2-Methoxy antimycin reveals a unique mechanism for Bcl-x_L inhibition

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Abstract

Overexpression of Bcl-x_L in multiple cancers correlates with resistance to chemotherapy and radiation therapy, and provides a rationale for development of small-molecule Bcl-x_L inhibitors. Based on knockout studies, nonneoplastic cells also require Bcl-x_L survival functions, particularly when challenged with cytotoxic agents. We analyze the selective cytotoxicity of one Bcl-x_L inhibitor, 2-methoxy antimycin A, toward cells with excess exogenous Bcl-x_L in isogenic cell line pairs. This selectivity, characteristic of a gain-of-function mechanism, is not shared by other known Bcl-x_L inhibitors, including BH3I-2, HA14-1, ABT-737, gossypol, or the stapled BH3 helical peptide SAHB-BID. We show that Bcl-x_L overexpression induces a shift in energy metabolism from oxidative phosphorylation to glycolysis. Treatment with 2-methoxy antimycin A acutely reverses the metabolic effects of Bcl-x_L, causing mitochondrial hyperpolarization and a progressive increase in mitochondrial NAD(P)H. We identify an additional small-molecule Bcl-x_L inhibitor, NSC 310343, establishing a class of Bcl-x_L inhibitors with gain-of-function activity. In contrast to other Bcl-x_L inhibitors, combining gain-of-function Bcl-x_L inhibitors with a standard inducer of apoptosis, staurosporine, enhances selective cytotoxicity toward Bcl-x_L–overexpressing cells. These results provide an example of the intersection of bioenergetic metabolism and Bcl-x_L functions and suggest a metabolic basis for the gain-of-function mechanism of Bcl-x_L inhibitors. [Mol Cancer Ther 2007;6(7):2073–80]

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Introduction

The regulation of apoptosis depends on the balance of cellular proapoptotic and antiapoptotic factors. This vital decision is governed by the family of Bcl-2–related proteins, composed of both death- and survival-promoting members with conserved homology domains BH1 to BH4. The Bcl-2 protein family can be subdivided into three categories; the first two consist of antiapoptotic and proapoptotic multidomain proteins (1, 2). The third group, designated ''BH3-only'' proteins, consists of single-domain proapoptotic proteins that function to inhibit proteins in the first category or activate those in the second (3). In particular, the BH3-only proteins BIM and BID have been proposed to activate the multidomain BAX and BAK proteins in response to distinct apoptotic signals (4, 5). One way in which antiapoptotic Bcl-2 proteins prevent activation of BAX is by sequestering ''activator'' BH3-only proteins through direct binding (6).

The increased expression of antiapoptotic Bcl-2 family members in various cancers has stimulated the development of small-molecule inhibitors as potential cancer therapeutics (7). A prevailing model of how these inhibitors work is by disruption of protein-protein interactions between prosurvival and proapoptotic family members (8). If cancer cells are ''primed to die'' with preformed heterodimers of antiapoptotic and BH3-only proteins, Bcl-2 inhibitors may display activity as single agents (9). However, in the perhaps more usual situation in which cancer cells have excess Bcl-2 ''capacity,'' Bcl-2 inhibitors are likely to have little effect as single agents and may sensitize normal cells as well as cancer cells to standard anticancer therapies.

Alternative models of Bcl-2 survival function have been suggested, related to an ability to preserve mitochondrial functions during apoptotic stress (10). These activities can be grouped as ''housekeeping'' functions related to mitochondrial homeostasis (11). For example, Bcl-x_L inhibits mitochondrial swelling in cells treated with respiratory poisons (12), and isolated mitochondria expressing Bcl-2 are resistant to opening of the permeability transition pore triggered by atractyloside, an inhibitor of adenine nucleotide translocase, or oxidative stress (13). Bcl-2 proteins also affect mitochondrial bioenergetic functions. Bcl-x_L regulates coupled respiration by supporting ATP/ADP exchange across the outer mitochondrial membrane (14) and inhibits gating of the voltage-dependent anion channel (15, 16), whereas Bcl-2 prevents dissociation of hexokinase from mitochondrial sites caused by truncated BID or dexamethasone (17, 18).

In this study, we investigated the selective cytotoxicity of the Bcl-x_L inhibitor 2-methoxy antimycin A (2-MeAA) toward cells with higher Bcl-x_L expression in isogenic cell line pairs (19). This property is unique among other

reported Bcl-x_L or Bcl-2 inhibitors and may provide a therapeutic advantage for cancers with high levels of Bcl-x_L expression. Increased drug activity at high expression levels of the drug target can be viewed as a gain-of-function mechanism, as described for the immunosuppressive drugs cyclosporin A and FK506 and their immunophilin binding targets (20). We show that the gain-of-function mechanism of 2-MeAA acts to acutely reverse a housekeeping function of Bcl-x_L affecting partitioning of bioenergetic metabolism.

Materials and Methods

Cell Culture

Murine TAMH hepatocyte cell lines were cultured in complete medium, as previously described (21). Rat INS-1 cells were cultured in RPMI 1640 with 10% FCS (22).

