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COMPARISON OF AZA-ANTHRACENEDIONE-INDUCED DNA DAMAGE AND CYTOTOXICITY IN EXPERIMENTAL TUMOR CELLS

LORI A. HAZLEHURST,* A. PAUL KRAPCHO† and MILES P. HACKER*‡

*Department of Pharmacology, University of Vermont College of Medicine, and †Department of Chemistry, University of Vermont, Burlington, VT, 05405-0068, U.S.A.

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Abstract—Aza-anthracenediones are a new class of anti-cancer drugs, which demonstrate promising *in vitro* and *in vivo* activity. Our laboratory has synthesized a variety of structural analogs in which we determined previously that the positioning of the nitrogen within the backbone, as well as sidearm modification, results in dramatic differences in the potency of cytotoxicity. We reported previously that although DNA reactivity appears to be a necessary component for mediating cell death, it is not sufficient for predicting cytotoxicity of the aza-anthracenediones. We have chosen three aza-anthracenediones (BBR 2828, BBR 2778 and BBR 2378) to investigate the importance of DNA strand breaks and/or protein-concealed DNA breaks induced by aza-anthracenediones. We determined in the present study that, while all three drugs cause DNA breaks as determined by alkaline and neutral elution, as well as KCl–SDS precipitation, these breaks do not correlate directly with their potency as cytotoxic compounds. Further, we found significant differences in the types of DNA breaks induced by these drugs. Finally, we report that the persistence of protein–DNA complexes induced by all three drugs was similar and, therefore, cannot account for differences in the potency of cytotoxicity of the aza-anthracenediones. Thus, we postulate that, while the total number of drug-induced protein-concealed DNA breaks is an important indicator of drug toxicity, it is possible that the actual nature of the breaks may differ among the aza-anthracenedione congeners, and it is these differences in the actual proteins present in the DNA breaks that differentiate between aza-anthracenediones.

Key words: intercalation; DNA breaks; topoisomerase II; anthracenediones; resistance; cleavable complexes

The aza-anthracenediones are a new class of anti-cancer agents that have shown promising activity *in vitro* and *in vivo* against a wide spectrum of tumor cell lines [1–3]. This is an intriguing class of compounds because the biological activity is exquisitely sensitive to the positioning of the aza group within the backbone, as well as to the chemical nature of the sidearms. The interesting structure–activity relationship of these compounds has provided us with a powerful tool to probe the mechanism of action of this novel class of anti-tumor drugs.

The mechanism of action of anthracenediones remains uncertain. The putative targets of anthracenediones include DNA reactivity [4], topoisomerase II [5], protein kinase C [6, 7], helicases [8], and microtubules [9]. Anthracenediones contain a characteristic flat planar ring necessary for intercalation between base pairs. There is evidence that many intercalating agents induce DNA–protein cross-links [10, 11]. It has been postulated by several investigators that the protein involved in forming cross-links is topoisomerase II [12, 13]. Thus, topoisomerase II has been identified as a putative target for many intercalating agents including mitoxantrone, amsacrine and Adriamycin® [14–17].

Topoisomerases have been classified into two separate categories. Type I topoisomerases are ATP independent, cut only one strand of DNA, and can change the linking number by steps of one. Type II topoisomerases are capable of cutting both strands of DNA, change the DNA linking number by two, and require ATP binding for strand passage and hydrolysis for turnover [18]. Topoisomerase inhibitors have been characterized as stabilizing this normally transiently bound DNA–protein complex. This drug-stabilized protein–DNA complex has been referred to as the cleavable complex.

Under deproteinizing conditions, this cleavable complex can be detected as single- or double-strand breaks by using the elution technique developed by Kohn *et al.* [19]. This assay allows one to quantitate the number of protein-associated single- or double-strand breaks. However, the literature does not demonstrate a direct relationship between the number of single-strand breaks and the potency of cytotoxicity. This has been depicted by structure–activity relationships of anthracyclines [20] and anthracenedione derivatives [21] and by comparison of different intercalating agents [22].

We have reported previously that DNA binding appears necessary, but it is not sufficient for conferring cytotoxicity to the aza-anthracenediones [23]. For example, we observed an over 100-fold difference in the potency of cytotoxicity with 72 hr of continuous drug exposure between the 1-aza-anthracenedione (BBR 2828) and the 2-aza-anthracenedione with identical sidearms (BBR 2778, see Fig. 1 for structures) and only a 5- to 10-fold difference in affinity constants for salmon sperm DNA.

We have also demonstrated that these compounds appear to interact with DNA by intercalation [23]. However, the less active analog, BBR 2828, has a second mode of interaction that is observed at a lower salt concentration (<250 mM NaCl). This second mode of binding is also observed with mitoxantrone and is presumably due to stacking of the drug on the negative phosphate backbone [24].

Although the affinity for DNA does not correlate

‡ Corresponding author. Tel. (802) 656-2185; FAX (802) 656-4523.

directly with cytotoxicity, it is possible that the ability to initiate cleavable complex formation and/or the persistence of the cleavable complex formed does correlate with cytotoxicity. Preliminary work by us using the HL-60 cell line indicates that both BBR 2828 and BBR 2778 induce DNA damage, albeit at quite different drug concentrations [25]. This paper will extend our previous work on the importance of single- and double-strand breaks, DNA–protein cross-links and persistence of single-strand breaks in mediating cell death by the aza-anthracenediones. We further demonstrate that sidearm modification significantly altered the cytotoxicity profile and the characteristics of DNA damage induced by these drugs.

MATERIALS AND METHODS

Materials

All aza-anthracenediones and mitoxantrone were synthesized by Dr. A. Paul Krapcho, Department of Chemistry, University of Vermont, Burlington, VT, or Boehringer Mannheim Italia, Research Center, Monza, Italy. The structural formulae for these drugs are depicted in Fig. 1. Purity of all drugs was assured using NMR, HPLC, TLC and visible absorption spectra. Radioactive thymidine was purchased from ICN (Irvine, CA).

Cell culture

Cytotoxicity and DNA damage data were obtained in L1210 cells, a murine leukemia cell line, and S180 cells, a murine sarcoma cell line. L1210 cells were grown in McCoy's 5A medium (Gibco, Grand Island, NY) supplemented with 5% donor horse serum (Gibco). S180 cells were grown in McCoy's 5A medium (Gibco) supplemented with 10% donor horse serum. Both cell lines were incubated at 37° in a humidified incubator supplemented with 10% CO_2. CEM and CEM/VM-1 cells were provided by Dr. William Beck, St. Jude Children's Research Hospital, Memphis, TN. The CEM/VM-1 cells are resistant to drugs that inhibit topoisomerase II resulting from an altered topoisomerase II activity in these cells. Both cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum.

Cytotoxicity

Exponentially growing tumor cells were diluted in medium to a final concentration of 5×10^4 cells/mL and subsequently treated with various concentrations of drugs (dissolved in sterile water) for 2 hr. The cells were then washed twice in PBS and resuspended in fresh medium for an additional 72 hr. For each culture, cell concentrations were then calculated using a Coulter particle counter (Coulter Electronics, Hialeah, FL), and IC_{50} values were calculated as the concentration that inhibits cell growth by 50% with respect to non-treated control cells.

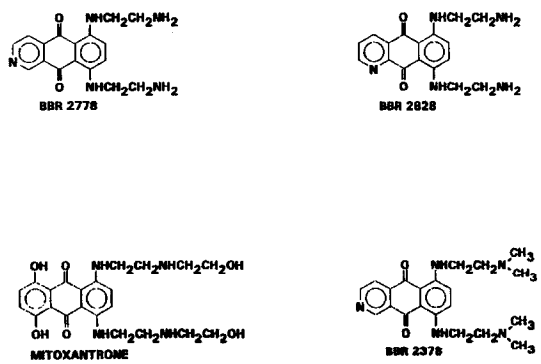

Fig. 1. Structural formulae for the aza-anthracenediones.

Alkaline elution

Alkaline elution assays were performed as described by Kohn [26]. Briefly, L1210 or S180 cells were labeled with either 0.2 μCi/mL [^{3}H]thymidine or [^{14}C]thymidine for 24 hr. The labeled cells were washed and resuspended in McCoy's 5A medium, and incubated for an additional 24 hr to ensure the random distribution of the radiolabeled thymidine. The ^{14}C-labeled population of cells was treated with various concentrations of drug for 2 hr in a humidified atmosphere of 5% CO_2. Subsequent to treatment, the cells were washed with ice-cold PBS buffer containing 6 mM Na_2 EDTA. The ^{3}H population of cells was washed with ice-cold PBS–6 mM Na_2 EDTA and subsequently irradiated with 400 rads to serve as an internal control for the rate of DNA elution from the filters. Polycarbonate filters (2 μm, Nucleopore) were prechilled with cold PBS–6 mM Na_2 EDTA, and approximately 2×10^5 cells from both the ^{3}H and ^{14}C-labeled population were gently placed on polycarbonate filters. Cells were then washed three times with ice-cold PBS–6 mM Na_2 EDTA and lysed for 30 min in a buffer containing 2% SDS, 20 mM Na_2 EDTA and 0.5 mg/mL proteinase K (pH 10.1). The lysate was washed subsequently with 20 mM EDTA (pH 10.1), and then eluted with 17 mL of an elution buffer containing 0.1% SDS, 20 mM free acid EDTA and tetrapropylammoniumhydroxide (RSA, Ardsley, NY), pH 12.2. Eight 90-min fractions were collected with a flow rate of 2.8 mL/90 min.

The filters were digested in protosol (NEN, Boston, MA) with gentle heat, and the filter holders were washed twice with 3 mL of 0.6 N NaOH. Ecolume (ICN, Costa Mesa, CA) containing 0.7% acetic acid was added to each vial, and the ^{14}C and ^{3}H were counted on a Packard 2500 TR liquid scintillation counter. The data were initially fit to a linear regression and plotted as the amount of ^{14}C retained on the filter as a function of the ^{3}H retained on the filter. The amount of ^{14}C retained on the filter when 40% of the internal control (^{3}H-labeled cells) was retained on the filter was determined, and this value was used to calculate rad equivalents. Rad equivalents were determined by the following equation:

$$\text{rad equivalents} = [\log(\text{treated/control})/\log(400 \text{ rads/control})] \cdot 400.$$

Persistence of single-strand breaks

L1210/0 cells (5×10^5 cells/mL) were treated with drug for 2 hr, washed three times in PBS, and resuspended in McCoy's 5A medium supplemented with 5% donor horse serum for 0, 2, 4, and 8 hr. The samples were then assayed for single-strand breaks as described previously.

Alkaline elution with/without proteinase K

The procedure was the same as alkaline elution except that conditions were changed to maximize protein binding to the filter, which included using a vinyl-acrylamide

filter (0.8 μm), the lysis buffer contained 2% sarkosyl instead of SDS, and the elution buffer contained no SDS [26]. The vinyl-acrylamide filter adsorbs proteins more efficiently than do the polycarbonate filters, and sarkosyl is a less stringent detergent and, thus, less likely to interfere with protein interactions on the filter.

Neutral elution

The procedure was the same as alkaline elution except for the following: the lysis buffer, 20 mM EDTA solution, and the elution buffer were all pH 9.6 to ensure that the DNA remained double-stranded. The internal control cells were irradiated at 10,000 rads to detect double-strand breaks [27].

KCl-SDS assay

The KCl–SDS assay was performed as described previously by Liu and co-workers [28]. L1210 or S180 cells were labeled with 0.2 μCi/mL [^{3}H]thymidine for 24 hr and then washed and resuspended in McCoy's 5A medium for an additional 24 hr. Various drug concentrations were added to approximately 5×10^5 cells/mL for 2 hr, and 3×10^5 cells/sample were then washed three times in solution A (0.4 G glucose, 8 g NaCl, 0.35 g $NaHCO_3$ and 1 mM Na_2 EDTA per liter, pH 7.4). The samples were resuspended in 1.2 mL lysis buffer consisting of 1% SDS, 5 mM EDTA, 0.4 mg/mL calf thymus DNA. To ensure complete lysis, cells were pipetted five times with a 22-gauge needle and heated to 65° for 15 min. From each sample, two 50-μL aliquots were taken to determine total radioactivity contained in the lysate. One milliliter of the lysate was transferred and subsequently precipitated with 250 μL of 650 mM KCl, vortexed vigorously for 5 sec, placed on ice for 10 min and centrifuged for 10 min at 1000 *g*. The pellet was then resuspended in wash buffer containing 10 mM Tris-HCl, 200 mM KCl, 1 mM Na_2 EDTA, 0.01 mg/mL calf thymus DNA, pH 8.0, and heated for 10 min at 60° in order to redissolve the pellet. After heating, the samples were vortexed vigorously, placed on ice for 10 min, and centrifuged. The pellet was then washed an additional two times and after the final wash the pellet was redissolved in 100 μL water at 65°. Samples were then transferred to a scintillation vial to which 4 mL of Ecolume was added, and each sample was counted on a Packard 2500 TR scintillation counter. The disintegrations per minute were divided by the average of the disintegrations per minute per milliliter of the lysate, and the data were then expressed as treated/control.

RESULTS

Cytotoxicity

We observed an approximate 30-fold difference in potency between BBR 2828 and BBR 2778 in L1210 and S180 cells (Table 1). The tertiary amine substituted 2-aza (BBR 2378) was approximately 10-fold more potent than the primary amine substituted 2-aza (BBR 2778) in both the L1210 and S180 cells. Mitoxantrone was over 40- and 3-fold more potent that BBR 2778 and BBR 2378, respectively, in L1210 cells. The S180 cells were inherently less sensitive to these drugs than were the L1210 cells. In the S180 cells, BBR 2378 and mitoxantrone were equipotent and BBR 2778 was approximately 10-fold less potent than mitoxantrone.

Table 1. Effect of drug treatment on L1210 and S180 tumor cell growth

Drug	IC_{50} (nM) L1210	S180
BBR 2778	337	1,430
BBR 2378	23	143
BBR 2828	10,520	>26,000
Mitoxantrone	8.2	113

L1210 or S180 cells were treated for 2 hr with various concentrations of drug. The cells were washed three times in PBS and then incubated for 72 hr in drug-free medium. The IC_{50} values were calculated as the concentration that inhibited growth by 50% with respect to non-drug-treated cells. The data shown are the averages of three experiments; the *SD* was less than 10% of the mean.

Alkaline elution

As shown in Fig. 2, there was an approximately 50-fold difference in potency between mitoxantrone and BBR 2778 in L1210 cells with respect to single-strand breaks. BBR 2378 had an interesting profile for single-strand breaks in that at the lower concentrations it was equipotent with BBR 2778; however, the drug-induced DNA breaks did not increase at concentrations of drug greater than 4 μM. Bell-shaped profiles are typical of many intercalating agents [20]; however, it is interesting to note that the maximum amount of single-strand breaks induced by BBR 2378 was significantly less than that induced by BBR 2778, yet BBR 2378 was 10-fold more potent with respect to cytotoxicity. BBR 2778 was approximately 10-fold more potent than BBR 2828 with respect to single-strand breaks, which was in keeping with the fact that BBR 2778 was approximately 30-fold more potent with respect to cytotoxicity in L1210 cells.

To test whether the relative profiles we observed with the aza-anthracenediones in L1210 cells were a consistent phenomenon, we tested the abilities of these compounds to induce single-strand breaks in S180 cells. In these cells there was an approximately 5- to 8-fold difference in potency of single-strand breaks induced by BBR 2778 and BBR 2828. BBR 2378, again, demon-

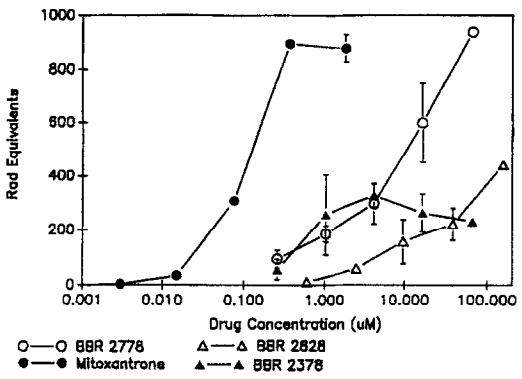

Fig. 2. Drug-induced DNA single-strand breaks in L1210 cells. Murine L1210 cells were treated for 2 hr with various concentrations of drug. Cells were then lysed and assayed for single-strand breaks by alkaline elution (pH 12.1) of DNA from filters. Values are means ± SD from 4 assays.

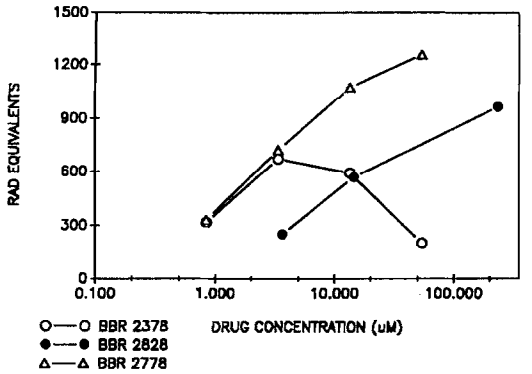

Fig. 3. Drug-induced DNA single-strand breaks in S180 cells. After 2 hr of drug treatment, cells were lysed and assayed for single-strand breaks by alkaline elution. Values are the means of 2–3 assays.

strated an interesting profile as there was an apparent reversal of strand breaks at the 66 μM concentration, which was not observed with either BBR 2778 or BBR 2828. As in the L1210 cells, BBR 2378 caused fewer total DNA breaks than either BBR 2778 or BBR 2828 (Fig. 3). It is interesting to note that although the S180 cells were less sensitive to the cytotoxic effects of these drugs than were the L1210 cells, they did have a greater number of drug-induced DNA breaks than the L1210 cells at each concentration tested.

To determine whether these single-strand breaks induced by the aza-anthracenediones in L1210 cells were protein concealed, L1210 cells were treated with equipotent concentrations of drug with respect to the number of single-strand breaks induced, and the drug-induced single-strand breaks in the presence or absence of proteinase K were measured. The vast majority (85–95%) of the single-strand breaks were protein concealed for all four drugs tested (Table 2).

Persistence of single-strand breaks

Another possible explanation for the differences in cytotoxicity among the aza-anthracenediones could relate to the persistence of the protein–DNA lesions induced by drug treatment. Fox and Smith [29] reported earlier that differences in cytotoxic potency of amsacrine and mitoxantrone could be attributed, at least in part, to such differences in persistence of the protein–DNA lesions. To test time, L1210 cells were treated with equipotent concentrations of drugs for 2 hr and placed in drug-free medium for up to 8 hr prior to assessing DNA damage by alkaline elution. As shown in Fig. 4, single-strand breaks initiated by mitoxantrone, BBR 2778, BBR 2828 and BBR 2378 were stable for at least 4 hr. While the amounts of DNA–protein lesions appeared to decrease to some degree for BBR 2778 and BBR 2828, neither amount was as unstable as those induced by amsacrine. The relative lack of persistence of the complexes formed by amsacrine is in agreement with results reported previously [5,29]. Our results suggest that differences in the persistence of single-strand breaks cannot account for the differences between potency of cytotoxicity between the aza-anthracenediones.

Table 2. Induction of protein-concealed DNA strand breaks by drug treatment

Drug	RAD equivalents +PK	RAD equivalents –PK
4 μM BBR 2778	424 ± 131	19 ± 26
4 μM BBR 2378	340 ± 119	15 ± 16
40 μM BBR 2828	396 ± 70	58 ± 35
0.075 μM Mitoxantrone	547 ± 116	16 ± 27

Approximately equipotent concentrations with respect to single-strand breaks were assayed ± proteinase K (PK), utilizing conditions that maximized protein absorption. Each condition is an average of three experiments. Values are means ± *SD* for each condition.

Neutral elution

Given the structural similarities between our compounds and mitoxantrone and the fact that mitoxantone has been shown to both inhibit topoisomerase II and induce double-strand DNA breaks, it was important to determine the ability of the aza-anthracenediones to induce similar DNA breaks. Neutral elution demonstrated a similar profile for mitoxantrone, BBR 2778 and BBR

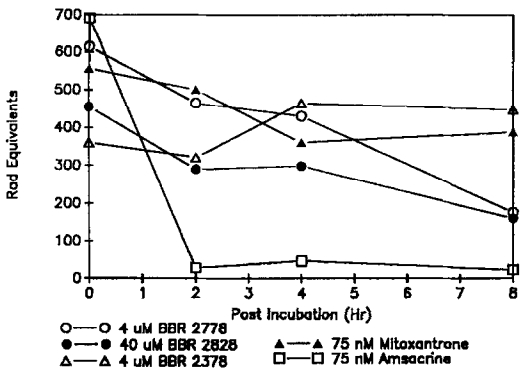

Fig. 4. Removal of single-strand breaks from drug-treated L1210 cells. L1210 cells were treated with approximately equitoxic concentrations of drug for 2 hr. Cells were then removed from drug and incubated for up to 8 hr in drug-free medium prior to lysis and alkaline elution. Values are means of two experiments.

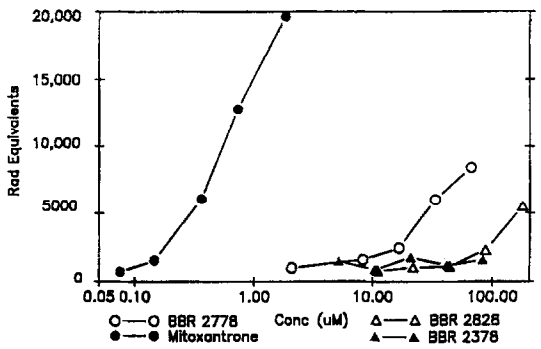

Fig. 5. Drug-induced DNA double-strand breaks in L1210 cells. Cells were treated for 2 hr with various concentrations of drug. Cells were then lysed and DNA was eluted from the filters, using a pH 9.6 buffer, to determine the extent of double-strand breaks induced. