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Synergism of the Anthracene-Derivative Anti-Cancer Agent Bisantrene with Nucleoside Analogs and A Bcl-2 Inhibitor in Acute Myeloid Leukemia Cells

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Abstract

Bisantrene is an anthracene derivative with anti-tumor activity. Unlike the anthracyclines, it has virtually no clinical cardiotoxicity. We hypothesized that its combination with nucleoside analogs and the BCL-2 inhibitor ABT199 would provide improved cytotoxicity in AML cell cultures. Cells were exposed to drugs for 48 hrs and analyzed by MTT assay for cell proliferation, annexin V assay for apoptosis, Western blotting for changes in the status of protein levels and modifications, and flow cytometry for changes in Reactive Oxygen Species (ROS) and Mitochondrial Membrane Potential (MMP). Exposure of OCI-AML3 and MOLM14 cells to bisantrene+nucleoside analog(s) (cytarabine, cladribine, fludarabine/Flu or clofarabine/Clo)+ABT199 resulted in increased cytotoxicity, apoptosis and synergism with combination indices<1. Increased levels of cleaved PARP1 and caspase 3, caspase 3 enzymatic activity, DNA fragmentation and ROS, and decreased MMP with the three- or four-drug combinations, all suggested activation of intrinsic apoptosis. A similarly enhanced activation of apoptosis was observed in leukemia patient-derived cells exposed to (bisantrene+Flu+Clo+ABT199). Exposure of AML cell lines to nucleoside analogs prior to addition of bisantrene exerted a higher level of cytotoxicity than when the reverse sequence was utilized. These results provide a rationale for clinical trials using these drug combinations for conventional (re-)induction therapy or as part of a pretransplant program leading to hematopoietic stem cell transplantation for high-risk AML patients. They also highlight the importance of considering the sequencing of cytotoxic agents when designing combination regimens for the treatment of acute leukemia.

Keywords: Bisantrene; Nucleoside analog; ABT199; Venetoclax; Acute myeloid; Leukemia; Anthracycline

Introduction

Treatment of Acute Myeloid Leukemia (AML) is a formidable challenge for hematologists. The complexity of its genetic and epigenetic abnormalities underscores the importance of finding drugs that efficiently control tumor-cell proliferation as well as counteracting inherent and acquired drug resistance. A widely-used approach to achieving increased cytotoxic efficacy in the treatment of AML has been to combine drugs with different mechanisms of action, but the patients' overall survival remains poor. More efficacious drugs clearly need to be identified, preferably paired with mechanistic studies of how to optimize their use in combination with other available agents.

The antineoplastic activity of anthracyclines was discovered in the early 1970's [1]. These drugs intercalate into DNA and form a stable anthracycline-DNA-topoisomerase II ternary complex, which inhibits the enzyme from relaxing the DNA supercoil torsion, resulting in inhibition of DNA and RNA synthesis [2]. This inhibition of topoisomerase II results in the accumulation of DNA double-strand breaks and cell cycle arrest, which ultimately causes apoptosis. Daunorubicin and its derivative hydroxy-daunorubicin/doxorubicin are the most commonly used anthracyclines for treating hematological disorders, but their associated cardiotoxicity has limited their usefulness [3-6].

Bisantrene is an anthracene derivative with greatly reduced cardiotoxicity compared with daunorubicin and doxorubicin. It was introduced for the treatment of leukemia in the 1980s, and it was approved in France for the treatment of AML in 1990. In spite of its excellent anti-tumor efficacy and the virtual absence of cardiovascular toxicity shown in earlier clinical trials [7-9], bisantrene was lost in the market place following the acquisition of its parent pharmaceutical company by a larger corporation with different priorities. With recent discoveries of new promising drugs for AML, combinations of bisantrene with these novel agents warrant further investigation, both for their efficacy and for their negligible cardiotoxicity, a problem that is particularly troublesome in children who are more sensitive than adults to the cardiotoxic potential of anthracyclines. The conventional 7+3 induction chemotherapy (7-day infusion of cytarabine (Ara-C) + 3 days of daunorubicin/doxorubicin/idarubicin) for AML has been used for decades, but its associated remission and survival rates are low, especially in older patients [10], who are incidentally commonly subject to comorbidities such as cardiac ailments. We hypothesized that a combination of bisantrene with Ara-C or with later-generation nucleoside analogs, which are less prone to the deamination and dephosphorylation events that confer resistance to Ara-C [11], may provide safer and more efficacious treatment options for acute leukemia.

Aside from Ara-C, nucleoside analogs used to treat AML include Fludarabine (Flu), Cladribine (Clad) and Clofarabine (Clo) in combination with other drugs [12-16]. A combination of bisantrene with any of these nucleoside analogs may result in a higher efficacy, and thus ultimately confer a better prognosis for AML patients.