Drug Treatment and Cell Viability Assay

Cells were seeded at 6×10^3 per well in 96-well plates and treated as indicated 24 h after seeding. Viability was determined as resazurin fluorescence (44 μmol/L; R&D Systems) 6 h after addition using a Fluoroscan Ascent plate reader (ex, 544 nm; em, 590 nm; ThermoElectron). All results were normalized to DMSO controls. 2-$MeAA_1$, BH3I-1, and BH3I-2 (ChemBridge); HA-14 (Maybridge); NSC 310343 (National Cancer Institute); staurosporine (Kamiya); and etoposide (Sigma) were prepared as stock solutions in DMSO and stored at −20°C. BID $SAHB_A$ was provided by Loren Walensky (Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA).

NAD(P)H and Tetramethyl Rhodamine Methyl Ester Fluorescence Measurements

Cells were grown to subconfluence on Bioptechs ΔT dishes (Bioptechs) and incubated with 10 nmol/L tetramethyl rhodamine methyl ester (TMRM; Invitrogen) for 30 min before analysis. At this concentration, mitochondrial TMRM fluorescence is not quenched as verified by addition of uncoupling agents. Simultaneous measurements of NAD(P)H and mitochondrial TMRM fluorescence were made using a Zeiss LSM-510 inverted scanning confocal microscope with tunable IR laser for two-photon excitation. NADH and NADPH have identical fluorescence spectra with peak single photon excitation at 340 nm and emission at 465 nm. Measurements of NAD(P)H and mitochondrial TMRM fluorescence were made using 140-fs pulses of 730-nm light. Both measurements remained stable during 1 h of illumination at 10-min intervals due to the highly focused excitation volume and low average power of the excitation beam (7 mW at the sample). Cells were imaged using a 40× Zeiss Plan-Apochromat oil immersion objective. NAD(P)H and TMRM fluorescence were collected with 390 to 465 nm and 565 to 615 nm bandpass filters, respectively. Confocal optical sections were collected at 1-μm intervals along the *Z* axis to generate three-dimensional stacks. Stacks were rendered as volumes using Volume With Velocity software (Improvision). NAD(P)H autofluorescence intensity was determined on three-dimensional renderings of individually masked cells and assigned as mitochondrial or cytoplasmic based on colocalization with TMRM fluorescence channels.

Competitive Bcl-x_L Binding Assay

Fluorescence binding studies were done as previously described (23). Briefly, 3 μmol/L 1-anilinonaphthalene-8-sulfonic acid (ANS; Sigma) was mixed with 3 μmol/L recombinant human Bcl-x_L protein in 10 mmol/L phosphate, 0.14 mol/L NaCl, 2.7 mmol/L KCl (pH 7.4). ANS fluorescence was excited at 374 nm and emission was recorded from 400 to 600 nm. NSC 310343 was added to a final concentration of 3 μmol/L and changes in ANS fluorescence were measured.

Results

A Gain-of-Function Mechanism of Cytotoxicity Is Unique to 2-MeAA among Reported Bcl-x_L Inhibitors

In isogenic TAMH cell line pairs, the cytotoxicity of 2-$MeAA_1$ is higher (7-fold lower EC_{50}) for cells stably transfected with Bcl-x_L (TAMH-Bcl-x_L expressing a 4- to 5-fold higher level of Bcl-x_L protein) compared with vector-transfected control cells (TAMH-neo; ref. 19). Increased expression of Bcl-x_L did not affect Mcl-1 expression (data not shown). Expression of a Bcl-x_L mutant protein with wild-type antiapoptotic function but reduced binding affinity for 2-MeAA, A142L, did not increase the sensitivity of TAMH hepatocytes to 2-MeAA, validating Bcl-x_L as a critical target of 2-MeAA (Fig. 1A; ref. 24). Increasing expression of Bcl-x_L also sensitized INS-1 rat insulinoma cells to 2-$MeAA_1$ (Fig. 1B). In each of these cases, Bcl-x_L overexpression affords the expected protection from staurosporine (Fig. 1).

Bcl-x_L–Overexpressing Cells Are Not Sensitized to Other Small-Molecule Bcl-x_L Inhibitors

Given that Bcl-x_L expression in TAMH cells was increased by introduction of a plasmid cDNA without selection for its antiapoptotic function, a primed-to-die model is not expected to explain the increased sensitivity of TAMH-Bcl-x_L cells to 2-MeAA. To confirm that this model did not apply to TAMH-Bcl-x_L cells, we tested multiple different small-molecule Bcl-x_L inhibitors against the TAMH isogenic cell line pair (Fig. 2A). Gossypol, currently in clinical trials as an inhibitor of Bcl-x_L, Bcl-2, and Mcl-1, was equally cytotoxic to both cell lines (25). ABT-737, a

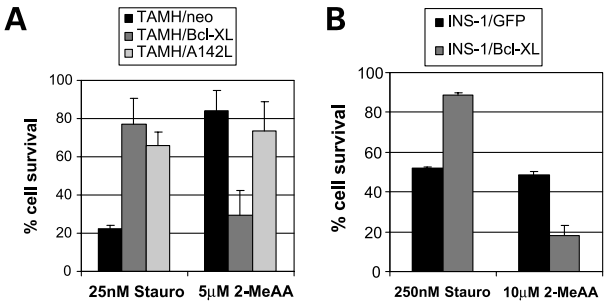

Figure 1. Gain-of-function mechanism of 2-MeAA cytotoxicity. Cell viability at 24 h after treatment with staurosporine (*Stauro*) or 2-MeAA1. TAMH (**A**) and INS-1 (**B**) cell lines stably expressing Bcl-x_L and vector controls. TAMH-A142L expresses the A142L mutant Bcl-x_L mutant protein. *Columns,* mean of quadruplicate assays; *bars,* SD.

