 4 weeks [39]. Patients with MYD88 mutation and wild type CXCR4 had better response to ibrutinib treatment than those with MYD88 wild type or WHIM-like CXCR4 mutations: 100% OR for patients with MYD88L265PCXCR4WT vs. 85.7% OR MYD88L265PCXCR4WHIM vs 71.4% OR MYD88WTCXCR4WT. The estimated 2-year progression-free and overall survival rates among all patients were 69.1% and 95.2%, respectively. Grade > 2 treatment related toxicities were observed. Based on the promising results, the Food and Drug Administration (FDA) approved ibrutinib for the treatment of patients with WM in 2015.

BTK expression was detected in approximately 20% of patients with classic Hodgkin lymphoma (HL) [40]. A single-agent ibrutinib at a dose of 560 mg was investigated in 2 primary refractory classic HL patients [41]. Two months after the

Table 6.1 Summary of phase 2 and 3 clinical trials of Ibrutinib in B cell lymphomas

Phase	Drug	N	Tumor subtypes	Response	Toxicity	Dose	Clinical trial NCT#	Year	Ref.
1b/2	Ibrutinib	85	Relapsed CLL	71% ORR; 15–20% PR; 75% PFS; 83% OS	Predominantly grade 1 or 2: Transient diarrhea, fatigue, and upper respiratory tract infection	420 mg 840 mg	NCT 01105247	2013	[36]
2	Ibrutinib	111	Relapsed/ refractory MCL	68% ORR; 21% CR; 47% PR; 13.9 months of MPFS; 58% OS at 18 months	Most grade 1 or 2: mild or moderate diarrhea, fatigue, and nausea; Grade 3 or grade 4: infrequent, neutropenia (in 16% of patients), thrombocytopenia (in 11%), and anemia (in 10%)	560 mg	NCT 01236391	2013	[35]
2	Ibrutinib	63	Relapsed/ refractory WM	57.1% PR; 77% ORR for patients with wild-type CXCR4 vs 30% with WHIM-like CXCR4 mutations	Grade > 2 treatment related toxicities include: thrombocytopenia; neutropenia; stomatitis; atrial fibrillation; diarrhea; hematoma; hypertension and epistaxis	420 mg	NCT 01614821	2015	[39]
2	Ibrutinib	40	Relapsed/ refractory FL	37.5% ORR; 12.5% CR; 25% PR; 52.6% ORR for rituximab-sensitive vs 16.7% for rituximab-refractory (P = .04); a lower ORR for chemotherapy-refractory; 50% ORR for low- or intermediate-risk FLIPI; 14 months of MPFS; 20.4% 2 yr PFS; 79.0% 2 yr OS	42.5% of patients experienced at least 1 grade 3–4 AE	560 mg	NCT 01849263	2018	[44]

2	Ibrutinib	110	Chemo-refractory FL	20.9% ORR; 10.9% CR; 4.6 months of MPFS; 63% 1 yr OS	Grade 1/2: Diarrhea, fatigue, and cough. 48.2% serious AEs; Grade 1/2: Diarrhea, fatigue, and cough; 48.2% serious AEs. 3.6% hemorrhage; 9.1% atrial fibrillation; 6.4% discontinued; 1 pt dose reduction	560 mg	NCT 01779791	2016	[45]
2	Ibrutinib	70	Relapsed/ refractory DLBCL	40% ORR in ABC vs 5.3% in GCB; 8% CR in ABC vs 0% in GCB; 32% PR in ABC vs 5.3% in GCB 2.5 months PFS in ABC vs 1.28 in GCB	N/A	560 mg	N/A	2012	[49]
3	Ibrutinib vs Ofatumumab	195 vs 196	Relapsed/ refractory CLL or SLL	Improved PFS; 90% vs 81% OS; 42.6% vs 4.1%;	57% of the patients had at least one adverse event of grade 3 or higher.	420 mg	NCT 01578707	2014	[50]
3	Ibrutinib vs chlorambucil	136 vs 133	previously untreated CLL	Significantly longer PFS; 98% vs 85% OS at 24 months; 84% lower risk death; 86% vs. 35% ORR; 4% vs 2% CR; improved hematologic variables	Grade 3 diarrhea (4%), grade 3 hypertension (4%), atrial fibrillation (6%) and grade ¾ hemorrhage (4%)	420 mg	NCT 01722487	2015	[51]
3	Ibrutinib vs Temsirolimus	139 vs 141	Relapsed/ refractory MCL	57% reduction in the risk of disease progression or death; 14.6 vs 6.2 months MPFS; 41% versus 7% PFS at 2 yrs; 72–77% vs 40–42% ORR; 19% CR vs 1% CR	Diarrhea (29%), cough (22%), and fatigue (22%), atrial fibrillation (4% vs 1%), major bleeding (10% vs 6%)	560 mg	NCT 01646021	2016	[55]

CLL: Chronic lymphocytic leukemia; MCL: Mantle cell lymphoma; WM: Waldenström's macroglobulinemia; FL: Follicular lymphoma; DLBCL: Diffuse large B-cell lymphoma; ABC: Activated B-cell; GBC: Germinal center B cell; SLL: small lymphocytic lymphoma; ORR: Overall response rate; CR: Complete response; PFS: Progression-free survival; MPFS: Median progression-free survival; PR: Partial response; R/R: Relapsed/refractory; Ref.: References.

initiation of ibrutinib, positron emission tomography-computed tomography (PET-CT) showed near-complete regression of disease in one patient with subsequent disease progression. Another patient had a CR, which was still ongoing more than 6 months later. The activity of ibrutinib in patients with classic HL warrants prospective assessment. A phase 2 multicenter trial to evaluate the efficacy and safety of ibrutinib in patients with R/R classical HL is ongoing (NCT02824029).

Primary FL cells have been found to maintain enhanced BCR pathway signaling when compared to normal B cells [42]. Sixteen patients with FL were treated with ibrutinib in the phase 1 study [43]. Of the cohort of 16 patients, 11 patients were treated at doses where full occupancy of BTK was achieved by ibrutinib. The OR rate was 55% and the median duration of response was 12.3 months and the median progression free survival (PFS) 13.4 months. Based upon drug occupancy and clinical responses, a phase 2 Consortium Trial of ibrutinib in R/R FL was conducted [44]. ORR was 37.5% with a complete response rate of 12.5%, median progression-free survival (PFS) of 14 months, and 2-year PFS of 20.4%. Response rates were higher among patients with rituximab-sensitive disease (52.6%) compared with those who had rituximab-refractory disease (16.7%; P = .04). Chemotherapy-refractory patients also had a lower ORR than chemotherapy-sensitive patients. Patients with low- or intermediate-risk FLIPI (the Follicular Lymphoma International Prognostic Index) had a trend toward a higher response rate compared with high-risk FLIPI (50% vs 25%; p = .19). The median PFS was 14.0 months and the 2-year PFS and OS were 20.4% and 79.0%, respectively. Similar results were found in a phase 2 study of ibrutinib in patients with chemoimmunotherapy-refractory FL (the DAWN study), which showed a significantly lower response rate of 20.9% in chemotherapy-refractory FL with a median PFS of 4.6 months, 10.9% CR and 63% 1 year OS [45].

Due to activating mutations in CD79B, MYD88, and CARD11, the BCR signaling, the toll-like receptor (TLR) and the NF-κB pathways are often constitutively activated in ABC-DLBCL compared with germinal center B-cell (GCB) type DLBCL [46–48]. A phase 2 multicenter study was performed to determine if ibrutinib would be more efficacious in ABC-DLBCL compared with GCB-DLBCL [49]. The ORR in patients with ABC type was 40%, whereas overall response rate in the GCB type was only 5%. The CR is 8% in ABC-DLBCL *vs.* 0% in GCB-DLBCL; PR is 32% in ABC-DLBCL *vs.* 5.3% in GCB-DLBCL; PFS is 2.5 months in ABC *vs.* 1.28 in GCB [49]. Furthermore, ibrutinib had activity in patients with and without CD79b mutations, suggesting an alternative mechanism of BCR pathway dependence. This study indicates that further study of ibrutinib should be aimed at the ABC type of DLBCL with attention to the different somatic mutations [49].

Based on the early promising results of the phase 2 trial on CLL, a multicenter, open-label, randomized, phase 3 trial (RESONATE) was opened to the study of ibrutinib *vs.* ofatumumab in patients with relapsed or refractory CLL or SLL [50]. Ibrutinib significantly improved the PFS and the OS (90% *vs.* 81%; p = .005) and ORR (42.6% *vs.* 4.1%; p < .001). Patients with a 17p13.1 deletion also had a

markedly improved PFS with ibrutinib compared with ofatumumab. In this trial, ibrutinib was associated with a slightly increased risk of grade 3/4 (57% *vs.* 47%) adverse events (AE) compared with ofatumumab. Based on the superior efficacy of ibrutinib compared to ofatumumab in difficult-to-treat patients with R/R CLL or SLL, the FDA expanded the approval of ibrutinib to include treatment of CLL patients with 17p deletion. In the following phase 3 RESONATE-2 study, the efficacy and safety of ibrutinib was compared with chlorambucil in patients 65 years of age or older with previously untreated CLL [51]. Consistent with the high-risk group, ibrutinib resulted in significantly longer PFS than that with chlorambucil with 98% OS at 24 months *vs.* 85% with chlorambucil. The relative risk of death with ibrutinib was 84% lower than that with chlorambucil ($p = .001$). The ORR was significantly higher in the ibrutinib group than in the chlorambucil group (86% vs. 35%; $p < .001$). CR occurred in 4% of the patients in the ibrutinib group and in 2% of those in the chlorambucil group. The hematologic variables were significantly improved in the ibrutinib treated group. Grade III diarrhea (4%), grade III hypertension (4%), atrial fibrillation (6%) and grade III/IV hemorrhage (4%) were more common in the ibrutinib treated group. These results support the use of ibrutinib as a first-line agent in CLL.

Temsirolimus is an inhibitor of the mechanistic target of rapamycin (mTOR) pathway that has been used to treat patients with relapsed MCL with 22–40% ORR and a median OS of 12.8 months [52–54]. A randomized phase 3 clinical trial led by the European MCL Network compared ibrutinib with temsirolimus in patients with R/R MCL [55]. With a median follow-up of 20 months, ibrutinib treatment resulted in a 57% reduction in the risk of disease progression or death compared with temsirolimus ($p < .0001$). The median progression-free survival (MPFS) was 14.6 months for the ibrutinib group *vs.* 6.2 months for the temsirolimus group. At a 2 year landmark, the PFS rate is 41% versus 7% and the ORR was 72–77% *vs.* 40–42% with a 19% CR *vs.* 1% CR. Median OR was not reached for ibrutinib versus 21.3 months for temsirolimus. The reported AEs were consistent with previous studies, including diarrhea (29%), cough (22%), fatigue (22%), atrial fibrillation (4% with ibrutinib *vs.* 1% with temsirolimus) and major bleeding (10% with ibrutinib *vs.* 6% with temsirolimus).

We investigated the efficacy of ibrutinib alone and in selective adjuvant combinations against BL *in vitro* and in a human BL xenografted immune-deficient NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mouse model [56]. Our data demonstrated that phospho-BTK level was significantly reduced in BL cells treated with ibrutinib ($p < .001$). Moreover, we observed a significant decrease in cell proliferation as well as significant decrease in half maximal inhibitory concentration (IC_{50}) of ibrutinib in combination with dexamethasone, rituximab, obinutuzumab, carfilzomib, and doxorubicin ($p < .001$). *In vivo* studies demonstrated ibrutinib-treated mice had a significantly prolonged survival compared to vehicle controls ($p < .02$). Our findings demonstrate the significant *in vitro* and preclinical *in vivo* effects of ibrutinib in BL. Based on our preclinical results, there is an ongoing clinical trial comparing OS in children and adolescents with R/R BL treated with chemoimmunotherapy with or without ibrutinib (NCT02703272).

Second-Generation Inhibiors of BTK

Ibrutinib binds to Cys-481 of the BTK but it also binds to several other kinases [57]. These off-target effects of ibrutinib contribute to its activity and toxicity such as bleeding [50]. Therefore, second-generation BTK inhibitors such as acalabrutinib (ACP-196), Tirabrutinib (ONO/GS-4059, GS-4059), spebrutinib (CC-292, AVL-292), and BGB-3111 are being developed with more selective kinase activity profiles (Table 6.2).

Table 6.2 Summary of clinical trials of second-generation BTK inhibitors

Inhibitor name	Off-targets	Phase of trials	N	Tumor subtypes	Overall response rate	Approved indication	Ref.
Acalabrutinib	NO	1/2	61	R CLL	95% ORR	NO	[165]
					85% PR		
					10% PR with lymphocytosis		
					5% SD		
					For del(17)(p13.1): 100% ORR		
					89% PR		
					11% PR with lymphocytosis		
					With prior idelalisib therapy: 100% ORR		
					75% PR		
					25% PR with lymphocytosis		
Acalabrutinib	NO	2	124	R/R MCL	81% ORR	YES for R/R MCL	[62]
					40% CR		
					72% medians for duration of response		
					67% PFS		
					87% OR		
Tirabrutinib (ONO/GS-4059)	NO	1	90	R/R CLL; MCL; DLBCL; FL; WM; MZL; SLL	PFS in CLL, MCL, and DLBCL: 874, 341, and 54 days, respectively	NO	[66]
					CLL: 96% ORR		
					MCL: 91.7% ORR, 50% PR, and 41.7% CR		
					DLBCL: 35% ORR, 29% PR, 6.45% CR		
BGB-3111	NO	1	25	CLL, MCL, WM, DLBCL, FL, MZL, HCL	64% OR (16/25), including 1 CR and 6 SD	NO	[67]

Acalabrutinib

Acalabrutinib (ACP-196) binds covalently to BTK with greater *in vivo* potency and selectivity than ibrutinib [58]. *In-vitro* studies demonstrated that acalabrutinib and ibrutinib had similar molecular and biologic consequences in primary CLL cells but different effects on lymphocyte-specific protein tyrosine kinase (LCK) and proto-oncogene tyrosine-protein kinase Src phosphorylation in primary T-lymphocytes [59]. The IC_{50} of acalabrutinib for the BTK protein is 5.1 nmol/L vs 1.5 nmol/L of ibrutinib, indicating a weaker BTK inhibition than ibrutinib. However, ACP-196 demonstrated higher selectivity for BTK than ibrutinib when profiled against a panel of 395 non-mutant kinases (1 μM) in a competitive binding assay [60]. Importantly, ACP-196 did not inhibit epidermal growth factor receptor (EGFR), interleukin-2-inducible T-cell kinase (Itk) or tyrosine-protein kinase TXK (Txk) [60]. The phase 1/2 ACE-CL-001 trial of acalabrutinib monotherapy in patients with relapsed CLL showed that acalabrutinib was well tolerated and no major hemorrhage or atrial fibrillation was noted [61]. The clinical activity of acalabrutinib was rapid and robust. With a median follow up of 14.3 months, the ORR was 95% with 85% PR, 10% PR with lymphocytosis and 5% stable disease. The ORR was 100% for patients with del(17)(p13.1) with 89% PR, 11% PR with lymphocytosis. In the 4 patients with prior idelalisib therapy, the response rate was 100% (PR, 75%, PR with lymphocytosis, 25%). A direct comparison of acalabrutinib with ibrutinib in a phase 3 study (NCT02477696) is active and on the way to recruit patients with high-risk CLL.

Acalabrutinib is also active in clinical trials as a single agent or in combination for the treatment of other lymphomas including MCL (NCT02213926), FL (NCT02180711), WM (NCT02180724), and DLBCL (NCT03205046). In 2015, a phase 2 trial (ACE-LY-004) was conducted on patients with R/R MCL (NCT02213926) [62]. One hundred twenty-four patients with R/R MCL were enrolled in this trial. At a median follow-up of 15.2 months, the ORR was 81% and CR was 40%. The Kaplan-Meier estimated medians for duration of response, PFS, and OR rates at 12 months were 72%, 67%, and 87%, respectively. Primarily grade 1 or 2 adverse events were the most common. Consistent with CLL trials, atrial fibrillation and worse hemorrhage events were rare. The results demonstrated that acalabrutinib treatment provided a high rate of durable responses and a favorable safety profile in patients with relapsed or refractory MCL. Based on the promising results in the ACE- LY-004 trial and other clinical data, acalabrutinib was granted Breakthrough Therapy Designation by the FDA in 2017 for patients with MCL who have received at least one prior therapy.

Tirabrutinib

Tirabrutinib (ONO/GS-4059) is an irreversible inhibitor with a greater selectivity for BTK than for LCK, proto-oncogene tyrosine-protein kinase Fyn (FYN), tyrosine-protein kinase LynA (LYNA), and Itk [63]. *In-vitro* studies showed that IC_{50} of ONO/

GS-4059 to BTK was 2 nmol/L and it induced apoptosis at nanomolar concentrations in the activated DLBCL cell lines [64]. ONO/GS-4059 treatment resulted in inhibition of tumor growth in an ABC-DLBCL xenograft model [65]. These promising preclinical data prompted clinical evaluation of ONO/GS-4059. In a multicenter phase 1 dose escalation study, 90 patients with R/R B cell malignancies including CLL/SLL, MCL, DLBCL, FL, MZL, and WM, were enrolled in a 3 + 3 dose-escalation study [66]. The overall estimated mean PFS in CLL, MCL, and DLBCL were 874, 341, and 54 days, respectively. CLL patients had a 96% ORR. Of these patients, 13 had loss of TP53 and 21 had unmutated immunoglobulin heavy-chain variable region (IGHV) gene segments. All 12 patients with TP53/17p deletion or TP53 mutations had a response, with 9 remaining on study. MCL patients had 91.7% ORR, 50% PR, and 41.7% CR. DLBCL patients had 35% ORR, 29% PR, and 6.45% CR. A striking feature of this study was that ONO/GS-4059 across all disease subsets showed a low incidence of associated toxicities. ONO/GS-4059 may have significant advantages over other selective kinase inhibition in terms of reduced toxicities.

BGB-3111

BCB-3111 is another more selective, irreversible BTK inhibitor with higher BTK specificity than ibrutinib [67, 68]. In biochemical and cellular assays, BGB-3111 demonstrated nanomolar BTK inhibition activity and showed less off-target kinase inhibition against a panel of kinases [68]. Both in the MCL and ABC-DLBCL tumor cells xenografted models, BGB-3111 demonstrated dose-dependent antitumor effects and prolonged the overall survival of xenografts [68]. Additionally, BGB-3111 demonstrated at least ten-fold weaker than ibrutinib in inhibiting rituximab induced antibody-dependent cellular cytotoxicity (ADCC), supporting the combination therapy with anti-CD20 antibodies in lymphoma [68]. In the phase 1 trial of BGB-3111, 24 patients with advanced lymphoma (CLL, MCL, WM, DLBCL, FL, MZL) and 1 with Hairy cell lymphoma (HCL) were enrolled [67]. Sixty-four percent (16/25) of patients had objective responses, including 1 CR and 6 SD. Drug-related AEs and dose-limited toxicities (DLT) were not reported. These preliminary phase 1 results suggest that BGB-3111 is safe and highly clinically active but clinical efficacy remains to be further determined.

Molecular Mechanisms of BTK Inhibitors' Resistance

Despite the promising clinical responses of BTK inhibitors especially ibrutinib in a variety of B-cell lymphomas, cases of primary and secondary resistance were recognized [69]. Clinically, ibrutinib resistance presents in two forms: primary resistance in which patients demonstrate lack of response at initial therapy due to disease transformation (Richter transformation, an aggressively ibrutinib-resistant disease), and secondary resistance which is characterized by an initial disease response but it

is subsequently lost due to the cell's ability to bypass the target via alternative pathways or acquired mutations in the target or its pathway [15, 69, 70]. Understanding the molecular mechanisms (Fig. 6.2) underlying BTK inhibitors' resistance is of paramount importance. The reported resistant mechanisms of BTK inhibitors, especially ibrutinib resistance are summarized in Table 6.3.

