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ABT-737 overcomes Bcl-2 mediated resistance to doxorubicin–DNA adducts

Michal Ugarenko [a,1], Abraham Nudelman [b,2], Ada Rephaeli [c,3], Ken-Ichi Kimura [d], Don R. Phillips [a,1], Suzanne M. Cutts [a,*]

[a] Department of Biochemistry, La Trobe University, Victoria 3086, Australia
[b] Chemistry Department, Bar Ilan University, Ramat Gan 52900, Israel
[c] Felsenstein Medical Research Center, Sackler School of Medicine, Tel Aviv University, Beilinson Campus, Petach Tikva 49100, Israel
[d] Department of Biological Chemistry and Food Science, Iwate University, Morioka, Iwate 020-8550, Japan

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ABSTRACT

Doxorubicin is an anthracycline anticancer agent that functions primarily by inhibiting topoisomerase II, but also forms covalent DNA adducts depending on the cellular availability of formaldehyde. The combination of formaldehyde-releasing prodrugs (such as AN-9) with doxorubicin has been shown to result in synergistic doxorubicin–DNA adduct formation and synergistic apoptosis in HL-60 leukemic cells, offering the potential for lower concentrations of doxorubicin to be used clinically in order to minimize side-effects. However, the overexpression of Bcl-2 confers resistance to doxorubicin/AN-9 DNA adduct forming treatments, thus limiting the therapeutic potential of this drug combination. The small molecule inhibitor, ABT-737, which binds to and inhibits Bcl-2, Bcl-xL and Bcl-w, was used in combination with doxorubicin/AN-9 treatments to overcome resistance to doxorubicin–DNA adducts in Bcl-2 overexpressing HL-60 cells (HL-60/Bcl-2). The combination treatment of doxorubicin and AN-9 (and all single agent controls) failed to induce an apoptotic response in HL-60/Bcl-2 cells, however, the addition of low nanomolar (sub-lethal) concentrations of ABT-737 was able to greatly increase apoptosis levels. Various control compounds were used to demonstrate that the mechanism of cell kill in response to the 'triple treatment' (doxorubicin, AN-9 and ABT-737) is dependent on DNA adduct formation. Therefore, the ability of ABT-737 to inhibit Bcl-2 renders previously resistant HL-60 cancer cells highly sensitive to doxorubicin–DNA adducts, leading to a classical apoptotic response. In conclusion, the data obtained provides promising evidence that the anticancer activity of doxorubicin–DNA adducts can be substantially enhanced in Bcl-2 overexpressing cancers with the use of the small molecule Bcl-2 inhibitor, ABT-737.

1. Introduction

The anthracyclines (including doxorubicin, daunorubicin, idarubicin and epirubicin) are a group of antibiotics that possess anticancer activity against a broad spectrum of cancers [1]. Doxorubicin (Fig. 1A) is commonly utilized in combination chemotherapy with drugs that have a complementary mode of action to minimize drug resistance and maximize tumor cell kill [2,3]. Despite its wide use in the clinic, doxorubicin is limited by cardiotoxic side-effects and tumor cell resistance [4].

The primary mechanism of action of doxorubicin (and other anthracyclines) appears to be the poisoning of the enzyme topoisomerase II which results in double-strand DNA breaks, and the failure to repair these breaks leads to apoptosis [5]. More recently however, it has been demonstrated that doxorubicin also forms covalent adducts with DNA and these lesions are more cytotoxic than those induced by topoisomerase II impairment [6]. The adducts are formed predominantly at 5′-GC-3′ sites in DNA [7] where the doxorubicin sugar group (daunosamine) is covalently linked to the N-2 amino group of guanine via an aminal (N–C–N) bond [8–11]. The central carbon atom in the aminal bond is derived from formaldehyde, hence formaldehyde is an absolute requirement for adduct formation [10,12]. The resulting drug–DNA monoadduct (Fig. 1B) is further stabilized through intercalation and hydrogen bonding with the second strand of DNA [10]. Apoptosis resulting from doxorubicin–DNA adduct formation does not depend on topoisomerase II status, thus reflecting an

Abbreviations: AML, acute myelogenous leukemia; CDK, cyclin-dependent kinase; CLL, chronic lymphocytic leukemia; CPM, counts per minute; DTT, dithiothreitol; FACS, fluorescence-activated cell sorting; PEG, poly-ethylene glycol; SCLC, small-cell lung carcinoma; WT, wild-type.

* Corresponding author. Tel.: +61 3 94791517; fax: +61 3 94792467.
E-mail addresses: mugarenko@students.latrobe.edu.au (M. Ugarenko), Nudelman@mail.biu.ac.il (A. Nudelman), adarep@post.tau.ac.il (A. Rephaeli), s.cutts@latrobe.edu.au (S.M. Cutts).
[1] Tel.: +61 3 94791182; fax: +61 3 94792467.
[2] Tel.: +972 3 5318314; fax: +972 3 5318314.
[3] Tel.: +972 3 9376126; fax: +972 3 9228096.

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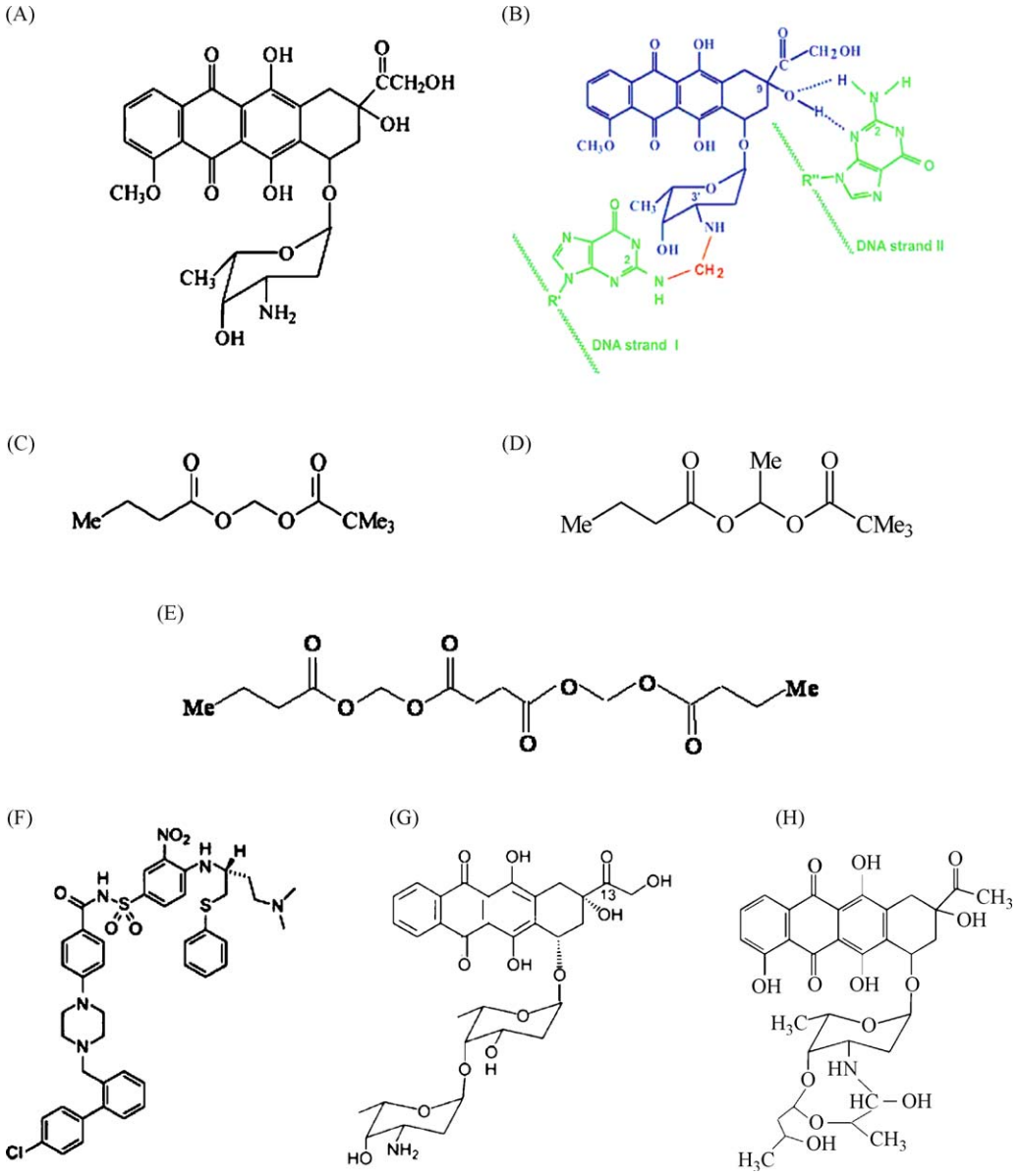

