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Cross Talk Between Cellular Redox State and the Antiapoptotic Protein Bcl-2

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Abstract

Significance: B cell lymphoma-2 (Bcl-2) was discovered over three decades ago and is the prototype antiapoptotic member of the Bcl-2 family that comprises proteins with contrasting effects on cell fate. First identified as a consequence of chromosomal translocation (t 14:18) in human lymphoma, subsequent studies have revealed mutations and/or gene copy number alterations as well as posttranslational modifications of Bcl-2 in a variety of human cancers. The canonical function of Bcl-2 is linked to its ability to inhibit mitochondrial membrane permeabilization, thereby regulating apoptosome assembly and activation by blocking the cytosolic translocation of death amplification factors. Of note, the identification of specific domains within the Bcl-2 family of proteins (Bcl-2 homology domains; BH domains) has not only provided a mechanistic insight into the various interactions between the member proteins but has also been the impetus behind the design and development of small molecule inhibitors and BH3 mimetics for clinical use.

Recent Advances: Aside from its role in maintaining mitochondrial integrity, recent evidence provides testimony to a novel facet in the biology of Bcl-2 that involves an intricate cross talk with cellular redox state. Bcl-2 overexpression modulates mitochondrial redox metabolism to create a ''pro-oxidant'' milieu, conducive for cell survival. However, under states of oxidative stress, overexpression of Bcl-2 functions as a redox sink to prevent excessive buildup of reactive oxygen species, thereby inhibiting execution signals. Emerging evidence indicates various redox-dependent transcriptional changes and posttranslational modifications with different functional outcomes.

Critical Issues: Understanding the complex interplay between Bcl-2 and the cellular redox milieu from the standpoint of cell fate signaling remains vital for a better understanding of pathological states associated with altered redox metabolism and/or aberrant Bcl-2 expression.

Future Directions: Based on its canonical functions, Bcl-2 has emerged as a potential druggable target. Small molecule inhibitors of Bcl-2 and/or other family members with similar function, as well as BH3 mimetics, are showing promise in the clinic. The emerging evidence for the noncanonical activity linked to cellular redox metabolism provides a novel avenue for the design and development of diagnostic and therapeutic strategies against cancers refractory to conventional chemotherapy by the overexpression of this prosurvival protein. *Antioxid. Redox Signal*. 00, 000–000.

Keywords: B cell lymphoma 2, Bcl2, reactive oxygen species, ROS, oxidative stress, BH domains

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Introduction

B cell lymphoma 2: the founding member of a functionally disparate family

B CELL LYMPHOMA 2 (Bcl-2) is the prototypical Bcl-family protein and a major regulator of cell death. Bcl-2 was discovered following the cloning of leukemic cells bearing the t(14;18) (q32;q21) chromosome translocation (175). Subsequent chromosome mapping identified *BCL2* as a putative gene involved in the pathogenesis of B cell and follicular lymphomas (174). McDonnell *et al.* (115) demonstrated that this interchromosomal translocation resulted in the overexpression of Bcl-2 and extended the life span of B cells.

Bcl-2 is primarily localized in the mitochondria (67), however, its localization has also be extended to the nucleus and endoplasmic reticulum (ER). Functionally, Bcl-2 is characterized as an antiapoptotic protein due to its role in inhibiting proapoptotic proteins; these include the Bcl-2 family members Bak and Bax (28). On apoptotic stimulation, death receptor- or drug-induced, a cascade of caspase activation results in the induction of mitochondrial outer membrane permeabilization (MOMP). In the case of death receptor signaling, direct executioner caspase activation is induced in certain cell types where the death initiating signaling complex is efficiently formed (Type 1 or extrinsic pathway); however, in other cell types, the initiator caspase activation is relatively weak to autonomously engage the executioner caspase(s), and therefore, the signal is routed through the mitochondria for efficient death execution (Type 2 or intrinsic signaling). The latter is also the preferred mode of execution triggered on exposure of cells to DNA damaging agents, γ-irradiation, as well as other forms of chemotherapy. Importantly, the recruitment of mitochondria is a function of the proapoptotic members of the Bcl-2 family that, on apoptotic stimulation, translocate to the mitochondria forming oligomeric complexes (40) that compromise the permeability of the outer membrane (15), thereby facilitating the egress of death amplification factors such as cytochrome *c* (83), Smac/direct IAP binding protein with low pI (DIABLO) (1), and apoptosis inducing factor (75). The antiapoptotic members of the Bcl-2 family, in particular Bcl-2 and Bcl-xL, prevent MOMP by competing with and inhibiting the oligomerization of the proapoptotic members, such as Bax and Bak (117, 135, 190). As such, the apoptosis inhibitory function of Bcl-2 is strongly associated with the Type 2 death signaling pathway (Fig. 1).

Bcl-2 expression has also been shown to regulate autophagy, a process of self- consumption induced on starvation or other stress states. Autophagy has emerged as a central player in the removal of damaged organelles as well as a source of nutrients for cells under starvation, hence the association with a prosurvival phenotype. Autophagy has also been linked to cell death signaling in response to certain noxious stimuli. Interestingly, being a survival promoting protein, Bcl-2 has also been shown to have a critical role in regulating autophagy (97). Pattingre *et al.* demonstrated the antiautophagic role of Bcl-2, promoting cell survival, which was dependent on its interaction with Beclin1 (139). This interaction utilized the BH1 and BH2 domains of Bcl-2. Beclin1 expression is low to absent in many cancer cells, leading to loss of autophagy and promotion of cell survival. Cancer cells expressing Beclin1 exhibited higher levels of LC3-induced autophagy, which was inhibited upon overexpression of Bcl-2. The expression of Bcl-2 mutants (G145A/W188A) resulted in the loss of Bcl-2:Beclin1 interaction, as well as the initiation of cellular autophagy. Interestingly, this was only observed when Bcl-2 was localized to the ER, not the mitochondria (139). The interaction was also found to be dependent on JNK1-mediated phosphorylation of Thr69, Ser70, and Ser87. This starvation-dependent phosphorylation resulted in a dissociation of Beclin1 from Bcl-2 and the induction of autophagy (182) (Fig. 2). The inhibitory effect of Bcl-2 on apoptosis and autophagy lends credence to the hypothesis that apoptosis and autophagy work in tandem to regulate processes involved in cellular transformation and its progression.

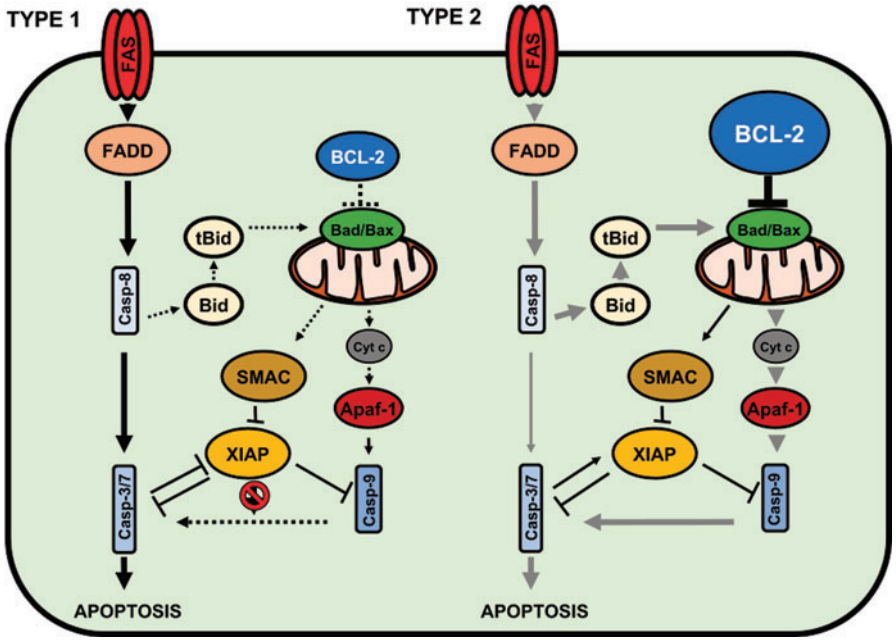

FIG. 1. Schematic of type 1 and type 2 death receptor pathways. Type 1 (*bold black line*) FAS (CD95) death signaling pathway signals independent of the intrinsic pathway. Following FAS ligand binding, signals are transduced through FADD-mediated caspase-8 activation and caspase-3/7 activation for cells to go under apoptosis. Type 2 (*bold gray line*) death signaling through FAS is directed through caspase-8-mediated Bid activation. Bcl-2 is the primary inhibitor of antiapoptosis in type 2 signaling. Bad/Bax can direct apoptosis by the release of cytochrome *c*, and the activation of Apaf-1 and caspase-9. *Dotted lines* indicate no pathway involvement. Bcl-2, B cell lymphoma 2. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

