REVIEW ARTICLE

Regulation of mitochondrial metabolism: yet another facet in the biology of the oncoprotein Bcl-2

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The Bcl-2 (Bcl is B-cell lymphocytic-leukaemia proto-oncogene) family comprises two groups of proteins with distinct functional biology in cell-fate signalling. Bcl-2 protein was the first member to be discovered and associated with drug resistance in human lymphomas. Since then a host of other proteins such as Bcl-xL, Bcl-2A1 and Mcl-1 with similar anti-apoptotic functions have been identified. In contrast, the pro-apoptotic Bcl-2 proteins contain prototypic effector proteins such as Bak and Bak, and the BH3 (Bcl-2 homology)-only proteins comprising Bak, Bid, Bim, Puma and Noxa. A complex interplay between the association of pro-apoptotic and anti-apoptotic proteins with each other determines the sensitivity of cancer cells to drug-induced apoptosis. The canonical functional of Bcl-2 in terms of apoptosis inhibition is its ability to prevent mitochondrial permeabilization via inhibiting the translocation and oligomerization of pro-apoptotic proteins such as Bax; however, more recent evidence points to a novel mechanism of the anti-apoptotic activity of Bcl-2. Overexpression of Bcl-2 increases mitochondrial oxygen consumption and in doing so generates a slight pro-oxidant intracellular milieu, which promotes genomic instability and blocks death signalling. However, in the wake of overt oxidative stress, Bcl-2 regulates cellular redox status thereby preventing excessive build-up of ROS (reactive oxygen species), which is detrimental to cells and tissues. Taken together, the canonical and non-canonical activities of Bcl-2 imply a critical involvement of this protein in the processes of tumour initiation and progression. In the present paper we review these functionally distinct outcomes of Bcl-2 expression with implications for the chemotherapeutic management of cancers.

Key words: apoptosis, B-cell lymphocytic-leukaemia proto-oncogene 2 (Bcl-2), mitochondrion, reactive oxygen species (ROS).

INTRODUCTION

The role of mitochondria in programmed cell death has been studied extensively over the last few decades. It is now well established that stress signals induce changes in mitochondrial membrane permeability, resulting in the release of cytochrome c from the mitochondrial intermembranous space and activation of a proteolytic cascade of cysteine proteases called caspases [1]. This pathway is a key component of the apoptotic machinery which plays an indispensable role in development, tissue homeostasis and immunity, the deregulation of which is often observed in cancers as an evasive mechanism against immune surveillance and other anti-cancer defence networks. One of the key proteins involved in blunting the apoptotic execution machinery is Bcl-2 (Bcl is B-cell lymphocytic-leukaemia proto-oncogene), an anti-apoptotic protein commonly up-regulated in many solid tumours and haematological malignancies [2].

Bcl-2 was originally identified as an oncoprotein generated by a translocation between chromosomes 18 and chromosome 14 present in approximately 85% of follicular lymphomas and 20% of diffuse B-cell lymphomas [3]. Being one of the earliest oncproteins to be discovered, it is among the most notorious anti-apoptotic proteins involved in malignant transformation. Owing to its anti-apoptotic properties, Bcl-2 is overexpressed in a variety of cancers, thereby allowing them to escape the built-in defences against tumour formation, as well as to accumulate further mutations which cause progression to metastatic malignancy [4]. To that end, co-expression of Bcl-2 and the proto-oncogene c-Myc has been reported to promote aggressive B-cell tumour formation from haemopoetic stem cells [5]. Similarly, Bcl-2 transgenic mice were shown to promote hyperplasia and form T-cell lymphomas, thus further establishing the involvement of Bcl-2 in tumorigenesis [6].

The potent tumorigenic activity of Bcl-2 is classically attributed to its protective effect on the integrity of the OMM (outer mitochondrial membrane), through the physical sequestration of its pro-apoptotic counterparts (Bax and Bak) and the prevention of MOMP (mitochondrial outer membrane permeabilization). Interestingly, previous evidence has revealed that Bcl-2 could also affect cell-fate decisions by modifying cellular redox status. ROS (reactive oxygen species), such as superoxide ($O_2^−$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($OH$), have been shown to function as agents of cell death [7–9]. Through the fine-tuning of intracellular levels of ROS, Bcl-2 was demonstrated to maintain intracellular redox status at a level optimal for cell survival. To that end, we recently reported a novel role for Bcl-2 in the regulation of mitochondrial respiration by means of a physical interaction with COX Va (the Va subunit of cytochrome c oxidase; complex IV) [9a]. Through this novel regulatory role, Bcl-2 was further shown to modulate mitochondrial ROS levels as...