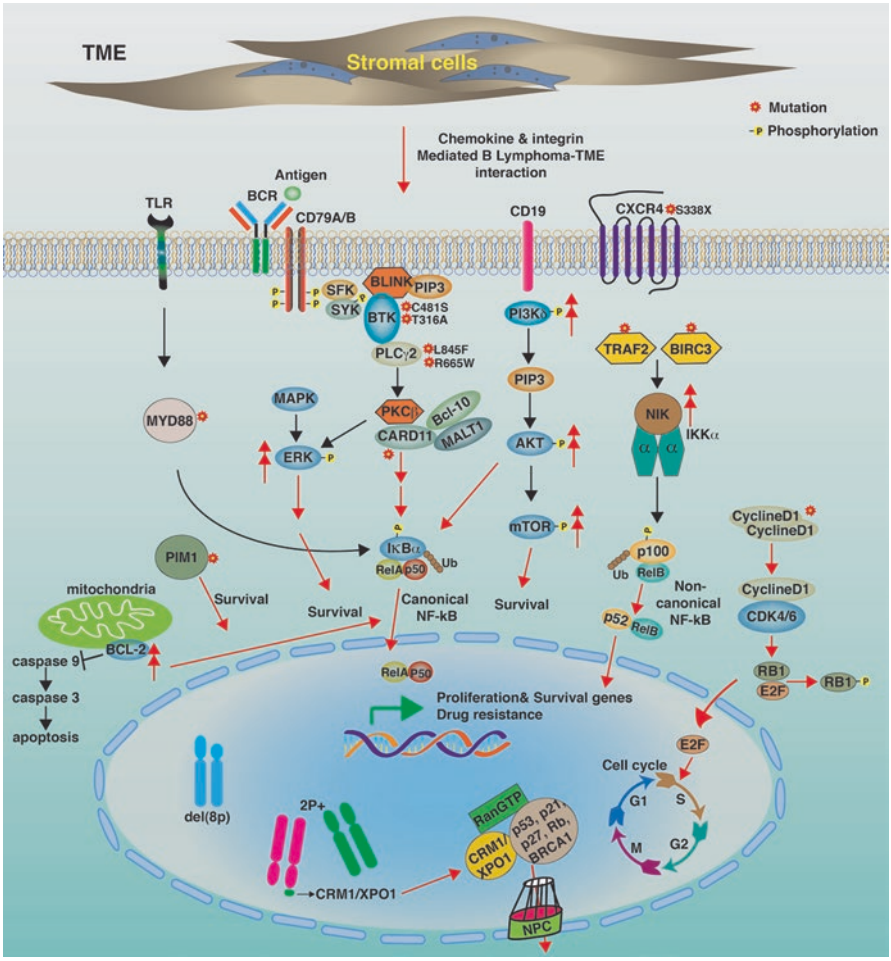

Fig. 6.2 Mechanisms of resistance of BTK inhibitors in B-cell lymphomas. Primary resistance of BTK inhibitors may be caused by sustained activation of other oncogenic pathways such as PI3K-AKT/mTOR, MAPK/ERK independent of BTK. Acquired resistances include mutations in BTK, PLCγ2, CARD11 and the activation of alternative NF-kB or PI3K/mTOR pathways. MYD88 and CXCR4 mutations in WM patients trigger pro-survival NF-kB signaling, activate AKT and ERK and promote resistance to ibrutinib. Chromosomal abnormality such as del(8p) and 2p+ has been documented in acquired ibrutinib resistance. Overexpression of CRM1/XPO1 is involved in nuclear export of a number of tumor suppressor proteins such as p53 and BRCA1, which is associated with drug resistance. Mutations in CCND1 stabilize cycline D1 and subsequently activate cyclin-dependent kinase (CDK) 4 to phosphorylate and inactivate retinoblastoma (Rb) protein. This event leads to G1/S cell cycle progression, cell proliferation and ibrutinib resistance. Last, TME–lymphoma interactions activate integrin b1-integrin-linked kinase (ILK)/PI3K-AKT-mTOR to mediate ibrutinib resistance

Table 6.3 Summary of the reported mechanisms of BTK inhibitors' resistance

BTK inhibitor	Diseases	Resistance mechanism	Report year	Ref.
Ibrutinib	CLL	Mutations: BTKC481S, PLCγ2 (R665W, L845F, S707Y)	2014	[72]
Ibrutinib	CLL	Mutations: PLCγ2 (S707F, M1141R, M1141K and D993H)	2016	[89]
Ibrutinib	CLL	Mutation: BTKT316A	2016	[76]
Ibrutinib	CLL	Mutations: EIF2A, RPS15, EP300, MLL2	2016	[89]
Ibrutinib	CLL	Del(8p)	2016	[89]
		TRAIL receptor haploinsufficiency		
Ibrutinib	CLL	2p+	2017	[166]
		XPO1 overexpression		
Ibrutinib	Mouse Eμ-myc B cells	Myc amplification	2017	[92]
Ibrutinib	MCL	Activation of the alternative NF-kB pathway	2013	[100]
Ibrutinib	MCL	Mutations in TRAF2, BIRC3, Activation of alternative NK-kB pathway	2014	[100]
Ibrutinib	MCL	Mutations in CARD11	2016	[87]
Ibrutinib	MCL	Mutations in primary resistance: A20, BIRC2, epigenetic modifiers, EGFR family;	2016	[78]
		Mutations in acquired resistance: PLCγ2, CARD11, epigenetic modifiers, NF-kB and PI3K/mTOR pathways		
Ibrutinib	MCL	PI3K–AKT activation	2014	[102]
Ibrutinib	MCL	ERK1/2 and AKT activation	2014	[103]
Ibrutinib	MCL	Upregulating the c-Myc and mTOR signaling pathways and metabolic pathways	2017	[88]
Ibrutinib	ABC-DLBCL	Mutations in PIM1	2016	[80]
Ibrutinib and ONO/GS-4059	ABC-DLBCL	NF-kB pathway activation	2017	[129]
Ibrutinib	DLBCL	Upregulation of IAP, survivin, cIAP2, BCL2 and BCL6, PI3Kα and PI3Kβ;	2017	[105]
		Downregulation of PTEN		
Ibrutinib	WM	CXCR4S338X mutation leads to activation of both AKT and ERK	2015	[85]
Ibrutinib	WM	Mutations: BTKC481S, CXCR4, CARD11, PLCγ2	2017	[83]
		Upregulation of Bcl-2 and AKT	2017	[106]

Mutational Resistance in BTK

The development of mutations within the drug target that alter drug sensitivity is an important mechanism of acquired resistance to ibrutinib. Whole-exome sequencing (WES), Sanger sequencing, and Ion Torrent deep sequencing of pre-treatment and

relapse samples from six CLL patients confirmed a cysteine-to-serine mutation at BTK position 481 (C481S) in five of the six patients [71, 72]. No patient at baseline had evidence of mutations in either BTK on the basis of WES and Ion Torrent sequencing. This finding was further confirmed by another study using peripheral blood cells and cell free DNA samples from ibrutinib naïve and treated CLL patients with custom DNA or locked nucleic acid (LNA) oligos in a wild-type blocking polymerase chain reaction, followed by Sanger sequencing and Next-generation sequencing (NGS) methods [73]. Functional characterization demonstrated that mutant BTK has significantly lowered affinity to ibrutinib than nonmutant BTK [72]. When transfected to cells with mutant BTK, ibrutinib was significantly less effective at blocking BTK auto-phosphorylation and downstream signaling than nonmutant BTK [72]. The data from the mutational analyses, signal transduction and gene expression profiling strongly suggest C481S mutation confers resistance to ibrutinib leading to increased BCR signaling at patient's relapse [74]. These functional studies suggest that the C481S mutation in BTK confers resistance to ibrutinib by preventing irreversible drug binding [72, 75]. Another mutation in BTK that is associated with ibrutinib resistance was identified at the center of the positively-charged binding pocket in the SH2 domain with a threonine to alanine change at BTKT316 site [76]. Unlike the C481, T474 and L528 mutations in the kinase domain to either directly attenuate or hinder ibrutinib binding, structure analysis revealed that T316A does not directly interfere with ibrutinib binding. *In vitro* cellular and molecular studies demonstrated that ibrutinib did not inhibit the cell proliferation of the transfected lymphoma cells with BTKT316A mutation and the degree of phosphorylation inhibition in p-BTK (Y223), p-PLCγ2, p-AKT and p-ERK following ibrutinib treatment was significantly less in C481S and T316A mutant cells than in wild type cells [76]. This data firmly established that the BTKT316A mutant is as capable as BTKC481S to confer ibrutinib resistance. The resistant BTK mutations were not detectable at the baseline before ibrutinib exposure [72, 75]. It might be limited by the sensitivity of the detection methods that may not identify small numbers of BTK mutant CLL cells in the presence of large numbers of nonmutant CLL cells. To investigate this possibility, Fam'a, R. et al. used an allele-specific polymerase chain reaction (AS-PCR) which is highly sensitive and can detect 1 mutant allele per 1000 wild-type alleles, to assess the occurrence of small subclones harboring the C481S codon mutations in ibrutinib-naive CLL patient samples [77]. Among CLLs that have not been exposed to ibrutinib, the BTK C481S variant was not detected, indicating the ibrutinib resistance in CLL is not mutation driven resistance [77].

Mutational Resistance in Other Proteins than in BTK

Mutations in the prosurvival pathways to bypass BTK appear to be another common mechanism of resistance. The samples from relapsed CLL patients were detected to have gain-of-function mutations targeting PLCγ2, a direct downstream target of

BTK phosphorylation [72]. When transfected with PLCγ2 with the L845F mutation, or with the R665W mutation into human embryonic kidney (HEK) 293 T cells and DT40 cells, which lack endogenous PLCγ2 expression, upon activating BCR signaling, phosphorylation of extracellular signal-regulated kinases (ERK) and AKT was less inhibited by ibrutinib than nonmutant cells [72]. In the phase 3 MCL 3001 (RAY) trial, mutations were identified in NF-kB signaling pathways, both canonical (e.g., A20) and noncanonical (e.g., BIRC2); in epigenetic modifiers; and in the epidermal growth factor receptor (EGFR) family in primary resistance to ibrutinib [78]. Mutations were found in PLCγ2, CARD11, epigenetic modifiers and alternate NF-kB or PI3K/mTOR pathways in the MCL patients with acquired resistance after a short treatment duration [78]. Serine/threonine kinase (PIM1) encodes a serine/threonine kinase that is a critical regulator of tumorigenesis in a number of hematologic malignancies [79]. Interestingly, in 48 DLBCL patient samples with available genomic profiling, PIM1 mutations appeared more frequently in patients with ABC-DLBCL than those with GCB-DLBCL [80]. PIM1 mutations were also identified in patients with poor response to ibrutinib, indicating PIM1 mutations are associated with intrinsic ibrutinib resistance in ABC-DLBCL. *In vitro* studies demonstrated that introducing one of these mutations into an ABC-DLBCL cell line is sufficient to induce ibrutinib resistance through stabilizing the protein and enhancing NF-κB signaling [80]. The combination of pan-PIM inhibitors such as AZD-1208 with ibrutinib results in greater efficacy than ibrutinib as a single agent and can circumvent resistance. Activating somatic mutations in MYD88 and CXCR4 are present in 90–95% and 30–40% of WM patients, respectively [81–83]. MYD88 mutations trigger pro-survival NF-kB signaling through BTK [84]. The WHIM-like CXCR4 (S338X) somatic mutation activates AKT and ERK and promotes resistance to ibrutinib [85]. In a multicenter study that administered ibrutinib to rituximab refractory WM patients, patients with CXCR4 mutations showed delayed responses, and the 1 patient with WT MYD88 showed no response to ibrutinib [86]. Xu et al. utilized Sanger sequencing, highly sensitive AS-PCR assays and targeted NGS to identify mutations associated with clinical progression in WM patients treated with ibrutinib [83]. Their study revealed that 5.1% patients on ibrutinib without clinical progression had BTKC481S mutation. And BTKC481 mutations are associated with mutated CXCR4 [83]. Akin as in CLL, BTKC481 mutations were not detected in baseline samples or ibrutinib-naïve WM patients. Additional mutations in ibrutinib resistant WM samples were identified in CARD11 and PLCγ2 [83]. CARD11 is a scaffold protein required for BCR induced NF-kB activation. Its mutation may result in a constitutive activation of B-cell receptor (BCR)/NF-κB signaling and render the mutant cells resistant or sensitive to some of the BCR/NF-κB inhibitors [47]. CARD11 mutations were observed in 5.5% of MCL samples [87]. When overexpressed *in vitro*, CARD11 mutants conferred resistance to ibrutinib, providing new insights for ibrutinib resistance in MCL and continuous activation of NF-kB pathway.

In addition to acquisition of these mutations, other mechanisms of resistance, such as upregulation of potentially druggable survival pathways [88], clonal evolution of genetic alterations [89, 90], presence of BCL-6 abnormalities [91], complex

karyotype [91], TP53 abnormality [90, 91], MYC amplification [90–92] and baseline del(17p) [90, 91], are associated with an increased risk of acquired resistance to ibrutinib.

Chromosomal Abnormality

Deletions in chromosomes have been documented in acquired ibrutinib resistance such as large deletions in the short arm of chromosome 8 [89]. Deletions of chromosome 8p were reported as a recurrent event in B-NHL and tumor necrosis factor related apoptosis inducing ligand receptors (TRAIL-R) were identified as dosage-dependent tumor suppressor genes in this region whose monoallelic deletion can impair TRAIL-induced apoptosis in B-cell lymphoma [93]. In ibrutinib resistant CLL patients, del(8p) was not present at baseline, but was detected at the time of progression on ibrutinib, indicating ibrutinib therapy favors the selection and expansion of CLL subclones carrying del(8p) [89]. The region of del(8p) was confirmed to encompass TRAIL-R [89]. Treatment with TRAIL decreased cell viability in a greater proportion of non-del(8p) CLL samples compared to the del(8p) CLL samples (16% *vs.* 5%), indicating monoallelic deletion of chromosome 8p was sufficient to abrogate the positive or negative effects of TRAIL on cell viability *in vitro*. The expected sensitivity to TRAIL in the pre-treatment samples and resistance in the relapse samples further confirmed the role of del(8p) in protection from TRAIL-induced apoptosis [89]. Some ibrutinib resistant CLL patients acquired additional putative driver mutations in eukaryotic translation initiation factor 2A (EIF2A), 40S ribosomal protein S15 (RPS15), the histone acetyltransferase EP300 (Y1397F) and the chromatin regulator MLL2 (the mixed lineage leukemia 2) without detectable mutations in BTK and PLCγ2 genes, which likely confer proliferative advantage and bypass the BTK pathway [89].

The gain of the short arm of chromosome 2 (2p+) was reported as a frequent chromosomal abnormality in CLL [94, 95]. Using single nucleotide polymorphisms array and fluorescence in situ hybridization approaches, chromosome region maintenance1/Exportin-1 gene (CRM1/XPO1) was identified to be overexpressed in the tested 2p + CLL samples [95]. CRM1/XPO1 is a ubiquitous nuclear export receptor protein that regulates intracellular nuclear export of many substrates, including both proteins and ribonucleic acid (RNA) [96]. CRM1/XPO1 is often overexpressed in cancer cells and its overexpression is involved in nuclear export of a number of tumor suppressor proteins such as p53, BRCA1, retinoblastoma, forkhead box O (FOXO), cell cycle inhibitors (p21, p27) and other drug targets [96]. CRM1/XPO1 overexpression and its mediated export has been associated with poor prognosis and resistance to therapy in various cancers [96]. Relapsed 2p+/CLL patients after treatment showed a similar or increasing percentage of cells carrying a XPO1 gain compared with the patients at the time of diagnosis, indicating the potential relevance of XPO1 in CLL drug resistance [95]. *In vitro* ibrutinib induced significantly lower programmed cell death in the 2p+/CLL cells compared with the 2p−/CLL control

cells, indicating XPO1 overexpression associated with 2p + is associated with ibrutinib resistance in the 2p+/CLL cells [95]. Further studies are needed to investigate if the combination of selinexor, a selective inhibitor of XPO1 currently in Phase 1/2 clinical trials, with ibrutinib can enhance cell death in the 2p+/CLL cells.

Activaiton of Prosurvival Pathways

Canonical and Non-canonical NF-kB Signaling Pathways

NF-kB signaling is an integral important part of the BCR signaling pathway in B cell lymphoma [97]. In canonical NF-κB pathway, NF-κB activation relies on inducible degradation of inhibitor of kappa B (IκBs), leading to nuclear translocation of various NF-κB complexes, predominantly the p50/RelA dimmer [98]. While, in a non-canonical (alternative) NF-κB pathway, the RelB/p52 NF-κB complex activation uses a mechanism that relies on the inducible processing of p100 instead of degradation of IκBα [99]. The deregulated non-canonical NF-κB signaling has associated with hematologic malignancies [99]. In a study of ibrutinib-resistance in MCL, Rahal et al. revealed that the resistant MCL cell lines depended on the alternative NF-kB pathway rather than on the canonical pathway [100]. RNA sequencing and single nucleotide polymorphism arrays showed recurrent mutations in TNF receptor associated factor 2 (TRAF2) or baculoviral IAP repeat containing 3 (BIRC3) in 15% of these individuals in ibrutinib-insensitive cell lines. The BIRC3 mutations were not only less efficient at destabilizing NIK (also known as NF-kappa-B-inducing kinase, mitogen-activated protein 3 kinase 14 or MAP3K14) but also markedly impaired in their ability to suppress p52 production [100]. And these MCL cell lines with alternative NF-κB pathway alterations are dependent on the NIK signaling both *in vitro* and *in vivo*, suggesting that NIK inhibition may offer a novel, targeted therapeutic strategy for this ibrutinib-resistant population of patients [100].

PI3K-AKT/mTOR Pathway

PI3K-AKT/mTOR activation represents a crucial downstream event of BCR/pre-BCR signaling [101]. The relapse-specific C481S mutation is often absent in patients with primary resistance or progression following transient response to ibrutinib, suggesting alternative mechanisms of resistance in MCL [102]. Chiron et al. found that primary ibrutinib resistance or transient response seems not to stem from defective ibrutinib inhibition of BTKWT in MCL cells but rather may involve sustained PI3K–AKT activation [102]. Ma et al. found that inhibition of ERK1/2 and AKT, but not BTK phosphorylation, correlates well with the extent of cell death to BTK inhibition in MCL cell lines as well as in primary tumors [103]. RNA-Seq and gene set enrichment analysis (GSEA) revealed the marked upregulation of

components of the c-Myc and mTOR signaling pathways in the ibrutinib-resistant MCL patient samples, indicating that the activation of the pathways may mediate ibrutinib resistance [104]. The role of PI3K-AKT pathway in ibrutinib resistance is also reported in DLBCL and WM. DLBCL Ibrutinib resistance cell lines were generated by continuous culturing of parental DLBCL cell lines in increasing concentrations of ibrutinib [105]. In the resistant cells, besides the increased expression of inhibitors of apoptosis (IAP) family members, survivin, cIAP2 (cellular inhibitor of apoptosis protein 2) and oncogenic BCL2 and BCL6, the deoxyribonucleic acid (DNA) damage repair pathway, and the checkpoint kinase 1 (CHK1), PI3K isoforms PI3Kα and PI3Kβ were upregulated with decreased expression of PI3Kδ and phosphatase and tensin homolog (PTEN) which is a PI3K negative regulator. When treating these resistant cells with the PI3Kβ/δ isoform targeting Drug KA2237, metabolic activity (survival) and surviving of these cells were reduced [105]. Although ibrutinib is highly effective in WM, no complete remissions in WM patients treated with ibrutinib have been reported to date, indicating the WM cell's ability to maintain their survival under ibrutinib-induced stress [106]. Paulus et al. developed ibrutinib resistant WM cell lines to identify the potential mechanisms of ibrutinib resistance in WM cells [106]. These cells exhibited decreased survival dependency on BTK-mediated signaling, but phospho-AKT level was increased in ibrutinib resistant WM cells. When the resistance cells were treated with clinical-grade allosteric pan-AKT inhibitor, MK2206, pAKT level was marked reduced and apoptosis was enhanced as indicated by poly [ADP-ribose] polymerase 1 (PARP-1) cleavage. Remarkably, when cells were treated concurrently with ibrutinib and MK2206, pBTK and pAKT levels were significantly reduced with more robust cleavage of PARP-1 and resistant tumor cell viability was synergistically reduced. This data demonstrated that drug combination strategies encompassing BTK + AKT/PI3K inhibition may potentially overcome ibrutinib resistance in WM [106].

B-Cell Lymphoma-2 (BCL-2) Family Members Mediated Resistance

BCL-2 was initially discovered as a part of the t(14;18) chromosomal translocation in patients with NHLs [107]. The dysregulation of *BCL-2* leads to high levels of Bcl-2 protein in B-cells, which alters the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family [108]. The resulting inhibition of apoptosis is thought to lead to chemoresistance [108]. Recent studies show that Bcl-2 is involved in ibrutinib resistance. CLL patient samples treated *ex vivo* with ibrutinib or acalabrutinib and the primary samples from CLL patients on clinical trials of both drugs show enhanced mitochondrial Bcl-2 dependence without significantly altering overall mitochondrial priming [109]. The Bcl-2 family regulators profiles restored to pre-treatment levels in the samples of CLL patients that developed ibrutinib resistance [110]. Treatment of DLBCL cells with ibrutinib increased Bcl-2 expression and combination treatment with Bcl-2 inhibitors and ibrutinib

completely inhibited tumor growth in murine models of ABC-DLBCL [111]. In ibrutinib resistant WM cell lines, apoptosis regulators Bcl-2 and Mcl-1 expression were increased [106]. With Bcl-2 inhibitor, venetoclax compromised mitochondrial function in ibrutinib-resistant WM cells by increasing mitochondrial outer membrane permeability (MOMP) with induction of apoptosis [106]. These data demonstrated that drug combination strategies encompassing BTK + Bcl-2 inhibition can potentially overcome ibrutinib resistance.

Cell Cycle Deregulation

Cell cycle regulator Cycline D1, encoded by CCND1, binds and activates cyclin-dependent kinase (CDK) 4 to phosphorylate and inactivate retinoblastoma (Rb) protein [112]. This event leads to G1/S cell cycle progression and cell proliferation [112].

High-throughput sequencing has consistently revealed CCND1 was frequently mutated in MCL [113]. Recently, Mohanty et al. found some recurrent mutations located in the N-terminus of CCND1, which interfere with T286 phosphorylation and lead deregulated CCND1 turnover and increased protein levels [114]. More importantly, these mutated CCND1-expressing MCL cells were more resistant to ibrutinib [114]. In another study, it was found that tissue-specific proliferation of ibrutinib resistant MCL cells was driven by the activation CDK4 [102]. Cyclin-dependent kinase 4 specific inhibitor palbociclib prolonged early G1 arrest and sensitized resistant MCL cells to ibrutinib killing, suggesting a strategy to override acquired ibrutinib resistance [102].

Tumor Microenvironment Mediated Resistance

Tumor microenvironment (TME) is known as a critical regulator of immune escape, progression, metastasis of cancer, and tumor resistance to various therapies [115]. The complex cell-signaling relationship between MCL cells, TME and ibrutinib resistance, is currently under investigation but it is less studied in other types of B-cell lymphoma [116]. Zhao et al. recently revealed how the TME contributes to the development of acquired ibrutinib resistance in MCL [117]. They found that co-culture of MCL cells with lymph node stromal cells or bone marrow stromal cells significantly increased pBTK, pERK and pAKT in MCL cell lines and primary MCL cells. Ibrutinib resistant MCL cells had a marked increase in adhesion to stromal cells and enhanced clonogenic growth in the presence of ibrutinib. Combining kinomics, longitudinal drug screening with *ex vivo*, *in vivo* TME, and patient-derived xenograft models, Zhao et al. identified a major kinase network involving PI3K-AKT-mTOR/integrin b1-integrin-linked kinase (ILK) as a central hub for TME–lymphoma interactions mediating ibrutinib resistance [117]. When PI3K inhibitor dactolisib or mTOR inhibitor AZD8055 was combined with ibrutinib, cell

survival, b1 expression, cell adhesion and clonogenic growth were substantially inhibited in all ibrutinib resistant MCL lines and in patient samples of acquired ibrutinib resistant MCL. AZD8055 in combination with ibrutinib induced remarkable inhibition of ibrutinib resistant MCL, reduction of pAKT, pS6K1, p4EBP and b1 expression levels and reduced cell adhesion to stromal cells in these xenograft tumor cells [117]. Their finding suggested that combined disruption of BCR signaling and central pathways resulting from kinome reprogramming is critical for overcoming ibrutinib resistance in MCL.