Fig. 1. Structures of (A) doxorubicin; (B) the doxorubicin–DNA adduct, with the central carbon in the aminal linkage derived from formaldehyde shown in red; (C) AN-9; (D) AN-158; (E) AN-193; (F) ABT-737; (G) MEN-10755; (H) barminomycin. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

independent mechanism of cell kill and highlighting that formaldehyde availability switches the mechanism of doxorubicin action from topoisomerase II impairment to the formation of more cytotoxic DNA adducts [6].

Doxorubicin–DNA adducts have been detected in breast cancer cells (using accelerator mass spectrometry) after treatment with sub-micromolar doxorubicin (25–200 nM) [13]. This is attributed to endogenous formaldehyde levels which are often higher in tumor cells (1.5–4 μM) compared to normal cells [14,15], as well as formaldehyde production from the oxidation of doxorubicin itself [12]. While evidence indicates that doxorubicin–DNA adduct formation occurs in tumor cells using clinically relevant concentrations of doxorubicin as a single agent, there has been interest in increasing the level of adducts with the use of exogenous formaldehyde. The formaldehyde-releasing prodrug AN-9 (pivaloyloxymethyl butyrate; Fig. 1C) is cleaved by intracellular esterases to release formaldehyde, butyric acid and pivalic acid [16]. AN-9 functions as a histone deacetylase inhibitor due to its ability to release butyric acid [17], and displays anticancer activity as a single agent both in vitro [18,19] and in vivo [16,20], and has been well-tolerated in a Phase II clinical trial [21]. AN-9 has also been used in combination with doxorubicin, resulting in synergistic doxorubicin–DNA adduct formation [6,22] and synergistic induction of apoptosis [6,23]. This synergy is due solely to the released formaldehyde [22]. Furthermore, it has been shown that the combination of daunorubicin and AN-9 increased the survival of mice with monocytic leukemia [20].

One of the major issues surrounding current cancer therapy is chemoresistance. In particular, many cancer cells overexpress anti-apoptotic proteins such as Bcl-2 which allows cells to survive in the presence of death signals induced by chemotherapeutic compounds [24,25]. Recent evidence implicates an indirect model for Bax/Bak activation where the hydrophobic grooves of the anti-apoptotic proteins Bcl-2, Bcl-XL, Bcl-w and Mcl-1 bind to the BH3 domains of pro-apoptotic Bax/Bak, thus keeping Bax/Bak in check and preventing the initiation of the apoptotic cascade [26,27]. Upon various apoptotic stimuli, BH3-only proteins (e.g. Bid, Bim, Bad, Noxa, Puma) become activated and bind to the anti-apoptotic

proteins, thus displacing Bax/Bak and allowing apoptosis to proceed [28,29].

Since the overexpression of Bcl-2 and other anti-apoptotic proteins has been implicated in tumor progression and maintenance [30], and drug resistance phenotype [31], this has prompted the development of strategies to target and inhibit anti-apoptotic proteins to overcome the block in apoptosis [32]. Recently, Abbott Laboratories developed a small molecule inhibitor, ABT-737 (Fig. 1F), which has a high affinity for Bcl-2, Bcl-XL and Bcl-w (Ki $\leq$ 1 nM), but not for Mcl-1 or A1 [33]. The compound acts as a BH3 mimetic by inserting into the hydrophobic groove of the anti-apoptotic proteins, thus preventing their ability to inhibit apoptosis [33] and allowing Bax/Bak to trigger mitochondrial outer membrane permeabilization and caspase activation.

ABT-737 is cytotoxic as a single agent in follicular lymphoma [33], chronic lymphocytic leukemia (CLL) [33,34], acute lymphocytic leukemia [35], acute myelogenous leukemia (AML) [36], and small-cell lung carcinoma (SCLC) [37] by inducing Bax/Bak dependant apoptosis. It has also been demonstrated that while ABT-737 is able to kill primary AML and CLL cells, non-malignant cells are not sensitive to ABT-737 [34,36]. ABT-737 displays synergistic cytotoxicity with radiation and several genotoxic agents including doxorubicin and etoposide [33] and has been shown to overcome Bcl-2 resistance to Imatinib in Bcr/Abl + leukemic cells [38]. Based on these promising in vitro results, ABT-737 has been applied to numerous mouse models where it has been well-tolerated and has caused complete regression of established xenograft SCLC tumors [33] and extended survival of mice in an AML model [36].

In the present study, we show that HL-60 cells overexpressing Bcl-2 are resistant to doxorubicin/AN-9 adduct forming treatments, and this resistance can be overcome with the addition of ABT-737. We report that the use of low nanomolar concentrations of ABT-737 is highly synergistic with doxorubicin/AN-9 in HL-60/Bcl2 cells. Cell kill induced by the 'triple treatment' (doxorubicin, AN-9, ABT-737) is dependent on DNA adduct formation and can potentially be increased with prodrugs that release higher levels of formaldehyde. Overall, we report that the clinical potential of doxorubicin/AN-9 treatments can be increased with the addition of ABT-737, thus allowing previously resistant cancer cells to be effectively killed in response to the triple treatment.

2. Materials and methods

2.1. Cell lines

The HL-60 promyelocytic leukemic cell line (HL-60/WT) and the mitoxantrone resistant HL-60/MX2 cell line which does not express topoisomerase IIβ and exhibits reduced topoisomerase IIα expression, were obtained from the American Type Culture Collection (Rockville, MD). HL-60 cells overexpressing Bcl-2 (HL-60/Bcl2) and the parental empty vector control cell line (HL-60/Puro) were obtained as a gift from Dr Gino Vairo (CSL Limited, Melbourne, Australia) and contain a stably inserted plasmid expressing puromycin resistance. HL-60/Bcl2 and HL-60/Puro cells were maintained in the presence of 2 μg/mL puromycin (Sigma, St. Louis, MO). All HL-60 cell lines were routinely passaged in RPMI 1640 media supplemented with 10% FCS (Trace Scientific, Melbourne, Australia) and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Chemicals

Doxorubicin was a gift from Pfizer (formerly Farmitalia, Milan, Italy), and radiolabeled [14-^{14}C]-doxorubicin (55 mCi/mmol) was obtained from GE Healthcare Biosciences (Little Chalfont, UK) and both were dissolved to a 1 mM stock solution in Milli-Q water and stored at −20 °C. Barminomycin was isolated and characterized as described [39], dissolved in methanol and stored at −20 °C, and diluted in PBS before use. The prodrugs AN-9, AN-158 and AN-193 were synthesized as previously described [16]. ABT-737 (A-779024; absolute configuration is "R") and its enantiomer (A-793844; absolute configuration is "S") were synthesized and kindly provided by Abbott Laboratories (Abbott Park, IL), dissolved in DMSO to produce a 5 mM stock solution and stored at −20 °C. MEN-10755 was a gift from Menarini Richerche SpA (Pomezia, Italy). The caspase inhibitor ZVAD-fmk was purchased from Promega (Madison, WI).