Abbreviations used; AIF, apoptosis-inducing factor; ANT1, adenine nucleotide translocase 1; Bcl, B-cell lymphocytic-leukaemia proto-oncogene; BH, Bcl-2 homology; COX, cytochrome c oxidase; DIABLO, direct IAP (inhibitor of apoptosis)-binding protein with low pl; DISC, death-induced signalling complex; ETC, electron transport chain; MOMP, mitochondrial outer membrane permeabilization; NAC, N-acetylcysteine; OMM, outer mitochondrial membrane; PTPC, permeability transition pore complex; ROS, reactive oxygen species; Smac, second mitochondrial-derived activator of caspase; SOD, superoxide dismutase; TNF, tumour necrosis factor; VDAC, voltage-dependent anion channel.

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well as downstream cell-fate signalling. In the present review we attempt to provide an overview of the cancer-promoting activity of Bcl-2 in light of its canonical anti-apoptotic activity and the new non-canonical function linking Bcl-2-mediated resistance to apoptosis to regulation of mitochondrial metabolism. In particular, we delineate the significance of Bcl-2 in regulating respiration-dependent ROS production under periods of oxidative insults and its relevance to cancer development and/or progression.

**BLOCKING MITOCHONDRIAL APOPTOSIS: THE CANONICAL ACTIVITY OF Bcl-2**

The genetically conserved pathway of apoptosis can be triggered by a variety of stress signals such as DNA damage, withdrawal of growth factors, damage to the cytoskeleton or external death receptor ligation [10]. Apoptotic execution is marked by the activation of downstream caspases which leads to chromatin condensation, nuclear fragmentation, cytoskeleton degradation and formation of blebs called apoptotic bodies which bud off from the membrane to be engulfed by phagocytes [11]. There are two main different, but converging, pathways, the death receptor or extrinsic and the mitochondrial or intrinsic pathways, which mediate the cell death signalling cascade. The extrinsic pathway involves signal transduction at the plasma membrane by the TNF (tumour necrosis factor) receptor superfamily, such as CD95/Apo1/Fas or TNF receptor ligation with their respective ligands CD95L/Apo1L/FasL or TNFα leading to receptor trimerization and formation of the DISC (death-induced signalling complex). DISC causes proximity-induced cleavage of pro-caspase 8 which activates the primary downstream executioner caspase 3 [12].

Alternatively, the intrinsic pathway involves the release of various mitochondrial apoptotic factors such as cytochrome c, AIF (apoptosis-inducing factor), Smac (second mitochondrial-derived activator of caspase)/DIABLO [direct IAP (inhibitor of apoptosis)-binding protein with low pI] and HtrA2/Omi from the mitochondria into the cytosol [13]. As a result, cytochrome c binds to Apaf-1 (apoptotic protease-activating factor 1) and pro-caspase 9 to form the apoptosome to activate caspase 9 [13]. The release of cytochrome c and the other mitochondrial apoptotic proteins is dependent on a highly regulated event called MOMP; also known as the ‘point of no return’ in the mitochondrial pathway of apoptosis [14]. MOMP and the ultimate execution of the mitochondrial apoptotic pathway is governed by the Bcl-2 family of proteins, a class of proteins grouped together based on the structural homology of the BH (Bcl-2 homology) domains discovered in these proteins.

Members of the Bcl-2 family are functionally classified into either pro-apoptotic or anti-apoptotic. Bcl-2, Bcl-xL, Mcl-1, Bcl-2A1 and Bcl-w are among the major members of the anti-apoptotic Bcl-2 proteins discovered to date [15]. These proteins are structurally distinct from the pro-apoptotic Bcl-2 proteins because of the presence of all four BH domains (BH1–4) [15]. The pro-apoptotic Bcl-2 proteins on the other hand are divided into the BH3-only proteins (Bim, Bad, Bid, Noxa and Puma), as well as the effector proteins (Bax and Bak), which contain only BH1–3 domains. However, structural alignment studies of globular Bcl-2 family proteins suggest that a BH4 motif could be present in the tertiary structure of both Bax and Bak as well [16]. The execution of the mitochondrial apoptotic machinery is a function of the ratio between the functionally antagonistic Bcl-2 proteins (such as Bax/Bcl-2), with the fate of the cell being determined by the tilt in the ratio towards one or the other [17].