Another promising drug for treating AML is ABT199/venetoclax, a BH3-mimetic small molecule that binds to and inhibits the anti-apoptotic B-cell Lymphoma-2 (BCL-2) protein, causing cells

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to undergo apoptosis [17,18]. It is FDA-approved for chronic lymphocytic leukemia/small lymphocytic lymphoma [19]. Preclinical studies have demonstrated the cytotoxicity of ABT199/venetoclax in leukemia cells [20-21]. Its combination with a hypomethylating agent resulted in a favorable overall response rate in high-risk elderly AML patients [22-23], which led to its recent FDA approval in combination with azacitidine or decitabine for the treatment of AML patients ≥ 75 years of age.

Based on the reported individual cytotoxicity of bisantrene, nucleoside analogs, and ABT199 in AML cells and their clinical efficacies, we hypothesized that their combination(s) would provide improved cytotoxicity in AML cell cultures. This study reports the synergistic cytotoxicity of bisantrene+nucleoside analog (Ara-C, Flu, Clad or Clo)+ABT199 towards AML cells and their possible mechanisms of action, and it also highlights the importance of the sequence of drug exposures to optimize the anticancer effects. Our results provide a basis for evaluating combinations of these agents for AML patients in clinical trials.

Materials and Methods

Cell lines and chemicals

AML cell lines used in this study included OCI-AML3 and MOLM14, both of which were obtained from the laboratory of Dr. Michael Andreeff (UT MD Anderson Cancer Center, Houston, TX, USA), as well as KBM3/Bu2506, an alkylating agent-resistant human AML line with (low-grade) cross resistance to doxorubicin [24]. The MOLM14 cell line is positive for the presence of internal tandem repeat-FLT3 (ITD-FLT3) and KBM3/Bu2506 is p53-null. Cells were cultured in RPMI 1640 medium (Mediatech, Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Inc., Flowery Branch, GA, USA) and 100 U/mL penicillin and 100 µg/mL streptomycin (Mediatech) at 37oC in a fully humidified atmosphere of 5% CO2 in air. Absence of mycoplasma contamination was confirmed using the EZ-PCR mycoplasma detection kit (Biological Industries, Cromwell, CT, USA). Bisantrene-HCl was provided by Race Oncology Ltd. (Melbourne, VIC, Australia) through IRISYS (San Diego, CA, USA). Cytarabine, Clo and ABT199/venetoclax were obtained from SelleckChem (Houston, TX, USA). Stock solutions of bisantrene and Ara-C were dissolved in sterile water and phosphate-buffered saline (PBS), respectively, while Clo and ABT199 were dissolved in dimethyl sulfoxide (DMSO).

Patient cell samples

Peripheral blood samples from patients were collected after obtaining written informed consent. Mononuclear cells were purified using lymphocyte separation medium (Mediatech) and cryopreserved in liquid nitrogen until used. For drug exposure, frozen purified cells were thawed at 37oC, washed with culture medium, and incubated overnight in suspension in the same complete RPMI 1640 culture medium used for cell lines, prior to drug treatment. All studies were performed according to a protocol approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center, in accordance with the Declaration of Helsinki.

Cytotoxicity and apoptosis assays

Cell suspensions (6 mL of 0.5 × 10^6 cells/mL) in T25 flasks were exposed to drugs or solvent alone for 48 h, aliquoted (100 µL) in triplicate into 96-well plates and analyzed immediately for inhibition of proliferation by the MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Briefly, 30 µL of 2 mg/mL MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) in PBS was added per well and incubated for 3-4 h at 37°C. The solid reaction product was dissolved by adding 100 µL of solubilization solution (0.1 N HCl in isopropanol containing 10% Triton X-100), mixing, and incubating at 37°C for at least 1 hr. Absorbance at 570 nm was measured using a Victor X3 (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA) plate reader. The number of MTT-positive cells was determined relative to the solvent control cells.

Like the MTT assay, apoptosis was determined immediately after the 48-h drug exposure by flow-cytometric measurements of phosphatidylserine externalization with Annexin-V-FLUOS (Roche Diagnostics, Indianapolis, IN, USA) and a fluorescent DNA-binding marker 7-aminoactinomycin D (BD Biosciences, San Jose, CA, USA) using a Muse Cell Analyzer (EMD Millipore, Billerica, MA, USA). Drug combination effects were estimated based on the Combination Index (CI) values [25] calculated using the CalcuSyn software (Biosoft, Ferguson, MO, USA). This program was developed based on the median-effect method: CI<1 indicates synergy, CI ~1 indicates additivity, and CI>1 suggests antagonism.