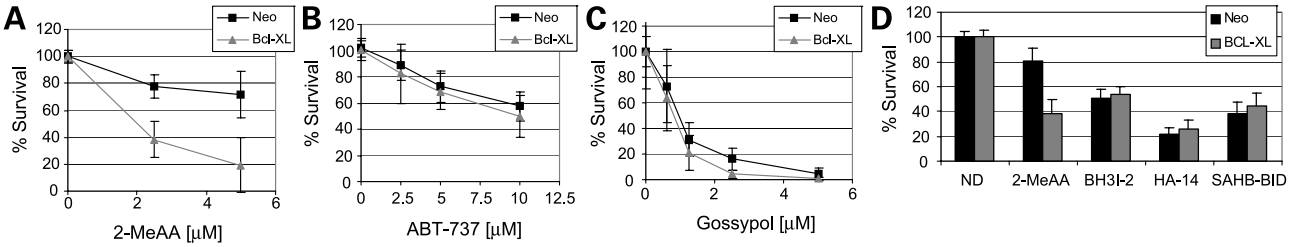

Figure 2. Gain-of-function mechanism is specific to 2-MeAA. Cell viability at 24 h after treatment with 2-MeAA1 (**A**), ABT-737 (**B**), and gossypol (**C**). **D,** cell viability after treatment with 2-MeAA1 (5 μmol/L), BH3I-1(1 μmol/L), HA-14 (5 μmol/L), and SAHB-BID (10 μmol/L). *Points* and *columns,* means of at least three separate experiments with quadruplicate assays; *bars,* SD.

compound with subnanomolar binding affinities for Bcl-x_L and Bcl-2, had only modest cytotoxicity for both cell lines (Fig. 2B and C; ref. 26). Neither ABT-737 nor gossypol showed selective cytotoxicity for Bcl-x_L–overexpressing cells at any concentration tested. Similarly, none of the other Bcl-x_L inhibitors tested, including BH3I-1, BH3I-2, and HA14-1, exhibited a gain-of-function mechanism of cytotoxicity (refs. 27, 28; Fig. 2D). Finally, we treated cells with SAHB-BID, a hydrocarbon-stapled BID BH3 peptide shown to enter cells efficiently (29). Vector-transfected TAMH cells with low levels of Bcl-x_L were slightly more sensitive to SAHB-BID than Bcl-x_L–transfected cells. Thus, most small-molecule inhibitors of Bcl-x_L do not act via the gain-of-function mechanism of cytotoxicity observed with 2-MeAA. Because these inhibitors were identified by protein-based screens for BH3 peptide mimetics or virtual docking screens for the BH3 peptide–bound hydrophobic groove, the lack of selective cytotoxicity for TAMH-Bcl-x_L cells observed for the peptide-based inhibitor SAHB-BID is consistent with these results.

Bcl-x_L Overexpression Alters Basal Partitioning of Energy Metabolism in TAMH Cells

In the absence of a primed-to-die model, we sought an alternative explanation for the increased sensitivity of Bcl-x_L–overexpressing cells to 2-MeAA. Expression of several Bcl-2 family members has been associated with altered regulation of energy metabolism. The proapoptotic BAD protein is required for mitochondrial translocation of glucokinase, and Bad-deficient mice exhibit defects in glucose tolerance (30). Transgenic targeting of Bcl-x_L to pancreatic β cells inhibits mitochondrial bioenergetic responses to glucose, pyruvate methyl ester, and α-ketoisocaproate (31).

We investigated the effect of Bcl-x_L overexpression on TAMH cell energy metabolism. TAMH-Bcl-x_L cells exhibited increased glucose consumption (4.3 ± 0.2 versus 3.8 ± 0.2 nmol/min/10^6 cells) and a higher ratio of lactate production to glucose consumption ($Y_{L/G}$, 1.65–1.57) compared with control TAMH cells (Table 1). Oxygen consumption rates of TAMH-Bcl-x_L cells were 30% lower than TAMH-neo cells (35 ± 5.5 versus 27 ± 5.2 nmol/min/10^7 cells in TAMH-neo and TAMH-Bcl-x_L cells; respectively; $P = 0.007$), indicating that Bcl-x_L overexpression is associated with a shift in bioenergetic metabolism away from oxidative phosphorylation to aerobic glycolysis. This shift does not seem to represent a fixed respiratory defect in TAMH-Bcl-x_L cells because these cells responded rapidly to changes in carbon substrate with reciprocal changes in respiration and glycolysis (Supplementary Fig. S1).[4] There was no difference in BAD expression between TAMH cells with high and low Bcl-x_L expression.