2.3. Western blot analysis

Cells were lysed and total protein from cell lysates were separated on 10% Bis–Tris gels by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 10% skim milk in PBS overnight at 4 °C and washed three times for 5 min in TBS containing 0.1% Tween 20 (TBS-T) before probing with primary and secondary antibodies. For Bcl-2 detection, anti-Bcl2 (1:500, Calbiochem, San Diego, CA) in TBS-T was applied overnight at 4 °C and anti-mouse (1:2000, Sigma) IgG HRP was used as the secondary antibody. To ensure equal loading of proteins, membranes were re-probed with an anti-actin antibody (Sigma). Bands were detected using Lumi-Light Western Blotting Substrate (Roche, Mannheim, Germany).

2.4. Sub-G1 FACS assay

HL-60 cells (2×10^5 per mL) were treated in 6-well plates for indicated times, pelleted (1200 rpm for 5 min) and fixed by resuspension in 70% ethanol for at least 30 min at 4 °C. After fixing, cells were pelleted (4000 rpm for 5 min), washed in PBS and centrifuged for a further 5 min. Cell pellets were resuspended in 250 μL of staining solution (2.5 μg/mL propidium iodide, 50 μg/mL RNase A in PBS) and incubated for 30 min at 37 °C in the dark. Samples were transferred to FACS tubes and stored on ice until analysed.

Analysis was performed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) employing FACSDiva software. Samples (10,000 events) were gated to distinguish small debris and doublets by employing a forward scatter versus side scatter dot plot and applying an appropriate gate. The gated events were plotted as a PI-A histogram and a marker region was set up to distinguish normal DNA content (G1, S, and G2 region) from sub-G1 or apoptotic DNA content. Quantitative data was obtained where the percentage of sub-G1 events was proportional to the percentage apoptosis for a given sample.

2.5. Caspase-3 activation assay

HL-60/Puro and HL-60/Bcl2 cells (2×10^5 per mL) were treated in 6-well plates for 6 h, pelleted, and lysed in chilled lysis buffer (10 mM EDTA, 0.5% Triton X-100, 10 mM Tris–HCl pH 8 in Milli-Q water) for 10 min at room temperature. DNA was sheared using a 23 gauge needle and samples were centrifuged at 13,000 rpm for 15 min at 4 °C. The caspase-3 substrate, Ac-DEVD-AFC (Calbiochem) was added to substrate buffer (0.1 M HEPES pH 7, 10% PEG-3350, 0.1% CHAPS, 10 mM DTT in Milli-Q water) to a final concentration of 50 μM. An aliquot of the cell lysate (20 μL containing approximately 25 μg total protein) was added to 80 μL of substrate mix and the resulting solution was mixed and added to a 96-well black, clear-bottom plate. Samples were incubated for 4 h in the dark and the fluorescence intensity (excitation 400 nm,

emission 505 nm) was recorded using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The fluorescence intensity obtained from a lysis buffer control sample was subtracted from cell lysate containing samples.

2.6. Morphology assay

HL-60/Puro and HL-60/Bcl2 cells (2×10^5 per mL) were treated in 6-well plates for 6 h, pelleted, fixed in 3.7% paraformaldehyde for 30 min, and washed in PBS. An aliquot of the cell suspension (30 μL) was added onto polylysine coated coverslips and incubated for 30 min at room temperature. The coverslips were washed twice in PBS and cells were permeabilized with the addition of 0.5% Triton X-100 for 5 min. Coverslips were washed again in PBS three times before the addition of Hoechst 33258 (1 μg/mL) and the coverslips were incubated for 30 min at 37 °C. The coverslips were rinsed in PBS to remove excess stain, mounted onto slides and examined using an Olympus BX-50 fluorescence microscope (Olympus, Tokyo, Japan). At least 200 cells per treatment were scored for apoptotic morphology based on the appearance of chromatin aggregation and fragmented nuclei.

2.7. Detection of [^{14}C]-doxorubicin–DNA adducts

HL-60 cells (3×10^5 per mL) were treated in 6-well plates with 1 μM [^{14}C] doxorubicin and 50 μM formaldehyde-releasing prodrugs (AN-9, AN-158, AN-193) for 4 h. Cells were harvested and the genomic DNA was isolated using a QIAmp blood kit (Qiagen, Hilden, Germany). Samples were subjected to two phenol extractions and one chloroform extraction to remove non-covalently bound drug and the DNA was ethanol precipitated in sodium acetate. The DNA pellet was resuspended in 100 μL TE buffer and the concentration of DNA was determined spectrophotometrically at 260 nm. Aliquots (90 μL) were added to 1 mL of ReadySafe Scintillation Cocktail (Beckman Coulter, Fullerton, CA). The level of [^{14}C]-doxorubicin incorporated into DNA was monitored using a Wallac 1410 Liquid Scintillation Counter and expressed as doxorubicin–DNA adducts per 10 kbp DNA.

3. Results

3.1. ABT-737 is cytotoxic as a single agent in HL-60 cells

To establish whether ABT-737 can overcome Bcl-2 mediated resistance to doxorubicin/AN-9 adduct forming treatments, HL-60 promyelocytic leukemic cells which constitutively overexpress Bcl-2 (HL-60/Bcl2) were used. Fig. 2A shows that the Bcl-2 protein levels were much greater (approximately 5-fold) in HL-60/Bcl2 cells compared to the empty vector control cell line (HL-60/Puro) and HL-60/WT cell line. The Bcl-2 overexpressed in the HL-60/Bcl2 cells was FLAG-tagged, hence the higher molecular weight of this band.

The effect of ABT-737 as a single agent was investigated in the three HL-60 cell lines. Using the sub-G1 FACS assay as a measure of apoptosis, HL-60 cells were treated with increasing doses of ABT-737. In HL-60/WT (Fig. 2B) and HL-60/Puro (Fig. 2C) cell lines the level of apoptosis increased gradually as the ABT-737 concentration increased, with 40–50% apoptosis achieved with approximately 100 nM ABT-737. In the HL-60/Bcl2 cells, in order to achieve the same level of cell kill (40–50%), approximately 10-fold higher concentration (1 μM) of ABT-737 was required (Fig. 2D). This difference was also observed in growth inhibition assays where the IC_{50} value for ABT-737 in HL-60/Bcl2 cells was approximately 10-fold higher compared to HL-60/Puro cells (~90 nM vs ~829 nM).