Protein analysis

Western blot analysis was performed to determine drug-induced changes in the level of key proteins and their modifications. Cells were incubated with the study drug(s) for 48 hrs, collected by centrifugation, washed with ice-cold PBS and lysed with lysis buffer (Cell Signaling Technology, Danvers, MA, USA). Total protein concentration was determined using the BCA protein Assay kit (Thermo Scientific, Rockford, IL, USA). The protein extracts were combined with the loading buffer, boiled for 5 min, and aliquots of equal amount of proteins were separated on polyacrylamide-SDS gels by electrophoresis. The proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The relevant antibodies were added and detected using the chemiluminescent substrate Immobilon (EMD Millipore). The antibodies used and their sources include cleaved PARP1 (Cell Signaling/5625), cleaved caspase 3 (Cell Signaling/9661), c-MYC (Cell Signaling/3402), γ-H2AX (Cell Signaling/9718), 3MeH3K27 (Active Motif/39155, Carlsbad, CA, USA), and β ACTIN (Sigma-Aldrich/A5316).

Analysis of production of Reactive Oxygen Species (ROS) and changes in Mitochondrial Membrane Potential (MMP)

Cells exposed to drug(s) for 48 hrs were analyzed for the production of ROS using CM-H2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester), an ROS indicator that diffuses into cells, where it is oxidized to a fluorescent product (Life Technologies, Grand Island, NY). Briefly, cells were aliquoted (0.5 mL) into 5 mL tubes and 2 µL of 1.5 mM CM-H2DCFDA (freshly dissolved in DMSO) was added. Cells were incubated at 37°C for 1 hr and immediately analyzed with a Gallios Flow Cytometer (Beckman Coulter, Inc., Brea, CA) using excitation/emission wavelengths of 492/520 nm. Geometric means of the fluorescence intensities were used in the analysis and the fold increase relative to the control was reported.

The JC-1 fluorescent probe (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) was used to determine changes in MMP using an MMP detection kit (Cayman Chemical Co., Ann Arbor, MI, USA). Cells were exposed to drug(s) for 48 hrs and 0.5 mL cell suspension was aliquoted into 5 mL tubes. Diluted (1:10

with cell growth medium, 40 µL) MMP-sensitive JC-1 reagent was added to each tube, incubated at 37°C for 20 min, and immediately analyzed by flow cytometry (λex=488 nm) using the 530 nm (FL-1 channel, green) and 585 nm (FL-2 channel, red) band-pass filters simultaneously. Healthy cells with functional mitochondria and high MMP exhibit red fluorescence (aggregated JC-1), whereas cells with disrupted mitochondria and low MMP show green fluorescence (monomeric JC-1).

Caspase 3 assay

Cells were exposed to drugs for 48 hrs, harvested and washed with ice-cold PBS. Total cell extracts were prepared using the Caspase-3 Colorimetric Activity Assay kit (Chemicon International, Temecula, CA, USA). Total protein concentration was determined as described above. Equal amounts of protein were analyzed for caspase 3 activity using the same kit.

Statistical analysis

Results are presented as the mean ± standard deviation of at least three independent experiments and statistical significance of the difference between two groups was determined by Microsoft® Office Excel program. P values ≤ 0.05 were considered statistically significant.

Results

The bisantrene+Ara-C+ABT199 combination exerts synergistic cytotoxicity towards AML cell lines

Ara-C is a common component of standard of care regimens for AML [10]. We determined its cytotoxicity towards OCI-AML3 and MOLM14 cell lines, alone or in combination with bisantrene and ABT199, using the MTT assay. Continuous exposure of OCI-AML3 cells for 48 hrs to 0.038 µM bisantrene, 0.26 µM Ara-C or 0.3 µM ABT199 resulted in ~19%, ~24% and ~21% inhibition of cell proliferation, respectively, versus control cells; combination of bisantrene and Ara-C resulted in ~32% inhibition of cell proliferation, and addition of ABT199 to this two-drug combination further and significantly inhibited cell proliferation by ~58% (Figure 1A). Similar results were observed in another AML cell line; exposure of FLT3-ITD-positive MOLM14 cells to 0.038 µM bisantrene, 0.45 µM Ara-C or 6.3 nM ABT199 resulted in ~21%, ~11% and ~23% inhibition of cell proliferation, respectively. Treatment with (bisantrene+Ara-C) and (bisantrene+Ara-C+ABT199) resulted in ~25% and ~35% inhibition of cell proliferation, respectively (Figure 1B).

The observed superior cytotoxicity of the (bisantrene+Ara-C+ABT199) combination based on the MTT assay is consistent with the increase in Annexin V-positive cells from ~17% (bisantrene) to ~27% (bisantrene+Ara-C) to ~65% (bisantrene+Ara-C+ABT199) in OCI-AML3 cells and from ~15% (bisantrene) to ~21% (bisantrene+Ara-C) to ~60% (bisantrene+Ara-C+ABT199) in MOLM14 cells, suggesting significant activation of apoptosis (Figure 1).

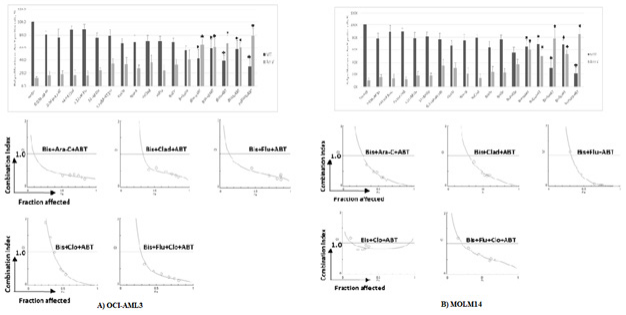

Figure 1: Cytotoxicity of bisantrene (Bis) in combination with various nucleoside analogs and the BCL-2 inhibitor ABT199. AML cells were exposed to the indicated concentrations of bisantrene, Cytarabine (Ara-C), Cladribine (Clad), Fludarabine (Flu), Clofarabine (Clo) and ABT199, alone or in combination, for 48 h prior to determination of relative cell proliferation by the MTT assay and apoptosis by the Annexin V (Ann V) assay. Asterisks (*) indicate a statistically significant difference (P<0.05) between drug combination with and without ABT199. The results are averages of at least three independent experiments. To determine drug synergism, cells were exposed to various drug combinations at constant ratio concentrations for 48 h prior to MTT assay. The relationships between the calculated Combination Indexes (CI) and Fraction affected (Fa) are shown below the bar graphs. CI<1.0 indicates synergism. The graphs are representatives of two independent experiments.

To quantitatively assess drug synergism, cells were exposed to different concentrations of individual drugs or to the three-drug combination at a constant concentration ratio and the MTT assay was performed after 48 h. Combination index (CI) values at increasing drug effects were graphically analyzed according to the Chou-Talalay method [25] as shown in Figure 1 (below bar graphs). At 50% inhibition of cell proliferation, or 0.5 Fraction a ffected(Fa), the calculated CI values for (bisantrene+Ara-C+ABT199) were 0.4 and 0.5 in OCI-AML3 and MOLM14 cell lines, respectively, indicating strong synergism (CI<1) of the three drugs.

The effects of bisantrene, Clad and ABT199 are synergistic in AML cells

Cladribine (Clad), an adenosine analog, is effective for treatment of refractory AML [26]. We sought to determine if Clad, like Ara-C, would provide synergistic cytotoxicity when combined with bisantrene and ABT199 in AML cells. Figure 1A shows that exposure of OCI-AML3 cells to 14 nM Clad resulted in ~13% inhibition of cell proliferation in the MTT assay compared to control cells. When Clad was combined with 0.038 µM bisantrene, inhibition of cell proliferation increased to ~30%; addition of 0.3 µM ABT199 further increased the inhibition of proliferation to ~41%. Exposure of MOLM14 cells to (Clad), (bisantrene+Clad) or (bisantrene+Clad+ABT199) resulted in ~10%, ~20% and ~30% inhibition of proliferation, respectively. Analysis of OCI-AML3 cells exposed to (bisantrene), (bisantrene+Clad) or (bisantrene+Clad+ABT199) showed ~17%, ~36% and ~62% Annexin V-positivity, respectively (Figure 1B). In MOLM14 cells, (bisantrene), (bisantrene+Clad) or (bisantrene+Clad+ABT199) exposure resulted in ~15%, ~15% and ~50% Annexin V-positive cells, respectively (Figure 1B). Again, the synergism of bisantrene, Clad and ABT199 is suggested by the CI values of much less than 1 seen in both OCI-AML3 and MOLM14 cells (Figure 1).

Bisantrene, flu and ABT199 provide synergistic cytotoxicity in AML cells

Fludarabine, like Ara-C and Clad, is a nucleoside analog which is indicated for treatment of leukemia patients. It is effective for induction of remission [14] and, because of its immunosuppressive properties [27], it is commonly used as part of pretransplant conditioning regimens for AML [13,28,29]. To determine its cytotoxicity with bisantrene in the absence or presence of ABT199, OCI-AML3 and MOLM14 cells were exposed to drugs individually or in combination. Figure 1A shows that exposure to individual drugs resulted in ~12%-21% and ~21%-23% inhibition of proliferation of OCI-AML3 and MOLM14 cells, respectively, in the MTT assay at the used concentrations; exposure to (bisantrene+Flu) resulted in ~30% (OCI-AML3) and ~36% (MOLM14) inhibition of proliferation; exposure to (bisantrene+Flu+ABT199) further and significantly inhibited proliferation by ~61% (OCI-AML3) and ~70% (MOLM14). Analysis of activation of apoptosis showed ~16%-35% Annexin V-positivity in OCI AML3 cells exposed to bisantrene, Flu, or ABT199 alone, ~23% when exposed to (bisantrene+Flu), and ~67% when exposed to (bisantrene+Flu+ABT199). Similar results were obtained in MOLM14 cells. Annexin V-positivity was ~15%-35% after exposure to the individual drugs, ~24% after exposure to (bisantrene+Flu) and ~79% after the (bisantrene+Flu+ABT199) combination (Figure 1B). The synergistic cytotoxicity of bisantrene, Flu and ABT199 is indicated by the CI values of much less than 1 seen in both OCI-AML3 (CI=0.5) and MOLM14 (CI=0.3) cells (Figure 1B). These results suggest that addition of ABT199 to the (bisantrene+Flu) combination significantly increased the cytotoxicity of the two-drug combination.