We also analyzed cellular NAD(P)H using two-photon excitation fluorescence microscopy. Mitochondrial NAD(P)H was determined by overlapping fluorescence with the mitochondria-localized TMRM dye. The ratio of mitochondrial to cytosolic NAD(P)H [$NAD(P)H_M/NAD(P)H_C$] was lower in TAMH-Bcl-x_L cells than TAMH-neo cells (Fig. 3A), consistent with reduced oxidative phosphorylation. These changes are reminiscent of the observed decrease in substrate-linked generation of mitochondrial NAD(P)H observed in pancreatic β cells expressing transgenic Bcl-x_L (31).

2-MeAA Treatment Results in a Rapid Reversal of Bcl-x_L–Associated Metabolic Phenotype

We next examined the effect of 2-MeAA treatment on mitochondrial energetics in each cell line. We repeatedly imaged NAD(P)H and TMRM fluorescence for up to 1 h after addition of 2-$MeAA_1$. Within the first 20 min after addition of 2-$MeAA_1$, TAMH-Bcl-x_L cells exhibited a striking and progressive increase in mitochondrial NAD(P)H (Fig. 3B). TMRM fluorescence increased over the same time course, indicative of mitochondrial hyperpolarization (Fig. 3B). In contrast, NAD(P)H and TMRM fluorescence intensity and subcellular distribution remained unchanged in control TAMH-neo cells for at least 1 h after 2-$MeAA_1$ treatment. These results suggested that 2-MeAA reversed the diversion of energy metabolism away from mitochondria observed with high levels of Bcl-x_L and, in fact, led to an overshoot in mitochondrial redox state.

Bioenergetic metabolism is not generally understood to modulate the cytotoxic effects of standard anticancer agents. We investigated whether this was also true for 2-MeAA, given that 2-MeAA–induced cytotoxicity seemed to correlate with both a pretreatment metabolic phenotype and a distinct metabolic response to treatment. The glucose

[4] Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Table 1. TAMH-Bcl-x_L cells exhibited increased glucose consumption, lactate extrusion, and a higher ratio of lactate production to glucose consumption

Cell	Glucose consumption (nmol/min/10^6 cells)	Lactate extrusion (nmol/min/10^6 cells)	$Y_{L/G}$
Bcl-x_L	4.3 ± 0.2	7.1 ± 0.6	1.65
Neo	3.8 ± 0.2	6.0 ± 0.5	1.57

analogue 2-deoxyglucose is a competitive substrate for hexokinase and increases cellular dependence on oxidative phosphorylation for ATP generation. Pretreatment with 2-deoxyglucose strongly potentiated 2-MeAA$_1$ killing of TAMH-Bcl-x_L cells (Fig. 3C). Similar effects were observed with oxamic acid, an inhibitor of lactate dehydrogenase (data not shown). In contrast, pretreatment with 2-deoxyglucose did not enhance staurosporine- or etoposide-induced cell death (data not shown).

Identification of a Novel Bcl-x_L Small-Molecule Inhibitor with Gain-of-Function Mechanism

2-MeAA is an analogue of antimycin A, a well-known inhibitor of ubiquinone-cytochrome *c* reductase (mitochondrial complex III). Methylation at the 2-position in the salicylate ring of antimycin A dissociates the effects on Bcl-x_L from complex III inhibitory activity (19, 32); however, the possibility remained that some of the cellular and metabolic effects of 2-MeAA could result from residual minor inhibitory effects of this compound on electron transport. We recently carried out a chemical library screen for additional Bcl-x_L inhibitors with 2-MeAA–like gain-of-function activity. From the National Cancer Institute Structural Diversity Set of 1,980 compounds (33), we identified several compounds that bind to the Bcl-x_L hydrophobic groove *and* exhibit selective cytotoxicity against Bcl-x_L–overexpressing TAMH cells.

Our investigation of one of these compounds, NSC 310343 (Supplementary Fig. S2),[4] shows that NCS 310343 binds to the Bcl-x_L hydrophobic groove (Fig. 4A) and shows an EC_{50} of ~6 to 10 μmol/L for Bcl-x_L–overexpressing TAMH cells, without cytotoxicity for control TAMH-neo cells at these concentrations (Fig. 4B). Pretreatment with 2-deoxyglucose sensitized TAMH-Bcl-x_L cells to NSC 310343, as observed for 2-MeAA$_1$ (Fig. 4C). Similar to 2-MeAA, NSC 310343 has no detectable effect on mitochondrial oxygen consumption rate at cytotoxic concentrations (Fig. 4D), indicating that the gain-of-function mechanism of cytotoxicity is not explained by off-target effects of 2-MeAA on mitochondrial bioenergetics (i.e., electron transport chain inhibition). Furthermore, these results show that 2-MeAA is not unique among Bcl-x_L inhibitors in exhibiting a gain-of-function cytotoxicity mechanism and suggest that other small molecules with this desirable activity may be identified with appropriate screening strategies.

Standard Apoptosis Stimuli Amplify the Gain-of-Function Mechanism

Because the antiapoptotic functions of Bcl-2–related proteins in normal cells are understood to protect against various types of stress, it is possible that small-molecule Bcl-x_L inhibitors could sensitize both normal cells with low levels of endogenous Bcl-x_L and cancer cells to standard anticancer agents.