Novel Approaches to Overcome BTK Inhibitors Resistance

Overcome Ibrutinib C481 Mutation Resistance with Non-Covalent Inhibitors of BTK

Based on the improved understanding of ibrutinib resistance, several strategies have been utilized to overcome BTK inhibitor especially ibrutinib resistance. The second-generation BTK inhibitors such as acalabrutinib are covalent, target-specific and have shown improved clinical responses. However, these covalent inhibitors often lose potency against BTK C481 mutations. One strategy to treat C481-mutant based ibrutinib resistance is to develop small molecule BTK inhibitors that do not depend upon binding to the C481 site for inhibition of BTK. Non-covalent inhibitors GDC-0853, SNS-062 (Vecabrutinib®), and GNE-431 have been evaluated in preclinical and clinical studies with potency against C481 mutant BTK [118–120]. GDC-0853 is a novel non-covalent, reversible, selective, orally bioavailable, and ATP-competitive inhibitor of BTK that effectively blocks BCR signaling in the treatment of B-cell malignancies including CLL [118]. *In vitro* studies showed that GDC-0853 reduced the activations of BTK, PLCγ2, AKT, and ERK. Unlike ibrutinib, GDC-0853 inhibited signaling of both WT and C481S mutated BTK in transfected HEK293T cell lines and preserved NK cell mediated ADCC with clinical anti-CD20 antibodies [121]. In a phase 1 trial, unlike ibrutinib, GDC-0853 was able to inhibit BTK C481S mutants in CLL and NHL patients, demonstrated by reductions in C-C motif chemokine Ligand 3 (CCL3), which is one of the biomarkers to assess systemic inhibition of BTK in B-cell lymphoma [118]. SNS-062 is another non-covalent inhibitor of BTK unaffected by the C481S mutation. Fabian et al. found that SNS-062 and ibrutinib demonstrated comparable activity in inhibiting BTK, decreasing the expression of B cell activation markers, and reducing CLL cell viability in in BTK wild type CLL cells [119]. More importantly, SNS-062 was not affected by BTK C481S mutation but the activities of ibrutinib and acalabrutinib were hindered. SNS-062 also showed 6 times more potent than ibrutinib and more than 640 times more potent than acalabrutinib against C481S BTK [119]. Finally, the investigators found that SNS-062 diminished stromal cell protection in CLL cells, suggesting the drug can reduce the protection from the TME to CLL [119]. Their findings support clinical investigation of SNS-062 in patients with acquired

resistance to covalent BTK inhibitors. A phase 1b study is currently recruiting B lymphoid cancers (NCT03037645). Non-covalent inhibitor GNE-431 also showed excellent potency against the C481S, C481R, T474I, and T474 M mutants with nanomolar potency in-vitro, in cells, and in whole blood [120]. These non-covalent inhibitors may provide a potentially effective treatment option to ibrutinib resistant patients, but further studies are needed to demonstrate their clinical response.

Utilize Alternate Kinase Inhibitors to Overcome Ibrutinib Resistance

Researchers have proposed and investigated several other salvage approaches to overcome BTK inhibitors' resistance, which include using alternate kinase inhibitors. Based on an *in vitro* CLL proliferation model, Cheng et al. demonstrated that the ibrutinib resistant CLL cells were sensitive to the inhibition of dasatinib (blocking multiple tyrosine kinases including LYN and BTK), and SYK inhibitors (Cerdulatinib (PRT062070) and PRT060318) and idelalisib (PI3Kδ inhibitor) [74]. In a recent study, the finding of the increased incidence of PI3Kα in DLBCL sheds light on the molecular basis of the intrinsic resistance of DLBCL to PI3Kδ inhibition observed in the clinic [122]. Copanlisib is a predominant PI3Kα/δ dual inhibitor [122]. It led to significantly reduced cell viability in-vitro in both ibrutinib-sensitive and -resistant ABC-DLBCL cell lines by suppression of p-AKT and blocking nuclear factor-κB activation driven by CD79mut, CARD11mut, TNFAIP3mut, or MYD88mut [122]. Copanlisib also demonstrated potent *in vivo* anti-tumor effect in ibrutinib-resistant CARD11mutand/or MYD88mut DLBCL mice models [122]. Dasatinib was identified as the most DLBCL-specific agent in a drug screen composed of 2160 FDA-approved drugs and other targeted drugs. Notably, dasatinib overcomes Ibrutinib-resistance caused by BTK C481S mutation through FYN suppression [123]. These results are consistent with the previous report.

Combine BTK Inhibitors with Other Oncogenic Inhibitors

The second strategy is to utilize drug combination that targets multiple components or multiple oncogenic pathways (Table 6.4). Most of the reported combination studies are in preclinical evaluation with promising results. For example, addition of ONO/GS-4059 + entospletinib (SYK inhibitor) or idelalisib, had an additive effect on induction of apoptosis in primary CLL cells. The addition of ABT-199 to entospletinib, ONO/GS-4059, or idelalisib showed additive to synergistic effects on induction of apoptosis in primary CLL cells, and increased the maximal level of apoptosis [124]. The safety and tolerability of the combination was evaluated in a Phase 1b clinical trial [125]. ONO/GS-4059 at up to 160 mg in combination with entospletinib up to 400 mg daily was safe and well tolerated [125]. The combination

Table 6.4 Summary of combination therapies with BTK inhibitors

BTK inhibitor	Combination reagents	Role of the combination reagent	Diseases	Study stage	Year	Ref.
ONO/ GS-4059	Idelalisib or Entospletinib or ABT-199	PI3Kδ inhibitor, SYK inhibitor, Bcl-2 inhibitor	CLL	Preclinical	2015	[124]
Acalabrutinib	ACP-319	PI3Kδ inhibition	CLL	Preclinical	2017	[126]
ONO/ GS-4059	Idelalisib	PI3Kδ inhibition	ABC-DLBCL	Preclinical	2017	[129]
ONO/ GS-4059	Entospletinib	SYK inhibitor	CLL	Preclinical	2015	[124]
ONO/ GS-4059	Entospletinib	SYK inhibitor	CLL; Non-GCB DLBCL; FL; WM; MCL; SLL; MZL	Phase 1b clinical	2017	[125]
Ibrutinib	AZD2014	mTOR1/2 inhibitor	ABC-DLBCL	Preclinical	2014	[128]
PLS-123	Everolimus	mTOR inhibitor	MCL	Preclinical	2018	[167]
Ibrutinib	Ulixertinib	ERK1/2 inhibitor	MYD88 mutated WM ABC DLBCL	Preclinical	2016	[132]
Ibrutinib	Pimasertib	MEK1/2 inhibitor	DLBCL MCL	Preclinical	2016	[168]
PLS-123	N/A	BTK/ PLC-γ2 dual inhibitor	B cell lymphoma	Preclinical	2015	[133]
QLX138	N/A	BTK/MNK dual inhibitor	B cell lymphoma	Preclinical	2016	[134]
MDVN1003	N/A	BTK/PI3Kδ dual inhibitor	B cell lymphoma	Preclinical	2017	[135]
Pyrimidine derivatives compounds	N/A	BTK/JAK dual inhibitor	B cell lymphoma	Preclinical	2018	[136]
Ibrutinib	Venetoclax (ABT-199)	Bcl-2 inhibitor	MCL, ABC-DLBCL, FL	Preclinical	2015, 2017	[138, 140]
Ibrutinib	Venetoclax	Bcl-2 inhibitor	MCL	Phase 2 clinical	2018	[139]
Acalabrutinib	Venetoclax	Bcl-2 inhibitor	CLL	Preclinical	2018	[169]
ONO/ GS-4059	Venetoclax (ABT-199)	Bcl-2 inhibitor	CLL	Preclinical	2015	[124]
Ibrutinib	Rituximab	Anti-CD20 antibody	High risk CLL	Phase 2 clinical	2014	[141]
Ibrutinib	Rituximab	Anti-CD20 antibody	R/R MCL	Phase 2 clinical	2016	[142]
Ibrutinib	Rituximab	Anti-CD20 antibody	Naïve FL	Phase 2 clinical	2016	[143]

(continued)

Table 6.4 (continued)

BTK inhibitor	Combination reagents	Role of the combination reagent	Diseases	Study stage	Year	Ref.
Acalabrutinib	Rituximab	Anti-CD20 antibody	R/R FL	Phase 1/2 clinical	2015	[144]
Ibrutinib	R-CHOP	Rituximab + cyclophosphamide, doxorubicin, vincristine, and prednisone	Naïve B-NHL	Phase 1b clinical	2014	[145]
Ibrutinib	Bendamustine + rituximab	a nitrogen mustard drug + anti-CD20 antibody	Naïve or R/R NHL	Phase 1/1b clinical	2015	[146]
Ibrutinib	Bendamustine + rituximab	a nitrogen mustard drug + anti-CD20 antibody	R/R CLL	Phase 3 clinical	2016	[147]
ONO/ GS-4059	Obinutuzumab	Anti-CD20 antibody	ABC-DLBCL	Preclinical	2017	[149]
Ibrutinib	CTL019	Anti-CD19 CAR T	MCL	Preclinical	2016	[152]
Ibrutinib	CD19/ CD3-scFv-Fc	Bispecific antibody	CLL	Preclinical	2018	[155]
Ibrutinib	ACY1215	HDAC6 inhibitor	MCL	Preclinical	2012	[156]
Ibrutinib	Panobinostat	HDAC inhibitor	ABC-DLBCL	Preclinical	2017	[157]
Ibrutinib	Palbociclib	CDK4 inhibitor	MCL	Preclinical	2014	[102]
Ibrutinib	PF-00477736	Chk1 inhibitor	MCL	Preclinical	2018	[158]
Ibrutinib	Bortezomib	Proteasome inhibitor	Bortezomib-sensitive or – resistant DLBCL MCL	Preclinical	2013	[160]
Ibrutinib	Carfilzomib	Proteasome inhibitor	MCL	Preclinical	2014	[161]
Ibrutinib	VS-6063	Focal adhesion kinase inhibitor	MCL	Preclinical	2018	[164]

of acalabrutinib with the PI3K-delta inhibitor ACP-319 significantly reduced CLL tumor proliferation and tumor burden in the peripheral blood and spleen with reduced NF-κB signaling and enhanced expression of BCL-xL and MCL-1 than single-agent therapy [126]. Vistusertib (AZD2014) is an ATP-competitive mTOR inhibitor, which can block the activity of both the mTORC1 (rapamycin-sensitive) and mTORC2 (rapamycin- insensitive) complexes and is highly selective against PI3K superfamily kinases [127]. The combination of ibrutinib and AZD2014 was shown to strongly induce apoptosis in ABC-DLBCL by regulation of 4EBP1 and cap-dependent translation (CDT) as well as Janus kinase (JAK) 3 / signal transducer and activator of transcription (STAT) 3, NF-κB, STAT3, and mTOR pathways [128]. The combination treatment of another mTOR inhibitor everolimus and the second-generation BTK inhibitor PLS-123 significantly induced cell apoptosis, blocked cell cycle

progression and synergistically downregulates activation of BCR, AKT/mTOR, JAK2/STAT3 and MAPK signaling in MCL cell lines *in vitro* and effectively inhibited MCL tumor growth *in vivo* in severe combined immunodeficiency SCID mice. These combinations promise to be attractive therapeutic approaches in patients. However, further investigations are needed on ibrutinib resistant tumor cells.

The combinations of BTK inhibitors with PI3K inhibitors, MEK1/2 inhibitor, ERK1/2 inhibitor, or PIM1 inhibitor have been investigated in BTK inhibitor resistant B lymphoma cells with promising results. The combination of ONO/GS-4059 and idelalisib was investigated in ibrutinib resistant DLBCL cells. The acquired ibrutinib resistant DLBCL cells, which had loss of A20 and BTK C481F mutation, were insensitive to both idelalisib and ONO/GS-4059 as single agents but were significantly inhibited with the combination of both agents [129]. The decrease in p-IκBα by the combination suggested that inhibition of MAPK and NF-κB pathways might be the mechanism that leads to the decreased cell viability seen with combination treatment in this resistant cell line [129]. A clinical trial is currently underway to evaluate the combination of idelalisib and ONO/GS-4059 (NCT02457598) [129]. Activation of the ERK pathway is a very frequent observation in mature B-cell lymphoid tumors [130] and is implicated in the resistance to ibrutinib [85]. Pimasertib is a highly selective and ATP non-competitive MEK1/2 inhibitor and currently being tested in clinical phase 1/2 trials [131]. The combination of pimasertib and ibrutinib induced apoptosis with an increase of cleaved poly ADP ribose polymerase (PARP) and is active in ABC-DLBCL xenografts. MYD88 mutated WM and ABC DLBCL cells with BTK C481S mutation showed persistent activation of PLCγ2-ERK1/2 signaling [132]. Ulixertinib (BVD-523, VRT752271) is a highly selective ERK1/2 inhibitor that is currently under clinical investigation. The combination of ulixertinib with ibrutinib produced higher levels of tumor cell killing than either agent alone, and significantly reduced interleukin 6 (IL-6) and interleukin 10 (IL-10) secretions which are associated with prosurvival signaling pathways [132]. The findings provide rationale for the investigation of ERK1/2 inhibitors in ibrutinib resistant MYD88 driven WM and ABC-DLBCL disease mediated by BTK mutations. As described in the early section, PIM1 inhibitor AZD-1208 may be a good choice to combine with ibrutinib to suppress ibrutinib resistance in ABC-DLBCL cells with mutations in PIM1 through NF-kB pathway [80].

Dual inhibitors have emerged as an attractive strategy by inhibiting the catalytic activity of BTK and other kinases such as PLC- γ2 kinase, Mitogen-Activated Protein Kinase interacting kinase (MNK kinase), PI3Kδ kinase and JAK3. PLS-123 displayed impressive potency against BTK Tyr551 and PLC-γ2 Tyr1217 phosphorylation [133]. It significantly reduced the phosphorylation of the BCR downstream signal pathways such as AKT/mTOR and MAPK [133]. Gene expression profile analysis further suggested that the different selectivity profile of PLS-123 led to significant downregulation of oncogenic gene tyrosine-protein phosphatase non-receptor type 11 (PTPN11) expression [133]. In addition, PLS-123 mediated TME to attenuate lymphoma cell adhesion and migration [133]. MNK kinase is one of the key downstream regulators in the RAF/MEK/ERK signaling pathway and controls protein synthesis via regulating the activity of

eukaryotic translation initiation factor 4E (eIF4E) [134]. Through a structure-based drug design approach, a potent BTK/MNK dual kinase inhibitor (QLX138) was discovered with covalent binding to BTK and non-covalent binding to MNK ability [134]. QLX138 enhanced the antiproliferative and apoptosis efficacies in-vitro against a variety of B-cell lymphoma cells, which respond moderately to BTK inhibitor in-vitro [134]. MDVN1003 is a first-in-class dual inhibitor of BTK and PI3Kδ kinases [135]. MDVN1003 induced cell death of a B-cell lymphoma cell line and it reduced tumor growth in a B-cell lymphoma xenograft model more effectively than either ibrutinib or idelalisib [135]. JAK3 plays an important role in survival of B cells by regulating the activity of STAT3 (the antiapoptotic transcription factors signal transducer and activator of transcription 3), STAT5 and the antiapoptotic PI3K-AKT pathway and its downstream targets [136].

Using diphenylpyrimidine derivatives (DPPYs) as scaffolds, Ge et al. synthesized a new class of DPPY derivatives bearing a variety of the flexible C-2 aniline side chains [136]. Some of the pyrimidine derivatives showed high inhibitory potency of BTK and JAK3. Flow cytometric analysis, and a xenograft model for *in vivo* evaluation indicated the efficacy and low toxicity of 2 derivatives in the treatment of B-cell lymphoma [136]. These primary studies indicate that simultaneous inhibition of BTK and other kinases' activity might be a new therapeutic strategy for B-cell lymphoma and may overcome BTK inhibitor resistance.

Combine BTK Inhibitors With BCL-2 Inhibitors

The BH3-only mimetic Venetoclax® (ABT-199) selectively inactivates BCL-2 and is a promising drug for treatment of BCL2-dependent cancers [137]. The preclinical study shows that the combination of ibrutinib and Venetoclax® displayed strongly synergistic effects in MCL cell lines and primary cells from recurrent MCL patients, mechanistically, by perturbation of p-BTK and p-AKT mediated survival signals and of BCL2 family proteins [138]. A single-group, phase 2 study of the combination was conducted in R/R MCL patients compared with historical controls [139]. The CR rate at week 16 was 42%, which was higher than the historical result of 9% at this time point with ibrutinib monotherapy ($p < .001$) [139]. Seventy-eight percent of the patients with a response were estimated to have an ongoing response at 15 months [139]. The estimated rates of PFS were 75% at 12 months and 57% at 18 months [139]. Minimal residual disease (MRD) clearance was confirmed by flow cytometry and allele-specific oligonucleotide polymerase chain reaction (ASO-PCR), 67% and 38% respectively [139]. The side effects were generally low grade [139]. Additionally, the combination of ibrutinib and venetoclax also synergistically suppressed cell growth in ibrutinib-resistant ABC-DLBCL and FL cells that overexpressed BCL-2 in a preclinical study [140].

Combine BTK Inhibitors With Immunotherapies (Anti-CD20 Antibodies, Anti-CD19 CAR T Cells, CD19/CD3 Bispecific Antibody)

The activity and safety of adding ibrutinib or acalabrutinib to rituximab based therapies (alone, with rituximab-cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), or with bendamustine) have been evaluated in early phase trials in patients with high-risk CLL, R/R MCL, naïve FL, R/R FL or R/R B-NHL [141–147] (Table 6.4). The encouraging results merit further investigation of the combinations in phase 3 trials in BTK resistant patients. ABC-DLBCL often has low response rate to BTK inhibitors ibrutinib or ONO/GS-4059 [66, 148]. The combination of ibrutinib combined with rituximab did not result in improved efficacy compared with respective monotherapy [149]. To overcome BTK inhibitor's resistance in ABC-DLBCL, the combination of ONO/GS-4059 with obinutuzumab (glycoengineered Type II CD20 antibody) or rituximab was evaluated for ABC-DLBCL in a preclinical study [149]. The combination was significantly better than the respective monotherapy with tumor growth inhibition of 90% for the obinutuzumab combination and 86% for the rituximab combination [149]. This result indicates that the combination of the second-generation inhibitor with rituximab and particularly obinutuzumab may be an effective treatment for resistant B-cell lymphoma.

Besides the combination with the antibody-based therapy, chimeric antigen receptor engineered T cells may be another good choice for the combination. Infusion of anti-CD19 CAR^+ autologous T-cells (CART19, CTL019) into patients with B-cell lymphomas such as CLL and DLBCL leads to dramatic clinical responses [150, 151]. Taking the advantage of the vastly different mechanisms of action of CTL019 and ibrutinib, in a preclinical study, the combination of ibrutinib with CTL019 augmented the antitumor effect compared to single agent and leaded to prolonged remissions in MCL xenografts [152]. Strikingly, a recent study found that anti-CD19 modified CAR-T cells induced 71% ORR in CLL patients after ibrutinib failure [153]. Since ibrutinib has been shown to improve T-cell function in CLL [154], a novel CD19/CD3-scFv-Fc bispecific antibody was developed to work as an adjunct with ibrutinib to target ibrutinib-resistant disease [155]. CD19/CD3-scFv-Fc was shown to have the ability to eliminate ibrutinib resistant CLL cells *in vitro* and *in vivo* [155].

Combine BTK Inhibitors with Inhibitors Targeting Other Cellular Processes

The inhibitors involved in histone deacetylation, cell cycle regulation, protein degradation, cell stress and TME also show promising anti-tumor effects when combined with ibrutinib for resistant lymphoma diseases (Table 6.4). Treatment with ibrutinib plus ACY1215, a selective histone deacetylase 6 (HDAC6) inhibitor,

resulted in a three-fold increase in apoptosis induction in MCL tumor cell lines, pointing to a synergistic effect of BTK and HDAC6 inhibition in MCL [156]. Panobinostat, a non-selective histone deacetylase inhibitor, inhibited MyD88-driven NF-κB activation, and enhanced ibrutinib efficacy in MyD88 mutant ABC-DLBCL [157]. The unrestrained proliferation of relapsed lymphoma cells after ibrutinib treatment suggests that simultaneous targeting of cell cycle regulators may override some mechanisms of resistance [102, 158]. Cyclin-dependent kinase 4 (CDK4) specific inhibitor palbociclib has been shown to prolong early G1 arrest and sensitized resistant MCL cells to ibrutinib killing [102]. Checkpoint kinase 1 (Chk1) inhibitor PF-00477736 also showed a synergistic anti-tumor effect with ibrutinib in-vitro in MCL cell lines that are sensitive or resistant to ibrutinib [158]. The ubiquitin-proteasome system degrades a variety of intracellular proteins, and plays an important role in maintenance of the balance between pro and anti-apoptotic proteins, and signal transduction regulation [159]. Synergistic interactions between ibrutinib and proteasome inhibitor bortezomib (the first approved therapeutic proteasome inhibitor) or carfilzomib (a selective proteasome inhibitor of the 20S proteasome) have been observed in a variety of DLBCL and MCL cells [160, 161]. However, further evaluations are needed for ibrutinib resistant cells. Additionally, heat shock proteins as molecular chaperones are exploited by tumor cells to buffer malignancy-associated cellular stress and facilitate the maturation, activation, and stabilization of many oncoproteins [162]. It was reported that heat shock protein 90 (HSP90) inhibitor AUY922 overcame nonclassical NF-κB signaling and BTK C481S in MCL. Focal adhesion kinase (FAK) functions downstream of integrins and mediate signals from the extracellular matrix to tumor cells to enhance tumor cell proliferation, survival and migration in response to stromal interaction [163]. A recent study shows the role of FAK in bone marrow stroma-mediated enhancement of MCL proliferation and survival and the combined treatment of ibrutinib and defactinib (VS-6063), a FAK inhibitor in ibrutinib resistant MCL cells, was highly synergistic, and overcame the resistance by abrogation of the NF-κB signaling pathway [164].

Conclusion

The BCR signaling pathway plays a crucial role in the development of B-cell lymphomas, providing a rationale to therapeutically target this pathway (Fig. 6.1). Several inhibitors targeting the members of this pathway have been developed and evaluated. Among these agents, BTK inhibitor ibrutinib with impressive clinical response and tolerability was the first to receive FDA approval for the treatment of patients with relapsed MCL and CLL. Ibrutinib has also shown promising activity in WM, FL and ABC-type DLBCL (Table 6.1). Ibrutinib has less kinase targeting specificity. It binds to BTK but also several other kinases. To improve the therapeutic effect, second-generation BTK inhibitors with more selective kinase activity profiles are developed and evaluated in early clinical trials (Table 6.2). With the promising clinical response and safer profile, acalabrutinib was granted Breakthrough

Therapy Designation by the FDA for patients with MCL who have received at least one prior therapy. Since many B-cell lymphoma depends on BCR signaling, the potential utility of BTK inhibitors will be tremendous. However, some patients show PRs or no response to BTK inhibitors at initial treatment and others developed disease progression and drug resistance during ibrutinib treatment. A better understanding of the resistant mechanism will allow accurate molecular classification of patients and assist in designing or choosing targeted therapies unique to that resistant mechanism. Advances in molecular genomics such as RNA-seq and whole genome sequencing have been instrumental in uncovering the ibrutinib resistant mechanisms. These mechanisms include mutational resistance in BTK and in other proteins, chromosomal abnormality, activation of prosurvival pathways, BCL-2 family members mediated resistance, and tumor microenvironment mediated resistance and potential other mechanims that are beyond our discussion in this review (Fig. 6.2 and Table 6.3). The resistant mechanisms of the second-generation BTK inhibitors are less studied and further investigation is needed to compare with ibrutinib resistance. Non-covalent inhibitors of BTK have been developed to bypass C481 mutation in ibrutinib. Extensive preclinical studies of utilization of the inhibitors of alternate kinases other than BTK in the BCR pathway, and the combination therapies of BTK inhibitors with other oncogenic inhibitors, or with inhibitors involved in histone deacetylation, cell cycle regulation, protein degradation, cell stress and TME are encouraging to move on to clinical trials to overcome ibrutinib resistance. Furthermore, the combinations of BTK inhibitors with the novel agents of immunotherapies such as anti-CD20 antibodies, anti-CD19 CAR T cells, CD19/CD3 bispecific antibodies hold great promise for eradicating resistance and achieving better clinical outcomes in patients with B-cell lymphoma.