In response to apoptotic stressors, the effector proteins could homo-oligomerize to form transmembrane pores at the OMM, thus promoting OMM permeabilization and the egress of various mitochondrial apoptotic factors into the cytosol. A double-knockout of Bax and Bak does not undergo OMM permeabilization under various apoptotic stimuli, particularly if the cell-death mechanism involves BH3-only proteins [18]. Interestingly, some studies have shown that MOMP is also regulated by a PTPC (permeability transition pore complex), which mainly consists of the VDAC (voltage-dependent anion channel), ANT1 (adenine nucleotide translocase 1) and/or cyclophilin D [19]. Although the exact mechanism of OMM permeabilization by the Bcl-2 family of proteins might be different from that of PTPC, interactions between the two have been reported. In particular, Bax has also been shown to interact with VDAC and/or ANT1 to form a PTPC, which could also promote the egress of mitochondrial apoptotic factors [19]. Bid has also been implicated in the formation of PTPC for induction of apoptosis [20]. Opposing the activity of the pro-apoptotic proteins is Bcl-2 and its anti-apoptotic relatives. The anti-apoptotic Bcl-2 proteins are generally localized at the OMM, although localization to the endoplasmic reticulum and nucleus has also been reported [17]. Bcl-2 and Bcl-xL, in particular, are widely known for their inhibitory activity over Bax/Bak, sequestering them at the OMM and thus preventing their oligomerization or their binding to VDAC [14]. By means of inhibiting the formation of PTPC and MOMP, overexpression of anti-apoptotic Bcl-2 proteins could therefore abrogate the effect of death-inducing triggers which target the mitochondria [17]. Moreover, the pro-apoptotic BH3-only proteins provide an additional dimension to the regulation of MOMP by the Bcl-2 family proteins. Depending on their mechanism of action, BH3 proteins are subdivided to either the ‘direct activators’ or the ‘sensitizers’. The direct activators (e.g. Bid and Bim) can either interact with the anti-apoptotic Bcl-2 proteins or directly interact and induce the oligomerization of Bax and Bak at the OMM [17]. On the other hand, the sensitizer BH3 proteins (such as Bad and Noxa) do not activate Bax/Bak directly, but bind to the anti-apoptotic Bcl-2 proteins, thus releasing Bax/Bak for MOMP [21]. The sensitizers might also displace the ‘direct activators’ that are bound to anti-apoptotic Bcl-2 proteins, thereby leaving them free to activate Bax/Bak [21]. It is clear, at least from a classical perspective, that MOMP and mitochondrial apoptotic execution is highly dependent on the interactions between the pro- and anti-apoptotic Bcl-2 proteins.

**Bcl-2 REGULATES ROS BY MODULATING MITOCHONDRIAL RESPIRATION**

**Bcl-2 and ROS in cell death**

Studies have revealed another level at which Bcl-2 opposes cell death, by regulating cellular redox status, which is distinct from its conventional activity of sequestering pro-apoptotic proteins [4,7,9,22–24]. However, being localized to the mitochondria, which are the hotbed for cellular metabolism and a major source of intracellular ROS, this role might be significant, particularly since the cellular redox status is critical in cell-fate determination. ROS have been traditionally regarded as agents of death, since their abnormal accumulation triggers oxidative damage of cellular macromolecules such as nucleic acids, lipids and proteins, ultimately leading to cell death [25]. However, contrary to the deleterious effects of overt oxidative stress, there is ample evidence to suggest that a slight pro-oxidant state (low levels of ROS) is a conducive environment for survival and proliferative signalling [7,23,26,27]. In this regard, a slight increase in the intracellular concentrations of O$_2^-$ and H$_2$O$_2$ has been shown to activate growth-related genes such as c-fos and c-jun [26], and is
Figure 1  Effect of ROS is dependent on both dose and species

Intracellular ROS could arise from various sources such as the mitochondria, NADPH oxidases, or even from exogenous triggers such as radiation and other pharmacological agents. This, however, is countered by a huge repertoire of antioxidant molecules as well as antioxidant enzymes within the cell. ROS-governed cell-fate decisions are therefore dependent on the balance between ROS production and the antioxidant defence machinery. The presence of a mild pro-oxidant environment in the cell promotes survival mainly by $O_2^-$-dependent oncogenic signalling, whereas a tilt in ROS levels dominated by $H_2O_2$ creates an intracellular milieu that favours apoptosis. On the other hand, an overwhelming production of ROS damages cellular macromolecules and leads to necrotic cell death. ASC, ascorbate; GSH, reduced glutathione; TOC, tocopherols; Trx, thioredoxins; Prx, peroxiredoxins.

