OXFORD

Carcinogenesis, 2017, Vol. 38, No. 6, 579–587

doi:10.1093/carcin/bgx016
Advance Access publication February 16, 2017
Review

REVIEW

Non-canonical roles of Bcl-2 and Bcl-xL proteins: relevance of BH4 domain

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Abstract

Bcl-2 protein family is constituted by multidomain members originally identified as modulators of programmed cell death and whose expression is frequently misbalanced in cancer cells. The lead member Bcl-2 and its homologue Bcl-xL proteins are characterized by the presence of all four conserved BH domain and exert their antiapoptotic role mainly through the involvement of BH1, BH2 and BH3 homology domains, that mediate the interaction with the proapoptotic members of the same Bcl-2 family. The N-terminal BH4 domain of Bcl-2 and Bcl-xL is responsible for the interaction with other proteins that do not belong to Bcl-2 protein family. Beyond a classical role in inhibiting apoptosis, BH4 domain has been characterized as a crucial regulator of other important cellular functions attributed to Bcl-2 and Bcl-xL, including proliferation, autophagy, differentiation, DNA repair, cell migration, tumor progression and angiogenesis. During the last two decades a strong effort has been made to dissect the molecular pathways involved the capability of BH4 domain to regulate the canonical antiapoptotic and the non-canonical activities of Bcl-2 and Bcl-xL, creating the basis for the development of novel anticancer agents targeting this domain. Indeed, recent evidences obtained on *in vitro* and *in vivo* model of different cancer histotypes are confirming the promising therapeutic potential of BH4 domain inhibitors supporting their future employment as a novel anticancer strategy.

Introduction

B cell lymphoma 2 (Bcl-2) protein family plays a leading role in regulating apoptosis, the highly conserved molecular process of programmed cell death physiologically implicated in the development and elimination of cells with aberrant or damaged DNA (1). Dysregulation of apoptosis is a common feature associated with oncogenesis, tumor maintenance and chemoresistance (2) and, furthermore, neurodegenerative disorders (3,4). The lead member *BCL-2* gene was originally identified from the breakpoint region of a recurrent chromosomal translocation in human follicular lymphoma (5). The capability of Bcl-2 protein to prevent the death of cytokine-deprived haematopoietic cells was the first evidence of its prosurvival function (6), but a great impulse in dissecting the functional role of Bcl-2 in apoptosis was given by the genetic characterization of programmed cell death in the development of *Caenorhabditis elegans* worm, in which *C. elegans ced-9* gene was identified as an homologue of mammalian *BCL-2* in protecting from programmed cell death (7,8). Bcl-2 is the main member of a protein family whose relatives are functionally classified as either antiapoptotic or proapoptotic, and deregulation of their expression is associated to development and chemoresistance of various tumors with different histotypes (9–11). Bcl-2 family proteins can be classified in three groups according to the presence of four Bcl-2 homology (BH) domains: antiapoptotic and proapoptotic multidomain members sharing BH1-4 regions and a third class characterized by the presence of the only BH3 domain (BH3-only proteins). In addition, some proteins belonging to Bcl-2 family, such as Bcl-2 and its homologue Bcl-xL, contain a C-terminal transmembrane domain that direct them to intracellular membranes, including mitochondria outer membrane, endoplasmic reticulum (ER) and nuclear envelope. In the

Received: June 29, 2016; **Revised:** January 29, 2017; **Accepted:** February 11, 2017

Abbreviations

AA	Ala–Ala
Bcl-2	B cell lymphoma 2
BH	Bcl-2 homology
DD	Asp–Asp
DSB	double-strand break
ER	endoplasmic reticulum
dNDP	deoxyribonucleoside diphosphates
HIF-1	hypoxia inducible factor 1
hRRM1	human ribonucleotide reductase 1
hRRM2	human ribonucleotide reductase 2
IP3R	inositol 1,4,5-trisphosphate receptor
MM	multiple myeloma
MMPs	metalloproteinases
NNK	nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NHEJ	non-homologous end joining
NF-kB	nuclear factor kappa B
NFAT	nuclear factor of activated T-cells
RyR	ryanodine receptor
VEGF	vascular endothelial growth factor