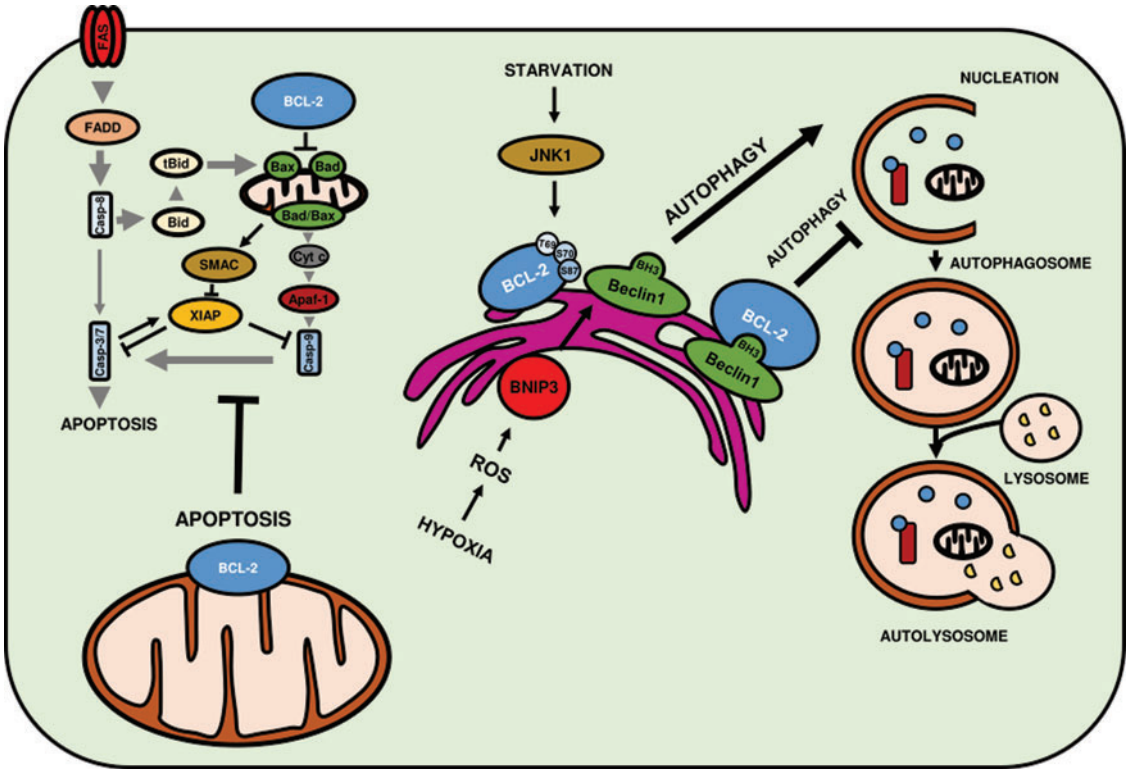

FIG. 2. Schematic of Bcl-2 role in apoptosis and autophagy. Bcl-2 localized to the ER directly interacts with Beclin 1 to inhibit autophagy. Starvation induced JNK1 activation leads to phosphorylation of Thr69, Ser70, and Ser87 of Bcl-2 disrupting the interaction with Beclin1 to induce autophagy. Interaction between the BH3 domains of Beclin1 and Bcl-2 results in the inhibition of autophagy. BNIP3 can interrupt the interaction between Bcl-2 and Beclin1 under conditions of hypoxia-induced ROS inducing autophagy. The autophagy pathway consists of nucleation, autophagosome assembly followed by fusion with the lysosome to result in the formation of an autolysosome. Bad/Bax forms heterodimers with Bcl-2 causing its inactivation and the initiation of apoptosis. BH, Bcl-2 homology domains; BNIP3, Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3; JNKs, c-Jun N-terminal kinases. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Expression in cancer and mutational landscape. Genomic alterations in *BCL2* are common in a variety of cancers. Data sets obtained from The Cancer Genome Atlas (TCGA) accessed by cBioPortal (24, 53) revealed a high percentage of copy number alterations across the majority of cancers surveyed. In this regard, gene amplifications and deletions are the primary alterations (Fig. 3A). Unsurprisingly, amplifications and mutations occurred with higher frequencies in B cell lymphomas. The expression of *BCL2* mRNA was also determined by accessing the same data sets across 21 cancer types. Bcl-2 is not a ubiquitously expressed protein and there seems to be no correlation between organ systems and Bcl-2 expression in cancers, although hematopoietic and lymphoid malignancies were consistently high (Fig. 3B). Although *BCL2* expression was low in carcinomas of the bladder and testicular cancer, it still holds promising predictive power for outcomes in patients (35, 44). Interestingly, the lowest expression was found in hepatocellular carcinoma, where numerous studies have implicated its role as delaying the progression of carcinogenesis by delaying cell cycle progression (148, 177).

The mutational landscape of *BCL2* extends to both hematopoietic and nonhematopoietic tumors. To assist the interpretation of the likely impact of these mutations on Bcl-2 function, we have prepared a putative Bcl-2 homodimer model, based on the crystal structure complex of Bcl-2 with a Bax BH3 peptide (PDB 2XA0) (86), the solution structure of Bcl-xL in its p53-bound conformation (PDB 2ME8) (50), and the crystal structure of the Bcl-xL domain-swapped homodimer (PDB 2B48) (131) (Fig. 3C). Characteristic gain of copy number and expression in diffuse large B cell lymphoma has been associated with R129H missense mutation. In the putative Bcl-2 homodimer, Arg129 appears to facilitate BH2 domain swapping; R129H may facilitate this more efficiently. The G128E mutation was identified in stomach cancer, although was not associated with a variation in copy number. This residue is on the Bcl-2 surface and is not involved in interactions; therefore, the influence of the Gly128 mutation on Bcl-2 function is unclear from the structure.

Numerous G47S mutations have been identified in stomach cancer. Gly47 is located in the large unstructured loop region between the BH4 and BH3 domains and occurs at the dimer interface in the domain-swapped homodimer; G47S would likely enhance homodimer stability. Mutations in melanoma include T96I and A224 V. Thr96 is located at the dimer interface; T96I will likely enhance homodimer stability. Ala224 is located in the TM region of Bcl-2; A224 V will likely embed in the membrane more efficiently. A more comprehensive overview of the mutational landscape is accessible from the TCGA. Further probing of the functional relevance of mutations in *BCL2* is required for the significance in various disease contexts.

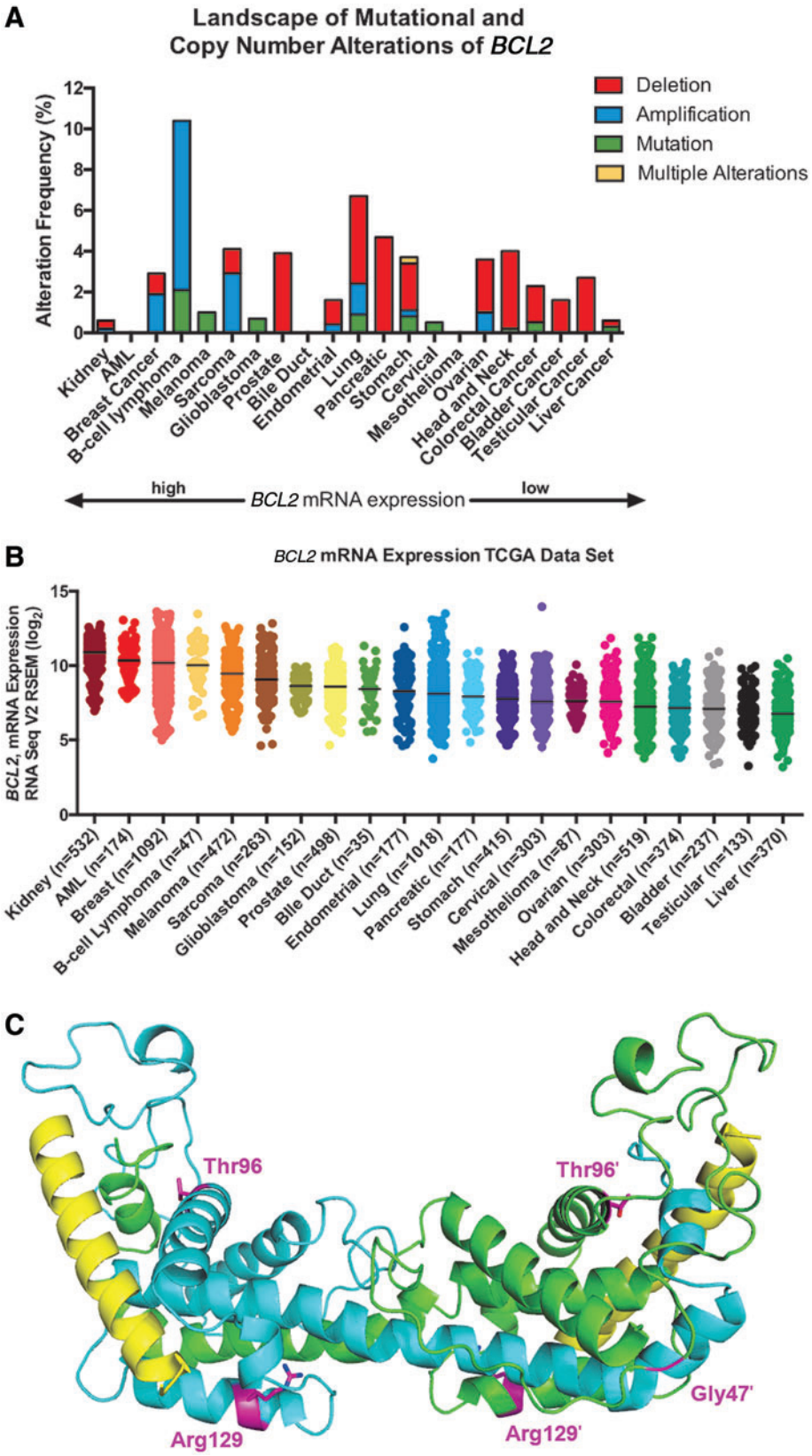

FIG. 3. ***BCL2*** **gene expression, mutational landscape, and copy number variations in cancer.** **(A)** TCGA data sets retrieved from cBioPortal indicate copy number and mutation alterations across 21 cancer types. Deletions (*red*), amplifications (*blue*), mutations (*green*), and multiple alterations (*yellow*) are shown as their alteration frequency in cancers sorted from high *BCL2* mRNA expression to low *BCL2* mRNA expression. **(B)** *BCL2* mRNA expression across 21 cancer types from TCGA data sets retrieved from cBioPortal. **(C)** Putative homodimer structure of Bcl-2 bound to Bax BH3 peptide with mutations highlighted. Color guide: *blue* - Bcl-2 molecule 1; *green* - Bcl-2 molecule 2; *yellow* - Bax BH3 peptides; *violet* - mutations identified in TCGA (not visible/highlighted: Gly47 in Bcl-2 molecule 1, Gly128 in either Bcl-2 molecule). TCGA, The Cancer Genome Atlas. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

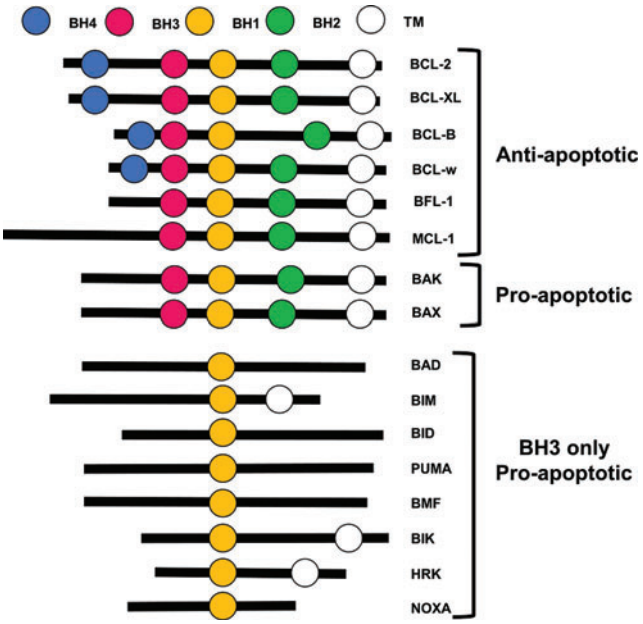

FIG. 4. Schematic view of Bcl-2 family proteins. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Structural knowledge of Bcl-2 and its interactions with peptides and small molecule inhibitors. The structure of Bcl-2 family proteins has been extensively characterized by X-ray crystallography and NMR spectroscopy, and in the majority of cases, structural details of interactions with other proteins and prospective inhibitors are known. Bcl-2 family proteins are characterized by the presence of one or more BH domains. The BH3 domain is the most highly conserved, being present in all members of the Bcl-2 antiapoptotic multidomain, proapoptotic multidomain, and BH3-only domain members (Fig. 4). The antiapoptotic members contain all BH1-BH4 domains, except MCL-1 (induced myeloid leukemia cell differentiation protein) and A1, which lack the BH4 motif. Bcl-2 itself features four BH domains (in sequence order, BH4, BH3, BH1, and BH2), which facilitate protein/protein interactions with proteins from and outside of the Bcl-2 family. The majority of the family proteins, excluding some of the BH3-only members (Bad, Bmf, Bid, Puma, and Noxa), possess a TM domain that is critical for homo- and hetero-oligomerization at the mitochondrial membrane (135, 191).