implicated in Rac-dependent oncogenic signalling by p21Ras [28]. The prevalence of a pro-oxidant state in many tumour types due to deficiencies in redox-regulating enzymes further corroborates the pro-survival role of ROS [26,28]. Studies showing diminished cell proliferation upon overexpression of catalase and SODs (superoxide dismutases) further support these findings [26]. It has also been shown that Bcr (breakpoint cluster region)-Ab1 (Abelson) kinase in chronic myelogenous leukaemia causes Nox-dependent ROS production that can activate Akt and GSK3β (glycogen synthase kinase 3β) and their downstream effectors, β-catenin and Mcl-1 [29]. Similarly other oncoproteins, such as NF-κB (nuclear factor κB) and AP-1 (activator protein 1), have been shown to activate ROS-dependent survival signalling [30]. Thus stress-associated cell-fate signalling is strongly influenced by the intracellular concentration of ROS, with lower concentrations displaying a distinct non-detrimental effect on cell physiology (Figure 1).

An added level of complexity to this decision-making process is the specific oxygen species that are produced following a stimulus. Our work over the years has highlighted the critical role of a tight balance between the two major ROS species, $O_2^-$ and $H_2O_2$, in cell-fate decisions. A tilt in the ratio towards $O_2^-$ leads to inhibition of apoptotic signalling, whereas an increase in intracellular $H_2O_2$ over $O_2^-$ facilitates death execution [7,23,27,31–33]. The increase in intracellular $H_2O_2$ downstream of a death signal has been linked to the mitochondria, where it could promote MOMP and release pro-apoptotic proteins such as cytochrome c, AIF and Smac/DIABLO [34]. Alternatively, released $H_2O_2$ could be inhibiting an ATP-dependent Na+/H+ antiporter that regulates cytosolic pH, thereby generating an acidic intracellular milieu [34]. Cytosolic acidification favours apoptosis through yet to be discovered mechanisms, although it has been documented that there is increased caspase 3 cleavage at a lower pH [35]. Indeed, although this apoptotic role of $H_2O_2$ forms the basis of ROS-producing anti-cancer drug therapies, $O_2^-$ functions as an oncogenic oxygen species with anti-apoptotic and proliferative effects [23]. Clearly any exogenous or endogenous stimulus that alters the tightly regulated ratio of ROS species within the cells could affect cell survival and death signalling.

So where does Bcl-2 fit into this intricate redox signalling system? Conventionally, Bcl-2 has been described as containing antioxidant properties, by virtue of which it can protect cells from $H_2O_2$- and menadione-induced cell death [9]. Upon overexpression, Bcl-2 was able to reduce oxidative-stress-mediated lipid peroxidation in two model systems of apoptosis, with a concomitant increase in antioxidant enzymes [9]. However, no evidence to date has established the presence of an intrinsic antioxidant capability in Bcl-2, rather studies have shown induction of a pro-oxidant milieu following Bcl-2 overexpression in tumour cells [7,23]. In particular, the anti-apoptotic nature of Bcl-2 suggested a link to the oncogenic $O_2^-$ species. This was established in leukaemia cell lines where the anti-apoptotic function of Bcl-2 was diminished when NADPH-oxidase-dependent $O_2^-$ production was inhibited with DPI (diphenyliodonium) or the use of a dominant-negative form of the GTPase Rac1 [23]. These reports uncover a novel side to the oncogenic potential of Bcl-2 through its ability to promote a pro-survival environment via increased $O_2^-$ generation.
Bcl-2, ROS and mitochondrial respiration

During oxidative phosphorylation, electrons are transferred across the various mitochondrial electron transport complexes before being finally passed on to the terminal electron acceptor, molecular oxygen, by COX. However, inefficient electron transfer often leads to the leakage of electrons from intermediate electron transporters (complex I and III) directly to molecular oxygen, resulting in the single-electron reduction of oxygen to O$_2^-$ anions [36,37]. Bearing this in mind, as well as the pro-oxidant activity of Bcl-2 at the mitochondria, it is thus possible to extend the function of Bcl-2 to the regulation of mitochondria bioenergetics and its associated by-production of O$_2^-$ from the ETC (electron transport chain).