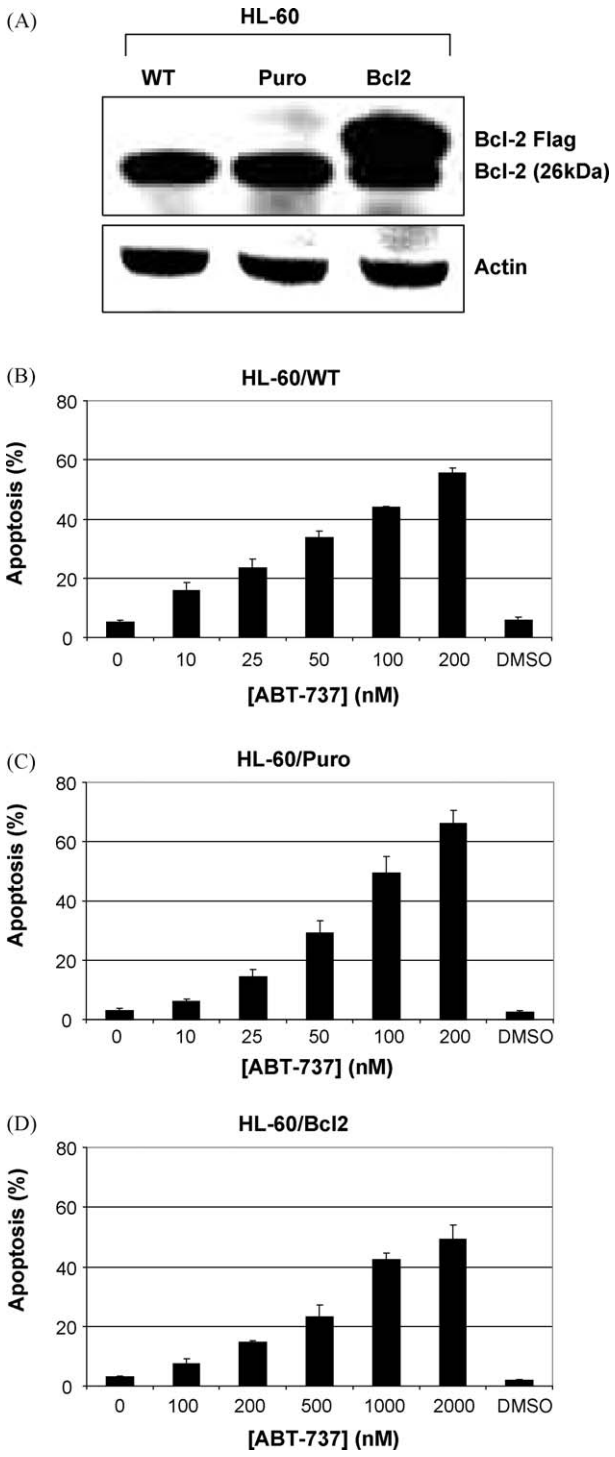

Fig. 2. ABT-737 induces cell kill as a single agent in HL-60 cells. (A) Western blot showing endogenous levels of Bcl-2 in HL-60/WT, HL-60/Puro and HL-60/Bcl2 cells with actin used as a loading control (two additional blots yielded equivalent results). HL-60 cells were treated with increasing concentrations of ABT-737 from 10 to 200 nM in (B) HL-60/WT and (C) HL-60/Puro cells and from 100 to 2000 nM in (D) HL-60/Bcl2 cells (DMSO was used as a vehicle control). Cells were treated for 6 h, after which flow cytometry was conducted to determine the percentage of apoptotic (sub-G1) cells. Error bars represent the standard error of the mean from three independent experiments.

These results demonstrate that nanomolar levels of ABT-737 were able to effectively kill HL-60 cells, highlighting its potential as an effective single agent in these cells. Furthermore, ABT-737 was able to kill HL-60 cells overexpressing Bcl-2, although a higher

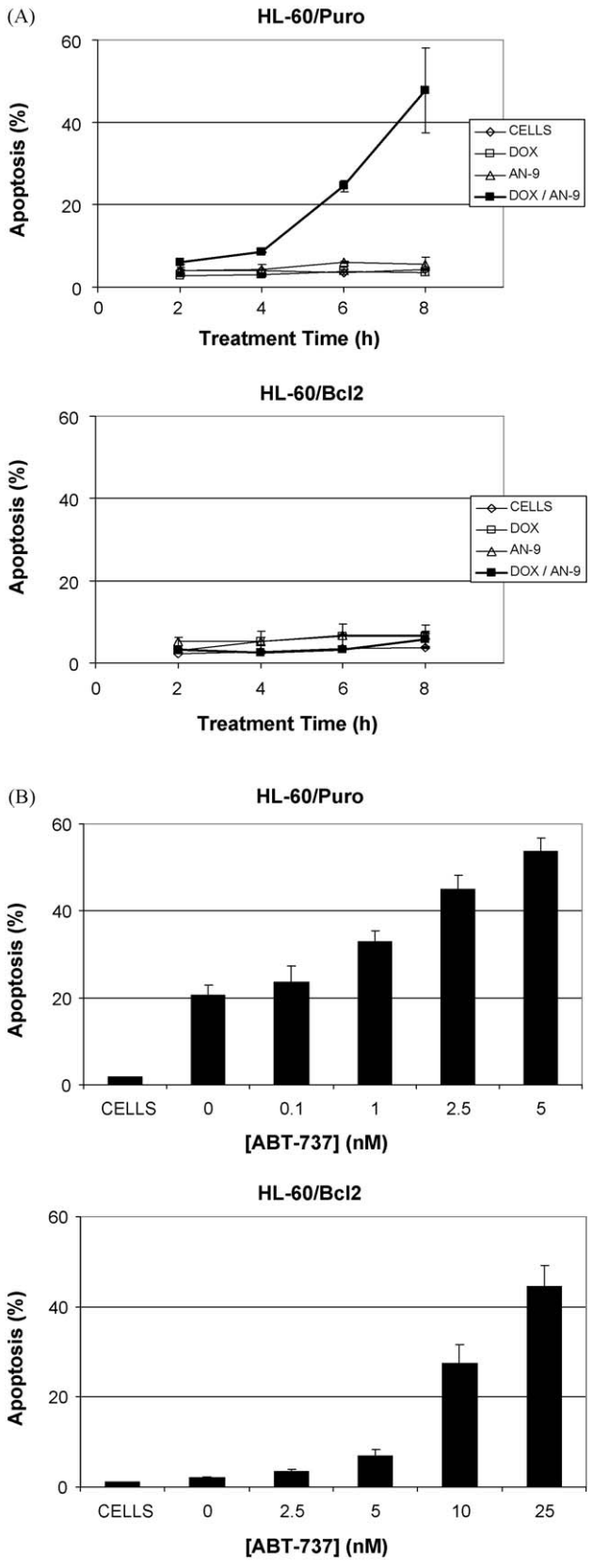

Fig. 3. Bcl-2 overexpression confers resistance to doxorubicin/AN-9 treatments which can be overcome by the addition of ABT-737. (A) HL-60/Puro and HL-60/Bcl2 cells were treated with doxorubicin (500 nM) and AN-9 (25 μM) and the combination for 2–8 h, after which flow cytometry was conducted to determine the percentage of apoptotic (sub-G1) cells ($n = 3$). (B) HL-60/Puro and HL-60/Bcl2 cells were treated with doxorubicin (500 nM), AN-9 (25 μM) and increasing concentrations of ABT-737 (0.1–5 nM for HL-60/Puro cells and 2.5–25 nM for HL-60/Bcl2 cells) for 6 h, after which the percentage of apoptotic (sub-G1) cells was determined. Error bars represent the standard error of the mean from three independent experiments.

concentration was required to neutralize Bcl-2 and allow the apoptotic cascade to proceed.