The structure of Bcl-2 was first determined by NMR spectroscopy using a Bcl-2 construct, in which the loop between the BH4 and BH3 motifs was replaced by the equivalently located and shorter loop from the related protein Bcl-xL (146). This construct has been used to provide the majority of Bcl-2 structures (Table 1). The overall structural similarity of Bcl-2 to Bcl-xL, the native (119) and peptide-bound (147, 161) structures of which were already known at the time, was revealed and provided the first indications as to the different peptide binding specificities of the two proteins.

The structure of Bcl-2 has since been resolved with a small range of peptide interactors, including fragments from naturally occurring proteins as well as designed peptides (Table 1). In all cases, binding is achieved *via* an α-helix (13, 74, 86) or α-helix-like (25) structure interacting with a groove formed by the BH3, BH1, and BH2 domains (Fig. 5). Furthermore, peptides are oriented within the groove in the same way in all structures, with the N-terminal oriented

TABLE 1. EXPERIMENTALLY DETERMINED BCL-2 STRUCTURES

PDB ID[a]	*Method*	*Resolution (Å)*	*Resolved portion of structure*[b]	*Bound molecules*[c]	*Reference*
1G5 M	NMR	N/A	3-34, 92-207	None	(146)
1GJH	NMR	N/A	3-34, 92-207	None	(146)
1YSW	NMR	N/A	3-34, 92-207	2-phenethylbenzothiazole analog of Compound **1**	(134)[d]
2O21	NMR	N/A	3-34, 92-207	2-phenethylbenzothiazole analog of Compound **1**	(18)[d]
2O22	NMR	N/A	3-34, 92-207	Compound **1b**	(18)[d]
2O2F	NMR	N/A	3-34, 92-207	Compound **43a**	(18)[d]
2 W3L	X-ray	2.10	9-35, 92–203	Phenylpyrazole **2**	(149)
2XA0	X-ray	2.70	10–31, 92–206	Bax BH3 peptide	(86)
4AQ3	X-ray	2.40	9-31, 92–203	Compound **18**	(141)
4IEH	X-ray	2.10	9-31, 92–204	Compound **6**	(173)
4LVT	X-ray	2.05	9-32, 92–204	Navitoclax (ABT-263)	(167)[d]
4LXD	X-ray	1.90	6-33, 92–204	Compound **2**	(167)[d]
4MAN	X-ray	2.07	8-29, 92–204	Indole analog of compound **2**	(167)[d]
5AGW	X-ray	2.69	8-31, 92–204	***α/β*-1**	(25)
5AGX	X-ray	2.24	8-32, 92–204	***α/β*-1-LIN**	(25)
5FCG	X-ray	2.10	6-34, 92–203	Peptide from HBx protein	(74)
5JSN	X-ray	2.10	7-32, 87–207	αBCL2 peptide	(13)[d]

[a]All structures obtained from Bcl-2:Bcl-x(L) chimera except 2XA0, 5FCG, and 5JSN, which were determined from full-length Bcl-2.
[b]All structures have been determined from the Bcl-2 construct in which the BH4-BH3 loop of Bcl-2 replaced with shorter loop from Bcl-xL, except PDB 5JSN. In the NMR structures, the structure resolved is continuous; the replaced loop is largely not resolved in X-ray.
[c]To avoid long systematic names, compounds are named according to their designation in their original publication; see the original publication cited in the corresponding row of the Reference column for specific compound details.
[d]This study also reports ligand complexes with Bcl-xL; see the respective publication for further details.
Bcl-2, B cell lymphoma 2; BH, Bcl-2 homology domains; N/A, not applicable.

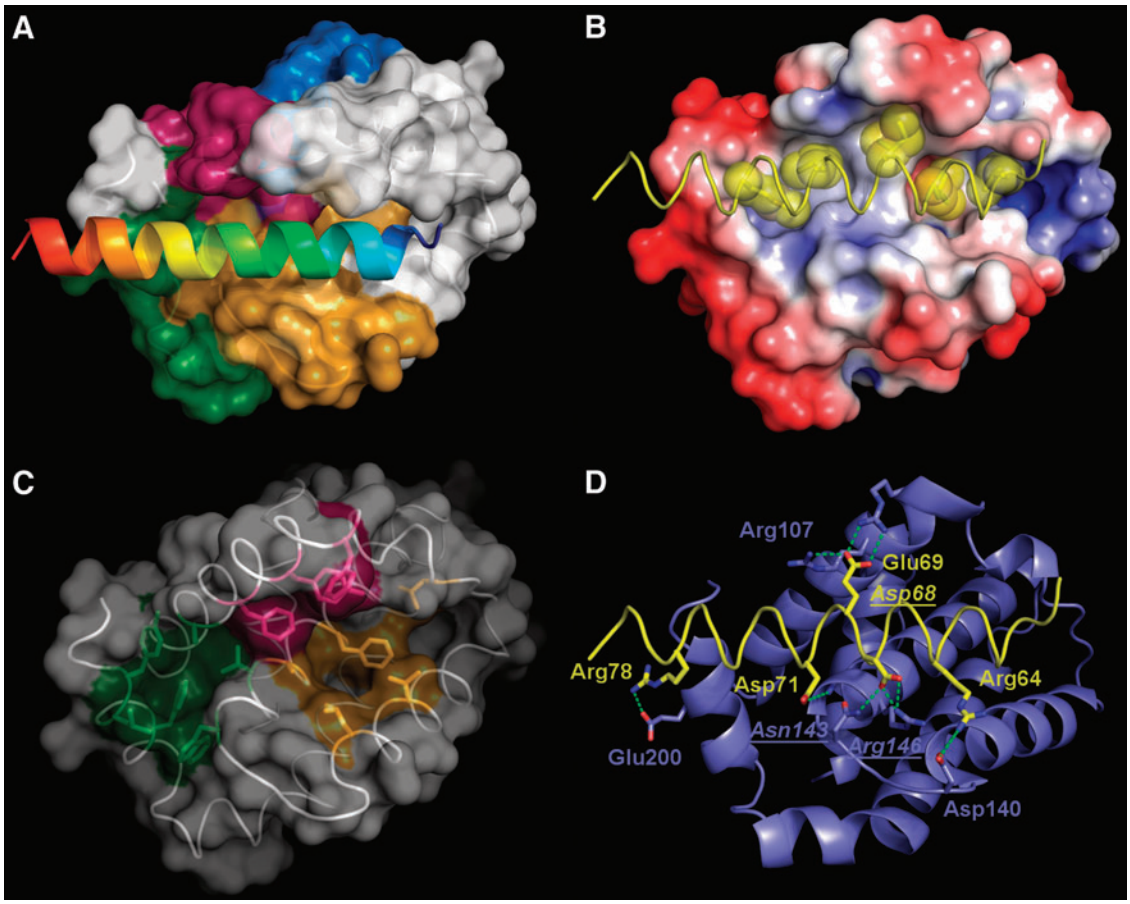

FIG. 5. Overview of the structural basis of Bcl-2 recognition of peptides. (A) Bcl-2 in complex with Bax BH3 peptide (PDB 2XA0). Bcl-2 shown as a surface, with the BH4 (*blue*), BH3 (*magenta*), BH1 (*yellow*), and BH2 (*green*) motifs highlighted. Bax BH3 peptide shown in cartoon view, colored as a *blue* to *red* rainbow from the N to the C terminus. **(B)** Electrostatic potential surface of Bcl-2 in complex with Bax BH3 peptide (*yellow*). Hydrophobic residues of the Bax BH3 peptide interacting with Bcl-2 shown as transparent spheres. Electrostatic potential shown as a *red-white-blue* gradient, from negative (*red*) to neutral (*white*) to positive (*blue*). **(C)** The three major binding sites within the Bcl-2 binding groove. **(D)** Hydrogen bonding interactions observed in the Bax BH3 complex with Bcl-2. Asn143 and Arg146 of Bcl-2, and Asp68 of Bax BH3 labeled in italics and underlined to indicate that these residues are involved in hydrogen bonds replicated in the majority of peptide complexes with Bcl-2. Electrostatic potential calculated using the PDB2PQR/APBS web server; figures generated in PyMOL. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

toward the BH1 domain and the C-terminal oriented toward the BH2 domain; peptide binding to Bcl-xL also displays the same directionality in all cases (7, 49, 93, 94, 132). Peptide binding to Bcl-2 appears largely mediated by a series of up to five hydrophobic residues (typically branched-chain amino acids) on one face of the peptide helix interacting with hydrophobic pockets along the Bcl-2 binding groove; this feature is present in all peptides cocrystallized with Bcl-2, both naturally derived and designed peptides. Three hydrophobic pockets of Bcl-2 with which peptides interact may be defined: a large pocket formed largely by BH1-derived residues (herein referred to as the ''BH1 pocket''; Leu119, Val133, Leu137, Ala149, and Phe153), a smaller pocket formed from near the end of the BH3 domain (herein referred to as the ''near-BH3 pocket''; Phe104, Tyr108, and Phe112), and a second large pocket formed from residues from the BH3, BH1, and BH2 domains (herein referred to as the ''BH2-incorporating pocket''; Ala100, Phe104, Trp144, Val148, Phe198, Leu201, and Tyr202). Peptides engaging all three pockets generally bind, from N to C terminal, the BH1 pocket with the most N-terminal hydrophobic residues (1–2 residues), the near-BH3 pocket with the middle hydrophobic residue, and then, the BH2-incorporating pocket with the final hydrophobic residues (1–2 residues). The HBx-derived peptide primarily engages the BH1 pocket, while the αBCL2 peptide uses a single leucine to engage the BH1 and near-BH3 pockets. In addition, a range of supporting hydrogen bonds are present in each structure, however, just two are present in all structures (except the HBx complex); these are the interactions of an acidic residue (typically aspartate) between the hydrophobic residues engaging the BH1 and BH2-incorporating pockets with Asn143 and Arg146 of Bcl-2. As the majority of hydrogen bonds in the peptide complexes with Bcl-2 are solvent exposed, these may be transient in typical cellular conditions. These hydrogen bonds are not replicated in any of the small molecules structurally characterized in complex with Bcl-2.