In this respect, our group has demonstrated that the pro-oxidant function of Bcl-2 is indeed via engagement of COX/complex IV, the rate-limiting complex of the ETC [7]. COX by itself exists as a dimer of 13 subunits each, with three core subunits encoded by the mitochondrial genome and ten others by the nuclear genome. The mammalian COX subunits Va and Vb are orthologues of the yeast COX IV and VI whose regulation is directly proportional to the concentration of oxygen in the cell and are considered essential for the assembly and stability of the enzyme complex [38]. Similarly, COX Va and subsequent COX Vb incorporation into the COX complex during assembly might have a role in COX enzymatic activity [39]. Interestingly, these COX subunits have been shown to be up-regulated in a variety of cancers and in particular the presence of the Vb subunit correlates with COX activity and is shown to inhibit cell death [40].

We reported previously that overexpression of Bcl-2, enforced or constitutive, resulted in increased intra-mitochondrial O$_2^-$ and a concomitant increase in mitochondrial respiration and COX activity [7]. Importantly, the same results were seen with coupled mitochondrial respiration, indicating that the observed increase in O$_2^-$ generation was not due to uncoupling, but instead due to an intrinsic increase in the electron flow across the ETC [7]. These studies suggest that Bcl-2 increases the mitochondrial burn rate of oxygen, thereby increasing the probability of electrons leaking on to oxygen to generate O$_2^-$. This effect on mitochondrial respiration was linked to a physical interaction of Bcl-2 with the Va subunit of the COX complex, and more importantly, an increased localization of COX Va to the inner mitochondrial membrane in cancer cells with significantly higher expression of Bcl-2 [9a]. Interestingly, Bcl-2 does not immunoprecipitate with COX Vb, but causes increased localization of COX Vb to the mitochondria as well. This could be explained by an upstream association of Bcl-2 with COX Va which changes its subcellular localization that might subsequently facilitate mitochondrial import of COX Vb, leading to higher COX activity and O$_2^-$ generation. Alternatively, COX Vb might be interacting with mitochondrial membrane proteins involved in import such as Tom20 and overexpression of Bcl-2 simply increases the import of COX Vb [9a]. These data suggest that Bcl-2 leads to increased COX Va and Vb localization to the mitochondria, thereby increasing COX activity, which could be responsible for the enhanced mitochondrial generation of O$_2^-$ (Figure 2). Of note, the effect of Bcl-2 expression on mitochondrial metabolism was not a function of a change in the expression of COX or its subunits [7], thus indicating that this effect was indeed a matter of improved assembly of the COX complex via Bcl-2-mediated import of COX Va and perhaps Vb subunits. Furthermore, RNAi (RNA interference)-mediated gene silencing of Bcl-2 reduced COX activity, diminished O$_2^-$ generation and mitochondrial localization of COX Va and Vb, lending further support to the role of Bcl-2 in modulating COX via Va and Vb import [7].

This gives a better insight into the normal pro-oxidant role of Bcl-2, but how does this functionality alter when the cell is under oxidative stress? And is there a link between the conventional antioxidant nature of Bcl-2 and its novel role as a O$_2^-$ generator? To that end, it is highly intriguing that the presence of Bcl-2 reversed these effects on mitochondrial oxygen consumption, COX activity and ROS production in cells following exposure to stimuli that induce oxidative stress in the mitochondria, such as the complex III inhibitor antimycin A, serum deprivation and hypoxia [7]. In fact the overall levels of O$_2^-$ generated were maintained at the same basal level in Bcl-2-overexpressing cells in contrast with the mock-transfected cells, which showed a steady increase in intramitochondrial O$_2^-$ [7]. This suggests that, although Bcl-2 has intrinsic pro-oxidant properties that help in creating a milieu favourable for cell survival and proliferation, under conditions of stress Bcl-2 can regulate mitochondrial respiration to prevent further augmentation of ROS and keep cell death at bay. Thus Bcl-2 functions as a regulator of oxidative stress, with a usual pro-oxidant function, which in the wake of stress alters the metabolic activity of the cell to reduce ROS levels and favour survival. This model, when taken in regard to the conventional antioxidant activity of Bcl-2, indicates that the previously seen antioxidant function could just be the ROS modulation of Bcl-2 observed physiologically.