3.2. Nanomolar levels of ABT-737 overcomes Bcl-2 mediated resistance to doxorubicin–DNA adducts

It is now well documented that the combination of doxorubicin with formaldehyde-releasing prodrugs results in adduct formation and a synergistic apoptotic response [6,22]. To demonstrate this synergy in the cellular system used in this study, HL-60/Puro and HL-60/Bcl2 cells were treated simultaneously with doxorubicin (500 nM) and AN-9 (25 μM) for 2–8 h (Fig. 3A). In both cell lines, doxorubicin and AN-9 alone did not induce cell kill above background levels, therefore, under these treatment conditions, the impairment of topoisomerase II by doxorubicin does not contribute to cell kill. In HL-60/Puro cells the combination of doxorubicin/AN-9 resulted in a synergistic induction of apoptosis after 6 and 8 h treatments, while in HL-60/Bcl2 cells the combination treatment did not induce cell kill above background levels even after 8 h. This demonstrates that overexpression of Bcl-2 confers resistance to adduct forming treatments in HL-60 cells by causing a block in the apoptosis pathway. This is consistent with the results of Swift et al. who showed that Bcl-2 overexpression inhibited DNA fragmentation, dsDNA breaks and apoptosis in response to doxorubicin/AN-9 treatments. The 6 h treatment time point was chosen for future experiments since a synergistic response occurred in HL-60/Puro cells but not in HL-60/Bcl2 cells.

To establish whether this Bcl-2 mediated resistance could be overcome by inhibiting Bcl-2, ABT-737 was used in combination with doxorubicin and AN-9 to form a 'triple treatment'. In HL-60/Puro cells where the combination of doxorubicin and AN-9 resulted in ~20% apoptosis, the addition of ABT-737 (0.1–10 nM) resulted in a gradual dose dependant increase in apoptosis with ~40% apoptosis achieved with 2.5 nM ABT-737 (Fig. 3B). The ability of ABT-737 to increase cell kill in response to adduct forming treatments was even further pronounced in HL-60/Bcl2 cells. These cells were completely resistant to doxorubicin–AN9 treatment after 6 h, however, the addition of 10 or 25 nM ABT-737 resulted in a synergistic increase in apoptosis, thus reflecting that the anti-apoptotic function of Bcl-2 can be effectively inhibited by ABT-737. It is important to note that the concentrations of ABT-737 that were able to enhance apoptosis levels were much lower than the corresponding IC_{50} values and did not induce apoptosis as a single agent (data not shown).

3.3. Triple treatment is synergistic in three independent apoptosis assays

To further validate the observation that nanomolar levels of ABT-737 could overcome the inherent resistance of HL-60/Bcl2 cells to adduct forming treatments, HL-60/Puro and HL-60/Bcl2 cells were treated with 2.5 and 25 nM ABT-737, respectively, and the level of apoptosis (sub-G1 assay) induced by the triple treatment (as well as all single and double agent treatments) is shown in Fig. 4A. In both cell lines, all three single agents at the concentrations used failed to induce apoptosis above background levels. The combination of doxorubicin/AN-9 was synergistic in HL-60/Puro cells with the addition of ABT-737 resulting in a further increase in apoptosis, whereas in HL-60/Bcl2 cells, apoptosis above background was only induced when ABT-737 was added to the doxorubicin/AN-9 combination.

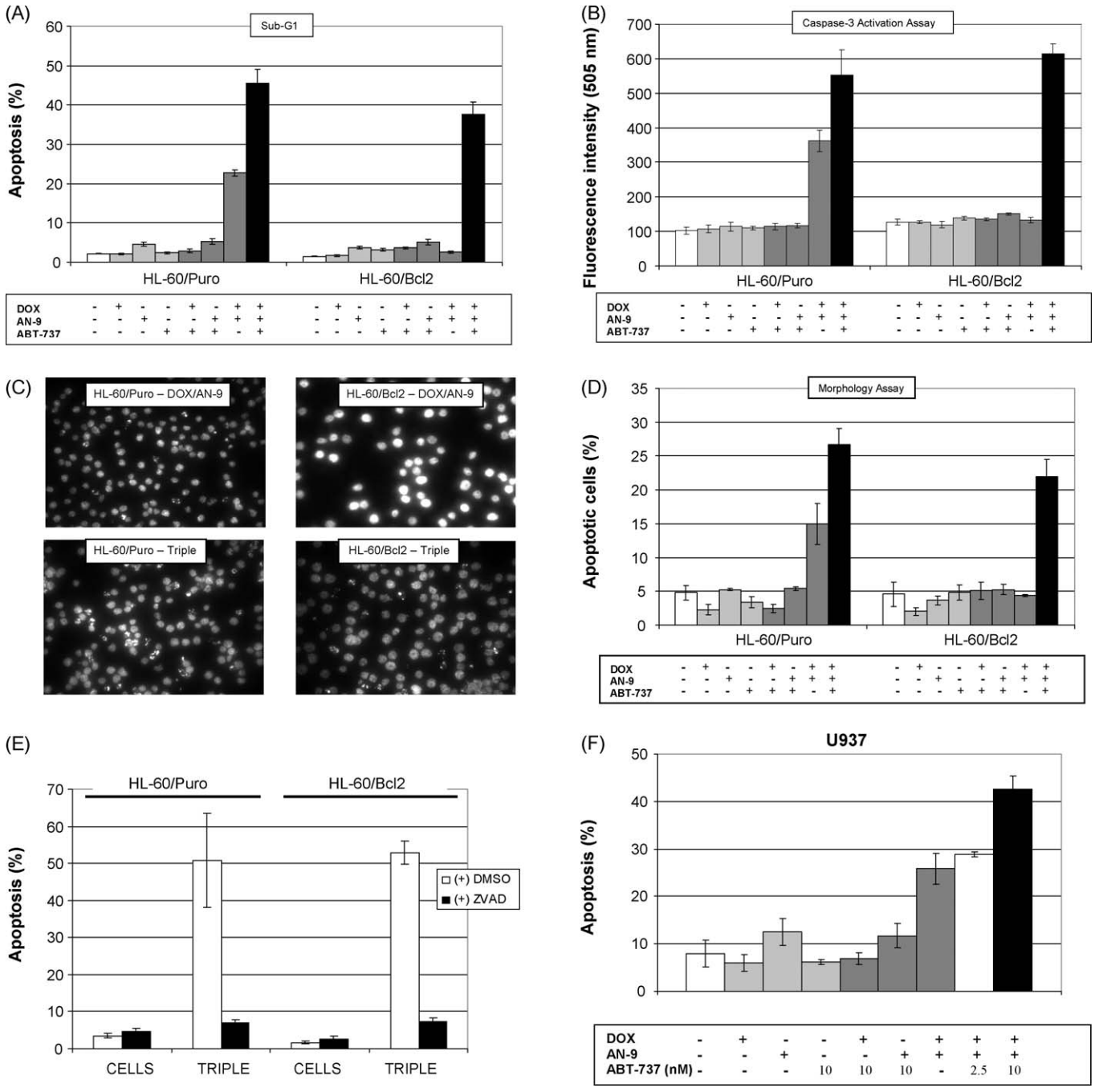

Fig. 4. ABT-737 potentiates the effects of doxorubicin/AN-9 treatments. HL-60/Puro and HL-60/Bcl2 were treated with doxorubicin (500 nM), AN-9 (25 μM) and ABT-737 (2.5 nM in HL-60/Puro cells and 25 nM in HL-60/Bcl2 cells) for 6 h to form a triple treatment (all single agent and combination controls are included), and the percentage of sub-G1 apoptotic cells (A), fluorescence intensity relative to caspase-3 activity (B), and percentage of cells displaying visible chromatin condensation (C, examples of images and D, quantitative data) were determined independently for each treatment ($n = 3$). (E) HL-60 cells were also pre-treated with 30 μM ZVAD-fmk caspase inhibitor for 1 h before applying the triple treatment (500 nM doxorubicin, 25 μM AN-9, and either 2.5 nM ABT-737 in HL-60/Puro cells or 25 nM ABT-737 in HL-60/Bcl2 cells) for 6 h, after which the percentage of sub-G1 cells was determined ($n = 2$). (F) U937 cells were also treated with the triple treatment (500 nM doxorubicin, 25 μM AN-9, and either 2.5 or 10 nM ABT-737) for 6 h and the percentage of sub-G1 apoptotic cells was determined ($n = 3$).