The structure of Bcl-2 has also been extensively characterized in complex with prospective small molecule inhibitors

(Table 1) (18, 134, 141, 149, 167, 173). Due to the similarities between the Bcl-2 and Bcl-xL binding pockets, many of these inhibitors have also been structurally characterized in complex with Bcl-xL; achieving selectivity for Bcl-2 over Bcl-xL is a major challenge in the design of clinically useful Bcl-2 inhibitors (8). Inhibitors structurally characterized in complex with Bcl-2 typically engage Bcl-2 in a remarkably similar way to the peptides, featuring long structures capable of binding to all three of the major pockets of the Bcl-2 binding groove; this also gives the majority of inhibitors molecular weights well over 500 Da, outside the typical range for small molecule drugs (103). Most inhibitors feature a small hydrophobic group, typically a single aromatic ring, bound to the BH1 pocket, a relatively straight structure comprising two rings connected in series, followed by a sulfonamide, lying between the BH1 and BH2-incorporating pockets, and a large flexible hydrophobic group interacting with the BH2-incorporating pocket (Fig. 6); these molecular features are largely carried over from the prototypical Bcl-2 inhibitor, ABT-737 (discussed in further detail in section IV: A Brief Overview of Bcl-2 Inhibitors: Past, Present, and Future). An exception to this is phenylpyrazole **2** (149), which does not engage the BH2-incorporating pocket; despite this, phenylpyrazole **2** exhibits ~10-fold selectivity for Bcl-2 over Bcl-xL, although its overall potency is eclipsed by ABT-737. Further modification of phenylpyrazole **2** yielded compound **18** (141), which does extend to the BH2-incorporating pocket and displays overall greater structural similarity to ABT-737.

Reactive oxygen species and their role in cell signaling

Reactive oxygen species (ROS) collectively refer to oxygen-derived (O_2) free radical and nonradical species (31). Radical ROS include superoxide anion ($O_2^{\cdot -}$), hydroxyl radical (HO•), peroxyl radical (ROO•), and alkoxyl radical (RO•) (61). Nonradical ROS include hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (153, 172). A tight balance between intracellular ROS and ROS regulating systems is critical for maintaining cellular homeostasis (38). Therefore, excessive generation of ROS or deficient antioxidant capacity alters cellular redox state with profound effects on cell growth, proliferation, and survival. Homeostatic levels of ROS are maintained by both enzymatic and nonenzymatic antioxidants. A depletion or overexpression of these antioxidants can alter cell fate. For example, overexpression of antioxidant enzyme SOD2 can protect cells from tumor necrosis factor (TNF)-induced apoptosis (110). This protected the cell from H_2O_2-induced cell death and promoted cell survival. On the contrary, SOD1 has been shown to be a potential therapeutic target in some cancers (56). These contrasting roles for similar antioxidant enzymes demonstrate that the tight balance of ROS levels can determine whether cells will survive or undergo apoptosis. The dogmatic view that any increase in intracellular ROS is linked to cell injury and death has been challenged by substantial experimental evidence attributing a secondary messenger function to a mild increase in ROS or a ''pro-oxidant'' milieu. Alterations in the cellular redox metabolism is linked to aging (105) as well as a host of pathological states, such as cancer (102), Alzheimer's disease (112), Parkinson's disease (29), diabetes (71), atherosclerosis (62), nonalcoholic fatty liver disease (17), and asthma (43).

Intracellular sources of ROS. The mitochondrion serves as an important source of intracellular ROS [elegantly reviewed in (30)], mainly generating $O_2^{\cdot -}$ from Complex I (NADH dehydrogenase) (60) and Complex III (cytochrome *c* reductase) (120), when electrons derived from NADH or $FADH_2$ leak out on to and reduce molecular oxygen (121). The mitochondrial $O_2^{\cdot -}$ levels are regulated by the action of manganese superoxide dismutase (MnSOD) in the inner matrix that generates H_2O_2 in the process (118). H_2O_2 in turn can be scavenged by catalase, glutathione peroxidases, and peroxiredoxins (85). A second important site of $O_2^{\cdot -}$ generation, best exemplified in phagocytic cells, is the NADPH oxidase (NOX) family of enzymes. NOX enzymes are made up of six subunits: a Rho guanosine triphosphatase (GTPase) and five phagocytic oxidase subunits (gp91, p22phox, p40phox, p47phox, and p67phox) (91). Additional sources of ROS include $O_2^{\cdot -}$ from xanthine oxidases (88), cyclooxygenases or prostaglandin-endoperoxide synthase (PTGS) (55), the cytochrome P450 enzyme family (14), and nonheme lipoxygenases (21).

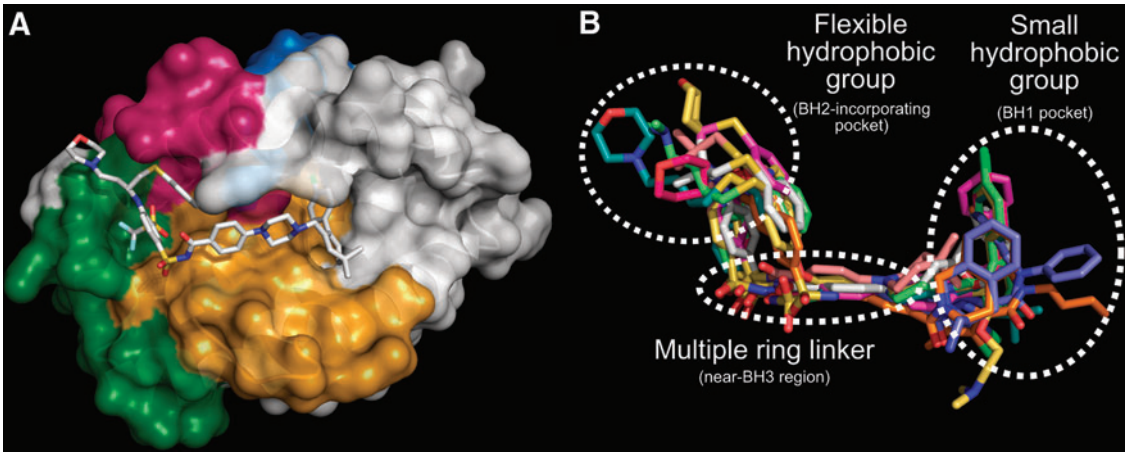

FIG. 6. Structural determinants of small molecule interactions with Bcl-2. (A) Bcl-2 in complex with navitoclax (ABT-263) (PDB 4LVT). **(B)** Overlay of all small molecules structurally characterized in complex with Bcl-2, highlighting key molecular features present in the majority of ligands. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

ROS as signaling molecules. It is now widely accepted that ROS function as important signaling molecules, implicated in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinase (MAPK), Keap1-Nrf2-ARE, PI3K-Akt, Notch, and Wnt signaling pathways (20, 192). The various functional consequences of an altered redox milieu have been associated with a critical balance between the two major ROS molecules, $O_2^{\cdot -}$ and H_2O_2 (34, 145, 156). Whereas an overwhelming increase in either of these species results in cell injury and death, a tilt in the ratio in favor of $O_2^{\cdot -}$ confers a survival advantage, while a significant shift toward H_2O_2 creates a permissive environment for death execution (84, 144). For example, H_2O_2 is known to suppress Wnt/β-catenin signaling, an important survival and growth pathway, through a variety of mechanisms, including targeting the interaction between nucleoredoxin and disheveled (Dvl) (52) and *via* oxidative modification of the zinc-coordinating cysteines of tankyrase resulting in its inactivation (78). Interestingly, ROS-mediated Wnt signaling regulation of *BCL2* involving GSK3β/β-catenin has been reported on chronic exposure to Cr(VI) compounds (166). NF-κB signaling is closely linked to the transcriptional regulation of Bcl-2(22). Furthermore, Nrf2-ARE signaling can also regulate *BCL2* transcription through the antioxidant response elements (ARE) located within the *BCL2* promoter under conditions of oxidative stress (129). Interestingly, there is evidence of considerable cross talk between Nrf2 and NF-κB (181). For a more detailed account of the disparate functional outcomes on changes in intracellular ROS, please refer to these two comprehensive reviews (153, 154).

The cross talk between cellular redox status and Bcl-2

Apart from the role of Bcl-2 as a major regulator of apoptosis and cell fate, there is convincing experimental evidence to indicate a close interplay between cellular redox status and Bcl-2 expression. Of note, there are reported observations supporting both a pro-oxidant activity and an antioxidant activity of Bcl-2 (68, 197). Not only does the expression of Bcl-2 impact intracellular redox milieu but also a reciprocal regulation of Bcl-2 expression and/or function has been associated with intracellular ROS (26). For example, Bcl-2 has been shown to modulate intracellular ROS through increased catalase and glutathione peroxidase/reductase expression (46), as well as increased total levels of NADPH and reduced glutathione (GSH) (47). On the flip side of it, there is emerging and significant evidence that Bcl-2 expression impacts mitochondrial ROS metabolism to stimulate $O_2^{\cdot -}$ production, through increased COX activity, thereby creating a pro-oxidant environment that favors cell survival (26, 145).

The mRNA expression of *BCL2* is also tightly correlated with the expression of genes involved in ROS detoxification and production (Fig. 7). Many peroxidase elimination enzymes, such as peroxiredoxins, and other redox proteins, such as thioredoxins, exhibit a strong negative correlation with *BCL2*. This relationship may be due to an intrinsic mechanism through which cellular redox levels are in a constant pro-oxidant state, facilitating cancer cell survival. This pro-oxidant environment has been established as critical for the stability and antiapoptotic function of Bcl-2 (107). This hypothesis is also supported by a strong positive correlation between $O_2^{\cdot -}$ producing enzymes NADPH oxidases, NOX2 and NOX4. Interesting to note is the strong negative correlation between *BCL2* and *SOD1* across 18 of the 21 cancers surveyed, while SOD2 only exhibits a negative correlation in tumors with higher expression of *BCL2*. *SOD3*, the extracellular superoxide dismutase, was found to be positively correlated with Bcl-2 expression. The negative correlation of *BCL2* with *SOD1* in multiple cancers supports the notion that Bcl-2 may also function to maintain the pro-oxidant intracellular milieu necessary for cell survival (107). These data suggest that the expression of *BCL2* is strongly correlated with the expression of many antioxidant enzymes and the tight interplay between regulating intracellular redox levels plays an important role in cell fate, especially in the context

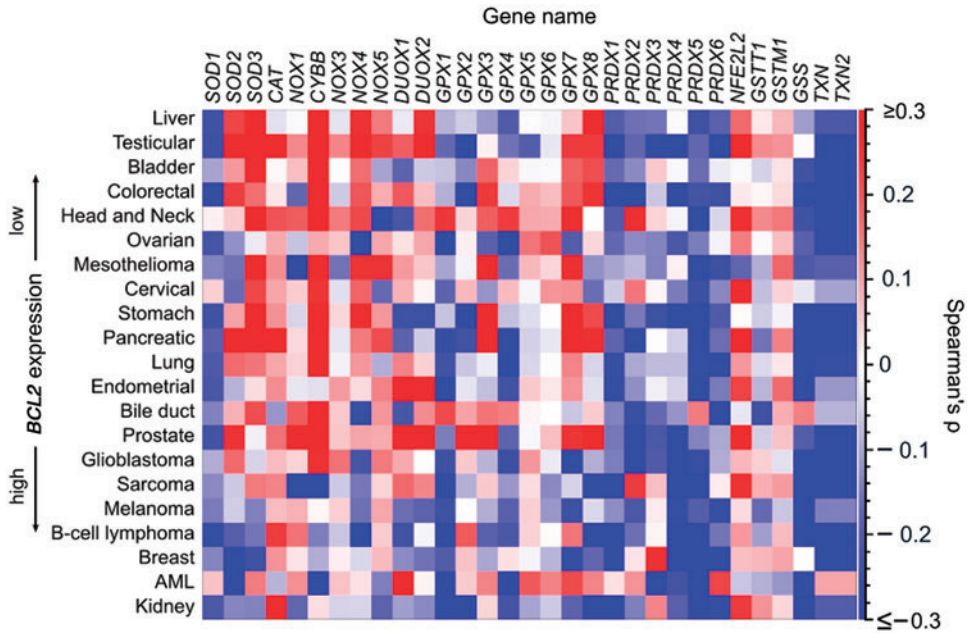

FIG. 7. Correlation between *BCL2* and antioxidant genes. Cancers were sorted from Bcl-2 high to low mean mRNA expression as obtained from TCGA data sets. Correlations (Spearman's ρ) are indicated as *red-white-blue* gradient (positive-none-negative). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

of carcinogenesis. A list of key publications in terms of Bcl-2 and the regulation of Bcl-2 by ROS have been described in Table 2.