Acknowledgements This work was supported by the grants from Pediatric Cancer Research Foundation (MSC) and New York Medical College Translational Science Institute, Children Health Translational Research Grant (YC). YC reviewed the literatures, developed the design of the paper and wrote the manuscript. MSC and AB critically revised the manuscript and have approved the final version for publication. The authors would like to thank Erin Morris, RN, and Virginia Davenport, RN for their excellent assistance with the preparation of this manuscript.

Disclosure of Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 7
Resistance to Checkpoint Blockade Inhibitors and Immunomodulatory Drugs

Anthony N. Audino and Mitchell S. Cairo

Abstract Cancer therapy has evolved from surgery and radiation to multi-agent chemotherapy, and although we have seen decreased mortality and increased cure rates, most of this therapy has continued to focus on the tumor itself, and not on the tumor microenvironment. Various cells within the tumor microenvironment have been implicated in leading to resistance to immune therapy. Through a complex system of steps, T-cells become activated after presentation of a specific antigen. Because continuous T-cell activation can lead to lymphoproliferation and unwanted autoimmunity, the human T-cell immune system has evolved into a process of checks-and-balances, referred to as immune checkpoints, that allows for co-inhibitory receptors to inhibit T-cell activation. Through the use of check point inhibitors, we have seen patients with cancers refractory to multiple treatments have durable responses, and in some, long term remissions. Some of the most studied inhibitors include Programmed Cell Death Protein 1 (PD-1) and Cytotoxic T Lymphocyte-Associated Antigen 4 (CTLA-4), although more have been identified. As we continue to explore possible treatment options for cancer, we must also be diligent in preemptively investigating how and why some patients will become resistant to these treatments, and what, if any, actions can be taken to circumvent this resistance.

Keywords Checkpoint blockade inhibitors, PD-1, CTLA-4 · Lymphoma · Resistance

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A. C. Xavier, M. S. Cairo (eds.), *Resistance to Targeted Therapies in Lymphomas*, Resistance to Targeted Anti-Cancer Therapeutics 21,
https://doi.org/10.1007/978-3-030-24424-8_7

Abbreviations

APC	Antigen Presenting Cells
ASCT	Autologous Stem Cell Transplant
BV	Brentuximab Vedotin
CAF	Cancer Associated Fibroblasts
COG	Children's Oncology Group
CTLA-4	Cytotoxic T-Lymphocyte Associated Antigen-4
FDA	Food and Drug Administration
HL	Hodgkin Lymphoma
HSC	Hematopoietic Stem Cells
ICAM	Intracellular Activation Motifs
ICOS+	Inducible Costimulatory
IDO	Indoleamine 2, 3-Droxygenase
ITAM	Immunoreceptor Tyrosine Based Activation Motifs
LAG-3	Lymphocyte Activation Gene 3
MDSC	Myeloid Derived Suppressor Cells
MHC	Major Histocompatibility Complex
MHC I	Major Histocompatibility Complex Class I
MHC II	Major Histocompatibility Complex Class II
NSCLC	Non-small Cell Lung Cancer
ORR	Objective Response Rate
OS	Overall Survival
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Cell Death Ligand 1
PD-L2	Programmed Cell Death Ligand 2
PFS	Progressive Free Survival
R/R	Relapsed/Refractory
TAM	Tumor Associated Macrophages
TCR	T-Cell Receptors
TIM-3	T-cell Immunoglobulin Mucin 3
Treg	Regulatory T-cells

Introduction

Cancer therapy has evolved over the last several decades from surgery and radiation to multi-agent chemotherapy, and although we have seen decreased mortality and increased cure rates, most of this therapy has continued to focus on the tumor itself, and not on the tumor microenvironment. As cancer therapy continues to advance, the cancer scientific community has begun to recognize that this microenvironment plays an active role in tumor development and progression, via recruitment of other cell through the release of signals and chemokines [1]. With this knowledge, many have speculated that the tumor microenvironment may play a role in resistance to

certain therapies, such as immune checkpoint blockade, and a reason why some patients may not have a continued durable response. Due to this, new therapies that focus on targeting the tumor microenvironment, in addition to the tumor itself, may be lead to better outcomes.

The tumor microenvironment is a dynamic milieu of immune cells that interact with each other and with the tumor, providing an atmosphere of chronic inflammation where the tumor can continue to grow and thrive [2]. Various cells within the tumor microenvironment have been implicated in leading to resistance to immune therapy, including regulatory T-cells (Treg), myeloid derived suppressor cells (MDSC), tumor associated macrophages (TAM) and cancer associated fibroblasts (CAF) [3, 4]. Various tumors, including Hodgkin lymphoma (HL), distinguished by the presence of Reed-Sternberg cells, can produce chemo-attractants that not only select for the infiltration of Treg cells instead of T-helper or T-effector cells, but also reprogram tumor infiltrating T-cells to less active Treg cells [5]. The Treg cells have the ability to suppress effector T-cells by secreting their own inhibitory cytokines, such as IL-10, IL-35, and transforming growth factor β [6]. The presence of MDSCs within the tumor microenvironment has not only been implicated in decreasing efficacy of checkpoint inhibitors, but also in leading to tumor cell invasion, metastases, and promoting angiogenesis [6]. MDSC also express Indoleamine 2,3-dioxygenase (IDO), which promotes naïve T-cells into Treg cells [3]. TAMs have been shown to directly suppress T-cell function response via their expression of suppressor ligands, such as programmed death-ligand 1 (PD-L1) [7]. They are also known to secrete certain chemokines that suppress T-effector function directly and indirectly with the recruitment of Treg cells [7]. Finally, CAF can affect the success of therapy by directly excluding T-cells into the microenvironment via production of chemokines, or by impeding the entry of T effector cells into the microenvironment by producing an extracellular matrix that the cells cannot physically enter [1, 3].

This review will focus on the components of immune cell regulation within the tumor microenvironment and potential mechanisms of resistance to immune checkpoint blockade.

T-Cell Lymphocyte Physiology

T-lymphocytes, initially derived from pluripotent hematopoietic stem cells, transverse the thymus gland in order to start the T-cell maturation process. This process consists of the addition of cell surface markers and rearrangement of a series of germline genes which encode the T-cell receptor (TCR) [6, 8]. Initially, T-lymphocytes will express both CD4 and CD8, but ultimately the majority of T-cells will differentiate into either CD4 or CD8 T-cells as they migrate through the thymus prior to entering the peripheral blood circulation [9]. CD4 cells, also known as T-helper cells, typically interact with major histocompatibility complex class II (MHC II) molecules while CD8 lymphocytes, known as cytotoxic T-cells, interact

with major histocompatibility complex class I (MHC I) molecules on the surface of antigen presenting cells (APC) [10, 11].

T-Cell Receptor

The TCR is a molecule that resides on the surface of the T-lymphocyte. The TCR recognizes antigens or peptides that are presented by an APC via the major histocompatibility complex (MHC). The majority of TCRs consist as a complex of a heterodimer of α and β chains. The complex itself has a large extracellular domain, but only a short intracellular domain that is not able to produce an adequate signal transduction. It is therefore noncovalently bound to another complex known as CD3 on the cell surface. CD3 is a co-receptor made up of four units that contain intracellular activation motifs (ICAM) [8]. Another unit considered to be part of the CD3 complex, Zeta (ζ), consists of a short extracellular domain and longer intracellular domain, also containing ICAM. Assembly of all these components, which takes place in the endoplasmic reticulum, is required to produce a stable complex that can be exported to the cell surface. In addition to the TCR, other co-receptors are needed in order to establish proper binding. Helper T-cells will have a CD4 co-receptor as part of the complex, while cytotoxic T-cells will have a CD8 co-receptor [12]. It is these CD4 and CD8 co-receptors which help the TCR recognize peptides that are presented by the APC via the MHC. It is this recognition that begins the process of T-cell activation.

T-Cell Activation

Activation of T-cells is a process that requires multiple steps. The first step of this process requires an interaction between the T-cell receptor and the antigen being presented via the MHC molecule. This process is very specific in regards to T-cells, meaning that MHC I molecules will only be recognized by CD8+ T-cells and MHC II molecules will be recognized by CD4+ T-cells. It is this interaction that causes a downstream affect via several kinases and phosphatases, such as Lck, FYN, CD45 and Zap70, which ultimately leads to the phosphorylation of immunoreceptor tyrosine based activation motifs within the cytoplasm of T-cells [8, 13] (Fig. 7.1). The second step in the process requires stimulation of co-receptors, such as CD28. The ligands of the CD28 co-receptor, B7–1 and B7–2, are expressed on the APC as well as stromal cells [6, 14]. It is the second step in this process that it is needed in order to make the T-cells fully functional and not anergic. The binding of CD28 with B7–1 and B7–2 has been shown to be required to promote T-cell activation, clonal expansion and acquisition of effector functions [15]. Once the T-cell has recognized the specific antigen signal, the last step in activation process takes place. In the last

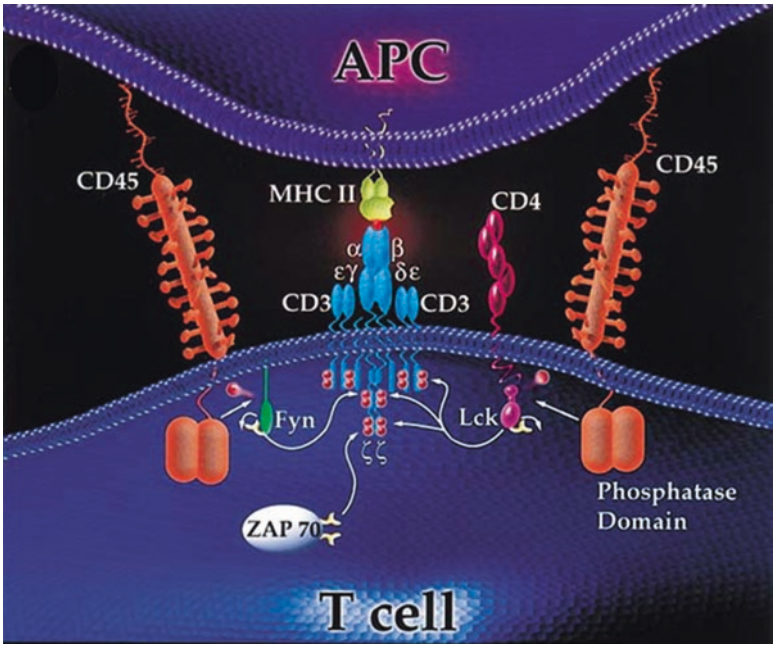

Fig. 7.1 T-cell activation. The role of the TCR/CD3 complex and the CD4 receptor in the initiation of early protein tyrosine phosphorylation. On binding to the peptide/MHC complex, the earliest recognizable event is activation of the Src-kinases, Lck and Fyn. This requires removal of a C-terminal phosphate *(red dot)* by the tyrosine phosphatase, CD45. This allows the kinase to unfold and to phosphorylate ITAM motifs (*blue rectangles* in the intracellular domains of CD3δ, ϵ, γ, and ζ). Tandem ITAM phosphorylations are required for the recruitment of ZAP-70, which attaches by a pair of SH2 domains (yellow half circles) [13]

step, cytokines (IL-2, IL-7, IL-15) produced by the APC induce the T-cell into functional activation.

T-Cell Inhibition

Because continuous T-cell activation can lead to lymphoproliferation and unwanted autoimmunity, which would be detrimental to the host, the human T-cell immune system has evolved into a process of checks-and-balances, referred to as immune checkpoints, that allows for co-inhibitory receptors to inhibit T-cell activation [15]. Some of the most studied inhibitors include programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), although there are more than twenty that have been identified, including lymphocyte activation gene 3 (LAG-3), T-cell immunoglobulin mucin 3 (TIM-3), VISTA, KIR, TIGIT, BTLA and CD39 (Fig. 7.2 and Table 7.1) [15, 16].

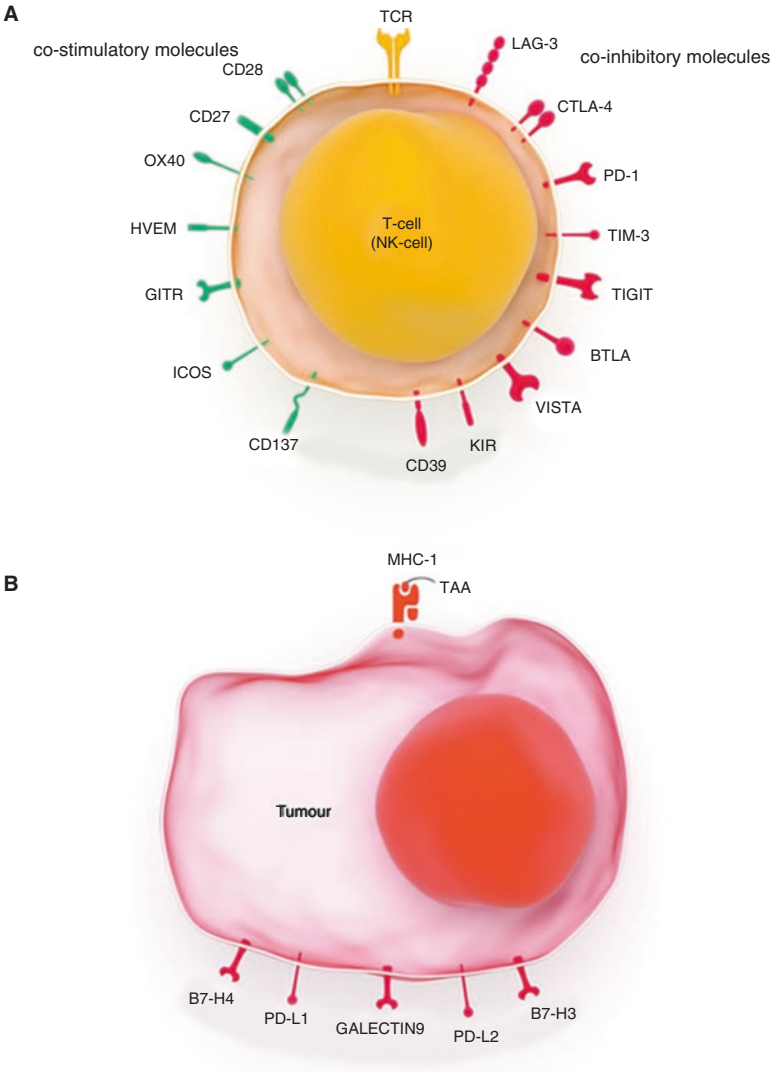

Fig. 7.2 Co-stimulatory and co-inhibitory molecules on T-cells (**a**) and co-inhibitory ligands on tumor cells (**b**). Potential targets of ICI on lymphocytes and tumor cells. (**a**) Activated T-cells (and natural killer cells to a certain extent) express multiple co-stimulatory and co-inhibitory checkpoint molecules on their surface, all of which are potential targets for immunomodulation by checkpoint agonists (co-stimulatory molecules) or inhibitors (co-inhibitory molecules). (**b**) Tumor cells evade the host immune system by expressing ligands for co-inhibitory checkpoint molecules on T cells, hence targeting these ligands leads to inactivation of inhibitory pathways and reactivation of tumor-specific T cells. TCR: T-cell receptor; MHC-I: major histocompatibility complex I; TAA: tumor-associated antigen; LAG-3: lymphocyte-activation gene 3; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; PD-1: programmed cell death protein 1; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; TIGIT: T-cell immunoreceptor with Ig and ITIM domains; BTLA: B- and T-lymphocyte attenuator; VISTA: V-domain immunoglobulin suppressor of T-cell activation; KIR: killer cell immunoglobulin-like receptor; ICOS: inducible T-cell co-stimulator; GITR: glucocorticoid-induced TNFR-related protein; HVEM: Herpesvirus entry mediator, PD-L1: programmed death-ligand 1; PD-L2: programmed death-ligand 2 [16]

Table 7.1 FDA approved checkpoint inhibitors [6, 16, 22–25, 27, 29–32, 34, 37, 40, 43, 45, 46, 64, 65]

	Target	Year approved	Indication	Efficacy	Side effects (Grade 3/4)
Ipilimumab	CTLA-4	2011	Unresectable or metastatic melanoma; expanded to pediatric patients ≥12 years of age in 2017	Meta-analysis of 10 prospective studies of 1861 patients; 3 year survival rate of 22% with some having 10 year durable response	Pruritus, mucositis, immune mediated colitis, diarrhea, hepatotoxicity, endocrinopathies.
Nivolumab	PD-1	2014	Metastatic melanoma, NSCLC, lymphoma, renal cell carcinoma, head and neck squamous cancers, hepatocellular carcinoma, metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer	Melanoma: ORR of 40% and 1 year OS 72.9% Hodgkin lymphoma: ORR ranging from 63–75%	Rash, pruritis, fatigue, diarrhea, nausea, pyrexia, colitis, hepatotoxicity, hypothyroid, arthralgia, dyspnea
Ipilimumab + Nivolumab	CTLA-4/ PD-1	2015	Metastatic melanoma	3 year OS 58%	Diarrhea, hepatotoxicity
Pembrolizumab	PD-1	2017	Metastatic melanoma, PD-L1+ NSCLC, head and neck squamous cancers	Melanoma: 2 year OS 55% Hodgkin Lymphoma: ORR 65%, with 16% CR and 48% PR	Fatigue, dyspnea, nausea, vomiting, diarrhea, and rash
Atezolizumab	PD-L1	2016	Urothelial cell carcinoma, NSCLC	Urothelial cell: ORR 28% NSCLC: Longer PFS	Infusion-related reaction, pneumonia, hypoxia, fatigue, anemia, musculoskeletal pain, hepatotoxicity, dysphagia, and arthralgia, pneumonitis, hepatitis, colitis, and thyroid disease

(continued)

Table 7.1 (continued)

	Target	Year approved	Indication	Efficacy	Side effects (Grade 3/4)
Avelumab	PD-L1	2017	Metastatic Merkle cell carcinoma	Previous treatment: ORR 33% (11.4% CR and 21.6% PR) Treatment naïve: ORR 60% (13.8% CR and 48.3% PR)	Infusion-related reaction, pneumonitis, colitis, hepatitis, adrenal insufficiency, hypo- and hyperthyroidism, diabetes mellitus, and nephritis
Durvalumab	PD-L1	2017	Urothelial cell carcinoma, NSCLC	Pre-treatment NSCLC: ORR 28%, longer PFS	Infection, pneumonitis, hepatitis, colitis, thyroid disease, adrenal insufficiency, and diabetes

CTLA-4, cytotoxic T lymphocyte-associated antigen 4; PD-1, programmed cell death protein 1; PD-L1, programmed cell death protein ligand; ORR, objective response rate; NSCLC, non-small cell lung cancer; PFS, progression-free survival; CR, complete response; PR, partial response

Check Point Inhibitors

CTLA-4

The first report of CTLA-4 being present on activated T-cells was in 1987 by Brunet and colleagues [17]. It was not until 1996 that Brunet et al. demonstrated that CTLA-4 blocking monoclonal antibodies were active in animal tumor models [18]. Initially, due to its similarities to CD28, CTLA-4 was thought to participate in the stimulation process of T-cells, but it was verified a few years later that it instead had the opposite role and functioned as an inhibitor to T-cell activation [15, 19]. Further investigation revealed that CTLA-4 induced T-cells enter an anergic state, as if the second step of T-cell activation had not occurred [19].

CTLA-4 is expressed on activated T-cells and competes with CD28, as they both share the common ligand, B7–1 [5]. It has been shown that the affinity and avidity for B7–1 in CTLA-4 is greater than CD28 [15, 20]. The interaction between B7–1 and CTLA-4 allows for the downregulation of T-cell activation, which in a normal host, results in the prevention of autoimmunity and tissue damage [15, 16, 21]. CTLA-4 induces T-cell inactivation of two distinct mechanisms. First, CTLA-4 competitively binds with B7–1 and B7–2, resulting in decreased ability for CD28 to interact with these ligands, and therefore allowing for stimulation of anti-tumor T-cells (Fig. 7.3a). Secondly, CTLA-4 has been shown to inhibit various intracellular

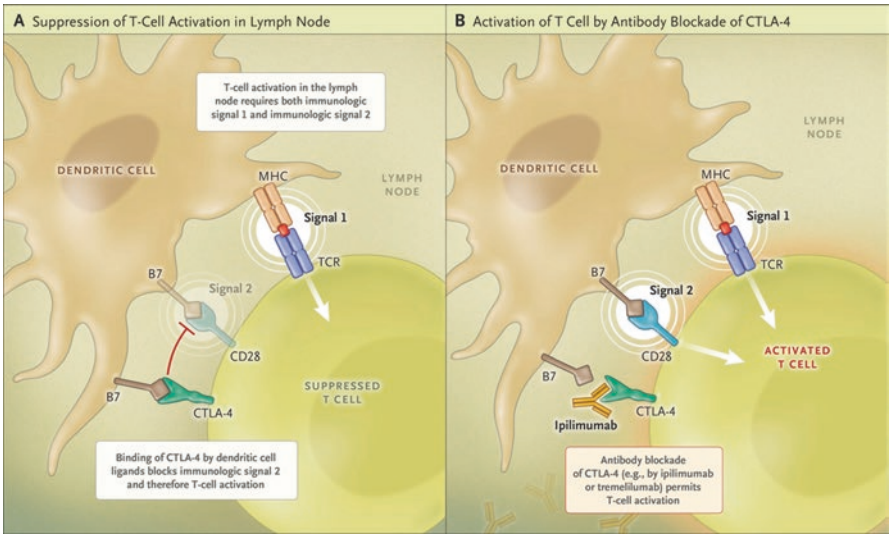

Fig. 7.3 Mechanism of T-cell activation through TCR and inhibition through CTLA-4 (**a**) and blockade by antibodies to CTLA-4 (**b**). Two immunologic signals are required for T-cell activation in the lymph node: stimulation of the T-cell receptor (TCR) by the MHC (immunologic signal 1), and stimulation of CD28 by the B7 costimulatory molecules (immunologic signal 2). However, binding of the B7 costimulatory molecules to CTLA-4 blocks immunologic signal 2, and therefore blocks T-cell activation. Antibody blockade of CTLA-4, for example, by ipilimumab, derepresses signaling by CD28, permitting T-cell activation [63]

signaling pathways, including NF-kB, AP1, MAPK, ERK and c-Jun NH2 terminal kinase signaling, leading to compromised Interleukin 2 (IL-2) production [15, 20].