Two other independent apoptosis assays (caspase-3 activation and morphology assays) were also performed to demonstrate that the classical hallmarks of apoptosis were observed in response to the triple treatment. After 6 h treatment, caspase-3 activation was evident (fluorescence intensity above background levels) in HL-60/Puro cells treated with the doxorubicin/AN-9 combination but not in HL-60/Bcl2 cells (Fig. 4B). Also, the addition of ABT-737 in the triple treatment further increased caspase-3 activity in HL-60/Puro cells and overcame Bcl-2 resistance in HL-60/Bcl2 cells. Similar results were also obtained in the morphology assay (Fig. 4C and D) in which cells were scored as being apoptotic based on the presence of chromatin condensation detected by Hoechst staining. Distinct chromatin aggregation was visible in HL-60/Puro cells treated with doxorubicin/AN-9 for 6 h (Fig. 4C, top left image), whereas the nuclei of HL-60/Bcl2 cells (Fig. 4C, top right) appeared normal. Only in the presence of ABT-737 (triple treatment) did chromatin aggregation become evident in HL-60/Bcl2 cells (Fig. 4C, bottom right).

These three independent apoptosis assays (DNA fragmentation, caspase-3 activation, and morphology assays) all demonstrated that ABT-737 was able to overcome the apoptosis block in cells in

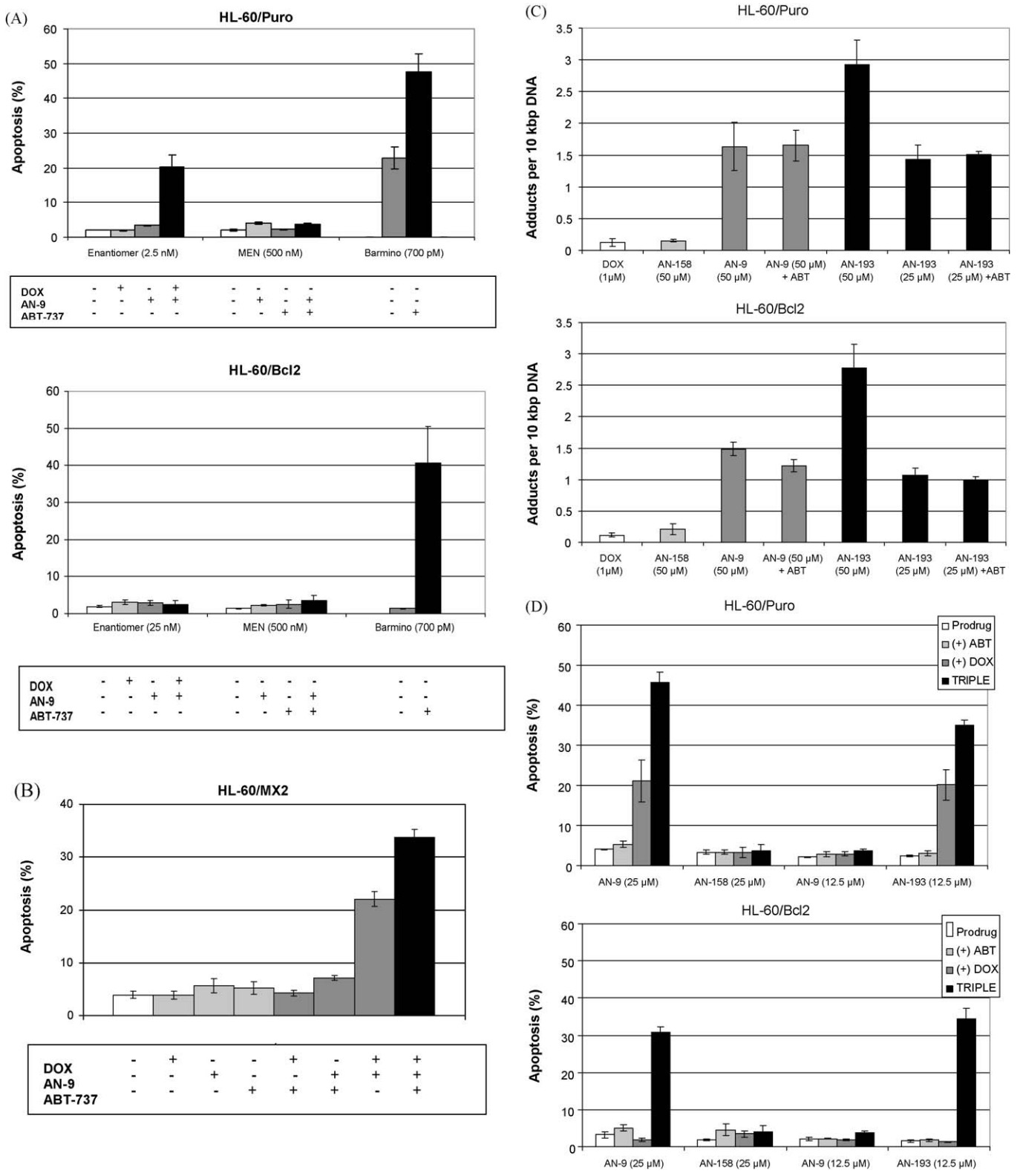

Fig. 5. Cell kill in response to the triple treatment involves formaldehyde-dependent doxorubicin–DNA adduct formation. (A) HL-60/Puro and HL-60/Bcl2 cells were treated for 6 h with the ABT-737 enantiomer (2.5 nM in HL-60/Puro cells and 25 nM in HL-60/Bcl2 cells), MEN-10755 (500 nM), and barminomycin (700 pM), both alone and in the combinations listed, and the level of apoptosis (percentage sub-G1) measured ($n = 3$). (B) HL-60/MX2 cells were treated with doxorubicin (500 nM), AN-9 (25 μM) and ABT-737 (2.5 nM) for 6 h to form a triple treatment (all single agent and combination controls are included), and the percentage of apoptosis (percentage sub-G1) determined ($n = 3$). (C) HL-60/Puro and HL-60/Bcl2 cells were treated for 4 h with [^{14}C]-doxorubicin (1 μM) in combination with AN-158 (50 μM), AN-9 (50 μM), and AN-193 (25 and 50 μM), in the presence and absence of ABT-737 (2.5 nM in HL-60/Puro cells and 25 nM in HL-60/Bcl2 cells), after which the DNA was extracted, scintillation counting performed, and the level of doxorubicin–DNA adducts quantitated ($n = 3$). (D) HL-60/Puro and HL-60/Bcl2 cells were treated with the triple treatment (500 nM doxorubicin, and 2.5 nM ABT-737 in HL-60/Puro and 25 nM ABT-737 in HL-60/Bcl2 cells) using the prodrugs AN-9 (25 and 12.5 μM), AN-158 (25 μM), and AN-193 (12.5 μM) and the level of apoptosis (percentage sub-G1) determined. Error bars represent the standard error of the mean from three independent experiments.

which Bcl-2 was overexpressed, thus restoring sensitivity to doxorubicin/AN-9 treatments.