Bcl-2 serves as a protector against oxidative insult. The protective role of Bcl-2 on an overt oxidative stress was originally discovered after Bcl-2-overexpressing cells demonstrated increased resistance to H_2O_2 and menadione-induced cell death (68). Subsequently, contradicting evidence has emerged to suggest that Bcl-2-mediated cellular protection from H_2O_2 was both independent (3) and dependent (73) on NF-κB activation. The latter study indicated that Bcl-2 induces constitutively active NF-κB signaling, resulting in the upregulation of γ-glutamylcysteine ligase, the first enzyme in the biosynthetic pathway of GSH (73). Transcriptional regulation of *BCL2* by NF-κB is now well characterized (22, 63). Bcl-2 is able to provide protection against other oxidizing species, including potassium cyanide, *tert*-butyl hydroperoxide (165), and γ-irradiation (122, 196).

The protective role of Bcl-2 also includes safeguarding against nitric oxide (NO)-induced apoptosis (116). Bcl-2-overexpressing murine macrophages exposed to NO donors, *S*-nitrosoglutathione (GSNO) and spermine-NO, and activators of inducible NO, lipopolysaccharide and interferon-γ, demonstrated apoptotic resistance to that of their *neo* control cells (116). While it has often been hypothesized that the mechanism by which Bcl-2 protects against oxidative insult is an intrinsic antioxidant property (68, 69, 95, 157), current evidence suggests that the antioxidant activity of Bcl-2 is merely a secondary effect (36, 54). Bcl-2 may be thought of as an ''antioxidant-by-proxy'' when in complex with GSH (197); this is discussed in detail in the following section (Mitochondrial-Dependent Regulation of Cellular Redox by Bcl-2). Cox and Hampton (36) demonstrated that Bcl-2 was able to protect cells against apoptotic (<200 μM), but not necrotic (>200 μM), doses of H_2O_2. These cells also showed no difference in the levels of GAPDH and peroxiredoxin 2 oxidation, while exhibiting evidence of increased micronuclei formation and genomic instability. This suggests that the antiapoptotic and pro-oncogenic role of Bcl-2 is through preventing the elimination of cells damaged by oxidation (36). Besides the apparent antioxidant activity of Bcl-2, recent evidence also suggests that Bcl-2 promotes cell survival by providing a pro-oxidant environment capable of supporting tumorigenesis (26, 170, 189).

Mitochondrial-dependent regulation of cellular redox by Bcl-2. Mitochondrial metabolism and its redox environment are highly dependent on Bcl-2 and GSH (111). In this respect, Bcl-2 regulates the mitochondrial pool of GSH and may act as a redox sensor. GSH may be displaced from Bcl-2 *via* competitive binding of BH3-only proteins (*e.g.*, Bim) and BH3 mimetics (*e.g.*, ABT-737) and induces mitochondrial dysfunction, oxidative stress, and cell death (197). Recently, it was demonstrated that Bcl-2 and 2-oxoglutarate carrier (OGC), a glutathione transport molecule, directly interact in the presence of GSH. This suggests that Bcl-2 and OGC participate in the transport of GSH to increase the glutathione

Table 2. List of Milestone Publications for Bcl-2 and Its Interplay with Reactive Oxygen Species

Key publications in the interplay between Bcl-2 and ROS

Authors	*Year*	*Journal*	*Publications*
Tsujimoto *et al.* (175)	1984	*Science*	Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation
Hockenbery *et al.* (67)	1990	*Nature*	Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death
Hockenbery *et al.* (68)	1993	*Cell*	Bcl-2 functions in an antioxidant pathway to prevent apoptosis
Oltvai *et al.* (135)	1993	*Cell*	Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death
Yin *et al.* (191)	1994	*Nature*	BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax
Muchmore *et al.* (119)	1996	*Nature*	X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death
Petros *et al.* (146)	2001	*PNAS*	Solution structure of the antiapoptotic protein bcl-2
HIldeman *et al.* (66)	2003	*PNAS*	Control of Bcl-2 expression by reactive oxygen species
Clement *et al.* (33)	2003	*Cell Death and Differentiation*	Decrease in intracellular superoxide sensitizes Bcl-2-overexpressing tumor cells to receptor and drug-induced apoptosis independent of the mitochondria
Oltersdorf *et al.* (134)	2005	*Nature*	An inhibitor of Bcl-2 family proteins induces regression of solid tumours
Cox and Hampton (36)	2007	*Carcinogenesis*	Bcl-2 over-expression promotes genomic instability by inhibiting apoptosis of cells exposed to hydrogen peroxide
Chen and Pervaiz (26)	2007	*Cell Death and Differentiation*	Bcl-2 induces pro-oxidant state by engaging mitochondrial respiration in tumor cells
Chen and Pervaiz (27)	2010	*Cell Death and Differentiation*	Involvement of cytochrome c oxidase subunits Va and Vb in the regulation of cancer cell metabolism by Bcl-2
Velaithan *et al.* (178)	2011	*Blood*	The small GTPase Rac1 is a novel binding partner of Bcl-2 and stabilizes its antiapoptotic activity
Low *et al.* (107)	2014	*Blood*	Ser70 phosphorylation of Bcl-2 by selective tyrosine nitration of PP2A-B56delta stabilizes its antiapoptotic activity

GTPase, guanosine triphosphatase; PP2A, protein phosphatase 2, ROS, reactive oxygen species.

mitochondrial pool, and a mechanism through which the mitochondria are protected from oxidative stress (184). It has been shown that Bcl-2 overexpression relocalized GSH to the nucleus, altering nuclear redox and blocking caspase activation to promote cell survival (179). Glutathione homeostasis regulated by Bcl-2 has been shown in MCF-7 breast cancer cells, where Bcl-2 overexpression increases glutathione content, although this was found to be independent of changes in gene expression related to glutathione synthesis. The inhibition of glutathione synthesis was able to overcome Bcl-2-induced cisplatin resistance (160).

The mechanisms by which Bcl-2 regulates mitochondrial respiration are vital for the understanding and interpretation of the overall cellular redox environment (106). Bcl-2 expression has been linked to the activity of cytochrome *c* oxidase (COX), the rate-limiting enzyme in mitochondrial electron transport chain (ETC), and thus vital in the regulation of mitochondrial respiration (26). Leukemic lymphoblastic cells (CEM cell line) overexpressing Bcl-2 exhibited higher levels of mitochondrial $O_2^{\cdot-}$, oxygen consumption, and higher COX activity, while the opposite was seen after the introduction of siRNA directed at *BCL2* (26, 27). It should be pointed out that the effect of Bcl-2 on mitochondrial COX activity and oxygen consumption is dependent on the redox milieu of the mitochondria. In this regard, under states of normoxia, Bcl-2 upregulates mitochondrial respiration and COX activity through increased import and assembly of COX subunits Va and Vb (27).

Evidence also suggests a possible interaction between Bcl-2 and COX Va, and higher expression of Bcl-2 promoted the mitochondrial translocation of COX Va. On the contrary, during states of overt oxidative stress induced on pharmacologically inhibition of mitochondrial ETC complexes, Bcl-2 overexpression elicited a negative effect on mitochondrial respiration and COX activity. The latter is corroborated by a recent report demonstrating that conditional *BCL2* knockout in murine pancreatic β cells exhibited increased superoxide dismutase (SOD) activity, increased mitochondrial respiration, and ultimately, cell death (2). The regulatory effect of Bcl-2 on mitochondrial respiration has also been reported in neuronal cells (77), in $SOD1^{G93A}$ mouse models of amyotrophic lateral sclerosis (138), rat ascites hepatoma (165), and hepatocytes (176).

Bcl-2-dependent increase in mitochondrial $O_2^{\cdot-}$ levels was also associated with the downstream activation of signal transducer and activator of transcription 3 (STAT3), which was mediated through a function of Rac1 (79). Overexpression of Bcl-2 induced phosphorylation of STAT3 on Tyr705, which was mediated by increased $O_2^{\cdot-}$. Furthermore, constitutively active mutants of STAT3 increased mitochondrial $O_2^{\cdot-}$ production (79). This study demonstrates the numerous signaling pathways that Bcl-2 can influence in regulating mitochondrial redox metabolism.

Bcl-2 family members have also been shown to mediate the switch between mitochondrial fusion and fission, which in turn suggests an influence of the family on mitochondrial metabolism and bioenergetics (183). Mammalian proteins Drp-1 and Fis-1 have been shown to be crucial in mitochondrial fission, while Mfn1/2 and OPA1 have been shown to play a role in fusion events. Bcl-2 family members Bax and Bak are required for mitochondrial fusion, where Bax interacts directly with Mfn2 (80). Bax has also been shown to colocalize with both Drp1 and Mfn2 during apoptosis and promotes mitochondrial fragmentation (164). Mammalian Bcl-2 is yet to be identified in having a role in either profusion or fission events (159), although it has also been described in the *Caenorhabditis elegans* homologue CED-9 (108). The study by Lu *et al.* found that CED-9 interacts with DRP-1 to promote mitochondrial fission. One could then speculate that Bcl-2 may function as a receptor for DRP-1 in a mammalian system to promote changes in mitochondrial dynamics and metabolism, although this is yet to be proven (159).

Taken together, it appears that the regulation of mitochondrial ROS extends into a variety of cellular contexts, and therefore, an alternative therapeutic strategy against refractory cancers could be to target key players involved in the regulation of mitochondrial metabolism to favorably modulate the cellular redox milieu for death execution (142).

Oxidative stress induced transcriptional regulation of *BCL2*. Early studies assessing the relationship between oxidative stress and *BCL2* expression indicated that acute oxidative stress induced by bright light in retinal rod receptor cells and cyclosporin A in human endothelial cells reduced *BCL2* expression (104). Later studies in t(14;18) lymphoma cells revealed that *BCL2* expression is tightly linked to NF-κB activation by the presence of cyclic AMP response element (CRE) and Sp1 binding sites (63). In prostate cancer cells, the overexpression of p50/p65 subunits of NF-κB increased expression of *BCL2*, and likewise, stimulation with TNF-α resulted in an increase in *BCL2* promoter activity (22). In U937 cells, NF-κB has also been implicated in ROS-induced upregulation of *BCL2* (37). *BCL2* transcriptional regulation has also been linked to Sonic hedgehog signaling through gli-1 and Wnt/β-catenin signaling by Wnt3a stimulation in osteoblast progenitor cells (5).

ROS have been shown to regulate *BCL2* gene expression indirectly, through which variations in cellular ROS levels lead to both an increase or decrease in *BCL2* gene expression depending on the cell context. Hildeman *et al.* (66) demonstrated that the addition of antioxidant manganese (III) tetrakis (4-benzoic acid)porphyrin (MnTBAP) to T cells *in vivo* significantly increased *BCL2* gene expression, although did not regulate other Bcl-2 family members, including Bcl-xL, Bad, and BimEL. MnTBAP also decreased intracellular ROS levels and inhibited death execution. ROS production was shown to be upstream of *BCL2* downregulation and to involve a Bim-independent mechanism. These results were mirrored by retroviral expression of catalase, which increased *BCL2* expression and rescued cells from oxidative stress-induced cell death (66). Similarly, H_2O_2 induced downregulation of Bcl-2 protein and gene expression, while concurrently upregulating Bax in cardiac myocytes (113). While the majority of studies have found that *BCL2* was downregulated when exposed to oxidative stress, Kaufmann *et al.* found that aged rats had increased *BCL2* expression in the hippocampus and cerebellum, which was a consequence of oxidative stress (81).

Bcl-2 regulation through oxidative stress was also demonstrated in hippocampal neurons, where the addition of H_2O_2 and glucose oxidase resulted in a decrease in *BCL2* gene and protein expression (150). The downregulation of Bcl-2 and concurrent increase in oxidative stress-induced apoptosis were able to be rescued through the addition of *N*-acetylcysteine (NAC), which subsequently increased Bcl-2 expression (151).

Transcriptional regulation of *BCL2* is partly controlled by cyclic AMP response element binding protein (CREB) (185). Oxidative insult through H_2O_2 or glucose oxidase decreased CREB activity on the *BCL2* promoter, which was reversed in the presence of NAC and manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP) (150). In rat liver, the induction of oxidative stress with triiodothyronine (T3) resulted in increased Bcl-2 expression, which was abrogated with the addition of α-tocopherol (vitamin E) (48). These studies demonstrate that Bcl-2 not only regulates the cellular redox milieu but oxidative stress itself can reciprocally regulate Bcl-2 expression as well.

Posttranslational modifications of Bcl-2 mediated by oxidative stress. The regulation of apoptosis by Bcl-2 has been shown to be mediated through various kinases, ligands, and oxidative stress signals due to changes in the expression and function of antioxidant enzymes. The regulation of Bcl-2 by posttranslational modification induced by oxidative stress is summarized in Figure 8. Bcl-2 suppression of apoptosis is partly facilitated through its direct interaction with extracellular signal-related kinase (ERK) 1/2 (109). This interaction can be regulated through oxidative stress, through which increased H_2O_2 induces Bcl-2 cysteine oxidation and the disruption of the ERK1/2-Bcl-2 complex. Mutagenesis indicated that the key residues regulating this interaction were Cys158 and Cys229. Cys158 is located adjacent to the Bcl-2 BH1 domain and is buried in both the monomeric and putative dimer forms (Fig. 2C) of the protein, although may become exposed during the conformational change that would be required to achieve dimer formation; Cys229 is suggested to be located within the Bcl-2 transmembrane helix. A C158A and C229A double mutant was resistant to oxidation, as well as H_2O_2-induced ubiquitination and subsequent degradation (109). The degradation of Bcl-2 through the ubiquitin-mediated pathway is also regulated by TNF-α-induced oxidative stress. H_2O_2 and TNF-α-induced oxidative stress resulted in degradation of Bcl-2, which was linked to the dephosphorylation of Ser87. The resultant dephosphorylation after TNF-α stimulation was demonstrated to be protein phosphatase 2 (PP2A) and PP2B independent (16). H_2O_2 has also been shown to regulate PKC-mediated Bcl-2 phosphorylation through a phospholipase Cγ1-dependent (PLC-γ1) mechanism. *Plcg1* null cells exhibited reduced viability and reduced Bcl-2 phosphorylation, following an insult with low levels of H_2O_2 (10).

Furthermore, c-Jun N-terminal kinase (JNK) activation can induce apoptosis through phosphorylation and inactivation of Bcl-2 (188). In response to menadione-induced oxidative stress, JNK phosphorylation and degradation of Bcl-2 were increased, but could be blocked on addition of NAC (193). Contrary to this study, Kelkel *et al.* demonstrated

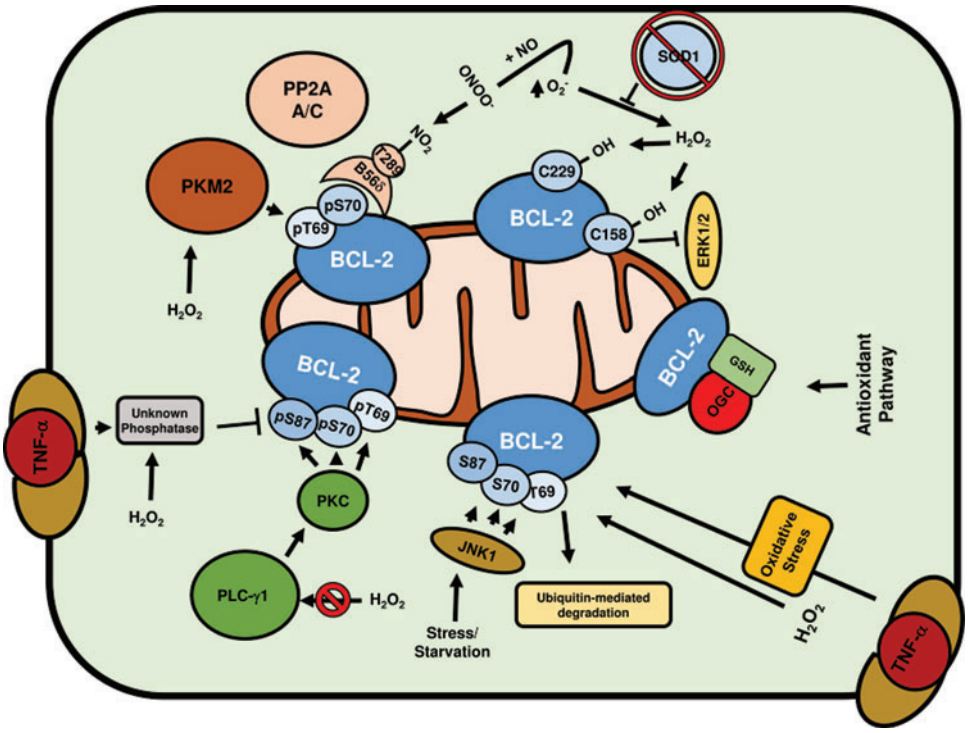

FIG. 8. Schematic of post-translational modifications of Bcl-2 due to oxidative stress. Posttranslational modifications of Bcl-2 occur under an insult of oxidative stress. Depletion or inhibition of SOD1 can initiate nitration of T289 on PP2A's B56δ subunit, preventing binding to the A/C subunits and the generation of the *holo*-enzyme. This prevents dephosphorylation of Ser70 of Bcl-2, stabilizing its antiapoptotic ability. Hydrogen peroxide can stabilize Bcl-2 through PKM2-mediated phosphorylation of T69, as well as causing its degradation through the ubiquitin-mediated pathway. PLC-γ1 mediates hydrogen peroxide resistance allowing phosphorylation of key residues by PKC and stabilization, while the opposite can also occur where hydrogen peroxide can stimulate dephosphorylation of Bcl-2. During cellular stress and starvation, JNK1 has also been shown to phosphorylate Bcl-2 at T69/S70/S87. Bcl-2 interacts with GSH and OGC to regulate the mitochondrial pool of GSH. GSH, glutathione; H_2O_2, hydrogen peroxide; NO, nitric oxide; $ONOO^-$, peroxinitrite; OGC, 2-oxoglutarate carrier; PLC-γ1, phospholipase Cc1-dependent; PPA2, protein phosphatase 2. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

that JNK activation and phosphorylation of Bcl-2 were independent, *via* the addition of the ROS-inducing agent diallyl tetrasulfide to U937 human histiocytic lymphoma cells (82). These opposing results suggest that redox-dependent post-translational modifications of Bcl-2 are highly dependent on the level of ROS. High levels of H_2O_2 primarily result in oxidation of Bcl-2 and degradation, while low levels initiate phosphorylation and stabilization of Bcl-2.

Functionally, ROS-mediated Bcl-2 phosphorylation has been linked to cell cycle transitions (39). The expression of phosphomimetic, gain-of-function Bcl-2 mutants (S70E and T69E/S70E/S87E) reduced intracellular ROS and inhibited G1/S cell cycle progression. The reduced G1/S phase progression was overridden by the addition of H_2O_2, through the downregulation of p27 and increased activation of Cdk2. Decreased ROS were attributed to increased SOD1 and catalase expression, which resulted in an antioxidant intracellular milieu on expression of the Bcl-2 phosphomimetics (39).

The relationship between the members of the SOD family and Bcl-2 has been explored in a number of cellular contexts. In spinal cord mitochondria, mutant SOD1 (G93A) and wild-type Bcl-2 have been shown to interact in the mitochondria, where they promote structural abnormalities and mitochondrial dysfunction. The mutant SOD1 causes a conformational change in Bcl-2, resulting in the exposure of the BH3 domain, which promotes mitochondrial toxicity (140). Low *et al.* (107) demonstrated that the stability and antiapoptotic function of Bcl-2 were enhanced in a pro-oxidant environment. Specifically, reducing SOD1 activity either by pharmacological means or siRNA directed at *SOD1* increased phosphorylation of Bcl-2 at Ser70 (pSer70). Increased levels of $ONOO^-$, as a result of increased $O_2^{\cdot -}$, induced selective nitration of Tyr289 of the B56δ subunit of PP2A. $ONOO^-$-induced nitration of PP2A at Tyr289 prevented the assembly of the holoenzyme involving the regulatory B subunit and the core A-C subunits. Importantly, SOD1 expression and Bcl-2 pSer70 were found to be negatively correlated *in vivo*; high Bcl-2 p70 could predict a poor patient prognosis. Furthermore, *in vitro* studies showed an association between high levels of Bcl-2 pSer70 and chemoresistance. This suggests the possibility for the use of redox modulators as chemosensitizers in the treatment of lymphomas (107). The study by Low *et al.* provided mechanistic insight to observations published previously by Zhao *et al.*, demonstrating that Bcl-2 pSer70 was required for protection against oxidative stress, which was abolished through the activation of PP2A (194).

Liang *et al.* (100) recently demonstrated that under H_2O_2-induced oxidative stress, Bcl-2 is stabilized through phosphorylation of Thr69 (pThr69) by pyruvate kinase M2 isoform (PKM2), promoting tumorigenesis in glioma cells. Oxidative stress induces mitochondrial translocation and a potential conformational change in PKM2, resulting in the phosphorylation of Bcl-2 at Thr69. This potential conformational change may be facilitated through the ATPase activity of HSP90α1 and permits the binding of PKM2 to Bcl-2. pThr69 prevents ubiquitination by a Cul3-based ubiquitin E3 ligase, thus preventing its degradation. This HSP90α1-PKM2-Bcl-2 axis is crucial in stabilizing the antiapoptotic function of Bcl-2 and driving tumorigenesis (100).

The Bcl-2:Beclin1 interaction has been demonstrated to be potentially redox dependent. Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3) is a proapoptotic protein, whose expression is regulated by HIF-1α. It functions as a redox sensor during times of prolonged hypoxia-induced oxidative stress. Dimerization of BNIP3 was shown to be sensitive to H_2O_2-induced oxidative stress (87). Hypoxia-induced BNIP3 expression has been demonstrated to disrupt the interaction between Bcl-2 and Beclin 1 to induce autophagy (12). These studies show that apoptosis and autophagy are clearly linked to specific Bcl-2 binding partners in various cellular components. To that end, *S*-nitrosylation of Bcl-2 negatively regulates autophagy; nitrosylation of Cys158 and Cys229 stabilizes the interaction between Bcl-2 and Beclin1, thereby inhibiting Beclin1 activity and as a result decreasing autophagic flux (187). This effect is reversed on addition of ABT-737, aminoguanidine (NO inhibitor), and a redox-inactive Bcl-2 double-mutant (C158A/C299A). Interestingly, these cysteine residues were previously shown to be prone to H_2O_2-induced oxidation (109).

Furthermore, *S*-nitrosylation of Bcl-2 was previously demonstrated to inhibit its ubiquitin-mediated proteasomal degradation. This NO-mediated effect was independent of Ser87 phosphorylation and decreased cellular apoptosis (9). These studies highlight the importance of NO-driven signaling and Bcl-2 in the regulation of autophagic and apoptotic signaling. NO has also been demonstrated to increase carbonylation of Bcl-2 (19), and IL-1β-induced NO production increased Bcl-2 carbonylation, which preceded its downregulation. The downregulation of Bcl-2 coincided with an increase in NO-mediated DNA fragmentation (19). This is contrary to the previously mentioned studies where NO was associated with resistance to apoptosis, suggesting that the regulation of cell fate by NO is dependent on the precise post-translational modification of Bcl-2.

Bcl-2 and peroxide eliminating enzymes. A study by Gouaze *et al.* (58) demonstrated that glutathione peroxidase-1 (GPx1) overexpressing cells were resistant to CD95-induced apoptosis. T47D cells overexpressing GPx1 exhibited lower levels of ROS and Bcl-2. Similarly, Clement *et al.* (33) showed that Bcl-2 overexpression blocked CD95-induced cell death, which was restored on NOX inhibition or overexpression of dominant-negative Rac1, decreasing overall levels of $O_2^{\cdot -}$. To that end, Rac1 has also been demonstrated to increase $O_2^{\cdot -}$ and inhibit apoptosis in melanoma and bladder carcinoma cells (143). Further studies provided evidence for an interaction between Bcl-2 and Rac1 in leukemic cells that increased intracellular $O_2^{\cdot -}$, thus creating a pro-oxidant environment that favors cell survival and/or inhibits apoptotic execution (178). Synthetic BH3 peptides and BH3 mimetics were able to block the interaction of Rac1 and Bcl-2, decrease $O_2^{\cdot -}$ production, and sensitize human leukemia cells to chemotherapeutics. Previously, it was also demonstrated that Rac1 was needed for the phosphorylation of Bcl-2 by the JNK/stress-activated protein kinase (JNK/SAPKβ) p54-SAPKβ (114).

Peroxiredoxins are functional antioxidant enzymes that control intracellular peroxide levels. *BCL2* mRNA expression has been shown to exhibit a primarily negative correlation with the five peroxiredoxins (*PRDX1*, *PRDX3*, *PRDX4*, *PRDX5*, and *PRDX6*) in a wide range of primary tumor samples. In lung cancer either a weak or negative correlation is observed (Fig. 7). In A549 lung cancer cells with gefitinib resistance (A549/GR), shRNA knockdown of *PRDX2* resulted

in a decrease in Bcl-2 expression and concurrent increase in peroxide levels in the cell, culminating in cell death (89). The change in correlation in gefitinib-resistant cells can be hypothesized to be directly due to JNK/ROS activation, both of which were altered compared to the A549 cells. H_2O_2 also protected cardiomyocytes from oxidative stress-induced apoptosis, which was associated with an increase in Bcl-2. H_2O_2-induced oxidative stress caused a decrease in the level of both Prx2 and Bcl-2, showing a strong correlation between intracellular peroxide levels and Bcl-2 expression (195). Bcl-2 expression has also been shown to be associated with members of the thioredoxin family. The use of antisense *BCL2* therapy in neuroblastoma cells demonstrated that decreasing levels of Bcl-2 were associated with increasing levels of thioredoxin. This seems to be consistent across a range of tumors (Fig. 7) and may involve a cellular compensatory mechanism when cells are undergoing oxidative stress (99).

Redox-dependent regulation of Bcl-2 in viral infection. Intracellular regulation of cellular redox has been shown to be an important factor in viral establishment, replication, and progression (11). A pro-oxidant environment has been demonstrated during infection with influenza (64), human immunodeficiency virus (HIV) (65), and hepatitis C (57). Bcl-2 has been demonstrated to influence cellular redox and alter viral replication in various settings. During influenza A replication, cellular levels of GSH are decreased, resulting in a pro-oxidant environment. Cells overexpressing Bcl-2 exhibited higher levels of GSH and produced less virus (126). Influenza A infection has also been shown to increase ROS in an NOX-4-dependent manner (6). Mechanistically, viral activation in Bcl-2^+ cells induced p38MAPK-mediated phosphorylation of Bcl-2 at Thr56 and Ser87, resulting in cell death. siRNA-mediated knockdown of *BCL2* resulted in increased influenza A replication and viral ribonucleoprotein (vRNP) export (125). In asymptomatic HIV-infected patients, H_2O_2 production was increased in monocytes with parallel downregulation in thioredoxin and Bcl-2. These reductions were suggested to be a result of significantly increased oxidative stress (45). These studies highlight the importance of redox regulation in altering Bcl-2-mediated cell fate in viral infections. The role of Bcl-2 in viral infections and its interaction with viral proteins is comprehensively reviewed by Alibek *et al.* (4) Redox in viral infections have previously been reviewed elsewhere (101, 123, 168).

A brief overview of Bcl-2 inhibitors: past, present, and future

As the discovery and development of Bcl-2 inhibitors have been recently comprehensively reviewed (8, 98), this section focuses primarily on molecules that have been or are currently under investigation through clinical trials (Fig. 9).

Oblimersen is an antisense oligonucleotide targeted to the *BCL2* mRNA, thus preventing Bcl-2 protein expression (41). It was the first molecule against Bcl-2 to be investigated clinically. Oblimersen has been extensively investigated in clinical trials in a wide variety of solid tumors and blood cancers, and in combination with a wide variety of cancer therapeutics, however, its use as a single agent or in combination have not been approved for any clinical indication (51). The related molecule SPC2996 has also been investigated in clinical trials, which failed to demonstrate effective downregulation of Bcl-2 expression (42). Oblimersen was demonstrated in PC3 cells to increase the oxidation of nuclear DNA, measured by 8-hydroxy-2′-deoxyguanosine staining,

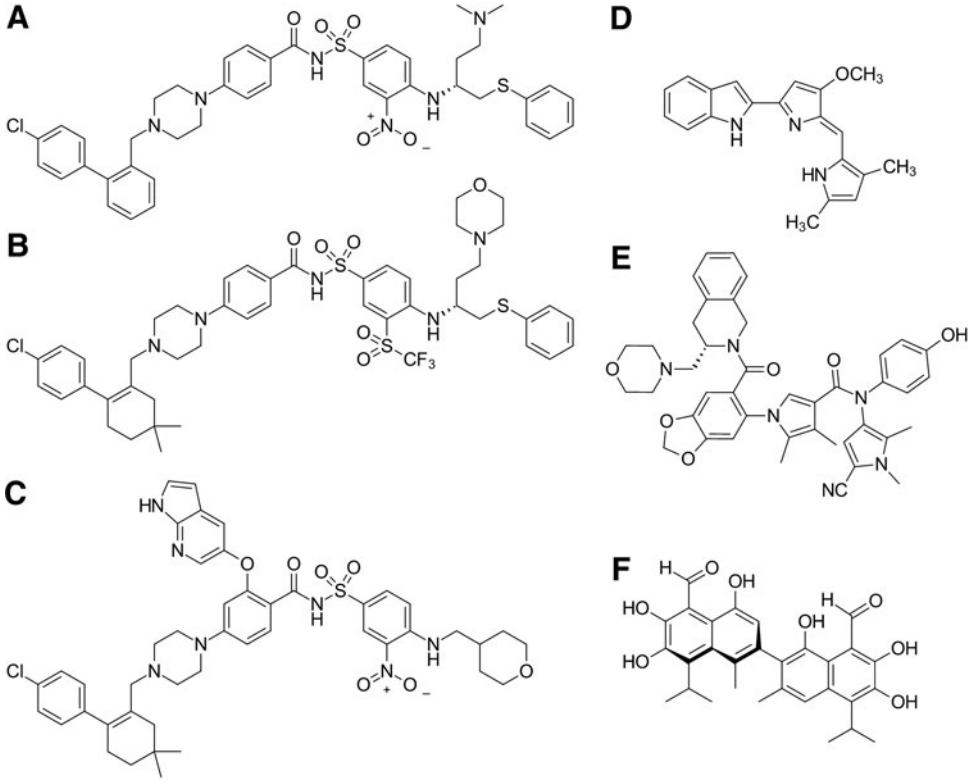

FIG. 9. 2D structures of small molecule Bcl-2 inhibitors. (A) ABT-737. **(B)** Navitoclax (ABT-263). **(C)** Venetoclax (ABT-199). **(D)** Obatoclax. **(E)** Representative molecule from the patent describing the production of S55746-related molecules. **(F)** (-)-Gossypol (AT-101).

suggesting that cell death was due to an ROS-dependent mechanism (90).