From a clinical standpoint, CTLA-4, sometimes referred to as "the godfather of checkpoint inhibitors", was the first checkpoint inhibitor ulitized [21]. The first studies revolved around patients with melanoma. One of the first agents, ipilimumab, administered alone or combined with other agents, such as a glycoprotein 100 peptide vaccine or dacarbazine, showed an objective response in patients with metastatic melanoma, leading to significantly longer overall survival (OS). Unfortunately, Grade 3 and Grade 4 adverse events were seen in as many as 15–56% of patients [22–25]. Their initial responses were encouraging, and as these patients were followed years out from their treatment, a durable response was noted in in approximately 20% of patients treated with this drug *vs.* placebo [6, 23].

As with melanoma, the first checkpoint inhibitor that was used in HL was also ipilimumab. This was initially done as a phase 1 study in fourteen patients who had relapsed or refractory (R/R) disease and had failed an allogeneic stem cell transplant. With a single dose, 2 of the 14 patients achieved complete remission (CR) [5, 16, 26]. This led to further research in larger studies. Further Phase 1/2 studies, combining ipilimumab with Brentuximab vedotin (BV) in eighteen R/R HL patients, showed an objective response rate (ORR) of 72%, with 50% of these patients being

in CR [16, 27]. Finally, in 2011, 15 years after the discovery that CTLA-4 blocking antibodies were successful in animal tumor models, the US Food and Drug Administration (FDA) approved ipilimumab as the first inhibitory checkpoint inhibitor in the treatment of Stage IV melanoma (Fig. 7.3b).

PD-1

PD-1 was identified shortly after CTLA-4 in 1992, and demonstrated inhibition to T-cells during long term antigen exposure or during times of inflammatory response or infection [15, 21, 24]. PD-1 has two main ligands that bind with it, PD-L1 and programmed death ligand 2 (PD-L2) (Fig. 7.4a). PD-L1 has been shown to be expressed in various cells, including hematopoietic cells, peripheral tissues and malignant cells, while PD-L2 seems to be exclusively associated with hematopoietic cells, including dendritic cells, macrophages and mast cells [15, 16, 21]. While CTLA-4 is induced early on in the activation process, PD-1 has been shown to be induced at the later effector phase, therefore protecting cells in the periphery. PD-1 accomplishes this by generating a signal to prevent phosphorylation of intracellular signals, reducing the activation of the T-cell [16, 28]. This is a common mechanism used by tumor cells to evade the immune system [6, 15].

Clinically, there are several PD-1 blocking antibodies under investigation for various tumor types (Fig. 7.4b). In 2016, the FDA approved pembrolizumab (an anti-PD-1 checkpoint inhibitor) for melanoma, after two pivotal studies, KEYNOTE-001 and KEYNOTE-002 [29]. The first study, KEYNOTE-001, a phase

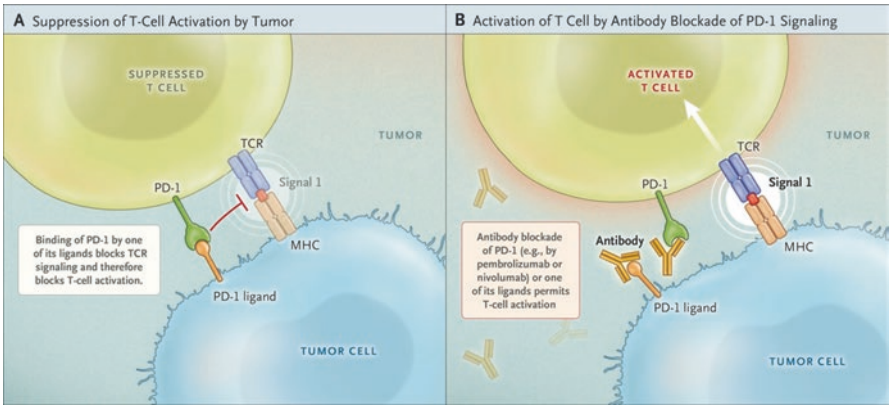

Fig. 7.4 Suppression of T-Cell activation through TCR by PD-1 binding to PD-1 Ligand (**a**) and activation of T-Cell by inhibitory PD-1 by antibody blockade (**b**). During long-term antigen exposure, such as occurs in the tumor milieu, the programmed death 1 (PD-1) inhibitor receptor is expressed by T-cells (**a**); it suppresses the effect of the TCR on T-cell activation. Blockade of PD-1 or its ligand (**b**) (e.g., by pembrolizumab or nivolumab) derepresses TCR signaling, thereby permitting T-cell activation [63]

I study, looked at 411 patients with advanced melanoma. Pembrolizumab showed a durable response in 34% of treated patients after 18 months. This response was maintained in 81% of patients, and median OS was over 2 years [30]. This was followed by KEYNOTE-002 which consisted of patients who had disease progression while being treated or after being treated with ipilimumab. This study compared pembrolizumab versus standard chemotherapy. The pembrolizumab arm showed superiority over standard chemotherapy with longer duration of response as well as lower number of Grade 3 and Grade 4 adverse events [31]. Finally, another study, KEYNOTE-006, comparing ipilimumab *vs*. pembrolizumab, again showed superiority of the latter, concluding that the new standard of care for advanced melanoma should be pembrolizumab [32].

As PD-1 blocking antibodies were being tested in patients with melanoma, studies were also being done in patients with R/R classical HL. Several of the first studies were done using pembrolizumab [33]. KEYNOTE-013 [34] was a Phase 1b study looking at the use of pembrolizumab in classical HL patients who had already failed treatment with BV. Thirty-one total patients were enrolled onto the study. The majority of the patients had already failed an autologous stem cell transplant (ASCT) and greater than four lines of previous therapy. Patients were given pembrolizumab on a biweekly schedule until disease progression was noted. Results revealed an ORR of 65%, with 16% of patients reaching CR and 48% of patients reaching a partial response. The progression free survival (PFS) and OS were 69% and 100% at 24 weeks, and almost half of the responders had a durable response at 1 year follow-up (Fig. 7.5a–e) [34].

The follow-up Phase 2 study, KEYNOTE-087, showed similar results [35]. This larger study had a total of 210 enrolled patients. These patients were split into 3 cohorts determined by their progression (cohort 1: patients who had failed ASCT and BV; cohort 2: patients who had failed salvage therapy *plus* BV but were ineligible for ASCT; cohort 3: patients who had failed ASCT but did not receive BV). Patients in this study received a higher dose of medication (200 mg versus 100 mg) and it was given every 3 weeks. The ORR in this study was 69%, with 22.4% of patients achieving CR [35].

Following the success of pembrolizumab, other PD-1 inhibitors have undergone investigation. A Phase 1 study, CheckMate 039, was one of the first studies to look at nivolumab as a possible agent in refractory hematologic malignancies [36]. This study had a total of 23 heavily pretreated patients, most of which had already failed ASCT and BV. Patients received therapy every 3 weeks. The ORR for this group was a rather impressive 87%, with 17% of patients achieving CR, 70% of patients achieving PR and the remainder of patients having stable disease [36].

The follow-up nivolumab study was a Phase 2 study, CheckMate 205, which also studied R/R HL patients [37]. This study enrolled 243 patients that were split into 3 cohorts based on exposure to BV (cohort 1: patients who had never received BV; cohort 2: patients who received BV only after ASCT; cohort 3: patients who received BV both before and after ASCT). Patients received therapy biweekly. Results showed an ORR ranging from 63–75% between all of the groups with a median PFS of 14.7 months [37].

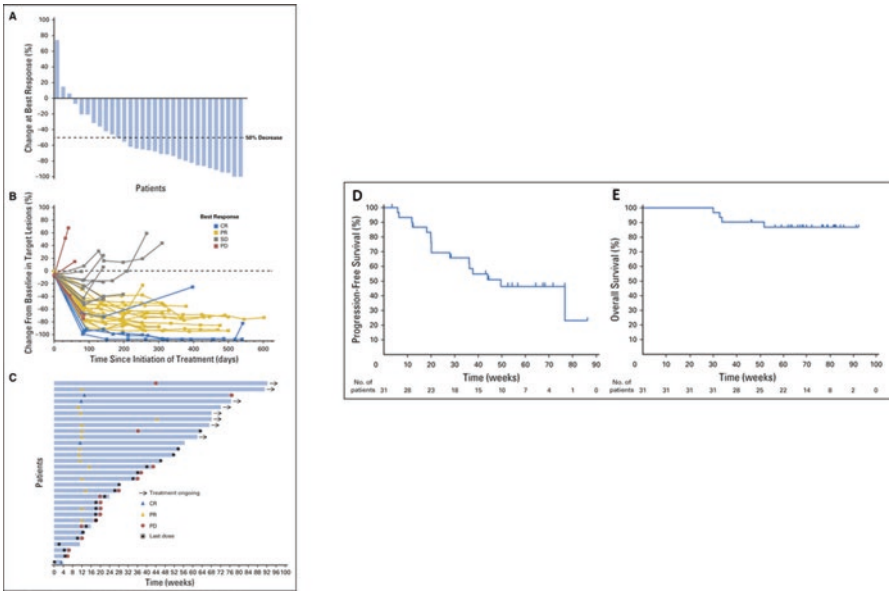

Fig. 7.5 Response to Pembrolizumab in adults with relapsed/refractory cHL waterfall maximal change (**a**); change from baseline in each Patient (**b**); response duration (**c**); probability of PFS (**d**) and OS (**e**) in adults with relapse/refractory cHL treated with Pembrolizumab. Response to treatment. (**a**) Maximum percentage change from baseline in target lesions. (**b**) Change from baseline in target lesions. (**c**) Treatment exposure and response duration. Three patients had a formal response assessment before the protocol-required time point of 12 weeks. One patient only received one dose of pembrolizumab, discontinued treatment because of toxicity at 4 weeks, and had nonprotocol scans to assess response, which showed CR. The other two patients had nonprotocol scans to confirm the clinical impression of progressive disease before the 12 week time point. Abbreviations: CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease [34]

Due to the overall success of these trials, in May of 2016, nivolumab was approved by the FDA for patients with relapsed classical HL and patients who progressed following ASCT and BV. Also, due to the high response rate and overall safe side effect profile, studies have expanded to the younger patients, including pediatric and adolescent and young adults with R/R HL. The Children's Oncology Group (COG) is now enrolling patients as young as 5 years of age and up to 30 years of age on a risk adapted phase 2 study that is using nivolumab and BV followed by BV and bendamustine for patients with R/R CD30 positive classical HL (NCT02927769) [38].

Combined Checkpoint Blockade Therapy

Due to the success of CTLA-4 and PD-1 inhibitors as monotherapy, researchers began to investigate possible approaches using a combination of both these inhibitors. In one of the first studies, CheckMate 069, 142 untreated melanoma patients

were randomized to combined therapy with ipilimumab and nivolumab *versus* ipilimumab alone. This study showed a significantly greater PFS and as well as ORR in the group who received combination therapy *versus* monotherapy [39]. This study went on to show a 2 year OS of 64% in the combination group versus 54% in the monotherapy group [40].

The next study went one step further with 3 cohorts where patients received combination therapy with nivolumab and ipilimumab, nivolumab alone or ipilimumab alone [40]. Results of this study revealed that in patients with advanced melanoma, a significantly longer 3 year OS in the combination group of 58%, versus 52% and 34% in the nivolumab and ipilimumab groups, respectively (Fig. 7.6) [40].

PD-L1

Shortly after studies using PD-1 inhibitors started showing promise, other possible checkpoint inhibitors started to be investigated. As discussed previously, PD-L1, a known ligand for PD-1, is expressed by various cells, including hematopoietic cells, peripheral tissues and malignant cells. Overexpression of PD-L1 and PD-L2 has been associated with alterations in chromosome 9p.24.1, and these alterations have been identified in the malignant Reed-Sternberg cells of classical HL [20, 41, 42]. Similar alterations have been found in primary mediastinal B cell lymphoma and diffuse large B cell lymphoma [20]. Roemer et al. used a 9p24.1 fluorescent in situ hybridization assay to determine incidence of alterations in patients with classical HL. Their results showed that almost all patients had some type of alteration in the PD-L1 and PD-L2 loci, with the majority consisting of copy gain and amplification [42]. They also found that the highest level of expression, ones with amplification, seemed to have a higher incidence in patients with advanced disease, whereas early stage disease had less amplification. Further investigation showed a difference in patient outcome based on percent amplification. By dividing patients into 3 groups, early stage-favorable, early stage-unfavorable and advanced stage, based on clinical features and percent amplification, they identified a significant decrease in PFS related to an increased percent amplification. This led to their speculation that a possible change in treatment may be the addition PD-L1 blockade to standard therapy in these patients [42].

When PD-L1 binds to PD-1, it results in the T-cell entering an exhausted state, making it unavailable to be targeted by T-cells, therefore promoting cancer growth. One of the first PD-L1 immune checkpoint inhibitors approved for bladder cancer was atezolizumab [28]. This checkpoint inhibitor gained FDA approval following a study of 310 patients with inoperable, locally advanced or metastatic urothelial cancer that had failed platinum therapy. In this study, the ORR was 28% for those patients who expressed ≥5% PD-L1 positive tumor infiltrating immune cells [43].

A few months after being approved by the FDA for bladder cancer, atezolizumab was then approved for non-small cell lung cancer (NSCLC) [43]. In a Phase 3 trial

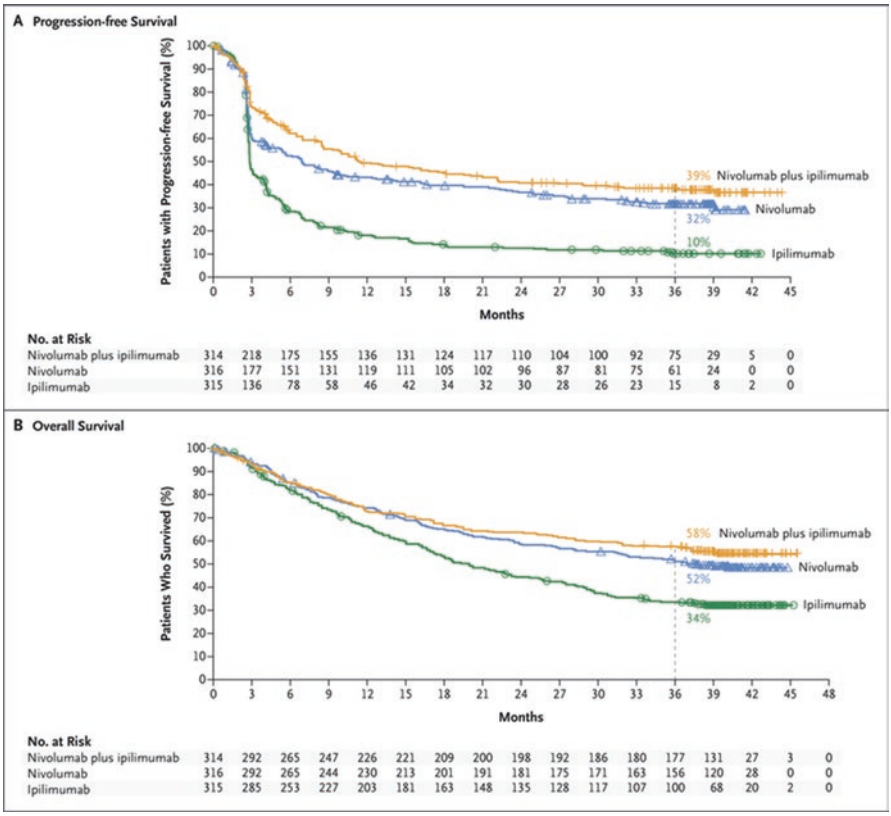

Fig. 7.6 Probability of PFS (**a**) and OS (**b**) in patients with advanced melanoma treated with ipilimumab, nivolumab or both. (**a**) Shows the Kaplan–Meier estimates of progression-free survival as assessed by the investigator. Patients were followed for a minimum of 36 months (dashed line). The median progression-free survival was 11.5 months (95% CI, 8.7–19.3) in the nivolumab-plus-ipilimumab group and 6.9 months (95% CI, 5.1–9.7) in the nivolumab group, as compared with 2.9 months (95% CI, 2.8–3.2) in the ipilimumab group. The rate of progression-free survival at 2 years was 43% in the nivolumab-plus-ipilimumab group and 37% in the nivolumab group, as compared with 12% in the ipilimumab group. The 3 year rate of progression-free survival was 39% in the nivolumab-plus-ipilimumab group and 32% in the nivolumab group, as compared with 10% in the ipilimumab group. (**b**) Shows the Kaplan–Meier estimates of overall survival. The median overall survival was not reached in the nivolumab-plus-ipilimumab group and was 37.6 months (95% CI, 29.1 to not reached) in the nivolumab group and 19.9 months (95% CI, 16.9–24.6) in the ipilimumab group. The overall survival rate at 2 years was 64% in the nivolumab-plus-ipilimumab group and 59% in the nivolumab group, as compared with 45% in the ipilimumab group. The 3 year rate of overall survival was 58% in the nivolumab-plus-ipilimumab group and 52% in the nivolumab group, as compared with 34% in the ipilimumab group. Symbols (tick marks, triangles, and circles) indicate censored data [40]

comparing atezolizumab *versus* docetaxel, there was a significant improvement in OS in the atezolizumab arm (13.8 months *versus* 9.6 months, P = 0.0003). The atezolizumab group was also shown to have less severe side effects than the docetaxel group [44]. Further Phase 3 studies compared the addition of atezolizumab to standard platinum based therapy *versus* standard therapy alone, and preliminary results are in favor of the addition of atezolizumab, which demonstrated longer survival (8.3 months *versus* 6.8 months, P < 0.0001) [45].

Finally, avelumab, another PD-L1 inhibitor, has been studied in multiple tumors. A previous Phase 1b study in patients with advanced stage lung cancer showed an ORR in 12% of patients as well as stable disease in 38% [45]. This inhibitor has also been studied in metastatic Merkel cell carcinoma, a rare neuroendocrine cancer originating from the skin. Two cohorts were studied – Cohort A: those patients who had failed prior therapy; Cohort B: chemotherapy-naïve patients. In cohort A, an ORR was seen in 33% of patients (11.4% with CR and 21.6% with PR) with approximately 74% of these patients having a durable response over 1 year. Cohort B had a greater ORR with 60% of patients seeing a response, 13.8% with CR and 48.3% with PR [46].

Resistance to Checkpoint Blockade

With the addition of checkpoint inhibitors as a possible treatment options for refractory/relapsed cancers, the scientific community has been reinvigorated in finding possible cures for some of the most difficult cancers to treat. Early studies have shown overall promising results in patients, with some patients even achieving a CR, something that could not be done with standard therapy. Unfortunately, although initial responses were positive in some patients, a number of patients did not seem to benefit at all from the same therapy. In some of the early melanoma studies, up to 60% of patients never benefited from treatment with a checkpoint inhibitor [2, 47, 48]. In addition, in those who did respond initially, up to 25% developed relapsed disease that no longer responded to the therapy [48]. Currently, studies are underway to determine why checkpoint inhibitors may benefit some patients, but not others, even though they have the same presumed cancer. Those who never benefited are thought to have a certain profile that may indicate a primary resistance, which may contain a component of adaptive immune resistance, while those with initial response and later loss of this response may have developed possible genetic mutations leading to an acquired resistance to treatment. Resistance to PD-1 checkpoint inhibitors can be due to both extrinsic and intrinsic factors relating to the tumor.

Extrinsic Factors

Lack of Tumor Infiltrating T-Cells

The basis of PD-1 immune checkpoint inhibitors treating tumor cells is that there are functional T-cells present within the tumor microenvironment in order to carry out the cytotoxic effects needed to destroy tumor cells. Recent studies have shown that some tumor microenvironments, especially at times of relapse, are lacking these ever important T-cells [2, 48, 49]. Interestingly, it was demonstrated by Zaretzky et al. that the T-cells, although present and abundant, were only present at the tumor margins, and not within the tumor itself, making them less effective in exerting cytotoxic activity [49]. Another recent study concluded that pre-existing $CD8^+$ T-cells were essential in anti-PD-1 therapy. This study showed that those patients whose pre-treatment tumor cells had a larger number of $CD8^+$ T-cells as well as PD-1/PD-L1 expression, both in the margins and within the tumor microenvironment, had the most tumor regression [50]. This has led to some investigators proposing different strategies for use of immune checkpoint inhibitors based on type of tumor microenvironment (whether tumors are $PD\text{-}L1^+$ and if they have tumor infiltrating $CD8^+$ T-cells) [51].

Others have also reported the significance of tumor infiltrating T-cells in the success of checkpoint blockade. One such study showed that sufficient T-cell infiltration, and not necessarily PD-L1 expression, was essential for an adequate response to checkpoint inhibition [52]. Due to this, they went on to postulate and show certain techniques that could be used in order to increase the population of infiltrating T-cells into the tumor microenvironment. By using the basis of previous studies that showed tumors can suppress chemokine production, Tang et al. have demonstrated that targeting LIGHT (also known as tumor necrosis factor superfamily member 14) can increase lymphocyte infiltration by activating lympotoxin β receptor (LTβR) signaling (Fig. 7.7). The activation of LTβR then induces the production of chemokines and adhesion molecules in tumor tissues which ultimately attract lymphocytes to the tumor [52]. They theorized that this technique could overcome checkpoint inhibitor resistance in some cases.

Still others have shown that it may not only be the quantity of tumor infiltrating T cells that are present, but also the type. A recent publication has shown that Anti-PD-1 and Anti-CTLA-4 checkpoint inhibitors use distinct cellular mechanisms, resulting in induction of different types of T-cells. While both these targeting agents induce subsets of exhausted-like CD8 T-cells, only CTLA-4 blockade induces the expansion of inducible costimulatory ($ICOS^+$) Th1-like CD4 T-cells [53]. This data was eloquently demonstrated by using immunogenic MC38 colorectal tumors from mice treated with anti-CTLA-4 and anti-PD-1 (Fig. 7.8). Interestingly, Wei et al. went on to demonstrate that the tumor infiltrating $ICOS^+$ CD4 T cells that were induced by anti-CTLA-4 and anti-PD- 1 had separate and distinct transcriptional responses, with only 3 being shared among the top 15 cellular pathways. While anti-PD-1 seemed to modulate mitochondrial and oxidative phosphorylation pathways, anti-CTLA-4 modulated pathways which involved cell cycle regulation (Fig. 7.9).