To confirm that cell kill involved caspase-dependent apoptosis (and not other means such as necrosis), the broad spectrum caspase inhibitor ZVAD-fmk was used to inhibit apoptosis. Cells were pre-treated with 30 μM ZVAD-fmk for 1 h before being treated with the triple treatment. Pre-treatment with ZVAD-fmk reduced the apoptotic levels to near background levels (Fig. 4E), indicating that cell kill in response to the triple treatment was mediated by caspase-dependent apoptosis.

To confirm that the cytotoxicity of the 'triple treatment' was not limited to only HL-60 cells, another leukemic cell line, U937 was used. The combination of doxorubicin and AN-9 (at the same concentrations and treatment time as used for HL-60 cells) was shown to be synergistic (Fig. 4F), and the addition of 10 nM ABT-737 (sub-lethal) was able to increase cell kill further in the triple treatment. The use of higher ABT-737 concentration in the triple treatment in the U937 cells compared to HL-60/Puro cells (10 nM vs 2.5 nM) is attributed to the fact that U937 cells express higher endogenous levels of Mcl-1 [36] and as such are more resistant to ABT-737 (data not shown).

These results demonstrate that ABT-737 is able to overcome Bcl2-mediated resistance to doxorubicin/AN-9 treatments, thus making previously resistant cells exquisitively sensitive to cell kill via adduct damage response pathways.

3.4. Cell death induced by the triple treatment is dependent on doxorubicin–DNA adduct formation

To confirm the molecular aspects of the interactions responsible for cell kill induced by the triple treatment, various control compounds were utilized. These compounds included the ABT-737 enantiomer (which binds with a much lower affinity to Bcl-2, Bcl-w and Bcl-XL), MEN-10755 (Fig. 1G; a doxorubicin analogue that contains an additional daunosamine sugar group which imparts steric constraints and prevents this compound from forming adducts in the presence of formaldehyde) and barminomycin (Fig. 1H; a pre-activated anthracycline that is able to form DNA adducts without the requirement for formaldehyde).

The addition of the ABT-737 enantiomer to doxorubicin/AN-9 (Fig. 5A) did not increase the level of apoptosis in either cell line relative to the doxorubicin/AN-9 combination (shown in Fig. 4A). This confirms that the correct configuration of the compound is required to enable high affinity binding to Bcl-2. MEN-10755 did not induce apoptosis when combined with AN-9 or AN-9/ABT-737 in either cell line (Fig. 5A). While the compound is able to induce cell kill as a single agent as effectively as doxorubicin by inhibiting topoisomerase II, its inability to form adducts in the presence of formaldehyde provides evidence that the main mechanism of cell kill induced by the triple treatment (doxorubicin, AN-9, ABT-737) is DNA adduct formation. Further evidence is provided by the use of barminomycin which induces apoptosis as a single agent (700 pM) in HL-60/Puro cells due to its ability to form DNA adducts without additional formaldehyde. However, as observed with the combination of doxorubicin/AN-9, the overexpression of Bcl-2 confers resistance to barminomycin which was overcome by ABT-737. Cell kill in response to doxorubicin/AN-9 and the triple treatment was also observed in topoisomerase II-deficient HL-60/MX2 cells, indicating that the mechanism of cell kill is independent of topoisomerase II inhibition (Fig. 5B). Furthermore, it was demonstrated using a γH2AX flow cytometry assay that the addition of ABT-737 in the triple treatment of both HL-60/Puro and HL-60/Bcl2 cells did not increase the level of double-strand DNA breaks. This indicates that any increase in cell kill caused by ABT-737 is not attributed to topoisomerase II dependent double-strand DNA breaks (data not shown).

To further characterize the mechanism of cell kill in response to the triple treatment, HL-60/Puro and HL-60/Bcl2 cells were treated with [^{14}C]-doxorubicin and prodrugs that release differing amounts of formaldehyde, and the resulting levels of DNA adducts were quantitated (Fig. 5C). In both cell lines, after 4 h treatment, only low levels of adducts were detected in response to doxorubicin alone and in combination with the prodrug AN-158 (structure shown in Fig. 1D) which does not release formaldehyde. Due to the lack of formaldehyde release and resulting lack of DNA adduct formation, the combination of AN-158 with doxorubicin and in the triple treatment (AN-158, doxorubicin, ABT-737) failed to induce apoptosis above background levels (Fig. 5D). The combination of the prodrug AN-193 (which releases two molecules of formaldehyde; Fig. 1E), with [^{14}C]-doxorubicin resulted in approximately double the level of DNA adducts per 10 kbp when compared with AN-9 (Fig. 5C) at the same concentration (50 μM). Using half the concentration of AN-193 (25 μM) resulted in similar adduct levels to 50 μM AN-9 in both cell lines (Fig. 5C), and resulted in comparable apoptosis levels when combined with doxorubicin and in the triple treatment in both cell lines (Fig. 5D). The presence of ABT-737 did not alter the adduct levels in these assays indicating that the compound does not interfere with the process of adduct formation or removal at early time frames in cells (Fig. 5C).

4. Discussion

The discovery that doxorubicin is able to form more cytotoxic DNA adducts in the presence of formaldehyde has allowed the use of lower concentrations of doxorubicin to achieve high levels of tumor cell kill in vitro [6,22]. Considering that the major limitation of doxorubicin in cancer treatments is dose-limiting cardiotoxic side-effects [40], the use of lower doses of doxorubicin is of great clinical interest. The synergistic cell kill observed using doxorubicin and formaldehyde-releasing prodrugs in numerous cancer cell lines to date has been very promising [6,22], and as such doxorubicin combined with AN-9/AN-193 is currently being assessed in mouse models of human solid tumors.

Recently it has been demonstrated that doxorubicin–DNA adducts occur in tumor cells treated with clinically relevant concentrations of doxorubicin as a single agent [13]. In order to potentiate adduct formation and maximize cytotoxicity we have co-administered doxorubicin with formaldehyde-releasing prodrugs, however, another group have described a formaldehyde–doxorubicin conjugate, doxazolidine, which forms doxorubicin–DNA adducts and displays a much higher toxicity compared to doxorubicin alone in breast cancer cells without an increase in toxicity to cardiomyocytes [41]. A stable, non-toxic prodrug of doxazolidine has been synthesized (pentyl PABC-Doxaz) which becomes cleaved intracellularly by carboxylesterases releasing active doxazolidine [42,43], thus highlighting a potential single agent doxorubicin–DNA adduct forming treatment. The use of either formaldehyde-releasing prodrugs or doxorubicin–formaldehyde conjugates provides various avenues of maximizing doxorubicin–DNA adduct formation in tumor cells which in the future may potentially be applied in the clinic.

The overexpression of anti-apoptotic proteins in cancer cells is a major factor in the inherent resistance of these cells to cytotoxic agents such as doxorubicin, and there has been great interest in inhibiting the action of these anti-apoptotic proteins. It has been shown that overexpression of Bcl-2 in HL-60 cells leads to a block in cell kill following treatment with doxorubicin/AN-9, thus limiting the clinical potential of this combination (Fig. 3A). In order to overcome this resistance, the BH3 mimetic ABT-737 was examined and was able to induce cell kill as a single agent in the nanomolar range (Fig. 2B–D). Evidence suggests that the main

factor that dictates cellular resistance to ABT-737 is the levels of Mcl-1, with cells with high Mcl-1 levels being more resistant to ABT-737 due to the low affinity that the compound has for this anti-apoptotic protein [37,44–46]. Mcl-1 has been implicated in keeping Bak in check, therefore, the inability of ABT-737 to bind to Mcl-1 prevents full Bak release and the induction of apoptosis is therefore impaired [26,47]. HL-60 cells express relatively low levels of Mcl-1, and as such are more sensitive to ABT-737 compared to another leukemic cell line, U937 which expresses higher Mcl-1 levels (data not shown).