Addition of Clo to bisantrene and ABT199 provides synergistic effects against AML cell lines

Among the three nucleoside analogs tested (Ara-C, Clad, Flu), the combination of Flu with (bisantrene+ABT199) provided the highest cytotoxicity level in OCI-AML3 and MOLM14 cells (Figure 1). Our previous pre-clinical [30] and clinical [12] studies demonstrated the efficacy of Flu in combination with another nucleoside analog, Clo (i.e., (Flu+Clo)). We, therefore, sought to explore the cytotoxicity of (bisantrene+Clo+ABT199) and (bisantrene+Flu+Clo+ABT199) in AML cell lines. Exposure of OCI-AML3 cells to (bisantrene+Clo) resulted in ~32% inhibition of cell proliferation in the MTT assay and ~33% Annexin V-positivity (Figure 1A). Addition of Flu to this two-drug combination further increased the degree of proliferation inhibition to ~45% of control and increased the number of Annexin V-positive cells to ~41%. Addition of ABT199 to (bisantrene+Clo) or (bisantrene+Flu+Clo) resulted in a further significant increase in the degree of proliferation inhibition to ~43% and ~69%, respectively, and increased Annexin V-positivity to ~61% and ~79%. Similar results were obtained in MOLM14 cells where exposure to (bisantrene), (bisantrene+Clo), (bisantrene+Flu+Clo), (bisantrene+Clo+ABT199) and (bisantrene+Flu+Clo+ABT199) resulted in ~21%, ~23%, ~45%, ~32% and ~78% inhibition of cell proliferation, respectively, and ~15%, ~23%, ~37%, ~52% and ~85% Annexin V-positivity (Figure 1B). Analysis of their interactions showed CI values of 0.4 for both (bisantrene+Clo+ABT199) and (bisantrene+Flu+Clo+ABT199) in OCI-AML3 cells and CI values of 0.8 and 0.6 for (bisantrene+Clo+ABT199) and (bisantrene+Flu+Clo+ABT199) in MOLM14 cells (Figure 1B), suggesting a synergistic increase in cytotoxicity when bisantrene, Flu and Clo were combined with ABT199.

The combination of bisantrene and ABT199 with nucleoside analog(s) activates the apoptosis pathway

The observed increase in Annexin V-positive cells suggests activation of apoptosis. We, therefore, sought to confirm this by determining changes in the cleavage of PARP1 and caspase 3 which are commonly used molecular markers for apoptosis activation. Exposure of OCI AML3 cells to (bisantrene+nucleoside analog(s)+ABT199) resulted in extensive cleavage of PARP1 and caspase 3 (Figure 2A). Although exposure of MOLM14 cells to ABT199 or (Flu+Clo) caused some cleavage of PARP1, (bisantrene+nucleoside analog(s)+ABT199) caused more substantial cleavages of both PARP1 and caspase 3 (Figure 2B). This activation of apoptosis and the observed decrease in cell proliferation (as indicated by the MTT assay) are consistent with a decreased level of the c MYC protein (Figure 2B). The increased phosphorylation of histone 2AX (γ H2AX) and methylation of histone 3 at Lys27 (3MeH3K27: Figure 2) suggest activation of the DNA-damage response and chromatin relaxation mediated by the three- or four-drug combinations.

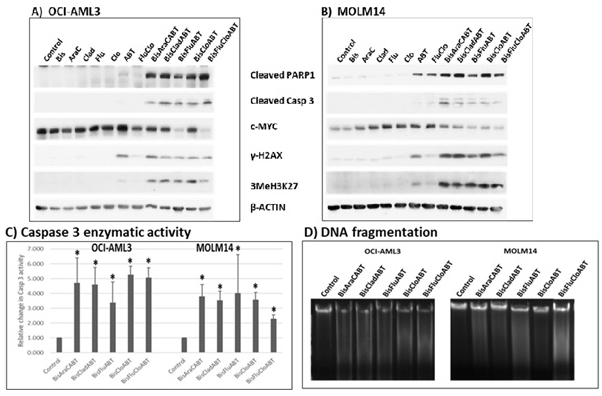

Figure 2: Effects of bisantrene, nucleoside analogs and ABT199 on the status of molecular markers of apoptosis. Cells were exposed to the indicated drugs for 48 h, harvested, and analyzed by Western blotting (A and B), caspase 3 enzymatic assay (C), and DNA agarose gel electrophoresis (D) Casp 3=caspase 3; other abbreviations are the same as in Figure 1.

To further analyze the importance of caspases in drug-mediated cell death, we determined the enzymatic activity of caspase 3 in cells exposed to various drug combinations. Exposure of OCI-AML3 cells to (bisantrene+nucleoside analog(s)+ABT199) resulted in ~3–5 fold increase in caspase 3 activity relative to the control cells; similar results were observed in MOLM14 cells with ~2–4 fold increase in caspase 3 activity (Figure 2C). Moreover, exposure of cells to these drug combinations increased DNA fragmentation, a biochemical hallmark of apoptosis [31], as determined by agarose gel analysis (Figure 2D), suggesting activation of caspase-dependent DNase.

(Bisantrene+nucleoside analog(s)+ABT199) combinations activate the production of ROS and decrease MMP

To better understand the cellular responses underlying drug-mediated cell death, we examined the production of ROS, which are known cell-death mediators, using the CM H2DCFDA fluorescent probe. Exposure of OCI AML3 cells to individual drugs increased the production of ROS by ~1–4 fold relative to the control, while two-drug combinations increased ROS by ~2–5 fold and three or four-drug combinations increased ROS by ~4–9 fold (Figure 3A). Similar results were observed in MOLM14 cells; exposure to individual or two-drug combinations resulted in ~1–2 fold increase in ROS while exposure

to (bisantrene+nucleoside analog(s)+ABT199) significantly increased ROS production by ~3.5–5 fold (Figure 3B). The results suggest that these drug combinations have perturbed the mitochondrial metabolism resulting in enhanced generation of ROS.

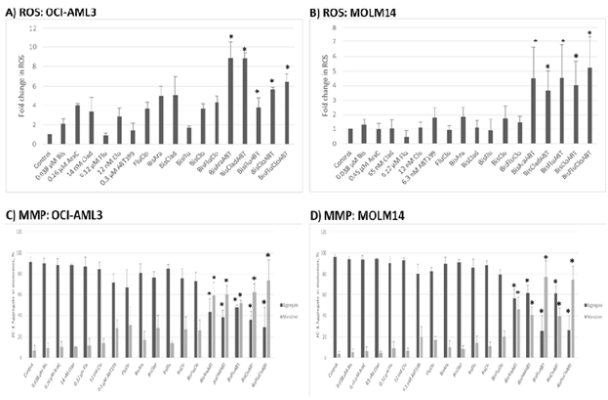

Figure 3: Drug-mediated changes in the production of Reactive Oxygen Species (ROS) and in Mitochondrial Membrane Potential (MMP). Cells were exposed to the indicated drug(s) for 48 h prior to flow cytometric analysis to determine ROS production and status of MMP as described under Materials and Methods. Asterisks (*) indicate a statistically significant difference (P<0.05) between drug combination with and without ABT199. The results are expressed as the mean ± standard deviation of at least three independent experiments.

To substantiate the effects of (bisantrene+nucleoside analog(s)+ABT199)combinations on the integrity of the mitochondria, the decrease in Mitochondrial Membrane Potential (MMP) was determined using JC-1 reagent. The aggregated form of JC-1 in the mitochondria emits a red fluorescence; a decrease in MMP causes translocation of the JC-1 probe to the cytoplasm, where it is converted into its monomeric form that emits a green fluorescence. As shown in Figure 3C, the control untreated OCI-AML3 cells showed ~92% aggregates and ~8% monomers. Exposure to individual or two-drug combinations resulted in ~9%-30% JC-1 monomers whereas (bisantrene+nucleoside analog(s)+ABT199) exposure resulted in ~52%-73% monomers, suggesting a significant leakage of the JC-1 reagent from the mitochondria to the cytoplasm. Similar results were obtained in MOLM14 cells; JC-1 monomers increased from ~4% in control cells to between ~5% and ~20% in cells exposed to individual or two-drug combinations; this further increased to ~39%-77% in cells exposed to (bisantrene+nucleoside analog(s)+ABT199) (Figure 3D). Overall, these results suggest extensive depolarization of the mitochondrial membrane in cells exposed to (bisantrene+nucleoside analog(s)+ABT199) combinations, which presumably caused the leakage of pro-apoptotic mitochondrial factors into the cytoplasm, thereby initiating the caspase-dependent cascade of events leading to apoptosis, consistent with the observed cleavage of caspase 3 and PARP1 (Figure 2 (A and B).

(Bisantrene+Flu+Clo+ABT199) combination has synergistic effects in patient leukemia cells similar to its effects in cultured cells

To assess the potential clinical implications of our observations, we isolated mononuclear cells (MNCs) from peripheral blood of patients with active myeloid and lymphoid leukemia and exposed the cells to individual drugs or (bisantrene+Flu+Clo+ABT199) combination. Figure 4 (upper panel) shows the characteristics of the patients whose MNCs were used in this study. We generally observed a lower degree of drug sensitivity of the primary leukemia cells than in the established cell lines used above, and we therefore used 0.2 μM bisantrene, 0.5 μM Flu, 20 nM Clo and 50 nM ABT199 for this study. Exposure of the isolated MNCs to (bisantrene+Flu+Clo+ABT199) generally increased the cleavage of PARP1 and caspase 3 (Figure 4), consistent with what was observed in the OCI-AML3 and MOLM14 cell lines (Figure 2 (A and B). These observations suggest that this four-drug combination is highly cytotoxic to both myeloid and lymphoid leukemia cells.