case of antiapoptotic proteins, BH1 and BH2 domains, together with BH3, are involved in the creation of a hydrophobic groove that is capable of binding BH3-only proapoptotic proteins, inhibiting their activity and leading to a prosurvival cell condition. A crucial role of BH3 domain, as the mediator of the physical interaction between proapoptotic and antiapoptotic members of Bcl-2 family, has emerged in the last years. Thus, many efforts have been made in investigating the therapeutic efficacy of compounds mimicking BH3 domain for the treatment of tumors characterized by overexpression of antiapoptotic Bcl-2 family proteins (10,12–14). Some BH3 mimetics have been evaluated in different phases of human clinical trials but, to date, most of them have not been approved in clinical practice for cancer therapy due to limited efficacy as single agent or in combination, presence of side effects and acquisition of cancer cell resistance (15). Venclexta (ABT-199) is the first Bcl-2 inhibitor recently approved by FDA for the treatment of patients with chronic lymphocytic leukemia who have chromosome 17p deletion and who have been treated with at least one prior therapy (16,17). Beyond the original identification of Bcl-2 antiapoptotic members as modulators of cell death, these proteins have been recognized to regulate other cellular functions, such as cell differentiation, senescence, autophagy, mitochondrial fusion and fission, tumor angiogenesis and metastatization (18) (Figure 1). Some of these functions have been reported to be mediated through the BH4 domain. Since in the last decades a great number of reviews described the role that Bcl-2 and Bcl-xL proteins play in canonical antiapoptotic functions, this aspect will not be discussed here. On the contrary, to the best of our knowledge, no reviews have been published discussing non-canonical new function of Bcl-2 family proteins. Very recently, a review described Bcl-2 family proteins as regulators of cancer cell invasion and metastasis focusing on mitochondrial respiration and reactive oxygen species (ROS) (18), but not on the relevance of BH4 domain in these pathways. Considering these evidences, in our review we will present findings only in the context of emerging new functions of Bcl-2 and Bcl-xL proteins, with particular regards to the role that BH4 domain plays in these functions. Finally, we will briefly discuss on the recent advantages in compounds targeting BH4 domain as a possible novel therapeutic approach to counteract tumor growth and progression.

Structure of BH4 domain in Bcl-2 family proteins

The BH4 region is characterized by a sequence of 18–20 aminoacids included in the N-terminal domain of Bcl-2 and Bcl-xL proteins, as well as in other antiapoptotic members of Bcl-2 family (1), such as Bcl-w (19) and Bcl-B (20). Also some proapoptotic members of Bcl-2 family, such as Bcl-2-related ovarian killer protein homolog B (Bok) (21), Bcl-xS (22) and Diva, also known as Boo (23), present the BH4 domain. While BH1, BH2 and BH3 sequences are highly conserved between the different Bcl-2 family members, BH4 domain displays less aminoacidic sequence homology (24). However, the sequence of BH4 domain is highly conserved between humans and other vertebrate species and some aminoacidic residues are present also in ced-9 sequence of both *C.elegans* and *C. briggsae*, as well as Bok of *Danio rerio* (25) (Table 1).

The BH4 domain is organized in an α-helical structure and, unlike BH1-3 domains, it is not involved in the formation of the hydrophobic loop responsible of Bcl-2 and Bcl-xL ability to elicit canonical antiapoptotic activity, binding either BH3-only proteins or other apoptotic regulators not belonging to Bcl-2 protein family (15). In addition, the cleavage of this domain in Bcl-xL and Bcl-2 by caspase 1 or 3 converts the two antiapoptotic proteins into proapoptotic ones that potently induce apoptosis (26,27). Experiments of BH4 domain deletion demonstrated that this region is responsible for non-canonical functions of Bcl-2, beyond the classical antiapoptotic activity, as described further and resumed in Table 2.

Role of Bcl-2 and Bcl-xL proteins in antiapoptotic properties and other cellular activities

Role of Bcl-2 and Bcl-xL proteins in apoptosis: involvement of BH4 domain

Since in the last years a great number of reviews have described the role that Bcl-2 and Bcl-xL proteins play as antiapoptotic regulators (1,9,10), here we will discuss only the relevance of BH4 domain in this pathway. Together with the classical binding

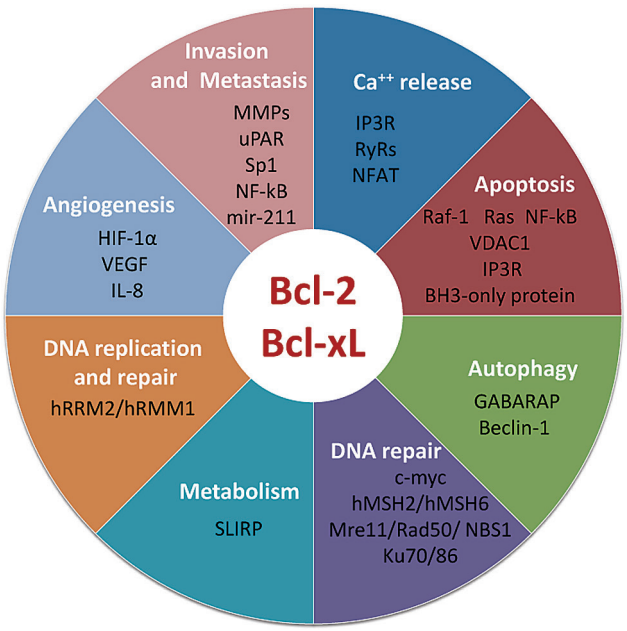

Figure 1. Novel targets and interaction proteins of Bcl-2 and Bcl-xL proteins involved in the different hallmarks of cancer.

Table 1. CLUSTAL alignment of the BH4 domain of different proteins in different organisms

Protein	Organism	Aminoacids	Sequence
Bcl-2	Human	11–30	NREIVMKYIHYKLSQRGYEW
	Mouse	11–30	NREIVMKYIHYKLSQRGYEW
	Bovin	11–30	NREIVMKYIHYKLSQRGYEW
	Chicken	11–30	NREIVLKYIHYKLSQRGYDW
	Rat	11–30	NREIVMKYIHYKLSQRGYEW
Bcl-$x_{L/S}$	Human	5–24	NRELVVDFLSYKLSQKGYSW
	Mouse	5–24	NRELVVDFLSYKLSQKGYSW
	Chicken	5–24	NRELVIDFVSYKLSQRGHCW
	Rat	5–24	NRELVVDFLSYKLSQKGYSW
	Pig	5–24	NRELVVDFLSYKLSQKGYSW
Bcl-w	Human	10–27	TRALVADFVGYKLRQKGY--
	Mouse	10–27	TRALVADFVGYKLRQKGY--
	Bovin	10–27	TRALVADFVGYKLRQKGY--
Bok	Human	32–51	-KALGREYVHARLLRAGLSW
Bok-B	Danio rerio	31–50	SKELCRDFIHSRITREGLSW
Ced9	*C. briggsae*	75–94	IQGFVVDYFTYRIAQNGLDW
	C. elegans	80–99	IEGFVVDYFTHRIRQNGMEW

Table 2. BH4 domain-mediated cellular functions of Bcl-2 and Bcl-xL proteins

Proteins	Function elicited	Protein interactor	References
Bcl-2	Apoptosis	Bax	(28,29)
		Raf-1	(30,31)
		Ras	(32)
	Hematopoietic differentiation	Raf-1	(33)
	Calcium trafficking/ apoptosis	IP3R	(34)
		calcineurin	(35)
		RyR	(36)
Bcl-xL		RyR	(37)
		VDAC1	(34,38–42)
Bcl-2	Autophagy	Beclin-1	(43)
		GABARAP	(44)
Bcl-2/Bcl-xL	Cell cycle	NFAT	(35)
Bcl-2	DNA replication	hRRM2	(45)
	DNA damage	c-myc	(46)
		hMSH6	(47)
		Ku70/86	(48)
		Mre11	(49)
	Mitochondrial RNA homeostasis	SLIRP	(50)
	Cell invasion	MMPs	(51,52)
	Tumor angiogenesis	HIF-1α	(52,53)

to BH3-only proteins, Bcl-2 protein inhibits apoptosis forming heterodimers with Bax, the proapototic member of Bcl-2 family, and the BH4 domain is the mediator of this function, as demonstrated by the overexpression of Bcl-2 protein lacking this domain (28,29). Interestingly, when the BH4 domain of Bcl-2 protein is replaced by the homologue aminoacidic sequence of BH4 domain of Bcl-xL, the overexpression of this chimeric protein is still able to protect from apoptosis induced by interleukin 3 deprivation, while the substitution with the BH4 sequence of bax protein fails to protect from apoptosis (28).

A great number of evidences regarding the relevance of BH4 domain of Bcl-2 and Bcl-xL antiapoptotic functions, are related to the relevance of this domain in the interaction with molecules not belonging to Bcl-2 family. The integrity of mitochondria is ensured by a mechanism involving Bcl-2 protein, which targets Raf-1 to this organelle determining a protection from drug-induced apoptosis but the BH4 domain deleted form of Bcl-2 fails to ensure this binding to Raf-1 and consequently does not exert antiapoptosis properties (30). In ventricular myocytes, Bcl-2 protects from apoptosis activating nuclear factor kappa B (NF-kB) pathway through a mechanism that involves Raf-1 kinase and is abrogated in the case of BH4 domain lacking (31). In endothelial cells, BH4 domain of Bcl-2 and Bcl-xL is required for inhibiting NF-kB pathway activation under inflammatory condition, since Bcl-2 mutant lacking BH4 is no longer able to inhibit the signalling (54). The interaction of BH4 domain with Raf-1 has also an impact on hematopoietic differentiation: both Bcl-2 and Bcl-xL proteins, through their distrinct BH4 domains, constrain a differentiation potential oriented towards erythroid or myeloid direction, respectively, modulating Raf-1 expression (33), as demonstrated by the expression of Bcl-2 mutant in which BH4 domain is replaced by its counterpart in Bcl-xL homologue protein. Overexpressing Bcl-2 protein mutants deleted for the different BH domains, the BH4 domain of Bcl-2 protein is identified as the mediator of the binding to the small GTPase Ras, the upstream regulator of Raf-1, determining a block of its function and leading to an inhibition of active Ras-dependent apoptosis (32). Also Bcl-xL protects cells from apoptosis regulating mitochondria function. Indeed Bcl-xL protein interacts with mitochondrial voltage-dependent anion channel 1 (VDAC1) and deletion of BH4 domain abolishes this binding, determining a reduction of calcium uptake (38,39). On the other hand, Bcl-2 protein has been deeply characterized for its capability to regulate calcium trafficking from ER to mitochondria and cytosol interacting with inositol 1,4,5-trisphosphate receptor (IP3R) (55): the result of this binding determines inhibition of calcium release and consequent protection from apoptosis (56). Later on, the conserved residue lysine 17 had been identified as the critical residue for BH4 domain-mediated binding of Bcl-2 to IP3R to inhibit calcium release and apoptosis (34). This residue is not conserved in BH4 domain of Bcl-xL protein (34), besides this Bcl-2 homologue is still able to exert its antiapoptotic activity modulating IP3R pathway binding the receptor in protein sequences different from the ones involved in the interaction with BH4 domain of Bcl-2 (34,40–42). Also Bok, a member of the Bcl-2 protein family that controls the intrinsic apoptosis pathway (21), is able to bind IP3R via its BH4 domain, albeit much more strongly than Bcl-2 and to a different IP3R region (57). Similarly to the interaction with the IP3R, Bcl-2 also ensures a correct calcium homeostasis targeting the ryanodine receptors (RyRs) through a binding involving its BH4 domain, as demonstrated by GST pulldown assay. In particular, the lysine 17 was identified to be determinant for eliciting this function by overexpression of a mutated Bcl-2^{K17D} protein which does not inhibit RyR-mediated calcium release as the same extent of full length Bcl-2 protein (36). Also Bcl-xL binds to RyRs, but both BH4 and BH3 domain are required for this interaction and the regulation of calcium homeostasis. Interestingly, the BH4 domain of Bcl-xL is necessary and sufficient to inhibit RyR-mediated calcium release (37). In hematopoietic cell lines, Bcl-xL protein is also able to regulate calcium oscillations modulating IP3R promoter activity, inhibiting the activity of the transcription factor Nuclear Factor of Activated T cells, cytoplasmic, calcineurin-dependent 2 (NFAT), leading to a protection from apoptosis (58). On the other hand, also Bcl-2 protein was found to suppress NFAT activity leading to an inhibition of apoptosis, binding and sequestering the active form of calcineurin, a calcium- and calmodulin-dependent protein phosphatase. Overexpressing different versions of Bcl-2 protein deleted of the

transmembrane domain, N-terminal or C-terminal regions the authors demonstrated that Bcl-2 protein required the integrity of its BH4 domain to bind calcineurin and to exert the inhibition of NFAT. Indeed, the BH4 domain is sufficient to exert this function (35).

Role of Bcl-2 and Bcl-xL proteins in autophagy: involvement of BH4 domain

The role of Bcl-2 family proteins as essential regulators of apoptotic cell death is an old concept, on the contrary their role in autophagy has only recently been appreciated (59). Autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome. Recent studies have clearly underscored that autophagy has a greater variety of physiological and patho-physiological roles, including intracellular protein and organelle clearance, development, cell death and tumor suppression (60). Indeed, it is very well established that elimination of cancer cells might not only occur via apoptosis but could also be mediated by other forms of cell death such as autophagic death. Some recent observations indicate that a decline of autophagic activity may be related to tumorigenesis (61,62). Autophagic cell death is mainly a morphologic definition (i.e. cell death associated with autophagosomes/autolysosomes) and it has been also recognized as a mechanism contributing to tumor cell eradication induced by chemotherapeutics under some circumstances (63). Intensive investigation in the last decades on the molecular mechanisms of apoptosis and autophagy has led to the identification of the several molecules involved in both autophagic and apoptotic pathways. In fact, autophagy not only can block the induction of apoptosis by inhibiting the activation of apoptosis-associated caspase, it also help to induce apoptosis (64). In this context, the interaction between the antiapoptotic protein Bcl-2 and the autophagy protein Beclin-1 represents a potentially important point of convergence of the apoptotic and autophagic machinery (59). In normal conditions, Beclin-1 is bound to and is inhibited by Bcl-2. This protein interaction is mediated by BH3 domain in Beclin-1 and by BH3 binding groove of Bcl-2. Nutrient starvation, which is a potent physiologic inducer of autophagy, stimulates the dissociation of Beclin-1 from Bcl-2, either by activating BH3-only proteins (such as Bad) or by post-translational modifications of Bcl-2 (such as phosphorylation) that may reduce its affinity for Beclin 1 and BH3-only proteins (59). An inhibitory autophagic function has been described also for other antiapoptotic Bcl-2 family proteins (65–67). A more recent paper has changed this view and providing the genetic and biochemical evidence that the prosurvival Bcl-2 protein family does not directly regulate autophagy, but any impact they have on autophagy is an indirect consequence of their inhibition of apoptosis mediators, such as Bax and Bak. In particular, this work supports a model that apoptosis induces autophagy and in which autophagy does not significantly induce cell death in the absence of Bax and Bak (68). Thus, within the signaling network of mammalian cells, Bcl-2 and Bcl-xL proteins are now considered an integrating node in control of both autophagy and apoptosis (69,70). Recent studies support a role of BH4 domain also in the regulation of autophagy. In particular, Ma's group demonstrated that BH4 domain is involved in the capability of Bcl-2 protein to interact with GABA Receptor Associated Protein (GABARAP), a molecule involved in autophagosome biogenesis (71), contributing to the crosstalk between apoptosis and autophagy (44). Through mapping Bcl-2/GABARAP interaction surface and performing pulldown and co-immunoprecipitation assays, this study also supports the notion that distinct regions and specific residues of Bcl-2 protein mediate its inhibitory effects on apoptosis and autophagy. Moreover, we previously reported that overexpression of Bcl-2 protein lacking its BH4 domain in melanoma cells determines a dramatic commitment to autophagy leading to an impairment of mice xenografts development which is dependent on the expression level of Beclin-1 (43). This observation could indicate that agents interfering with the BH4 domain of Bcl-2 protein, discussed later, may counteract tumor growth taking advantage of the functional role of Bcl-2 not only in apoptosis but also in autophagy.

Role of Bcl-2 and Bcl-xL proteins in cell cycle progression, DNA replication, genomic stability and mitochondrial messenger RNA expression: involvement of BH4 domain

In association with the role in apoptosis and autophagy, Bcl-2 and Bcl-xL have been characterized for being involved in other cellular processes (Figure 1) and in most of the cases BH4 domain is the key region involved in these functions.

Together with the capability to survive to cytokine-deprivation, Bcl-2 overexpressing cells stay in a condition of quiescence (6) or show a limited response to proliferation stimuli (72), thus suggesting a possible role of Bcl-2 in regulating cell cycle progression. Murine thymocites overexpressing Bcl-2 protein presented a prolonged G1 phase with a delay in S phase entry (73). The same phenomenon was observed in leukemia cell lines, in which Bcl-2 protein impaired NFAT-mediated transcription (74). Indeed, in different mammalian cells expression of not only Bcl-2, but also its homologs Bcl-xL and Bcl-w, similarly retarded progression to S phase, demonstrating that this cell cycle effect of Bcl-2 is manifested by other antiapoptotic molecules within the Bcl-2 family, and is not cell type specific (72,75). Interestingly, Bcl-2 appears to mediate its proliferative effect also by acting on specific cell cycle regulators. In fact, inhibition of G1/S transition by Bcl-2 overexpression is proceeded by the modulation of the level of proteins, such as p130 or p27, involved in the control of G1/S transition (76,77).

Some deletions or mutations that affect the antiapoptotic activity of Bcl-2 have also been reported to abolish its cell-cycle inhibitory activity. In particular, a Bcl-2 protein deleted of its 36 N-terminal residues, encompassing the BH4 domain, loses both antiapoptotic and antiproliferative activities (78). Expressing Bcl-2 protein with a single aminoacidic substitution, the conserved tyrosine 28 in the BH4 domain was then identified to be responsible for Bcl-2 protein-mediated inhibition of cell cycle progression but, most interestingly, this residue resulted to be dispensable for its antiapoptotic activity. Moreover, also mutation of the homologue residue in Bcl-xL protein, tyrosine 22, had the same effect (75).

Bcl-2 may also determine a delay in S phase due to a slower progression of replication fork leading to DNA replication stress through the involvement of the ribonucleotide reductase complex. Co-immunoprecipitation experiments employing recombinant Bcl-2 deleted of the distinct four BH domains demonstrated that Bcl-2 directly binds hRRM2 protein through its BH4 domain, disrupting the functional complex composed by the subunits human ribonucleotide reductase 1 and 2 (hRRM1/hRRM2), leading to a reduced conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates (dNDP), and consequently limiting the availability of dNTP precursors needed for the DNA synthesis. Indeed the BH4 deleted Bcl-2 protein fails to inhibit

cellular RNR activity, dNTPs synthesis, and DNA replication fork progression when compared to full length Bcl-2 protein (45).