Importantly, the regulatory effect of Bcl-2 on mitochondrial O$_2^-$ production appears to be independent of its canonical inhibitory activity on Bax/Bak oligomerization and MOMP. Since Bax/Bak-mediated MOMP has been shown to induce mitochondrial ROS production [20,42], it is plausible that the protection of Bcl-2 against mitochondrial O$_2^-$ could just be secondary to its well-known ability to inhibit Bax/Bak oligomerization, as well as the associated release of mitochondrial ROS. However, our recent findings suggested otherwise [24]. Using resveratrol-induced apoptosis in HCT116 cells as a model system, we demonstrated that resveratrol could induce MOMP and its associated by-production of O$_2^-$ from the ETC (electron transport chain).
and apoptosis, even in the absence of Bax/Bak, via the induction of an overwhelming production of mitochondrial $O_2^-$. Interestingly, Bcl-2 overexpression protected HCT116 cells against resveratrol-induced oxidative stress and apoptosis, despite the absence of Bax and Bak [24]. Furthermore, introduction of recombinant Bcl-2 to isolated mitochondria in a cell-free system devoid of both cytosolic Bax and Bak recapitulated the mitochondrial protective effect of Bcl-2 against resveratrol-induced oxidative stress [24]. Taken together, these pieces of evidence highlight the importance of the non-canonical role of Bcl-2 in protecting against oxidative insults, in addition to its classical inhibitory activity on Bax/Bak-induced MOMP and apoptosis.

The revelation of the non-canonical role of Bcl-2 in the regulation of mitochondrial electron transport activity via COX is not entirely surprising. Up-regulation of both COX Va and Vb has since been reported in a number of tumour cell lines, and COX Vb has even been shown to inhibit apoptosis under certain circumstances [40,43,44]. Intriguingly, p53 has also been reported to regulate mitochondrial respiration by modulating the expression level of SCO2 (synthesis of COX II), a nuclear-encoded gene necessary for the incorporation of the COX subunit II into the COX complex [45,46]. Liver mitochondria of p53$^{−/−}$ mice and isolated mitochondria of p53$^{−/−}$ colorectal cancer cells were reported to exhibit higher glycolytic flux but lower oxygen consumption rate and COX activity [46], and it was suggested that this could account for the metabolic switch from oxidative phosphorylation towards glycolysis in p53-null tumour cells, a phenomenon long recognized as the Warburg effect [45,47]. Although at first this might seem in headlocks with the effect Bcl-2 could have on metabolism given that tumours do not rely on oxidative phosphorylation, it could be viewed as an additional mechanism of increased energy production to support the demanding needs of the tumour, especially as cells undergo necrotic demise when the ATP levels become abnormally low. With an augmented rate of oxidative phosphorylation, tumour cells overexpressing Bcl-2 could in fact be bestowed with an added capability to optimize ATP generation for stress adaptation. In fact, this could potentially account for the increased dependence of many cancers such as malignant melanoma, lung, cervical, breast and ovarian carcinomas on oxidative phosphorylation and mitochondrial respiration as opposed to glycolysis [48–50]. As such, a novel mechanism for the survival advantage endowed by Bcl-2 overexpression could be a function of a mild pro-oxidant intracellular milieu coupled with bioenergetic proficiency that allow tumour cells to thrive even under non-favourable conditions.

### CLINICAL IMPLICATIONS: ROS-MEDIATED THERAPEUTICS

Unravelling the workings of Bcl-2 in the regulation of ROS is of immense clinical implications considering that ROS are often linked to genomic instability and cellular transformation, but their abnormal increase also serves as an execution signal, such as exposure to anti-cancer compounds. ROS are normally generated in the cell as a byproduct of cellular metabolism and respiration, but their abnormal accumulation is kept under check by the efficient and robust antioxidant defence systems. Therefore cellular redox status is a function of a tight balance between the rates of ROS generation and scavenging, and any defect or deficiency in the latter could result in overt oxidative stress with potentially lethal outcome.