Characteristics of patients whose cell samples were used in the study.

Patient	Age, yrs	Gender	Race	Diagnosis	Cytogenetics
1	32	Female	Caucasian	Acute myeloid leukemia	Not determined
2	46	Male	Hispanic	Myeloid dysplastic syndrome	46,XY,t(8;11)(q21q23),-5,add(7)(q22),+8,+11,+13,del(13)(q12q14)x2, del(15)(q11.2q15)-17,-20[10]
3	19	Female	Black	Mixed phenotype acute leukemia	45,XX,-5,-15,del(16)(p11.2p12.2),+der(7)t(7;9)(?q32)[10]/45,idem,add(17)(q25)[10]
4	62	Male	Caucasian	T-cell-prolymphocytic leukemia	46, XY FISH: ALK (-), JAK2 (-), BCR/ABL1 (-)

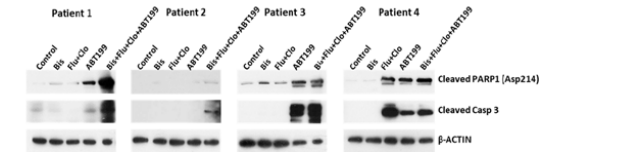

Figure 4: Efects of (bisantrene+Flu+Clo+ABT199) combination on patient-derived cells. Mononuclear cells were isolated from peripheral blood of patients with hematological malignancies and exposed to the indicated drugs for 48 h prior to Western blot analysis. The drug concentrations used for all patient samples were 0.2 μM Bisantrene (Bis), 0.5 μM Fludarabine (Flu), 0.02 μM Clofarabine (Clo) and 0.05 μM ABT199. Casp 3=caspase 3. The upper panel shows key characteristics of the four patients from whom the blood samples were isolated.

Efficacy in relation to drug sequence

The sequence of administration of drugs can be an important factor in the efficacy and interactions of 2 or more drugs [32]. For example, exposure of CG5 breast cancer cells to gemcitabine followed by doxorubicin was observed to be additive while the opposite sequence was antagonistic [33]. We therefore sought to determine the optimal sequence of exposure to bisantrene and nucleoside analogs. Continuous exposure of OCI-AML3 cells to bisantrene for 24 hrs followed by (Flu+Clo) for another 24 hrs resulted in ~39% inhibition of cell proliferation; the reverse sequence resulted in ~ 61% inhibition of proliferation (Figure 5A). Similar results were obtained in two other AML cell lines; exposure of MOLM14 cells to bisantrene followed by (Flu+Clo) resulted in ~42% inhibition of cell proliferation while the reverse sequence resulted in ~73% inhibition of proliferation, and exposure of KBM3/Bu2506 cells to bisantrene followed by (Flu+Clo) resulted in ~54% inhibition of proliferation while the reverse sequence resulted in ~73% inhibition of proliferation (Figure 5A). Analysis of apoptosis by Annexin V assay indicated similar differences, all of which were statistically significant. Exposure of OCI-AML3, MOLM14, and KBM3/Bu2506 cells to bisantrene followed by (Flu+Clo) resulted in ~31%, ~33%, and ~23% Annexin V-positive cells, respectively; the reverse sequence resulted in ~49%, ~62%, and ~41% Annexin V-positive cells, respectively (Figure 5B). Similar results were obtained when cells were washed with culture medium 24 hrs after the first drug(s) exposure and then resuspended in fresh medium containing the second drug(s) (data not shown). These findings suggest the relevance of exposing AML cells to nucleoside analogs prior to bisantrene to achieve a maximal synergistic interaction. Moreover, these findings are consistent with previous reports that exposure to nucleoside analogs resulted in the arrest of human leukemia cells in S-phase [34], which is known to be more susceptible to apoptosis upon exposure to topoisomerase II inhibitors [35].

Discussion

This study demonstrates that the efficacy of the anthracene derivative bisantrene towards AML cell lines can be enhanced when it is combined with one or more nucleoside analogs (Ara C, Flu, Clad or Clo) and with the BCL-2 inhibitor ABT199. The most potent cytotoxic combination was (bisantrene+Flu±Clo+ABT199). The observed synergism is associated with activation of the DNA-damage response and intrinsic apoptosis pathways.