To examine this possibility, we treated TAMH cell lines with staurosporine for 24 h to engage Bcl-x_L–dependent survival before treatment with various Bcl-x_L inhibitors

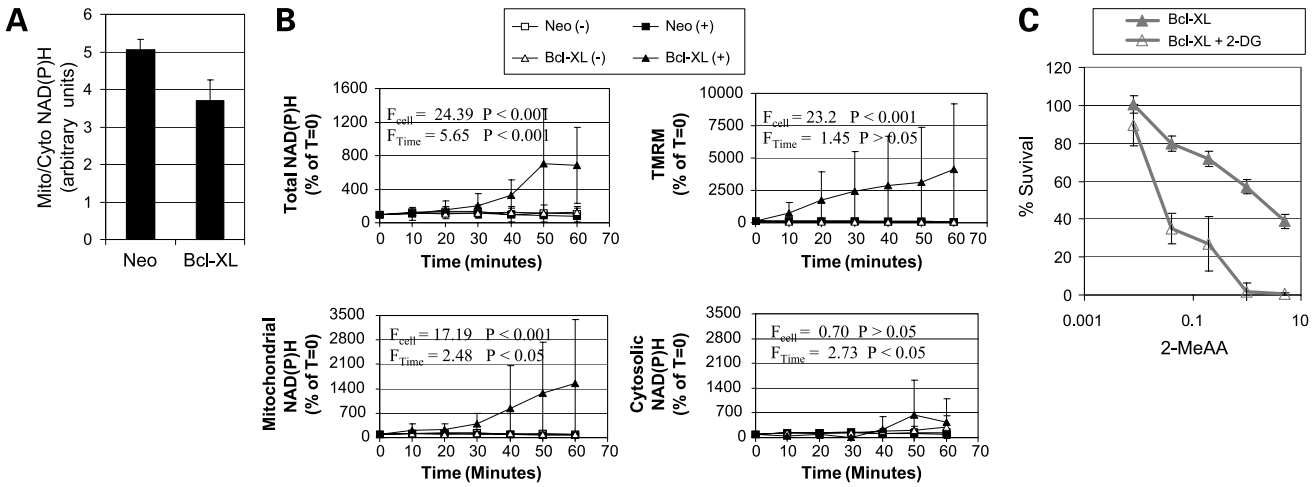

Figure 3. Mitochondrial NAD(P)H accumulation in TAMH-Bcl-x_L cells following 2-MeAA treatment. **A,** basal ratio of mitochondrial to cytoplasmic NAD(P)H autofluorescence for TAMH-Bcl-x_L and TAMH-Neo cells grown in 17.5 mmol/L glucose media. *Columns,* mean of three separate experiments; *bars,* SD. **B,** NAD(P)H autofluorescence and TMRM fluorescence following addition of 10 μmol/L 2-MeAA1 at time 0. A representative experiment is shown. *Points,* mean of eight randomly chosen cells for each treatment group; *bars,* SD. *F* ratios and significance of the 2-MeAA1 treatment effect were determined by two-way ANOVA for effect of cell line and time on the 2-MeAA – treated samples. **C,** pretreatment with 2-deoxyglucose sensitizes TAMH-Bcl-x_L cells to 2-MeAA. Cell viability of TAMH-Bcl-x_L cells treated with 10 mmol/L 2-deoxyglucose (*2-DG*) for 2 h followed by indicated concentrations of 2-MeAA1 for an additional 24 h. *Points,* mean of at least three separate experiments with quadruplicate assays; *bars,* SD.