Even when Bcl-2 is overexpressed (HL-60/Bcl2 cells), ABT-737 is still cytotoxic (but does require a higher concentration; Fig. 2D), thus highlighting the potential of this compound to overcome Bcl-2 related chemoresistance and in increasing cytotoxic responses when combined with other chemotherapeutics. Indeed the combination of ABT-737 with various DNA damaging agents (including doxorubicin, carboplatin and etoposide) has led to synergistic cancer cell death [33], especially if the genotoxic agents lead to the reduction of Mcl-1 levels [37]. The combination of doxorubicin with ABT-737 resulted in synergistic cell kill after 24 h treatment (Suppl. Fig. 1) in HL-60/WT cells but not in topoisomerase IIα-deficient HL-60/MX2 cells, reflecting a topoisomerase II dependent cell kill mechanism in the absence of formaldehyde and over longer treatment time. However this topoisomerase II-mediated effect was not observed at the early treatment times used in all subsequent triple treatment experiments.

The addition of low nanomolar concentrations of ABT-737 to doxorubicin/AN-9 treatments overcame resistance in Bcl-2 overexpressing HL-60 cells (Fig. 3B). The addition of ABT-737 to form a 'triple' treatment resulted in high levels of cell kill as monitored by DNA fragmentation (Fig. 4A), caspase-3 activation (Fig. 4B) and chromatin condensation (Fig. 4C and D), all of which are classical signs of apoptosis. This phenomena was not only limited to HL-60 cells since it was also demonstrated that the triple treatment was effective in U937 leukemic cells (Fig. 4F) and is therefore more broadly applicable. When the mechanism of cell kill in response to the triple treatment was investigated, it was found that the enantiomer (with the opposite configuration of the dimethylaminoethyl group; "S") did not increase cell kill (Fig. 5A) since it displays a much lower affinity for Bcl-2 [33]. Control compounds that do not result in DNA adduct formation (e.g. MEN-10755 and AN-158) did not induce cell kill when combined in a triple treatment with ABT-737, highlighting the absolute requirement and role of DNA adduct formation in this cell kill mechanism. On the other hand, barminomycin (which forms DNA adducts as a single agent) was synergistic with ABT-737 (Fig. 5A). Cell kill in response to the triple treatment was also shown to occur independently of topoisomerase II (Fig. 5B), confirming that the topoisomerase II inhibition function of doxorubicin is not involved in the observed cell kill mechanism.

When the level of DNA adducts was measured directly using a [^{14}C]-doxorubicin adduct assay, it was shown that the addition of ABT-737 to doxorubicin/prodrug treatments did not affect adduct levels (Fig. 5C), but did potentiate an apoptotic response (Fig. 5D). Once DNA adducts are formed, various damage response pathways become activated, eventually leading to the induction of the apoptotic cascade.[4] In response to DNA adducts, BH3-only proteins (in particular Puma and Noxa) may become activated (in a p53 independent manner since HL-60 cells are p53 null) leading to Bax/Bak release, caspase activation and cell kill [48]. In HL-60/Bcl2 cells it was shown that doxorubicin–DNA adducts formed to the same extent as in HL-60/Puro cells, indicating that adduct formation is unaffected. Therefore, it is expected that the same adduct response pathways would be activated in HL-60/Bcl2 cells that lead to apoptosis in HL-60/Puro cells. However, apoptosis does not occur in response to doxorubicin/AN-9 treatments in HL-60/Bcl2 cells (Fig. 3A) indicating that the overexpression of Bcl-2 prevents Bax activation thereby completely blocking the apoptotic cascade. It therefore appears that the Bcl-2 overexpressing cells are able to tolerate the presence of doxorubicin–DNA adducts and that the DNA may be repaired with time, although the exact repair mechanisms in response to adduct formation are only beginning to be understood [49]. The addition of ABT-737 leads to the inhibition of Bcl-2, Bcl-XL and Bcl-w, thus freeing Bax/Bak and leading to cytochrome c release, caspase activation, and high levels of cell kill.

This study has shown that HL-60 cells are highly sensitive to ABT-737 and the triple treatment, presumably due to the low Mcl-1 expression levels in these cells. However, cells with high Mcl-1 levels are more resistant to ABT-737 and therefore may be resistant to the triple treatment. Since Mcl-1 is also commonly overexpressed in cancer cells and is associated with cancer cell survival [50–52], the therapeutic potential of the triple treatment may be limited to cancer cells associated with low Mcl-1 expression. It has become clear that all anti-apoptotic proteins need to be inhibited to completely free Bax/Bak and allow effective induction of apoptosis [26,45,47]. Many strategies are currently being explored to knockdown or inhibit Mcl-1 levels in cells to increase sensitivity to ABT-737 and these include the use of shRNA [36,45], the CDK inhibitor roscovitine [44,45], and the MEK/ERK inhibitor PD98059 [36]. It may therefore be feasible in the future to combine the triple treatment with compounds/strategies that reduce Mcl-1 levels below a certain threshold to allow Bax/Bak release, thus broadening the potential use of the triple treatment to cancer cells which express high levels of both Bcl-2 and Mcl-1.

As with any treatment, the effects on normal cells and potential side-effects need to be considered. Since the expression of anti-apoptotic proteins is not limited to cancer cells, the inhibition of these proteins may be expected to trigger unwanted apoptosis in normal cells. However, it has been demonstrated by a number of groups that ABT-737 has limited effects on normal/non-malignant cells [34,36], and in vivo the only side-effects detected following ABT-737 treatment are lymphopenia and thrombocytopenia [33,36]. It is speculated that cancer cells exist in a 'primed state' where BH3-only proteins are constantly activated due to numerous physiological aberrancies including oncogene activation and cell cycle checkpoint violation [35,53]. As such, this may create a window where cancer cells are much more sensitive to Bcl-2 inhibitors compared to normal cells. For example, Konopleva et al. showed that ABT-737 was able to greatly reduce colony formation in primary patient derived AML progenitor cells but not in normal bone marrow cells [36]. Furthermore, the concentrations of ABT-737 used in the triple treatment are much lower than if ABT-737 was used as a single agent and this would be expected to minimize any ABT-737 related side-effects in vivo.

While pre-clinical testing with ABT-737 has been very promising both as a single agent and in various combination treatments, its low aqueous solubility and lack of oral bioavailability limit the therapeutic use of this compound. Recently a second generation BH3 mimetic, ABT-263, was developed which displays similar binding affinities to anti-apoptotic proteins as ABT-737, but has the advantage of being orally bioavailable [54]. Therefore, the combination of ABT-263 with doxorubicin/AN-9 treatments is expected to be as effective as the ABT-737 triple treatment utilized in this study but with the added advantage of being more flexible to dosing regimens in vivo.

In summary, the present study describes the combination of the DNA adduct forming treatment of doxorubicin/AN-9 with the Bcl-2 inhibitor ABT-737 to overcome Bcl-2 mediated chemoresistance. The combination of doxorubicin/AN-9 results in synergistic cell kill in HL-60 leukemic cells, however, Bcl-2 overexpression confers

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resistance to this combination which may limit the therapeutic potential of this treatment. The addition of nanomolar concentrations of ABT-737 is able to overcome this Bcl-2 resistance, leading to high levels of cell kill, thereby making previously resistant cancer cells susceptible to doxorubicin–DNA adduct forming treatments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.09.004.

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