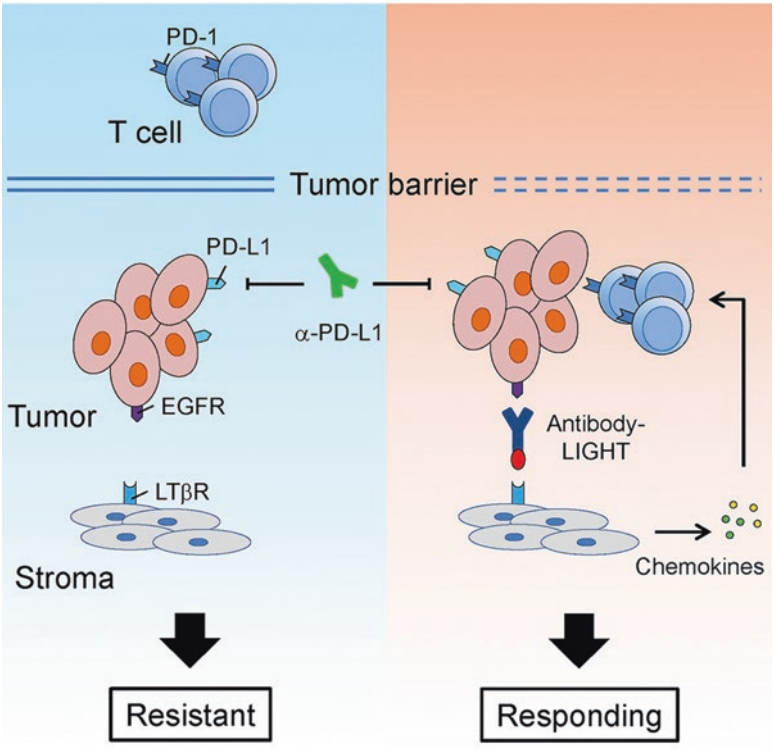

Fig. 7.7 Targeting non-T cell-inflamed tumor tissues by antibody-guided LIGHT. Targeting tumors with tumor necrosis factor superfamily member LIGHT activates lymphotoxin β-receptor signaling, leading to the production of chemokines that recruit massive numbers of T cells [52]

T-Cell Exhaustion

As stated earlier, the main function of PD-1 is to inhibit T-cells during long term antigen exposure or during times of inflammatory response or infection [15, 21, 24]. Chronic exposure to specific antigens can result in T-cell exhaustion, and more specifically, the level of expression of PD-1 signaling has been shown to define the level of exhaustion [47]. In the presence of exhausted T-cells, the PD-1^{high} phenotype appears to affect the efficacy of PD-1 inhibitors, making them resistant to this treatment. In contrast, exhausted T-cells in the presence of the PD-1^{low} or PD-$1^{intermediate}$ phenotype appear to respond to therapy, indicating that these cells can be "reinvigorated" and induced from their exhausted state in order to function against tumor cells [2, 47]. T-cells have also been shown to express other inhibitory immune checkpoints, such as LAG-3, TIM-3 and CTLA-4 possibly leading to severe exhaustion [6, 47]. Finally, the use of PD-1 independent pathways, such as release of IDO or adenosine from tumor cells has been shown to suppress T-cell function during treatment with PD-1 checkpoint inhibitors [6, 47].

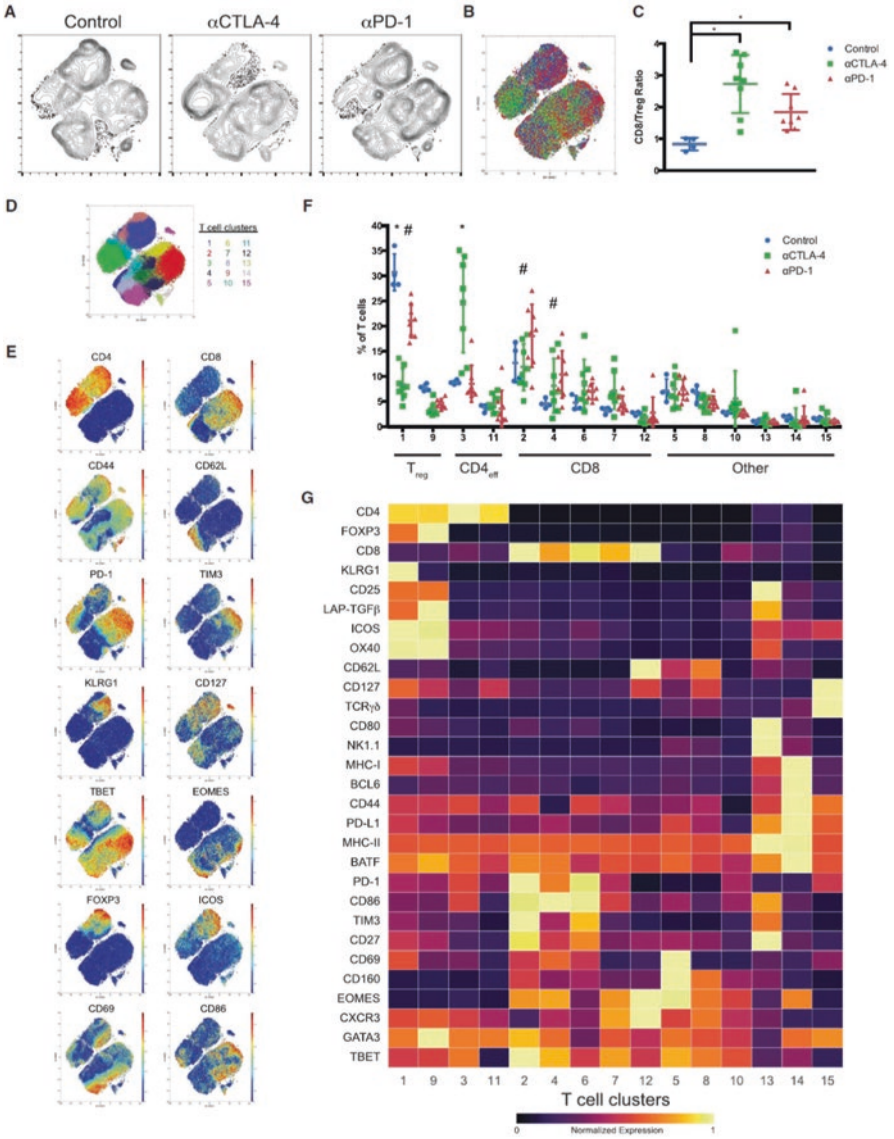

Fig. 7.8 Identification of checkpoint-blockade-responsive MC38 tumor-infiltrating T-cell populations. (**a**) Density t-SNE plots of an equal number of CD3ε$^+$ MC38 tumor-infiltrating T-cells from each treatment group; (**b**) overlaid t-SNE plot displaying equal number of events from each treatment group (control, blue; anti-CTLA-4, green; anti-PD-1, red); (**c**) plot of CD8/T_{reg} ratios displayed on a per-mouse basis with mean ± SD ($p < 0.05$, unpaired t test); (**d**) t-SNE plot of MC38 infiltrating T-cells overlaid with color-coded clusters; (**e**) t-SNE plot of infiltrating T-cells overlaid with the expression of selected markers; (**f**) frequency of T-cell clusters displayed on a per-mouse basis with mean ± SD (∗, control *versus* anti-CTLA-4; #, control *versus* anti-PD-1; $p < 0.05$, Dunnett's multiple comparison). T-cell compartments are denoted including CD8, T_{reg}, and CD4 effector ($CD4_{eff}$) and (**g**) heatmap displaying normalized marker expression of each T-cell cluster. Representative data from three independent experiments is shown [53]

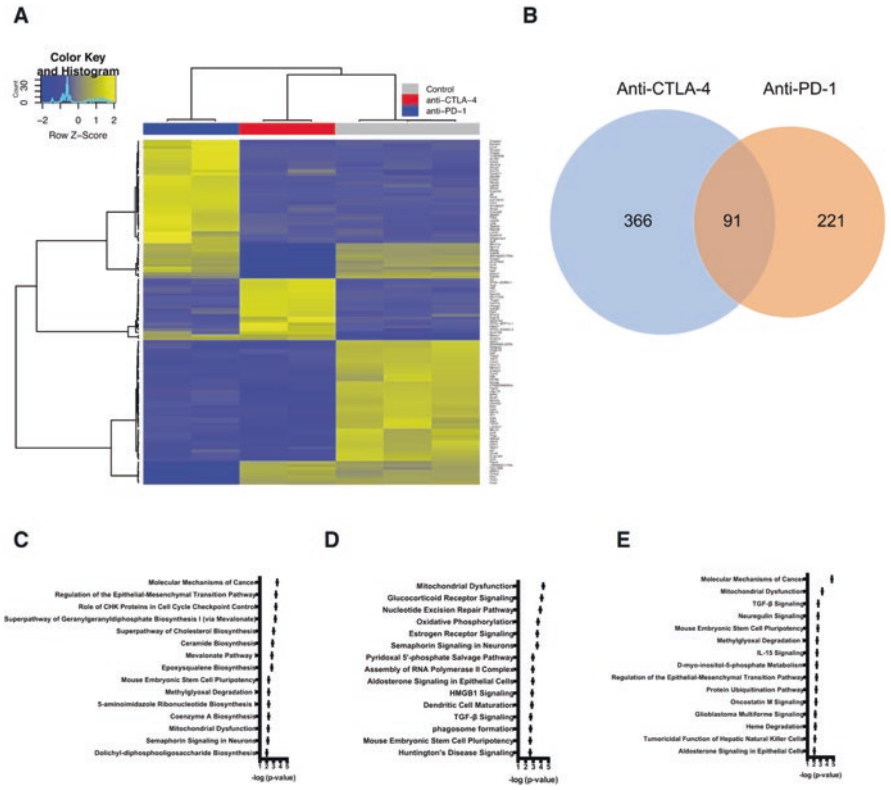

Fig. 7.9 Differential Transcriptional Regulation in MC38 Tumor-Infiltrating CD4 T Cells following Anti-CTLA-4 and Anti-PD-1. (**a**) RNaseq analysis of FACS sorted MC38 tumor infiltrating activated ICOS$^+$ CD4 T-cells. Heatmap of genes with significant differential mRNA expression identified by negative bionomial generalized linear models with likelihood ratio tests, comparing both treatment and control groups. Genes and samples are organized by two-way hierarchical clustering; (**b**) Venn diagram of genes identified as modulated significantly by only one or both anti-CTLA-4 and anti-PD-1 as determined by negative binomial generalized linear models with Wald tests, comparing each treatment to control samples. A 1% FDR cutoff was used for both statistical models; (**c**) The top 15 pathways modulated by anti-CTLA-4 compared to control identified by Ingenuity pathway analysis (IPA) of RNaseq expression analysis; (**d**) The top 15 pathways modulated by anti-PD-1 compared to control identified by IPA; (**e**) The top 15 pathways modulated by anti-CTLA-4 versus anti-PD-1 blockade as identified by IPA [53]

Innate Anti-PD-1 Resistance (IPRES) Signatures

Within the tumor microenvironment, there consists a milieu of cells, including other immune cells, stromal cells and an extracellular matrix [2]. It is these cells that help protect the tumor cell from being recognized as foreign. Twenty-six transcriptomic signatures, referred to as IPRES signatures, have been described as being related to PD-1/PD-L1 resistance [54]. These 26 signatures have been shown to express genes which are related to angiogenesis, wound healing, mesenchymal transition, cell adhesion, and extracellular matrix remodeling [2, 6, 54].

Intrinsic Factors

The cornerstone of PD-1 checkpoint inhibitors is the reactivation of T-cells and their ultimate recognition of tumor cell antigens, resulting in destruction of tumor cells. In order to evade this process, tumors have developed mechanisms of neoantigen loss, or total lack of antigen presentation.

Tumor Immunogenicity

Recent data has shown a correlation between immunogenic mutations and response to checkpoint inhibitors. Specifically, tumors with 5–10 somatic mutations per mega-base of DNA have shown the most response to anti-PD-1 therapy. This is in contrast to tumors, especially those with between 0.1–1 somatic mutation per mega-base of DNA, which have shown almost no response to therapy [55]. Tumors such as melanoma, renal cell carcinoma and non-small cell lung cancer, have shown a high immunogenic mutation load [56]. One study examined somatic nonsynonymous mutation burden in patients with NSCLC and its relation to PD-1 blockade [57]. Rivzi et al. concluded that those patients with a higher median number of nonsynonymous mutations were more likely to have a durable response. 73% of the patients with a high nonsynonymous burden experienced durable clinical benefits as compared to 13% with a low number of mutations. Rivzi et al. went on to demonstrate that both ORR was better, 63% versus 0% and PFS was better, 14.5 months versus 3.7 months, in this group (Fig. 7.10). It has also been demonstrated that some tumors can acquire the ability to decrease the number of neoantigens being presented, thus leading to decreased recognition by T-cells. One such study showed that in relapsed NSCLC patients, the loss of 7–18 putative mutation associated antigens lead to clones resistant to PD-1 therapy [58]. The peptides produced by these neoantigens promoted T-cell expansion in autologous T-cell cultures, thus loss of these peptides affected immune response [58].

Mutations of β-2-Microglobulin (β2M)

In addition to a quantitative loss of neoantigens, another mechanism of resistance is the loss of antigen presenting components, such as β2M, needed for presentation of the antigen to the T-cell. It has been well established that β2M is essential in the formation and transport of the MHC – I molecule to the surface of the presentation cell. Through its interactions with its alpha chain components, it allows for stability of the MHC I molecule during presentation of an antigen [59]. With a mutation of β2M, the end result is lack of antigen presentation, and ultimately lack of recognition by cytotoxic T-cells, allowing the tumor cell to evade apoptosis [6]. A recent study focusing on classical HL showed that of 108 tumor samples, 79% had decreased or absent expression of β2M/MHC I complex and concluded that those patients with a decrease in β2M/MHC I complex had a shorter PFS [60].

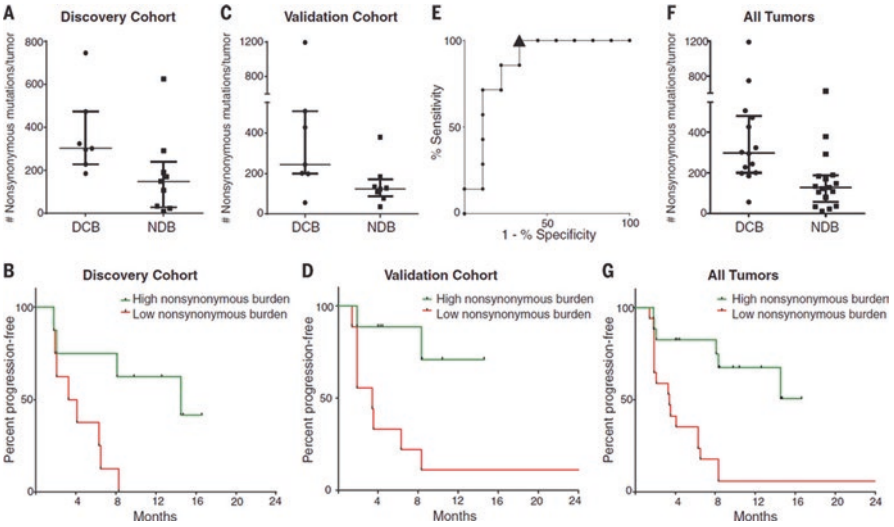

Fig. 7.10 Nonsynonymous mutation burden associated with clinical benefit of anti–PD-1 therapy. (**a**) Nonsynonymous mutation burden in tumors from patients with DCB (n = 7) or with NDB (n = 9) (median 302 versus 148, Mann-Whitney P = 0.02); (**b**) PFS in tumors with higher nonsynonymous mutation burden (n = 8) compared to tumors with lower nonsynonymous mutation burden (n = 8) in patients in the discovery cohort (HR 0.19, 95% CI 0.05–0.70, log-rank P = 0.01); (**c**) Nonsynonymous mutation burden in tumors with DCB (n = 7) compared to those with NDB (n = 8) in patients in the validation cohort (median 244 versus 125, Mann-Whitney P = 0.04); (**d**) PFS in tumors with higher nonsynonymous mutation burden (n = 9) compared to those with lower nonsynonymous mutation burden (n = 9) in patients in the validation cohort (HR 0.15, 95% CI 0.04–0.59, log-rank P = 0.006); (**e**) ROC curve for the correlation of nonsynonymous mutation burden with DCB in discovery cohort. AUC is 0.86 (95% CI 0.66–1.05, null hypothesis test P = 0.02). Cut-off of ≥178 nonsynonymous mutations is designated by triangle and (**f**) Nonsynonymous mutation burden in patients with DCB (n = 14) compared to those with NDB (n = 17) for the entire set of sequenced tumors (median 299 versus 127, Mann-Whitney P = 0.0008); (**g**) PFS in those with higher nonsynonymous mutation burden (n = 17) compared to those with lower nonsynonymous mutation burden (n = 17) in the entire set of sequenced tumors (HR 0.19, 95% CI 0.08–0.47, log-rank P = 0.0004). In (**a**), (**c**), and (**f**), median and interquartile ranges of total nonsynonymous mutations are shown, with individual values for each tumor shown with dots [57]

Mutation of Janus Kinase 1 and 2 (JAK1 and JAK2)

Another method of tumor escape exists with mutations of JAK1 and JAK2. In a recent publication by Zaretzky et al. [49], they examined 4 biopsy samples from diagnosis and relapse of melanoma patients who had initial response to PD-1 therapy, followed by relapse of disease. In 2 of these samples, it was noted that there was a loss of function mutation in JAK1 and JAK2. It has previously been well established that Interferons produced by tumor specific T-cells can recognize certain antigens, such as PD-L1 on tumor cells, resulting in an anti-tumor effect by cytotoxic T-cells [54]. By acquiring loss of function mutations in JAK1 and JAK2, the end result is decreased response to interferon gamma, resulting in decreased PD-L1 antigens and thus allows for tumor proliferation [48, 49].

Expression of CD73 and Production of Adenosine

CD73 expression has been identified in several types of cancer, including colon cancer, melanoma and leukemia, and has also been linked to poor prognosis in triple negative breast cancer [61]. Recent studies have shown that CD73 leads to a downstream production of adenosine, which ultimately leads to tumor-induced immune suppression via activation of A_{2A} receptors on T cells [62]. While therapy targeted against CD73 has not shown much effect, new studies looking at Adenosine Receptor 2A Blockade are showing an increase in the efficacy of immune therapy with PD-1 inhibitors. Beavis et al. showed that a dual blockade with PD-1 and A_{2A} enhanced expression of interferon-gamma by $CD8^+$ T cells leading to growth inhibition of tumors [62]. Because there are several A_{2A} antagonists that have undergone safety studies in other diseases, such as Parkinson's disease, it is possible that studies using them in combination therapy with checkpoint inhibitors may not be far away.

Conclusion

Cancer therapy has evolved over the last several decades and we have been fortunate to observe advancements in immunotherapy leading to successes in the treatment and cure of several cancers. While there remain some cancers that relapse or are refractory to therapy, there continues to be advancements in the field of tumor immunity with the development of novel drugs with different mechanisms of action, one such mechanism being the use of checkpoint inhibitor blockade. Through the use of such drugs, we have seen patients with cancers refractory to multiple treatments have durable responses, and in some, even go into a complete long term remissions. While this has been exciting, there has been a realization that not everybody will have this same response, and even those who do initially, may not have a continued durable response. As we continue to explore possible treatment options for cancer, we must also be diligent in preemptively investigating how and why some patients will become resistant to these treatments, and what, if any, actions can be taken to circumvent this resistance.

Acknowledgement This work was supported in part from the Pediatric Cancer Research Foundation and St. Baldrick's Foundation. The authors would like to thank Virginia Davenport, RN and Erin Morris, RN in their assistance in the preparation of this manuscript.

Disclosure of Conflict of Interest No potential conflicts of interest were disclosed.

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Chapter 8
Resistance to Bispecific T-Cell Engagers and Bispecific Antibodies

Stacy L. Cooper and Patrick A. Brown

Abstract Bispecific antibodies are an emerging novel therapeutic construct used to treat a variety of cancers. These drugs utilize a small fusion protein to link two single-chain antibodies, allowing for simultaneous binding of two different epitopes. Bispecific T-cell engagers (BiTE) are a subset of bispecific antibodies that bind the target antigen on the cancer cell while simultaneously binding a patient's endogenous T-cell. By bringing these two cells in close proximity, the patient's own immune system can be redirected to attack the cancer cell. Several mechanisms of resistance to these drugs exist, including extramedullary escape, loss of the target antigen, and inadequate endogenous immune response.

Keywords Bispecific antibodies · BiTE · Blinatumomab · Non-Hodgkin lymphoma · Acute lymphoblastic leukemia

Abbreviations

B-ALL	B Acute Lymphoblastic Leukemia
BiTE	Bi-Specific Antigen Receptor T-Cells
CAR-T	Chimeric Antigen Receptor T-cells
CNS	Central Nervous System
CR	Complete Response Rate
CRS	Cytokine Release Syndrome
DLBCL	Diffuse Large B-Cell Lymphoma
EM	Extramedullary
EFS	Event-Free Survival
FDA	Food and Drug Administration

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A. C. Xavier, M. S. Cairo (eds.), *Resistance to Targeted Therapies in Lymphomas*, Resistance to Targeted Anti-Cancer Therapeutics 21,
https://doi.org/10.1007/978-3-030-24424-8_8

HL	Hodgkin Lymphoma
MCH	Major Histocompatibility Complex
MLL	Mixed Lineage Leukemia
NHL	Non-Hodgkin Lymphoma
NK	Natural Killer
ORR	Overall Response Rate
OS	Overall Survival
PD-L1	Programmed Death Ligand 1
PD-1	Programmed Death Protein 1
R/R	Relapsed/Refractory
scFv	Single-chain Fragment Variable
TCR	T-Cell Receptor
Treg	Regulatory T-Cell

Introduction to Bispecific Antibodies and Bispecific T-Cell Engagers (BiTE)

Bispecific antibodies are novel therapeutic constructs able to recognize and concurrently bind two separate epitopes. Bispecific T-cell engagers (BiTEs) are a specific subset of bispecific antibodies that link the single-chain fragment variable (scFv) of a monoclonal antibody against the tumor antigen with a scFv from a monoclonal antibody directed against CD3 to bind an effector T cell. These two scFV are connected by a small linker protein that allows the fragments unrestricted rotation. By bringing the endogenous T-cell in close contact with the tumor cell, this allows the patient's own immune system to be redirected to attack the tumor cells through the recruitment and activation of polyclonal T-cells. Simultaneous binding of the T-cell and the tumor cell to the BiTE results in T-cell activation, manifested by upregulation of CD25 and CD69, as well as secretion of activating cytokines such as IL-2, IL-4, IL-6, IL-10, IFN-y and TNF-α. This temporary cytolytic synapse produces tumor cell death through perforin/granzyme-induced apoptosis. While both antibody fragments must be bound to cause cell death, this process is not dependent on the tumor antigen specificity of the T-cells, nor does it require costimulatory molecules [1]. Importantly, this antibody construct therefore allows this response to be independent of major histocompatibility complex (MHC), costimulatory molecules or T-cell receptor (TCR recognition) requirements, which are common escape mechanisms for many other antibody therapies [2, 3].