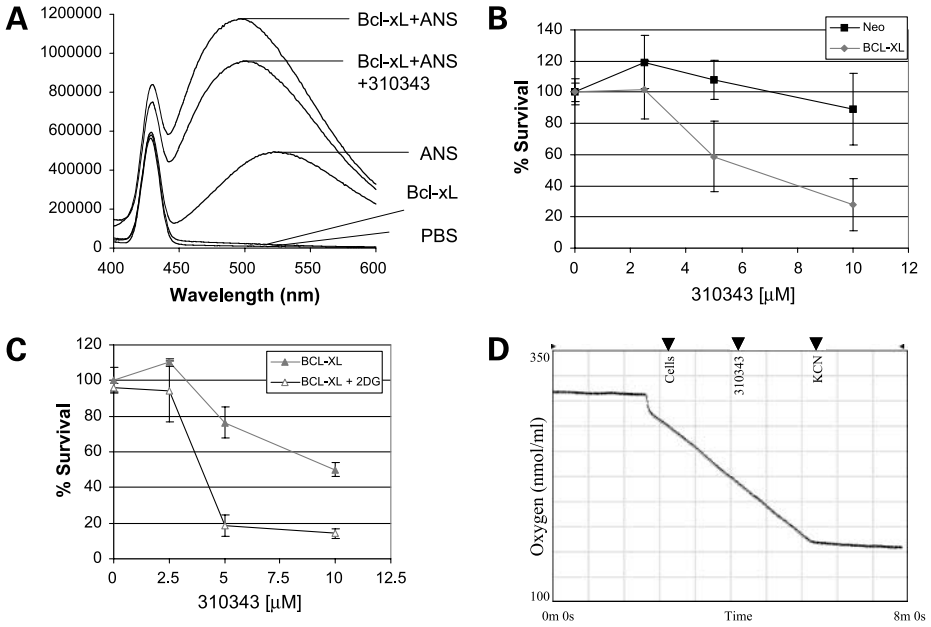

Figure 4. Gain-of-function activity with Bcl-x_L inhibitor NSC 310343. **A,** fluorescence emission spectra of ANS, Bcl-x_L, and the ANS-Bcl-x_L complex, with NSC 310343 inhibition of ANS binding. **B,** cell viability after treatment with NSC 310343 for 24 h in TAMH-neo and TAMH-Bcl-x_L cells. *Points,* mean of at least three separate experiments with quadruplicate assays; *bars,* SD. **C,** cell viability for TAMH-Bcl-x_L cells treated with 10 mmol/L 2-deoxyglucose for 2 h followed by indicated concentrations of 2-MeAA for an additional 24 h. Representative of three experiments. *Points,* mean of quadruplicate samples; *bars,* SD. **D,** oxygen consumption rate of TAMH-Bcl-x_L cells treated with NSC 310343. *Arrows,* addition of 1×10^7 cells/mL, NSC 310343 (final concentration, 10 μmol/L), and KCN (final concentration, 3 mmol/L).

(Supplementary Fig. S3A).[4] The combination of staurosporine with either 2-MeAA_1 (Fig. 5A) or NSC 310343 (Fig. 5B) produced greater than additive cell killing of TAMH-Bcl-x_L cells; however, less than additive cytotoxicity was observed for TAMH-neo cells. The increased cell killing effect of 2-MeAA_1 is due to Bcl-x_L inhibition because the A142L Bcl-x_L mutant with reduced binding affinity is sensitive to the combination treatment only at much higher drug concentrations (Fig. 5A). To determine whether the combination of 2-MeAA and staurosporine was synergistic, the combination index was calculated from isobologram analysis (Supplementary Fig. S3B).[4] The combination was synergistic in the TAMH-Bcl-x_L cells and antagonistic in the TAMH-neo cells (combination index = 0.157 and 1.763, respectively).

We also tested ABT-737 and gossypol against the TAMH cell lines in combination with staurosporine. Both TAMH-neo and TAMH-Bcl-x_L cells showed greater than additive cytotoxicity with ABT-737 and staurosporine, with a stronger effect for TAMH-neo cells (Fig. 5C). Cotreatment with gossypol did not produce additive effects (Fig. 5D). Furthermore, only the gain-of-function Bcl-x_L inhibitors exhibit selective killing of Bcl-x_L–overexpressing cells in combination with staurosporine (Fig. 5E). The relative insensitivity of TAMH-neo cells to 2-MeAA_1 or NSC 310343 combined with a standard apoptotic stimulus suggests that Bcl-x_L inhibitors with gain-of-function mechanisms might be used, with an enhanced therapeutic window, in combination drug regimens for cancers with Bcl-x_L–dependent chemoresistance.

Discussion

The antiapoptotic functions of Bcl-2, Bcl-x_L, and potentially other family members, including Mcl-1 and Bcl-w, confer marked resistance to the cytotoxic effects of available anticancer agents. In addition, Bcl-2 survival proteins are overexpressed in comparison with normal tissue counterparts in a significant subset of common cancers (34, 35). Basal Bcl-x_L expression is a strong negative predictor of cell survival with diverse classes of chemotherapeutic agents in the 60 cell lines included in the National Cancer Institute anticancer drug screen (36). Thus, small-molecule inhibitors of Bcl-2 and Bcl-x_L have drawn much interest as a goal for molecularly targeted cancer therapy. A major challenge in developing therapeutic inhibitors for Bcl-2–related survival proteins is the expression of these targets in many normal cell types (37, 38). The dependence of healthy tissues on the Bcl-2 family of antiapoptotic proteins for cell viability may narrow the therapeutic window for these agents. 2-MeAA represents the first of a novel class of inhibitors that display gain-of-function cytotoxicity, defined as enhanced killing in a cell line overexpressing a Bcl-2 survival protein target compared with an isogenic control cell line.

Based on the gain-of-function model, cancer cells with high endogenous levels of Bcl-x_L would be predicted to exhibit greater sensitivity to 2-MeAA than cancers with low Bcl-x_L expression. We have calculated average GI_{50} concentrations for 2-MeAA and three standard chemotherapeutic agents,[5] comparing the five cancer cell lines with the highest Bcl-x_L mRNA expression in the National Cancer Institute Anticancer Drug Screen to the five cell lines with lowest Bcl-x_L expression (36). In contrast to the negative correlation between Bcl-x_L expression level and standard agents, Bcl-x_L expression shows a positive correlation with