Many members of Bcl-2 family have been shown to play a role in modulating response to DNA damage, through different mechanisms (79). In the case of exposure to nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), one of the main component of cigarette smoke, Bcl-2 protein suppresses the repair of apurinic/apyrimidinic sites, the most frequent DNA damage determined by this molecule. Bcl-2 exerts this function through binding c-Myc in the nucleus, enhancing its transcriptional activity and repressing the expression of apurinic/apyrimidinic (AP) endonuclease, a protein playing an essential role in repairing AP sites. This effect is independent from Bcl-2 prosurvival activity, and BH4 domain is required for Bcl-2-dependent impairment of DNA damage repair as demonstrated by the overexpression of the BH4 deleted Bcl-2 mutant (46). Moreover, Bcl-2 inhibits NNK-induced DNA mismatch repair binding to hMSH6 through the same BH4 domain, as demonstrated by co-immunoprecipitation assay employing recombinant Bcl-2 protein full length or deleted of the different BH domain. The deletion of BH4 domain determines a disruption of hMSH2/hMSH6 heterodimer, whose main function is to correct mutations that escape the proofreading activity of DNA polymerase, in loss of the ability of Bcl-2 protein to suppress mismatch repair and hMSH6 activity (47).

Bcl-2 inhibits nonhomologous end joining (NHEJ) repair mechanism (80) and in particular it suppresses DNA double strand-break (DSB) repair by downregulating Ku DNA binding activity and disrupting Ku/DNA–PKcs complex, leading to increased genetic instability. Thus, exposure of human lung cancer cells to ionizing radiation determines a translocation of Bcl-2 protein into the nucleus where it binds Ku70/86 proteins. BH4 and BH1 domains are involved in the direct binding of Bcl-2 to both Ku70 and Ku86 as demonstrated by co-immunoprecipitations employing recombinant full length and deleted Bcl-2 proteins. Moreover both BH4 or BH1 domain deleted Bcl-2 proteins fail to accumulate in the nucleus following cell irradiation (48).

Both Bcl-2 and Bcl-xL protein are also involved in the regulation of homologous recombination (81), the other DNA repair pathway, beyond the NHEJ system, implied in resolving DSBs. Recently, Bcl-2 has been shown to interact with Mre11 nuclease through BH4 and BH1 domains, as demonstrated by co-immunoprecipitation experiments. This interaction disrupts the complex Mre11/Rad50/NBS1, impairing the DNA repair of DSBs induced by ionizing radiations (49).

The involvement of Bcl-2 protein in mitochondrial RNA biology was also demonstrated (82). In particular, we recently found that through its binding to SRA Stem-Loop Interacting RNA-binding protein, a protein widely expressed in human normal, as well as cancer tissues and cell lines (50), Bcl-2 was able to affect the expression of several RNA involved in mitochondrial homeostasis. Importantly, overexpression of a deleted form of Bcl-2 lacking the BH4 domain suppressed the positive effect on mitochondrial RNA expression mediated by Bcl-2 protein.

Role of Bcl-2 and Bcl-xL proteins in tumor progression and angiogenesis: involvement of BH4 domain

Many evidences demonstrate that Bcl-2 family members may have a strong impact on tumor progression, not only modulating tumor cell apoptosis, but also controlling cell migration and invasion, epithelial–mesenchymal transition (EMT) and metastatization of several tumor histotypes through the involvement of different signalling pathways and matrix-degrading enzymes (18,83). In details, Bcl-2 overexpression enhances the metastatic potential of breast carcinoma, glioma, neuroblastoma, squamous carcinoma and neuroblastoma cells, increasing the activity of some metalloproteinases (MMP), such as MMP-2 and MMP-9, and involving different signalling pathways (PI3K, p38, AP-1, ERK) (84–87). MMP induction is the mechanism through which our and other groups reported that Bcl-2 protein sustains also the migration and invasiveness potential of breast carcinoma and melanoma (51,83,88,89). We also identified urokinase plasminogen activator receptor/Sp1 axis as responsible of bcl-2 induced migration (90). Moreover, increasing evidence suggests that antiapoptotic bcl-2 family members also promote cell invasion by facilitating ROS production both by increasing mitochondrial respiratory activity, and blocking the inhibitory effect of multidomain pro-apoptotic members on ROS production (91). Other possible mechanisms through which antiapoptotic proteins promote cell invasion include the involvement of COX, Twist1, Myosin Va (92). Very recently, we demonstrated that Bcl-2 ability to increase cell migration in melanoma models was dependent on the expression of miR-211, a micro RNA prevalently expressed in the melanocyte lineage and acting as oncosuppressor (93). We also found that the deletion of BH4 domain impairs the capability of Bcl-2 protein to modulate MMP2 and MMP9 expression in breast carcinoma cells (51), and the metastatic potential of melanoma cells (52). Bcl-2 protein also enhances migration and invasion of colorectal cancer cells but the molecular mechanism involved in such phenomenon was not elucidated (94).

On the other hand, Bcl-2 protein may promote tumor progression through enhancing tumor angiogenesis, as demonstrated in different tumor histotypes by other and our groups (95–98). In particular, in the last years we reported that Bcl-2 protein cooperates with hypoxia, a common feature of solid tumors, to induce an increased secretion of vascular endothelial growth factor (VEGF) (99) through enhancing VEGF transcriptional activity and mRNA stability (100), and the activity of hypoxia inducible factor 1 (HIF-1) (89,101), the main transcription factor involved in VEGF promoter activation. Overexpressing Bcl-2 protein deleted of the BH4 domain, we found that BH4 domain is necessary for Bcl-2 protein-mediated enhancement of HIF-1/VEGF axis in hypoxia, independently from antiapoptotic function (53). As consequence BH4 domain is the key region required for Bcl-2 protein-dependent enhancement of angiogenesis (52).

New evidence from our and other studies suggests that, in addition to the regulation of apoptosis, also Bcl-xL regulates new functions that are genetically distinct from its effect on apoptosis (18,102–108). In particular, a pivotal role for Bcl-xL in *in vitro* and *in vivo* invasion of malignant glioma (102), colorectal cancer (94), and breast carcinoma (106,107) has been described. Moreover, gain-of-function studies in models of pancreatic cancer, demonstrated accelerated tumor formation and growth, while genetic ablation of bcl-xL attenuates invasiveness without affecting apoptosis or tumor growth (103,106). Bcl-xL ability to induce EMT has been also reported (105,106). Bcl-xL has also a prominent role in regulating survival of lung cancer stem cells (106,109) and in promoting melanoma and glioblastoma cell stemness characteristics (our unpublished results). We previously reported that modulation of Bcl-xL in human glioblastoma and melanoma cell lines regulates angiogenesis affecting CXCL8 expression (110) through a NF-kB-dependent mechanism (111). However, bcl-xL overexpression is not always sufficient for inducing its effects on tumor progression, and additional treatments (i.e. co-expression with particular oncogenes, exposure to hypoxia) are necessary in some cases (103,110), thus indicating

the relevance of cell type and environment in some functions of Bcl-2 family members. The mechanisms underlying these new functions of Bcl-xL protein has been recently identified, and the possible involvement of BH4 domain in their regulation has not been elucidated. Interestingly, most of them have been demonstrated to be independent from Bcl-xL canonical antiapoptotic activity and mitochondrial localization, and to require a novel nuclear function.

The clinical relevance of these studies at cellular level and using animal models derives from immunohistochemical analysis of tumor samples from patient with different cancer histotypes, being up-regulation of particular Bcl-2 family proteins, often observed during tumor progression (18).

Targeting BH4 domain as a therapeutic strategy: peptides and small molecules

Given the evidenced multiple functions of BH4 domain of Bcl-2 family proteins described so far, there has been a growing interest in identifying new therapeutic strategies for cancer and other pathological conditions (15).

Several findings support the possible therapeutic potential of cell permeable BH4 peptides, resembling the BH4 domain of Bcl-xL or Bcl-2, as antiapoptotic therapy in different pathological conditions. Recently, stapled peptides corresponding to the BH4 domain of Bcl-2 were employed to demonstrate that this region is able to determine a conformational block of Bax, inhibiting its activation (112) and leading to a preservation of mitochondria outer membrane. It was also evidenced that the exposure to a BH4 peptide of Bcl-xL was sufficient to control calcium signaling by modulating the IP3R activity in spinal cord astrocytes from a mouse model of amyotrophic lateral sclerosis (113). The same results were obtained in a murine model of Friedreich ataxia, a neurodegenerative disease characterized by a reduced expression of the mitochondrial protein frataxin: the BH4 peptide of Bcl-xL was able to restore calcium homeostasis in cultured frataxin-depleted neurons, increasing their viability (114). Moreover, the aminoacidic sequence of BH4 domain by itself showed a strong antiapoptotic activity and, thus protective effect, on several cell lines and tissues of different origins, including cardiac tissue (115–117), capillary endothelium (118,119), lymphocytes (120–123) and pancreatic islets (124). The peptides corresponding to BH4 domains of both Bcl-2 and Bcl-xL proteins are also able to modulate calcium trafficking through a mechanism involving the activity of the calcium/hydrogen antiporter activity of Bax inhibitor-1 (125), which regulates calcium homeostasis in ER and exerts an antiapoptotic function inhibiting Bax activation translocation to mitochondria (126).

The relevance of BH4 targeting in cancer progression has been also reported. Basically, two approaches targeting the BH4 domain are available nowadays: inhibiting peptides and small molecules. In particular, we found that the exogenous application of a cell-permeable peptide encompassing the aminoacidic sequence of the BH4 domain of both Bcl-xL or Bcl-2 proteins, fused to the protein transduction domain of HIV-1 TAT protein (TAT-BH4), acts like full-length *wild type* Bcl-2 mimicking Bcl-2 functions in terms of HIF-1/VEGF regulation in melanoma cells exposed to hypoxia. In fact, they are sufficient to increase HIF-1α protein half-life impairing HIF-1α protein ubiquitination, and to enhance VEGF secretion in melanoma cells exposed to hypoxia independently from antiapoptotic functions (53).

Based on the previously described interaction between the BH4 domain of Bcl-2 protein and IP3R receptor (127), TAT-pep2, a cell permeable peptide also known as Bcl-2/IP3 receptor disrupter-2, was designed. It spans 20 aminoacidic residues of IP3R protein involved in the interaction with BH4 domain, and demonstrated the capability to disrupt Bcl-2/IP3R interaction and to enhance anti-CD3 antibody-induced calcium release from ER, leading to apoptosis in leukemia cell lines (15,128). The same results were obtained employing a modified version of TAT-pep2, the TAT-$IDP_{DD/AA}$ peptide (129), in which the aspartyl protease site Asp-Asp (DD) was replaced with Ala-Ala (AA), a modification that may ensure more stability to the peptide. Interestingly, TAT-$IDP_{DD/AA}$ peptide has a limited apoptotic effect on normal peripheral blood lymphocytes cells (129), suggesting a selectivity of action toward chronic leukemia cells overexpressing Bcl-2 protein and/or IP3R2 receptor (130). Recently, this peptide demonstrated its efficacy in enhancing apoptosis also on *in vivo* models, not only as single agent (108) but also in combination with a panel of BH3 mimetics including ABT-263 (Navitoclax), ABT-199 (Venetoclax) (108) and HA14-1 (131).

The only example of non-peptide approach to target BH4 domain is the synthetic compound BDA-366, identified by high-throughput screening among 300 000 molecules of National Cancer Institute library (15,132,133). BDA-366 confirmed its efficacy *in vitro* on a panel of lung cancer cell lines and, as expected, it was strictly dependent on Bcl-2 protein expression, being lines expressing relatively higher levels of Bcl-2 more sensitive to the compound. BDA-366 shows a high specificity in terms of binding BH4 domain of Bcl-2 protein, excluding other BH domains of the same protein or other proteins in which BH4 is also present, such as Bcl-xL and Mcl-1. The binding of BDA-366 with the BH4 domain results in conversion of Bcl-2 from an antiapoptotic molecule into a death protein through a conformational change that exposes its BH3 death domain. Thus, thanks to this conformational change, BDA-366 induces apoptosis through a mechanism dependent on Bax protein. This action may be enforced by the capability of BDA-366 to disrupt Bcl-2/IP3R protein complex, determining an increase of calcium release and even if this additional mechanism by which BDA-366 might induce BH4-dependent apoptosis has not been demonstrated. Very interestingly, BDA-366 also induced autophagic cell death in lung cancer cell lines, resembling the effect of the expression of a BH4-deleted form of Bcl-2 protein, as we previously described (43). The promising efficacy of BDA-366 was also confirmed on *in vivo* models since it suppresses lung cancer xenografts growth through induction of apoptosis and potentiates the antitumor activity of RAD001, a mTor inhibitor. Moreover, the exposure of BH3 domain and the consequent activation of Bax was also observed in tumor xenografts derived from BDA-366-treated mice.

More recently, the ability of BDA-366 to induce apoptosis in multiple myeloma (MM) cell lines and primary MM cells has been demonstrated both *in vitro* and *in vivo*, without significant cytotoxic effects on normal hematopoietic cells or body weight (133).

Concluding remarks

Bcl-2 and Bcl-xL proteins have been characterized to be determinant in regulating tumor cell death, chemoresistance and progression and, as consequence, identified as potential drug targets in the design of new antitumoral therapies, mainly based on BH3 domain mimetic. Despite promising preclinical results, clinical trials on those compounds are quite unsuccessful (15). The identification of the key-role of BH4 domain in mediating the wide range of cellular activities of Bcl-2 and Bcl-xL proteins sustained the employment of BH4-targeting

compounds as a possible anticancer therapy, that would be characterized by the inhibition of a broad-spectrum of tumor cell functions. The first experimental evidences are confirming the promising therapeutic potential of BH4 domain inhibitors supporting their future employment as a novel anticancer strategy.

Funding

This work was supported by grants from the Italian Association for Cancer Research (DDB, 18560).
Conflict of Interest Statement: None declared.

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