Blinatumomab

Blinatumomab is a BiTE composed of two single-chain antibodies, with murine anti-CD3 on its C terminus and human anti-CD19 on its N terminus, covalently linked by a flexible peptide, thus simultaneously binding the patient's CD3+

endogenous T-cells as well as the CD19+ cells expressed by the malignant clone. CD19 is an attractive target for B-lineage hematologic malignancies, given its specificity for B-cells and near universal expression in these neoplasms. Its relatively small size compared to monoclonal antibodies, only 55kDa, allows for the target-effector cells to be brought into close proximity, and is also thought to improve penetration into lymphomatous regions of disease [4]. Once both cells are bound, T-cell mediated cytotoxicity occurs via perforin and granzyme release, forming channels within the cell membrane of the tumor cell through which granzyme passes to activated intracellular caspases and cause cell death via apoptosis. Following this cell death, the T-cell disengages from the blinatumomab-B cell complex and is able to target another malignant cell. Blinatumomab's low effector to target cell ratio ensures that the same T-cell can be redirected to lyse multiple malignant CD19+ cells [5]. The amount of disease burden at the initiation of therapy with blinatumomab has consistently been shown to be an important prognostic factor, with patients with >50% bone marrow blasts having significantly worse outcomes. This finding suggests that the ratio of effector to target cells is an important factor in optimal response [3].

Immunologic profiling of patients enrolled in the initial studies if blinatumomab for relapsed/refractory B-ALL was used to study the endogenous T-cell response to blinatumomab induced cytotoxic killing of the target cell. The peripheral blood T-cells in these patients were shown to consistently drop within hours of initiation of blinatumomab, with subsequent recovery to half baseline levels within 3 days and fully back to baseline by day 9. In many patients, this increase in T lymphocytes continued, reaching a mean maximal expansion by day 17 of treatment. This included polyclonal expansion of both CD4+ and CD8+ T-cells, with the greatest relative increase seen in effector memory T-cells (T_{EM}) and terminally differentiated memory cells (T_{EMRA}) cells. This temporary reallocation of the peripheral T-cells after initiation of blinatumomab appeared to be secondary to release of cytokines, specifically IL-2, IL-6, IL-10, IFN-y and TNFα [6].

Blinatumomab is generally well tolerated, with fever, headache and peripheral edema as the most common adverse events; and anemia, thrombocytopenia and hypokalemia as most common serious adverse events [7]. Two rare but serious side effects, cytokine release syndrome (CRS) and neurologic sequelae are usually reversible and manageable with supportive care [8]. Cytokine release syndrome is a potentially serious side effect related to a systemic inflammatory response, and manifests with fever, headache, and malaise and can progress to hypotension and multi-organ dysfunction. It is usually able to be managed with intravenous fluid resuscitation and the use of tocilizumab, an antibody targeting the IL-6 receptor, with severe refractory cases requiring the use of corticosteroids. Neurologic sequelae are another potential serious side effect of blinatumomab, manifesting with the entire spectrum of neurologic signs and symptoms, from headache and confusion to seizure and coma. It is managed with supportive care, as tocilizumab is not effective, with steroids are indicated in more severe, refractory cases that require intervention. Both CRS and neurotoxicity almost always resolve without any residual side effects [9].

The initial phase 1 studies of blinatumomab were performed with adult patients with R/R B-cell non-Hodgkin lymphoma (NHL), and demonstrated that the drug was safe and feasible to give, with the potential for efficacy particularly in patients with diffuse large B cell lymphoma (DLBCL) [10]. In a phase 2 study of blinatumomab in adults with heavily pre-treated R/R DLBCL, twenty-one evaluable patients had an overall response rate (ORR) of 43%, with complete response (CR) in 19%. Notably, three of the four complete responses were durable, long-term responses in the absence of any additional treatment [11].

Much of the subsequent clinical experience with blinatumomab has been with patients with pre-B acute lymphoblastic leukemia (B-ALL), another hematologic malignancy that almost always expresses CD19. The first phase 2 study in adults with R/R B-ALL enrolled 36 patients, with 69% achieving CR/CRi, almost all of which were MRD negative remissions [12]. A multi-institutional, phase 3 trial in adults with R/R B-ALL enrolled more than 400 patients, randomized to either blinatumomab alone or standard cytotoxic chemotherapy, with the patients receiving blinatumomab having significantly higher rates of CR, event free survival (EFS) and overall survival (OS) [7]. The first phase 1/2 study of blinatumomab in pediatric patients with R/R B-ALL showed a similar side effect profile with a CR rate of 40% [13]. Most recently, a single arm, phase 2 trial treated 118 adults with B-ALL who achieved an MRD positive (> 0.1%) CR, and demonstrated that blinatumomab therapy in these patients achieved MRD negativity in 78%, with 54% relapse-free survival at 18 months [14].

Based on these clinical trials, blinatumomab was first approved by the US Food and Drug Administration (FDA) in 2014 for adults with R/R Philadelphia chromosome negative B-ALL, and expanded in 2018 to include adults and children with B-ALL in first or second complete morphologic remission with MRD > 0.1% [15].

Clinical trials for blinatumomab are currently underway for several other CD19+ hematologic malignancies, including R/R indolent B cell NHL (NCT02961881), maintenance therapy after achieving CR after upfront DLBCL therapy (NCT03023878), maintenance therapy after autologous transplant for R/R DLBCL (NCT03072771), maintenance therapy after allogeneic transplant for R/R NHL (NCT NCT02807883), and in combination with lenalidomide for B-cell NHL (NCT02568553).

Other BiTE/Bispecific Antibodies Used in Lymphoma

Several other bispecific antibodies are currently in development for lymphoma. AFM11 is a tetravalent bispecific CD19/CD3 tandem antibody (TandAb), a molecule that is similar BiTE but with two bindings sites each for CD19 and CD3, which is thought to improve its potency over traditional BiTE molecules. Clinical trials are currently underway to study this drug in R/R NHL and B-ALL (NCT02106091 and NCT02848911, respectively) [16].

CD30 is a surface marker expressed in Hodgkin lymphoma (HL), and thus another promising target antigen for antibody directed therapy. Several bispecific

antibodies targeting CD30 have been studied since the 1990s with some promising efficacy in early trials, although manufacturing challenges halted their development [17, 18]. One of these, AFM13, is a bispecific anti-CD30/CD16A antibody that binds natural killer (NK) cells to mediate lysis of HL cells. In a phase 1 dose escalation study of 26 adults with heavily pre-treated R/R HL, AFM13 was found to be well tolerated, with 61% of achieving at least stable disease [19]. An ongoing phase 1b clinical trial is ongoing in adults with R/R HL that combines AFM13 with a PD-1 inhibitor in an attempt to augment its efficacy (NCT02665650).

Other bispecific antibodies currently in development for patients with NHL include RG6026 (which targets CD20/TCB) [20], mosunetuzumab (which targets CD3/CD19) [21], RO7082859 (which targets CD3/CD20, NCT03075696), and REGN1979 (which targets CD3/CD20, NCT02651662).

Mechanisms of Resistance and Relapse to BiTE and Bispecific Antibodies

While the mechanisms of resistance to bispecific antibodies and BiTE have yet to be fully elucidated, several major pathways have been identified. As discussed earlier in the chapter, the mechanism of action of bispecific antibodies is independent of MHC requirements, costimulatory molecules or TCR recognition, thus eliminating three common escape mechanisms seen in many other antibody-based therapies [2]. Of note, anti-mouse antibodies have never been detected in patients who have received bispecific antibodies, making this only a theoretical mechanism of resistance [22].

As blinatumomab is the bispecific antibody with the greatest clinical experience thus far, the understanding of resistance to BiTE and bispecific antibodies in general extends mainly from knowledge of the mechanisms known to affect blinatumomab. Therefore, the remainder of this discussion will primarily focus on the experience of resistance to blinatumomab.

Approximately half of all R/R B-ALL patients treated with blinatumomab are initially refractory, with half of the responders eventually relapsing through secondary mechanisms [23]. While not yet completely understood, three major pathways have been identified for this resistance: compartmental escape through extramedullary sites, loss of target antigen, and inadequate endogenous T-cell response.

Compartmental Escape to Extramedullary Sites

The central nervous system (CNS) and testes have long been identified as sanctuary sites that allow malignant cells to be protected from systemic chemotherapy. As a result of tumor microenvironment factors in these areas, overt or occult disease in these sites could allow for protection and immunity from the systemic blinatumomab.

In the long-term follow up studies of the phase 2 trial of blinatumomab in adult R/R B-ALL, two of the 21 evaluable patients who relapsed did so with extramedullary disease (one CNS, one testicular) [24]. Additionally, studies of patients treated with blinatumomab have shown that both a history of extramedullary B-ALL as well as active extramedullary B-ALL at the time of initiation of blinatumomab have been shown to be associated with poor outcomes [25].

In a retrospective analysis of a single center's 65 adult patients treated with blinatumomab for R/R B-ALL, among those patients who initially responded to blinatumomab but later relapsed, 40% relapsed with EM disease. Among those patients who were refractory to blinatumomab, 40% had extramedullary disease at the time of progression, including 5 patients with combined bone marrow and extramedullary involvement at the time of initiation of blinatumomab who had documented complete marrow remission at the end of the first cycle but with progression of the EM involvement [25].

Studies of blinatumomab in NHL provide further evidence for blinatumomab's decreased extramedullary efficacy, as the drug has been less effective in this patient population when to patients with B-ALL. However, interestingly, patients with mature B-NHL show improved outcomes with higher drug dosages used when compared to the dosages typically used in B-ALL, with only 1/15 patients achieving a CR at 15 ug/m^2/day, but 8/35 patients achieving CR when the dose was escalated to 60 ug/m^2/day [10]. This was confirmed with another study demonstrating that the 15 ug/m^2/day dose of blinatumomab was able to achieve CR in the bone marrow disease in patients with stage IV NHL, but a dose escalation to 60 ug/m^2/day was required for remission response in areas of nodal disease [26].

While the mechanism for this sanctuary site escape is not yet known, it is postulated that it could be secondary to poor extramedullary site penetration by T-cells and/or blinatumomab, as well as extramedullary microenvironmental factors that inhibit the action of the drug.

Loss of Target Antigen

Evolution of the malignant clone to cease expression of the target antigen is another mechanism of resistance to these antibodies. In patients treated with blinatumomab, loss of the CD19 target antigen has been reported, but is rare, occurring in approximately 10–20% of patients. In one of the largest retrospective reviews of 84 adult patients with R/R B-ALL treated with blinatumomab, surface marker expression was analyzed using flow cytometry and found that the overwhelming majority (92%) of non-responders and relapsed patients continued to express CD19 at high levels. Of the 38 refractory patients, only one patient lost CD19 expression after treatment, with two additional patients having a decrease in CD19 expression. Of the 30 patients who eventually relapsed after initial CR, four patients lost CD19 expression in their relapsed clone [23]. Consistent with this, in the initial phase 1/2 study of blinatumomab in pediatric patients with R/R B-ALL, 22% of the patients (5/70) experienced CD19 negative relapse or progression [27].

Two major pathways are proposed for antigen loss as a mechanism of resistance, with much of the data extrapolated from relapse after chimeric antigen receptor T-cell therapy (CAR-T) in R/R B-ALL. This modality of immunotherapy is similar to BiTE, but uses the patient's autologous T-cells that have been genetically engineered to express a chimeric antigen receptor to bind the tumor antigen. CD19+ CAR-T cells are the construct with the greatest clinical experience, and share many features of mechanism of action, side effects and pathways of relapse with blinatumomab.

One mechanism for CD19 negative escape is specific loss of the CD19 target antigen from the malignant B-cells. Studies of relapse after CAR-T therapy have found evidence that this epitope loss can be secondary to deletions as well as de novo frameshift mutations in the CD19 gene, as well as alternative splicing of the CD19 mRNA [28]. One study of adult patients with CD19 negative relapses after blinatumomab therapy identified another novel mechanism of CD19 loss, as one patient with CD19 negative relapse was found to have leukemia cells without mutation or deletion within the CD19 gene, and full length CD19 mRNA was identified. The team therefore hypothesized the resistance was secondary to abnormal trafficking of the antigen, and were able to identify an abnormal glycosylation of the blasts, which was traced to an abnormality in CD81 that prevented normal processing of CD19 within the Golgi complex [29]. Thus, many different mechanisms can potentially lead to loss of the CD19 antigen itself on the lymphoblasts.

The second mechanism for CD19 negative relapse is lineage switch, whereby the lymphoblasts are able to undergo alternate differentiation into myeloblasts that do not express CD19. While this was not demonstrated in the initial phase studies of blinatumomab, it has since been reported. This phenomenon is most well known in patients with MLL-rearranged leukemia, which is hypothesized to stem from a very early progenitor with lymphomyeloid differentiation potential, which allows for the switch from lymphoid to myeloid lineage when selective chemotherapy pressure is applied [30, 31].

However, while lineage switch is most commonly described as a mechanism of resistance in patients with MLL-rearranged leukemia, it has also been reported after blinatumomab therapy even in cases of patients without this MLL rearrangement [32]. Additionally, new evidence is emerging based on studies of subclones in pre-B ALL treated with CD19+ CAR-T, suggesting that lineage switch results from plasticity of the malignant cells, rather than clonal pressure [33].

Suboptimal T-Cell Response to BiTE

One mechanism of resistance that is unique to BiTE that does not affect other bispecific antibodies involves a poor response from the patient's endogenous T-cells. As the cytotoxicity of BiTE relies on tumor lysis mediated by the patient's immune system, suboptimal activation and expansion of these T-cells is becoming increasingly recognized as a major mechanism of resistance to this class of drugs.

The association between T-cell expansion and blinatumomab response has been well documented. The first evidence for this came from a phase 2 study of blinatumomab in 36 adult patients with R/R B-ALL, with response defined by morphologic CR. At the end of the first cycle, those who responded showed an increase in peripheral blood CD4+ and CD8+ T-cells by an average of 243% and 245% above baseline, compared to the non-responders whose CD4+ and CD8+ T cells remained at baseline at the end of the cycle. Consistent with this, responders also showed higher peak serum concentration of IFN-y, IL-6 and IL-10 [34]. Long term follow-up of these patients revealed a significantly more robust T-cell expansion in the ten long term survivors when compared to those who relapsed. Analysis of the T-cell kinetics demonstrated expansion of CD3+T cells in cycle 1 of those with survival more than 30 months, decreased but demonstrable T-cell expansion in those with initial CR but survival inferior to 30 months, and no evidence of T-cell expansion in patients refractory to blinatumomab. A similar trend was seen with CD3+ T_{EM} cells, which are particularly important for blinatumomab-mediated cytotoxicity [35].

Another immune factor that predicts response to blinatumomab is the percentage of regulatory T-cells (Tregs), immune cells that function to down-regulate effector T-cell responses. Studies in patients with solid tumors have consistently demonstrated high levels of intratumoral Tregs correlated with poor prognosis [36–38]. When T-cell subsets were analyzed in 42 adult patients with R/R B-ALL who received blinatumomab, no differences were seen in absolute number of total T-cells, or subsets of CD4+ and CD8+ T-cells. However, patients that were refractory to blinatumomab demonstrated a significantly higher percentage of Tregs in the peripheral blood when compared to patients who responded to blinatumomab. Subsequent *in vitro* experiments demonstrated that when Tregs were depleted from the samples from these non-responders, both CD4+ and CD8+ cells had increased proliferation [39].

Immune checkpoints are a complex system of inhibitory and stimulatory signals that allow for self-tolerance and prevention autoimmunity, as well as to limit the damage to nearby normal tissues when responding to a pathogen. Programmed Death Ligand 1 (PD-L1) and its receptor, Programmed Death Protein 1 (PD-1), are two critical checkpoint inhibitors that have been shown to have an emerging role in cancer immunotherapy. Binding of PD-L1 to PD-1 on T-cells results in inhibition of T-cell function, and lack of an immune response [40].

Upregulation of PD-L1 has been shown to result in T-cell dysfunction and inhibition of the adaptive immune response, and is becoming increasingly recognized as a pathway of immune evasion for BiTEs. In solid tumors as well as hematologic malignancies, high expression of PD-L1 has been associated with poor prognosis [41]. Antibodies targeting PD-1 and PD-L1 are thus promising targets for immunotherapy, as a way of bypassing this mechanism of resistance. Several of these have been approved by the FDA, including nivolumab (PD-1 inhibitor) in 2014, atezolizumab (PD-L1 inhibitor) in 2016, pembrolizumab (PD-1 inhibitor) in 2017, and durvalumab (PD-L1 inhibitor) in 2017.

The earliest report supporting this theory of T-cell mediated resistance to blinatumomab involved an adult patient with primary refractory B-ALL treated with

blinatumomab after several failed attempts at induction with conventional chemotherapy. With 30% bone marrow blasts in his marrow prior to starting blinatumomab, he was found to be refractory to blinatumomab as well, with 60% CD19+ blasts after the first cycle. Immunohistochemistry performed before and after blinatumomab demonstrated an increase in both PD-1 expression by marrow lymphocytes (5% positive *vs.* 15% positive) as well as PD-L1 by the tumor cells (2% positive *vs.* 40% positive). After treatment with blinatumomab, the patient's peripheral blood CD3+ T cells were collected and incubated with the patient's blasts, and compared to co-culture of the blasts with healthy donor T-cells, the endogenous T cells were found to have significant decrease in cell lysis (8.5% *vs.* 93.6%), with a concomitant decrease in the levels of IFN-y produced by the patient's T-cells compared to those from a healthy donor [42].

Another study screened the immunologic co-signaling molecules on ten CD19+ primary ALL cell lines as compared to normal bone marrow samples, to determine the stimulatory and inhibitory profiles of B lymphoblasts. PD-L1 had significantly higher expression on the lymphoblasts, with CD86 as the most pronounced marker of activation on these cells. Specifically, samples from patients refractory to blinatumomab were found to have higher expression of PD-L1 when compared to responders to blinatumomab and controls, with higher markers of T-cell exhaustion, such as PD-1 and TIM3, on the T-cells of these patients when compared to healthy donor controls. Based on these findings, peripheral blood mononuclear cells from healthy donors and leukemia patients were co-cultured with the patient's blasts and blinatumomab, with the presence and absence of antibodies against PDL-1 and/or CTLA-4. Proliferation of the T-cells was markedly increased with the addition of antibodies against PD-L1 alone, and with both PD-L1 and CTLA-4. This *in vivo* data led to the first report combining blinatumomab with checkpoint inhibition in a patient, when a 12 year-old girl with refractory ALL was refractory to blinatumomab monotherapy, with 45% marrow blasts at the end of the first cycle. Pembrolizumab, a PD-1 inhibitor, was added to her next cycle of blinatumomab, which was well tolerated and without significant toxicity. Marrow evaluation at the end of this cycle showed a morphologic CR, with the patient still alive at the time of manuscript publication [43].

Conclusions and Future Directions

BiTE and bispecific antibodies are a promising therapeutic platform for treating hematologic malignancies. The major mechanisms of resistance include extramedullary escape, loss of the target antigen, and for those antibodies relying on T-cell engagement, suboptimal response by the patient's endogenous T-cells. More research is needed to better understand the mechanisms of resistance to these forms of immunotherapy, and ways to overcome them. There is already some promising progress being made in this arena. To address the extramedullary escape, additional research is needed to determine the optimal dose for targeting tumor cells outside of

the bone marrow. These studies will also be augmented by ongoing research into the microenvironment of leukemias and lymphomas, to determine the differences and any therapeutic implications between the bone marrow niche and the extramedullary compartment. Regarding target antigen loss, there is robust research being done to determine ways to minimize this, including simultaneous targeting of several target antigens, as seen with early studies of bivalent CAR-T cells that target both CD19+ and CD22 [44]. And finally, in terms of augmenting the patient's endogenous T-cell response to BiTE, larger studies are ongoing to test whether the addition of checkpoint inhibitors can enhance the efficacy of this therapy (NCT02879695).

Disclosure of Conflict of Interest No potential conflicts of interest were disclosed by SC. P.B. has received medical writing support from Amgen for an unrelated manuscript.

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Chapter 9
Resistance to Chimeric Antigen Receptor T-Cell Therapy

Ana C. Xavier and Luciano J. Costa

Abstract Chimeric antigen receptor (CAR) T-cells are a form of adoptive immunotherapy constituted of autologous T-cells engineered with a receptors that is able to target tumor antigens. Treatment with CAR19 cells leads to rapid response in a significant proportion of patients with relapsed or refractory aggressive B-cell lymphomas. However, relapses post CAR-T cell therapy are common. In this chapter, we will discuss what is currently known about mechanisms of resistance to CAR-T cell therapy in B-cell lymphomas or leukemias.