[5] http://www.dtp.nci.nih.gov/docs/cancer/cancer_data.html

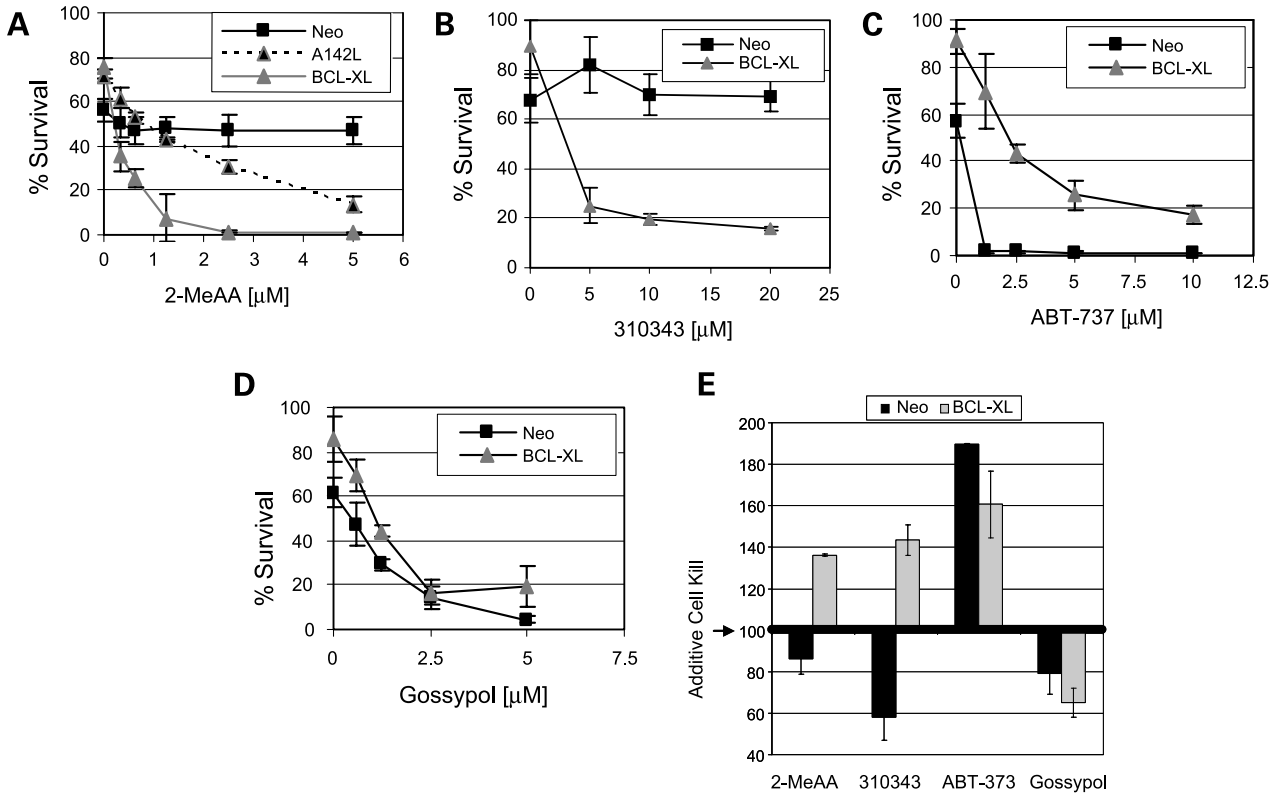

Figure 5. Gain-of-function mechanism of cytotoxicity synergizes with staurosporine. **A** to **D,** cell viability after pretreatment with 5 nmol/L staurosporine for 24 h, followed by indicated concentrations of 2-MeAA1 (**A**), NSC 310343 (**B**), ABT-737 (**C**), or gossypol (**D**) for additional 24 h. Representative of three experiments. *Points,* mean of quadruplicate samples; *bars,* SD. **E,** observed versus expected cell viability. Expected additive killing (staurosporine + Bcl-x_L inhibitor) is set at 100%. The following concentrations are represented for each inhibitor: 2-MeAA1, 2.5 μmol/L; NSC 310343, 5 μmol/L; ABT-737, 1.25 μmol/L; gossypol, 0.6 μmol/L.

sensitivity to 2-MeAA (Supplemental Table S1;[4] Fig. 4). In a recent article, Cao et al. (39) observed *in vitro* and *in vivo* sensitivity of mesothelioma cell lines with high expression of Bcl-x_L and Bcl-2 to 2-MeAA. Thus, this class of activity may expand the potential of Bcl-2 inhibitors beyond chemosensitization while also providing an improved therapeutic index.

Cancer cells with high endogenous levels of Bcl-2 family survival proteins may be primed to die due to constitutive activation of proapoptotic pathways (9). Small molecules that compete with BH3-only proteins for binding to Bcl-2 will displace BID or BIM, two activator BH3-only proteins capable of triggering BAX and BAK activation. Several results suggest that the 2-MeAA gain-of-function mechanism of cytotoxicity does not fit into the primed-to-die paradigm. First, introduction of exogenous Bcl-x_L into healthy cells does not lead to increased sensitivity to cell death stimuli as might be expected with basal activation of BH3-only proteins. Second, small-molecule Bcl-x_L inhibitors discovered on the basis of competitive displacement of BH3 peptides bound to Bcl-x_L (BH3I-1, BH3I-2, gossypol, ABT-737) do not exhibit a gain-of-function mechanism of cytotoxicity (25–27). Most significantly, a cell-permeable BH3 peptide with stabilized α-helical conformation also lacks selective cytotoxicity toward TAMH cells with high Bcl-x_L expression (29). These results, in sum, strongly suggest that the 2-MeAA gain-of-function mechanism of cytotoxicity does not depend on disruption of preformed Bcl-x_L heterodimers with activator BH3-only proteins.