The observed drug-induced apoptosis was likely initiated by the effects of bisantrene and nucleoside analogs in the nucleus where the drugs inflict damage to DNA, which is then communicated to the mitochondria through complex signaling pathways that decrease the levels of NAD+ and acetyl-CoA [36]. The nucleoside analogs, when phosphorylated, become incorporated into the DNA strands during synthesis, cause DNA damage and histone modifications, and induce chromatin remodeling. The relaxed chromatin likely becomes more susceptible to DNA intercalation by bisantrene, reminiscent of the susceptibility of relaxed chromatin to DNA alkylators as we previously reported [30]. Intercalation of small molecules into DNA is known to be more efficient in less constrained chromatin [37]. Such events are expected to facilitate the intercalation of bisantrene between base pairs of the DNA, thereby enhancing the inhibition of topoisomerase II. This model is consistent with the results of our drug sequence experiments; notably, continuous exposure of AML cells to (Flu+Clo) followed by bisantrene provided greater inhibition of proliferation compared with the reverse drug sequence (Figure 5).

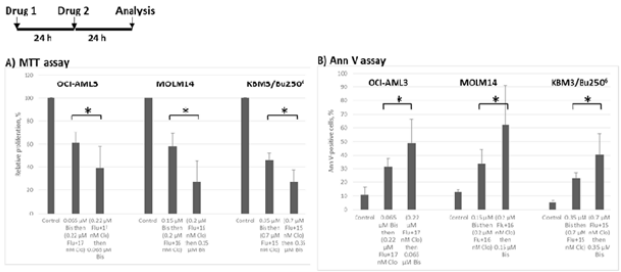

Figure 5: Effects of drug sequence on cytotoxicity. Cells were exposed to the first drug(s) for 24 h. Without washing the cells, the second drug(s) was/were added for another 24 h prior to analysis using MTT (A) and Annexin V (Ann V) (B) assays. Asterisks (*) indicate a statistically significant difference ($P<0.05$). The results are expressed as the mean ± standard deviation of five independent experiments.

The observed fragmentation of DNA (Figure 2D) in cells exposed to (bisantrene+nucleoside analog(s)+ABT199) indicates significant drug-induced formation of DNA strand breaks. The incorporation of nucleoside analogs into the growing strand of DNA is known to collapse the DNA replication fork and to inhibit DNA synthesis [38], which consequently causes DNA strand breakage. Anthracycline-mediated poisoning of topoisomerase II also induces DNA breakage by preventing the ligation of nicked DNA strands [2]. Anthracyclines can also undergo redox reactions to generate free radicals that in turn damage DNA [39]. All of these molecular events result in activation of the DNA-damage response as suggested by the phosphorylation of γ-H2AX (Figure 2 (A and B). It should be noted that treatment of human leukemia cells with topoisomerase II inhibitors is known to cause H2AX phosphorylation not only as a result of the drug-induced DNA damage per se but also because of the DNA fragmentation that occurs during cell death/apoptosis [40]; the relative contributions of these two responses to the γ-H2AX levels determined at the 48-h time point (Figure 2) cannot be distinguished without using more sophisticated assays than used here. Significant levels of DNA damage may cause cells to undergo apoptosis. Exposure of AML cells to a (bisantrene+nucleoside analog(s)+ABT199) combination resulted in increased Annexin V-positivity (Figure 1), cleavage of PARP1 and caspase 3 (Figure 2 (A and B), and caspase 3 enzymatic activity (Figure 2C), suggesting significant activation of the apoptotic pathway. Such cellular events likely resulted in the activation of caspase-dependent endonuclease [31] as shown by the observed DNA fragmentation (Figure 2D).

Depolarization of the mitochondrial membrane (Figure 3 (C and D), which likely causes the translocation of pro-apoptotic factors from the mitochondria into the cytoplasm, is also consistent with activation of apoptosis. This process is probably aggravated by the addition of ABT199 which binds to BCL-2 protein located on the outer membrane of the mitochondria; inhibition of BCL-2 is known to facilitate the release of pro-apoptotic proteins from the mitochondria [17]. Since BCL-2 also interacts with the Inositol Triphosphate Receptor (IP3R) protein to control Ca2+ release by the Endoplasmic Reticulum (ER) [41], its inhibition by ABT199 might have compromised Ca2+ homeostasis in the ER. In fact, we recently showed activation of the unfolded protein response in the ER in multiple myeloma cells exposed to an ABT199-containing drug regimen [42].

Conclusion

In summary, a combination of the anthracene derivative bisantrene with (a) nucleoside analog(s), without or with the BCL-2 inhibitor ABT199, exerts synergistic cytotoxicity towards AML cell lines by activation of the DNA-damage response and intrinsic apoptosis pathways. Our pre-clinical study provides a rationale for clinical trials, using these drug combinations for AML patients to induce remission through conventional therapy or to use such combinations as part of more complex strategy to stabilize and optimize relapsed (AML) patients, leading them more safely into (a) pre-transplant program(s) for allogeneic hematopoietic stem cell transplantation.

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