Keywords Lymphoma · Large B-cell · Diffuse; immunotherapy · Adoptive; drug resistance · Neoplasm

Abbreviations

ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
Axi-cel	Axicabtagene Ciloleucel
CAN	Copy-number Alteration
CAR	Chimeric Antigen Receptor
CARB	CAR-transduced B-cell leukemia

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A. C. Xavier, M. S. Cairo (eds.), *Resistance to Targeted Therapies in Lymphomas*, Resistance to Targeted Anti-Cancer Therapeutics 21,
https://doi.org/10.1007/978-3-030-24424-8_9

CR	Complete Response
CRS	Cytokine Release Syndrome
DLBCL	Diffuse Large B-Cell Lymphoma
Liso-cel	Lisocabtagene Maraleucel
LOH	Loss Of Heterozygosity
MCL	Mantle Cell Lymphoma
OS	Overall Survival
PFS	Progression-Free Survival
PMBCL	Primary Mediastinal B-Cell Lymphoma
PR	Partial Response
r/r	Relapsed/refractory
RR	Response Rate
SCT	Stem Cell Transplant
TCR	T-Cell Receptor
tFL	DLBCL arising from Follicular Lymphoma
Tisa-cel	Tisagenlecleucel
WES	Whole-genome sequencing

Introduction

CD19-directed genetically modified autologous T-cell immunotherapy is comprised of autologous T-cells collected from a patient and genetically engineered to encode an anti-CD19 chimeric antigen receptor (CAR) (Fig. 9.1) [1]. The structure of the anti-CD19 CAR T cell products (CAR19) recently evaluated in B-cell lymphoma trials are displayed in Fig. 9.2 [2]. Treatment with CAR19 cells leads to rapid response in the majority of patients with relapsed or refractory (r/r) aggressive B-cell lymphomas, including complete responses (CR). Notwithstanding the rapid initial response, a significant proportion of patients will eventually face disease

Fig. 9.1 (continued) transmembrane domain to intracellular signaling domains. Pro-inflammatory cytokines or co-stimulatory ligands expressed by the CAR T cells are depicted for the 4th generation. (**C**) Overview of so-called smart CAR T cell products. Pooled CAR T cell products consist of two or more single-targeting CAR T cell types with distinct antigen specificities. Multi-CAR T cells harbor several CAR molecules with different antigen specificities. A tandem CAR T ell expresses a CAR construct harboring two ligand-binding domains with different antigen specificities. In a conditional CAR T cell activation and co-stimulation are separated on two CAR constructs recognizing different target antigens. In the split CAR construct the ligand-binding or signaling domain is physically separated allowing controlled CAR T cell activation. iCAR T cells additionally express a receptor engineered to recognize an antigen expressed on normal tissue to provide an inhibitory signal in turn. In addition CAR T cells can be equipped with suicide genes or switches (e.g. iCasp9) allowing ablation of CAR T cells. (**D**) Left, status of published CAR T cell gene therapy trials or trials registered at clinicaltrials.gov including long-term follow-up studies. The status of one trial is unknown and not listed. The total number of clinical trials (dark blue bars) is compared to published clinical trials (light blue bars). The asterisk indicate zero trials. Right, phases of CAR T cell gene therapy trials. Long-term follow-up studies are not included. For nine trials, the phase classification is unknown. The asterisk indicate zero trials [1]

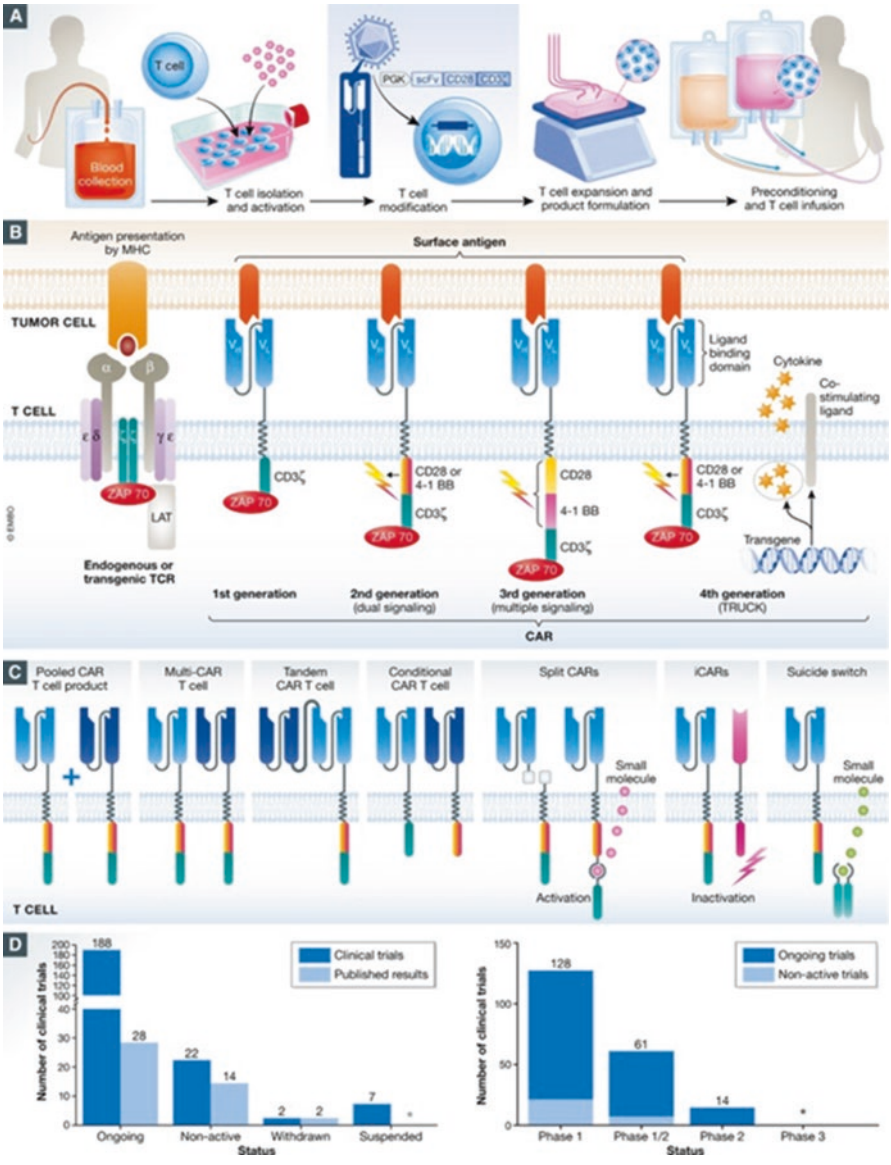

Fig. 9.1 CAR T cell therapy – principle and clinical trial overview. (**A**) The CAR T cell therapy process. T-cells are isolated from blood of the patient or a donor, activated, and then genetically engineered to express the CAR construct (an example shown in gray above the vector particle in violet). After *ex vivo* expansion of the CAR T cells, they are formulated into the final product. The patient undergoes either a conditional chemotherapy or the CAR T cell product is directly infused. (**B**) Schematic representation of a T-cell receptor (TCR) and four types of CARs being displayed on the surface of a T-cell while contacting their antigen (red) on the tumor cells. The single-chain variable fragment (scFv) as ligand-binding domain mediating tumor cell recognition in CARs is shown in light blue with the VH and VL domains being connected via a along flexible linker and

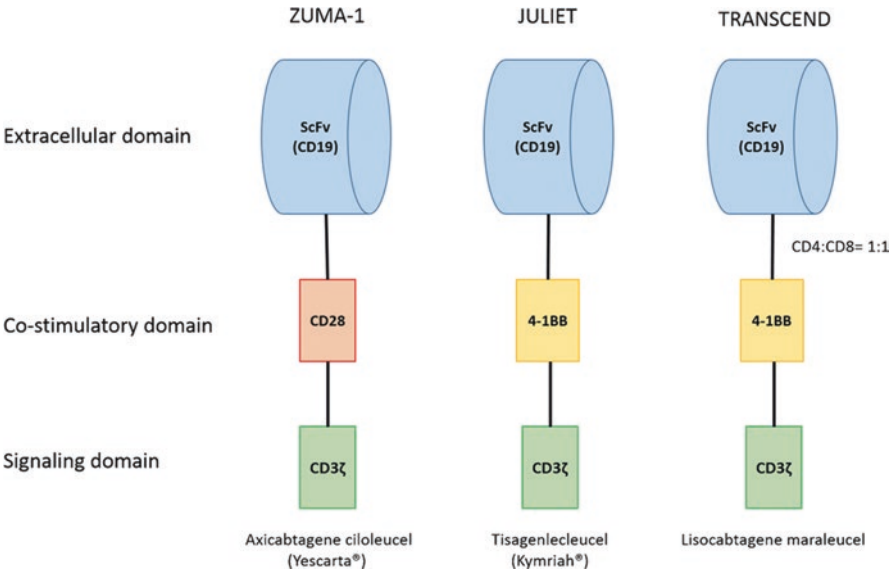

Fig. 9.2 Anti-CD19 CAR T cell products evaluated in pivotal trials in B-cell lymphomas. The intracellular domain of axicabtagene ciloleucel (ZUMA-1 trial) is composed of two signaling domains, CD3ζ and a co-stimulatory domain, CD28. Tisagenlecleucel (JULIET trial) and lisocabtagene maraleucel (TRANSCENT trial) use CD137 (4-1BB) as co-stimulatory domain. The co-stimulatory domain promotes the T-cell activation and persistence of CAR T cells [2]

relapses or progression, making crucial to understand mechanisms of treatment failure to CAR T cell therapy. Currently, CAR T cells with novel target antigens, such as CD22, CD20, κ-light chain for B-cell lymphomas, and CD30 for Hodgkin lymphoma and T-cell lymphomas are being investigated in several clinical trials (Fig. 9.1). While the era of CAR T cell therapy is in its infancy and there are large gaps in our understanding of the reasons cellular immunotherapy fails, a few mechanisms have become evident and will be the focus of our discussion in this chapter.

Anti-CD19 CAR T Cell Therapy in Lymphoma

Axicabtagene ciloleucel (axi-cel) was granted the US Food and Drug Administration (FDA) regular approval in 2017 for the treatment of patients with r/r large B-cell lymphoma after two or more lines of systemic therapy, including diffuse large B-cell lymphoma (DLBCL), high-grade B-cell lymphomas, and DLBCL arising from follicular lymphoma (tFL). The approval was based on the results of a seminal Phase 2 study published by Neelapu et al. [3]. In this multicenter study (ZUMA-1 trial), 111 patients with r/r DLBCL, primary mediastinal B-cell lymphoma

(PMBCL), or tFL were included. Patients received a target dose of 2×10^6 CAR19 cells per kilogram of body weight after conditioning regimen of low-dose cyclophosphamide and fludarabine. Axi-cel was administered to 101 patients, with objective response rate (RR) of 82%, and CR rate of 54%. Most CRs were durable, the overall rate of survival at 18 months was 52% [3]. At 27.1 months, the median overall survival (OS) was not reached, and the median progression-free survival (PFS) was 5.9 months [4]. Interestingly, CAR T cell levels during the first month of therapy seem to be associated with efficacy of the product [3, 5].

Tisagenlecleucel (tisa-cel) was approved by the US FDA in 2018 for adult patients with r/r DLBCL and tFL after at least two prior lines of therapy, including anthracycline and rituximab, or relapsing after an autologous stem cell transplant (SCT). Tisa-cel was tested in a Phase 2 multicenter study (JULIET trial) involving adult patients with r/r DLBCL [6]. A total of 93 patients received tisa-cel infusions and were included in the efficacy analysis of JULIET trial. The best overall response rate was 52%, including 40% of patients achieving CR and 12% achieving partial response (PR). At 12 months after the initial response, the rate of relapse-free survival was estimated to be 65% (79% among patients in CR) [6].

In addition to axi-cell and tisa-cel, lisocabtagene maraleucel (liso-cel) has also been studied in a Phase 1 multicenter study (TRANSCEND trial) [7]. The difference between liso-cell and tisa-cell or axi-cel is that liso-cel is a CAR T cell product administered in defined composition at a precise dose of CD8 and CD4 CAR T cells (Fig. 9.1). Adult patients with r/r DLBCL, PMBCL, tFL, or mantle cell lymphoma (MCL) were included and an interim analysis of the Phase 1 of the trial. Results showed that, with a median follow-up of 8 months, 80% of 73 patients treated achieved an objective response, and duration of response was not reached. The frequency of objective response at 6 months was 47% [7]. Main toxicity associated to CAR-T cell therapy includes development of cytokine release syndrome (CRS), neurotoxicity and B-cell aplasia (Table 9.1). For patients experiencing a relapse after an autologous or allogeneic stem cell transplant (SCT), administration of CAR T cell therapy seems to be safe and efficacious [8–10].

There is very limited experience with the use of CAR T therapy to treat lymphoma in pediatric and adolescent patients. Recently, Rivers et al. reported 5 pediatric patients (range 12–18 years) with r/r $CD19^+$ NHL (DLBCL, PMBCL, or gray zone B-cell) treated in an ongoing Phase 2 trial [11]. Patients received 1×10^6/Kg CAR19 cells as a 1:1 ratio of CD4 and CD8 cells, following lymphodepletion with fludarabine and cytarabine. One patients had history of auto/allo-SCT (PMBCL), 3 had had received immunotherapy (nivolumab or brentuximab vedotin). Similar to adult patients, the most common side effects were (mild) CRS (n = 4) and (mild) neurotoxicity (N = 2). At 3 weeks, anti-tumor response was observed in 4/5 patients, and 2/3 evaluable subjects were in CR at week 9. One subject had a $CD19^-$ progression at week 9, after initial response. One subject obtained CR, but eventually recurred with $CD19^+$ disease despite ongoing CAR-T cell persistence [11].

Table 9.1 Toxicity associated to anti-CD19 CAR T cell therapy described in pivotal lymphoma trials

≥ Grade 3 toxicity	Axicabtagene ciloleucel (%) [3]	Tisagenlecleucel (%) [6]	Lisocabtagene maraleucel (%) [7]
Any	95	89	NR
Pyrexia	14	5	NR
Neutropenia	78	32	NR
Anemia	43	39	NR
Hypotension	14	9	NR
Thrombocytopenia	38	12	NR
Nausea	–	1	NR
Fatigue	2	6	NR
Decreased appetite	2	4	NR
Headache	1	1	NR
Diarrhea	4	1	NR
Hypoalbuminemia	1	–	NR
Hypocalcemia	6	–	NR
Chills	–	0	NR
Tachycardia	2	3	NR
Febrile neutropenia	31	16	NR
Vomiting	1	–	NR
Hypokalemia	3	8	NR
Hyponatremia	10	–	NR
Constipation	–	1	NR
White-cell count decrease	29	31	NR
Hypophosphatemia		14	NR
Cytokine release syndrome	13	22.5	1
Neurologic event	28	12	13

NR, not reported

Mechanisms of CAR T Cell Resistance

Immune Scape from Antigen Loss

A significant proportion of relapses post CAR T cell therapy seem to be associated with immune scape from antigen loss of CD19, but the exact mechanisms of antigen loss in lymphoma therapy have yet to be understood. However, insights into possible mechanisms of antigen loss are being revealed by several studies done in pediatric and adult patients with B-acute lymphoblastic leukemia (ALL) suffering CD19 negative ($CD19^{-}$) B-ALL relapse after treatment with CAR19 cell therapy. Lack of CD19 expression has been shown to occur due to either mutations, alternative splicing in CD19, or by mutations in the B-cell receptor protein CD81. Those mechanisms are further discussed below.

CD19 Mutation and CD19 Alternative Splicing (exon 2 skipping)

The most known mechanism of CAR19 resistance is the emerging dominance of leukemic cells harboring isoforms of CD19 lacking the transmembrane domain or the targeted exon, under the selective pressure of CAR T cells. Sotillo et al. detected hemizygous deletions within chromosome 16 spanning the *CD19* locus and *de novo* frameshift and missense mutations in exon 2 of *CD19* in some relapse samples [12]. The investigators also described alternatively spliced *CD19* mRNA species, including one lacking exon 2, and demonstrated that exon 2 skipping bypasses exon 2 mutations in B-ALL cells and allows expression of the N-terminally truncated CD19 variant, which fails to trigger killing by CAR19 [12]. More recently, Fisher et al. analyzed the expression of CD19 isoforms in a cohort of subjects with $CD19^+$ B-ALL [13]. They demonstrated that an alternatively spliced *CD19* mRNA isoform lacking exon 2, and therefore the CAR19 epitope, but not isoforms lacking the transmembrane and cytosolic domains were expressed in the leukemia blasts at diagnosis and in the bone marrow of nonleukemia donors, suggesting that some of the CD19 isoforms contributing to CAR19 escape already preexist at diagnosis and could evolve as a dominant clone during CAR19 therapy [13].

Another mechanism of CD19 loss can be due to mutations in other genes that express other proteins of the B-cell receptor complex. To signal with the B-cell receptor, CD19 complexes with CD21, CD81, and CD225. Homozygous mutations in the *CD81* gene have been demonstrated to cause congenital immunodeficiency in humans [14]. Braig et al. demonstrated resistance to anti-CD19/CD3 BiTE therapy (blinatumomab) in patients with B-ALL via disrupted CD19 membrane trafficking [15]. At relapse post blinatumomab, patient's $CD19^-$ blasts were surface $CD81^-$, which led to non-CD19 processing and maturation in the Golgi complex [15]. Although not yet demonstrated, it is highly plausible that the similar mechanism play a role in antigen scaping in B-cell lymphomas.

Myeloid Switch

MLL-rearranged $CD19^+$ B-cell ALL are responsive to CAR19 therapy as demonstrated in a cohort of 7 patients who achieved CR after CAR T-cell therapy [16]. However, 2 patients relapsed, both with a myeloid phenotype leukemia approximately 1 months after CAR T cell infusion. One patient had no evidence of disease in the bone marrow after therapy on day 22 by flow cytometry, but karyotyping and FISH studies revealed persistent MLL rearrangement. On day 35, circulating blasts were present and expressed myeloperoxidase, CD4, and CD64 without CD19 or other B-cell lineage antigens, consistent with acute myeloid leukemia (AML). FISH for MLL rearrangement and IGH deep sequencing demonstrated that both B-ALL and AML were clonally related. Second patient was a young child with MLL-rearranged $CD19^+$ B-ALL who relapsed after 30 days of receiving CAR19 therapy with an abnormal myeloid population without B-lineage antigens but persistent

presence of MLL rearrangement [16]. CAR19 was detected in blood, and there was B-cell aplasia at the time of AML diagnosis. Deep sequencing of the *IGH* gene was negative for the rearrangement previously noted in the lymphoid blasts, suggesting myeloid relapse occurred form an immature stem cell clone [16]. Those two cases illustrate that myeloid switch is a mechanism of CAR T-cell resistance, likely due to presence of rearranged MLL, reprogramming or de-differentiation of previously committed B-cell lymphoid blasts (case 1) or myeloid differentiation of a noncommitted precursor or selection of a preexisting myeloid clone after CAR19 therapy (case 2).

Senescence and Exhaustion of CAR-T Cell Population

Yang et al. demonstrated that the presence of T-cell receptor (TCR) antigen can provoke loss in CD8+ CAR T cell efficacy associated with T-cell exhaustion and apoptosis [17]. Using an immunocompetent, syngeneic murine model of CD19-targeted CAR T cell therapy for B-ALL in which the CAR is introduced into T-cells with known TCR specificity, they demonstrated that loss of CD8 CAR T-cell efficacy associated with T-cell exhaustion and apoptosis when TCR antigen is present [17]. Long *et at.* also demonstrated that tonic CAR CD3-ζ phosphorylation, triggered by antigen-independent clustering of CAR single-chain variable fragments can induce early exhaustion of CAR T-cells that limit antitumor efficacy [18]. Interestingly, CD28 co-stimulation augments, whereas 4-1BB co-stimulation reduces, exhaustion induced by persistent CAR signaling [18].

Accidental Transfection of Tumor Cells and CD19 "Masking"

The manufacturing process of CAR-T cell requires collection of mononuclear cells from peripheral blood by apheresis and several steps to T-cell purification, expansion, and transfection of viral vector carrying the CAR. Absence of circulating tumor cells has not been considered a critical requirement for such therapy given that occasional malignant B-cells collected during apheresis would be selected out, not expanded and/or not transduced during the manufacturing process.

However, Ruella et al. recently described a B-ALL patient treated with CAR19 cell therapy who experienced a $CD10^{+}CD19^{-}$ ALL relapse caused by accidental transfection of tumor cells with CD19 "masking" [19]. Evaluation of the leukemia cells revealed that the B-leukemia cells were CAR-transduced B-cell blasts (CARB) by immunophenotyping, suggesting that malignant B-cells can survive the manufacturing process and be transfected with the lentivirus containing the CAR [19]. Such transfection was not inconsequential and the lack of CD19 expression was not caused by any known mechanism of antigen loss. In fact, CD19 mRNA transcripts were identified at the baseline and at relapse, and CD19 protein expression was also

detected by immunohistochemistry. Confocal microscopy demonstrated that colocalization of CAR19 and CD19 on the cell surface of the relapsed leukemia, leading to the hypothesis that the lack of detection of CD19 by flow cytometry was due to CAR10 binding *in cis* to CD19 on the cell surface and "masking" the epitope detection by flow cytometry. Antigen masking by transduction of B-ALL with CAR was demonstrated *in vitro* to be also possible during the manufacturing process of anti-CD22 CAR-T cell products [19]. CARB would initially be a minute fraction of the disease burden and not interfere with response at first. Over time, however, it gives rise to a resistant subclone manifesting clinically as leukemia relapse. So far, this mechanism of resistance has only been described in B-ALL and it seems to be a rare phenomenon, but it remains at least hypothetically possible in aggressive lymphomas as well.

Future Directions

One possible approach to overcome immune scape from antigen loss is the simultaneous or sequential targeting of more than one B-cell specific antigen. In fact, CD22-targeting CAR T cells are effective in r/r B-ALL and have recently been proven active in patients with relapse after anti-CD19 CAR T cell therapy [20]. Failure to anti-CD22 CAR T appears linked to the decrease in antigen density, rather than modification of the CD22 molecule [20]. The concomitant targeting of both CD19 and CD22 can potentially reduce the risk of relapse given that it would be unlikely that a single cell would develop simultaneous mechanisms of scape for both targets. Clinical trials are currently being performed with CAR T cell products targeting both CD19 and CD22.

Another possible way to overcome resistance to CAR T cell therapy would be trying to revert tumor-induced immunosuppression and immune exhaustion using immune checkpoint inhibitors. Chong et al. recently reported a case of a patient with DLBCL treated with PD-1 blocking antibody after progression post CAR19 therapy [21]. Overexpression of PD-L1 has been demonstrated in relapsed DLBCL samples post CAR19. Following PD-1 blockade with pembrolizumab, the patient had a clinically significant response and expansion of CART19 cells. Currently the use of pembrolizumab is being tested in a clinical trial setting in patients with $CD19^{+}$ lymphomas who failed post CAR19 therapy.

Acknowledgements The authors would like to thank Erin Morris, RN in her assistance in the preparation of this manuscript.

Disclosure of Conflict of Interest L.J.M has received research support from Celgene, Janssen, Amgen, and GlaxoSmithKline; honorarium from Celgene, Amgen, Karyopharm, GlaxoSmithKline, and Kite; and speaker fees from Amgen and Sanofi. A.C.X has no conflict of interest to disclose.

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A. C. Xavier, M. S. Cairo (eds.), *Resistance to Targeted Therapies in Lymphomas*, Resistance to Targeted Anti-Cancer Therapeutics 21,
https://doi.org/10.1007/978-3-030-24424-8