ABT-737, which is generally regarded as the prototypical small molecule Bcl-2 family protein inhibitor, was first reported in 2005 (134). ABT-737 was discovered through a SAR by NMR (structure-activity relationship by nuclear magnetic resonance) approach, through which small organic ''fragment'' molecules (<200 Da) were assessed for weak/moderate binding to Bcl-2 *via* NMR approaches (primarily saturation transfer difference spectroscopy and heteronuclear single quantum coherence spectroscopy), and subsequently ''assembled'' to generate molecules capable of tightly binding to Bcl-2. ABT-737 targets the hydrophobic peptide-binding groove of Bcl-family proteins nonselectively. The lack of oral bioavailability of ABT-737 has limited its clinical development, instead prompting the development of alternatives. Hepatocellular carcinoma (HCC) cells with high Bcl-2 expression demonstrated resistance to ABT-737 by suppressing the ROS-JNK pathway. In HCC cells expressing low levels of Bcl-2, apoptosis was induced after treatment with ABT-737 through increased ROS levels and activation of the ROS-JNK pathway (128). This study demonstrates that high levels of Bcl-2 can determine the effectiveness of ABT-737 in a ROS-dependent mechanism.

Navitoclax (ABT-263) is an orally bioavailable analog of ABT-737 that has progressed to Phase II clinical trials. Navitoclax features three modifications relative to ABT-737: (i) replacement of the nitro group with a triflyl group; (ii) replacement of the dimethylamine with a morpholine group; and (iii) replacement of the 4-chlorobiphenyl group with a 1-(4-chlorophenyl)-4,4-dimethyl-cyclohexene group (137). These modifications enhance oral bioavailability relative to ABT-737, while maintaining protein binding and cellular efficacy. Navitoclax elicits thrombocytopenia as a result of Bcl-xL inhibition (32, 158, 186), which limits its tolerated dose and has delayed its clinical development; nonetheless, trials involving navitoclax or combinations thereof are ongoing, largely against blood cancers.

Venetoclax (ABT-199, GDC-0199) is a Bcl-2-selective inhibitor derived from navitoclax, currently approved for the treatment of chronic lymphocytic leukemia. Venetoclax features three modifications relative to navitoclax: (i) replacement of the triflyl group with a nitro group (as in ABT-737); (ii) substitution of the *para*-aminobenzamide motif with a 1*H*-pyrrolo[2,3-*b*]pyridine-5-yloxy group; (iii) replacement of the morpholine and thiophenol arms with a methyl-tetrahydropyran group. Venetoclax retains the oral bioavailability of navitoclax, but is over 100-fold more selective for Bcl-2 over Bcl-xL, thus averting thrombocytopenia induced by navitoclax (167). Current clinical trials involving venetoclax are largely focused on broadening its application to include a wider variety of blood cancers, and involve both therapeutic combinations and venetoclax monotherapy (98).

Obatoclax (GX15-070) is a nonselective Bcl-2 family protein inhibitor, initially identified for its ability to inhibit Mcl-1 (127). Obatoclax features a comparatively simple scaffold compared to ABT-737 and related molecules; combined with its nonselectivity, this suggests that obatoclax may only interact with part of the binding groove present in Bcl-2 family proteins. Obatoclax has been evaluated for childhood cancers (both blood cancers and solid tumors), blood cancers in adults, and lung cancers in adults. Phase I studies indicated that the drug was well tolerated in patients with blood cancers (162), solid tumors (76), and exhibited modest single-agent activity (130). Obatoclax has been demonstrated to induce cell death in oral squamous cell carcinoma cells through a mitochondrial-dependent oxidative stress-induced mechanism (171). However, Phase II studies have generally failed to demonstrate its clinical usefulness (59, 136, 163). Its further development was discontinued in 2013.

S55746 (BCL-201, Servier-1) is an inhibitor developed by Servier selective for Bcl-2, built on the previously reported low-affinity, but highly Bcl-2-selective, phenylpyrazole molecule series (149). Preliminary results from a clinical trial of S55746 in patients with relapsed or refractory non-Hodgkin lymphoma suggest that S55746 monotherapy is safe, tolerable, and efficacious (92). Servier has previously reported S44563, a dual Bcl-2/Bcl-xL inhibitor, an analog of ABT-737 with conformational restriction on the phenylpiperazine region (124). Given this molecule's high similarity to ABT-737, it may also exhibit similarly poor oral bioavailability, which may have prompted the identification of alternative scaffolds and the ultimate development of S55746.

(-)-Gossypol (AT-101), a terpenoid phenol derived from the cotton plant, has been shown to act as a weak inhibitor of Bcl-2 and Bcl-xL (133). In addition to acting as a BH3 mimetic, AT-101 also appears to impair DNA repair mediated by APE1, a redox-active enzyme that is a known binding partner for Bcl-2 (152, 155). Unlike ABT-737 and related molecules, which directly prevent peptide binding to Bcl-2, (-)-gossypol induces a conformational change in Bcl-2 from an antiapoptotic to a proapoptotic state (96). (-)-Gossypol was evaluated in combination with androgen deprivation therapy for the treatment of castration-sensitive metastatic prostate cancer in a Phase II clinical trial (169); the results suggested this combination was not worth further development. (-)-Gossypol has also been evaluated for the treatment of progressive or recurrent glioblastoma multiforme; the majority of patients (62.5%) exhibited disease progression in the trial (72). (-)-Gossypol has been demonstrated to potentiate cell death induced by temozolomide (180); a Phase I clinical trial investigating this combination in the treatment of glioblastoma multiforme has been completed, but results are not currently available (23).

APG-1252 is a dual selective Bcl-2/Bcl-xL inhibitor currently under development by Ascentage Pharma Group. Limited details of this molecule have been made publicly available, however, as of this writing, recruitment is currently underway for a clinical trial of APG-1252 in patients with small-cell lung cancer and other solid tumors (70).

Perspective

It has been more than 30 years since the discovery of Bcl-2 and its association with drug resistance and aggressive hematopoietic malignancies. Over the years, the structural and functional biology of this remarkable protein has been unraveled. These pursuits have resulted in the discovery of a number of related proteins with opposing biological activities and grouped under a broader family, the Bcl-2 family. It is now well established that the balance between the pro- and

antiapoptotic members of the family is critical in cell fate decisions, which is a function of physical interactions between proteins from within and outside of the family. While the canonical antiapoptotic activity of Bcl-2 is associated with its ability to prevent MOMP by sequestering Bax and Bak from oligomerizing at the mitochondria, recent evidence also points to a redox-dependent regulation of cell fate by Bcl-2, which appears to involve its post-translational modification. Furthermore, the altered gene expression and mutational landscape in a host of human cancer, not limited to hematopoietic malignancies, underscore the importance of Bcl-2 as an important ''pro-oncogenic'' protein.

Based on its interacting domains, there has been a continuous focus on developing small molecule inhibitors to overcome chemotherapy resistance induced on Bcl-2 overexpression. Bcl-2 expression has been used as a biomarker for patient response to chemotherapy. Could we now extend this to patient profiles of Bcl-2 expression in terms of ROS high and ROS low, and would this have a stronger predictive power?

There is a large body of evidence that directly links Bcl-2 and cellular redox. Earlier observations suggested an antioxidant role for Bcl-2, however, more recent work indicates a remarkable dichotomy when it comes to Bcl-2 and its effects on cellular redox metabolism. These divergent effects appear to be a function of cellular redox status itself. As such, the effect might differ under states of normoxia, hypoxia, and oxidative stress. To complicate matters further, the same ROS can have seemingly opposite effects on the antiapoptotic activity of Bcl-2. H_2O_2 in one context can cause degradation through oxidation of cysteines and promote cell death, and on the other hand can cause increased stability and promote cell survival. Similarly, the effect of an increase in NO and its reaction product with $O_2^{\cdot -}$, $OONO^-$, could be associated with carbonylation or S-NO modification. Interestingly, an altered redox milieu impacts post-translational state of Bcl-2, such as its phosphorylation, which could have different functional consequences depending on whether the phosphorylation is multisite (inhibits its activity) or monosite as with serine 70 (stabilizes its activity). Based on these outcomes, it is imperative to clearly delineate the mechanisms behind the opposing effects of an altered redox state on Bcl-2 biology, particularly from the standpoint of its role in promoting chemotherapy resistance in cancer. This is even more important as many clinically approved drugs induce increases in intracellular ROS, which could fuel the process of carcinogenesis and its progression by promoting Bcl-2 stability.

A number of promising small molecules are being developed and are undergoing clinical evaluation, however, selectivity remains a major issue. The early generation of Bcl-2 inhibitors, based on BH3 domain interaction, did not show the level of stringency in terms of targeting the specific antiapoptotic protein within the family. More recent approaches, such as in the case of venetoclax, appear to have addressed this issue. One possible scenario still needs attention: the reciprocal upregulation of another antiapoptotic member on specific inhibition of one. For example, is it possible that specific inhibition of Bcl-2 might activate the compensatory upregulation of Mcl-1 or *Bcl-xL*? Future strategies could also leverage on the noncanonical function of Bcl-2 in terms of regulating cellular redox status. In this respect, one might envision the potential application of modalities that favorably tailor the cellular redox milieu for death execution as well as overcome the inhibitory effect on putative tumor suppressors, such as the phosphatase PP2A.

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Abbreviations Used

Bcl-2 = B cell lymphoma 2
BH = Bcl-2 homology domains
BNIP3 = Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3
COX = cytochrome *c* oxidase
CREB = cyclic AMP response binding protein
ER = endoplasmic reticulum
ERK = extracellular signal-regulated kinases
ETC = electron transport chain
GPx1 = glutathione peroxidase-1
GSH = reduced glutathione
GTPase = guanosine trisphosphate
HCC = hepatocellular carcinoma
HIV = human immunodeficiency virus
H_2O_2 = hydrogen peroxide
JNKs = c-Jun N-terminal kinases
MAPK = mitogen-activated protein kinase
MCL-1 = induced myeloid leukemia cell differentiation protein
MnTBAP = manganese (III) tetrakis (4-benzoic acid) porphyrin
MOMP = mitochondrial outer membrane permeabilization
NAC = *N*-acetylcysteine
NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells
NO = nitric oxide
NOX = NADPH oxidase
$O_2^{\cdot -}$ = superoxide anion
OGC = 2-oxyglutarate carrier
$ONOO^-$ = peroxynitrite
PKM2 = pyruvate kinase M2 isoform
PLC-γ1 = phospholipase Cc1-dependent
PP2A = protein phosphatase 2
ROS = reactive oxygen species
SOD = superoxide dismutase
STAT3 = signal transducer and activator of transcription 3
TCGA = The Cancer Genome Atlas
TNF = tumor necrosis factor