An alternative possibility is that Bcl-x_L expression generates secondary changes in cellular homeostatic mechanisms, which become unstable if Bcl-x_L function is acutely inhibited. The bioenergetic signature associated with Bcl-x_L overexpression in TAMH cells would fit this model. Several published studies support a link between cellular bioenergetics and Bcl-x_L function (12, 40). At high levels of Bcl-x_L transgene expression in pancreatic β cells, the ability of various metabolic substrates to stimulate mitochondrial membrane hyperpolarization, NAD(P)H, and ATP generation is suppressed, leading to impaired glucose tolerance and insulin secretion (31). In studies of uncoupled cardiac mitochondria, Bcl-x_L restricts ATP consumption by the F1F0-ATPase, consistent with inhibition of adenine nucleotide transport or ATPase function (41). Conversely, in the absence of glucose or other exogenous substrates, Bcl-x_L supports mitochondrial function and the ability to metabolize endogenous fuels (16).

Here we observed that high levels of Bcl-x_L in the TAMH hepatocyte cell line increase glucose conversion to lactate ($Y_{L/G}$) at the expense of oxidative phosphorylation [oxygen consumption rate and NAD(P)H_M/NAD(P)H_C]. This effect does not result from an irreversible impairment of mitochondrial function or reduction in capacity because substitution of pyruvate for glucose in culture media leads to a rapid increase in oxygen consumption rate and inhibition of lactate production by TAMH-Bcl-x_L cells (Supplementary Fig. S1[4] and data not shown). Acute inhibition of Bcl-x_L with 2-MeAA results in different metabolic responses depending on the level of Bcl-x_L expression. In cells expressing high levels of Bcl-x_L, progressive mitochondrial accumulation of reduced NAD(P)H was observed, reversing the effects of Bcl-x_L in the basal state. In contrast, for TAMH-Neo cells with normal endogenous levels of Bcl-x_L, mitochondrial and cytoplasmic NAD(P)H levels remained steady after treatment with 2-MeAA. Mitochondrial NAD(P)H redox state is a significant driving force for reactive oxygen species generation (42). As opposed to its parent compound, antimycin A, 2-MeAA does not inhibit mitochondrial electron transport (19), and direct effects on mitochondrial NAD(P)H oxidation would be expected to affect both TAMH-Bcl-x_L and TAMH-neo cells. Judged by the lack of effects of NSC 310343 on oxygen consumption in TAMH-Bcl-x_L cells, the gain-of-function mechanism of cytotoxicity is independent of electron transport inhibition.

Mitochondrial NADH is generated by mitochondrial oxidation of pyruvate and fatty acids and by transfer of cytosolic reducing equivalents through the malate-asparate shuttle. Both pyruvate transport and asparate/glutamate transporters in the malate-aspartate shuttles are electrogenic, involving H(+) cotransport for mitochondrial uptake of pyruvate and glutamate. The increased mitochondrial membrane potential observed in 2-MeAA–treated TAMH-Bcl-x_L cells may promote mitochondrial NADH accumulation. Addition of inhibitors of the mitochondrial pyruvate transport (cyano-4-hydroxycinnamic acid) or the malate-aspartate shuttle (aminooxyacetic acid) suppressed 2-MeAA–induced mitochondrial NAD(P)H accumulation in TAMH-Bcl-x_L cells (data not shown).

In view of the observation that 2-MeAA–induced cell killing and binding to Bcl-x_L protein are both suppressed by Bcl-x_L mutations in the hydrophobic groove binding site (43), 2-MeAA may act on Bcl-x_L proteins directly in the absence of bound BH3-only proteins. 2-MeAA inhibits the pore activity of Bcl-x_L and Bcl-2 in synthetic lipid membranes, a function that does not depend on proapoptotic binding partners (19, 23). This finding implies that small-molecule ligands to the hydrophobic groove of Bcl-2 antiapoptotic proteins may have effects beyond inhibition of protein-protein interactions. These effects may fall into the category of housekeeping functions, such as the role of BAD in glucose homeostasis, and extend beyond apoptosis. The question still remains on what extent housekeeping functions depend on interactions between proapoptotic and antiapoptotic Bcl-2 family members (11).

Various apoptotic stimuli, including staurosporine, elicit mitochondrial pathways of apoptosis and are opposed by Bcl-x_L overexpression (44, 45). Staurosporine, and possibly other types of apoptotic stress, seem to synergize with low doses of 2-$MeAA_1$ and NSC 310343 in selectively killing TAMH-Bcl-x_L cells. In contrast, whereas ABT-737 also showed enhanced cell killing when combined with staurosporine, TAMH-neo cells were sensitized to a greater degree, negating any therapeutic advantage.

Recently, 2-MeAA has been shown to synergize with cisplatin in treatment of mesothelioma cell lines both *in vitro* and *in vivo* (39). Thus, the gain-of-function mechanism of cytotoxicity associated with 2-MeAA and NSC 310343 may afford distinct advantages in selectively targeting cancer cells, particularly when combined with standard agents that trigger Bcl-x_L–dependent apoptotic pathways. Our future work will focus on investigating the effects of 2-MeAA in combination treatment with various chemotherapeutic agents. We anticipate that the mechanism of cell death triggered by these agents may determine the effectiveness of the combination treatments. Furthermore, investigating the metabolic changes that occur in TAMH-Bcl-x_L cells following treatment with staurosporine may provide further insights into the lethal mechanism of these Bcl-x_L inhibitors.

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