Many anti-cancer agents such as TRAIL (TNF-related apoptosis-inducing ligand), cisplatin, etoposide, daunouabcin and resveratrol, mediate their toxicity via a significant increase in intracellular ROS production [24,33,51,52] which induces MOMP and facilitates the release of death-amplifying proteins from the mitochondria. In fact the observed correlation between Bcl-2 overexpression and resistance of tumours to chemotherapy and radiotherapy, both of which can cause massive ROS production, provides evidence, albeit indirect, for a regulatory effect of Bcl-2 on cellular redox status. This is supported by our recent findings on the effect of Bcl-2 overexpression in a model of resveratrol-induced apoptosis in human leukaemia cells [24]. Although resveratrol induced an overwhelming mitochondrial $O_2^−$ production in the mock-transfected leukaemia cells leading to MOMP induction and apoptosis, Bcl-2-overexpressing leukaemia cells were rendered resistant to resveratrol-induced oxidative stress and cell death [24]. Moreover, Bcl-2 overexpression was shown to inhibit the effect of resveratrol on mitochondrial oxygen consumption rate and COX activity [24], thereby indicating that the level of Bcl-2 dictated the resistance of tumour cells to drug-induced ROS production, as well as its entailing death signals.

With Bcl-2 often being overexpressed in a variety of cancers [53], the remarkable biology of Bcl-2 regulating cellular redox status presents a real challenge for the therapeutic management of the disease. The ability of Bcl-2 to increase the flux of oxygen through the mitochondrial ETC predisposes to a slight pro-oxidant state, which on the one hand promotes genomic instability thereby favouring cellular transformation, and on the other hand induces antioxidant defence systems to prepare cells against oxidative-stress-induced death signals. By implication these functional attributes of Bcl-2 in the context of cancer biology suggest an important role in the process of transformation and tumour initiation through its canonical and/or non-canonical activities.

This therefore calls for the use of small-molecule Bcl-2 inhibitors in combination with other therapies for a more efficient treatment modality. To that end, recent studies have shown that ABT-737, a potent inhibitor of Bcl-2, exhibited synergistic killing of cervical cancer and T-lymphocytic leukaemia cells when used in combination with ROS-inducing chemotherapeutics, such as adaphostin and etoposide [54]. In particular, etoposide, when used in combination with ABT-737, exhibited more than a 2-fold increase in apoptotic activity as compared with single treatment of either etopoxide or ABT-737 [54], shedding light on the development of combination therapies using Bcl-2 inhibitors, together with other redox-altering compounds, in the treatment of various malignancies. Of note, pre-treatment with the ROS scavenger NAC ($N$-acetylcysteine), protected against ABT-737-induced cell death. These data suggest a mechanism of action of ABT-737 that could involve inhibition of the ROS modulatory activity of Bcl-2, in addition to its ability to facilitate Bax/Bak-induced apoptosis.

Along similar lines, another small-molecule Bcl-2 inhibitor, HA14-1, has been shown to be equally promising as well. HA14-1 was initially identified as a Bcl-2 inhibitor via computer modelling strategies [55]. Treatment of HL60 human leukaemia cells with HA14-1 alone was reported to elevate intracellular ROS levels [33], presumably via the inhibition of the ROS modulatory activity of Bcl-2. Therefore it is likely that combinatory treatment of HA14-1 would sensitize Bcl-2-overexpressing tumour cells to other ROS-inducing chemotherapeutics. True to this aspect, a study combining HA14-1 with flavopiridol resulted in an augmented level of apoptosis in human multiple myeloma cells which are normally resistant to individual treatment of either HA14-1 or favopiridol alone [56]. Despite being originally identified as a CDK (cyclin-dependent kinase) inhibitor, flavopiridol was shown to induce apoptosis via the activation of JNK (c-Jun N-terminal kinase), as well as the down-regulation of anti-apoptotic proteins such as Mcl-1 and Bcl-xL [57]. In addition, the toxic effect of flavopiridol was also associated with
ROS-mediated mitochondrial damage [56]. Importantly, the fact that scavenging of ROS via NAC pre-treatment reverses the synergistic effect of HA14-1 and flavopiridol strongly indicates ROS, at least in part, as the upstream mediator of flavopiridol-induced apoptosis [56]. As such, it is plausible that the synergistic activity of HA14-1 and flavopiridol is not solely due to the sensitization of cancer cells towards Bax/Bak-mediated apoptosis, but could also be a result of the ability of HA14-1 to interfere with the redox-modulatory effect of Bcl-2, thereby facilitating flavopiridol-induced apoptosis [56,57]. Clearly, a heightened level of Bcl-2 presents a major hurdle towards ROS-based chemotherapeutic strategies. Bcl-2 expression status of various malignancies should thus be of significant consideration amidst the design of future chemoprevention strategies.

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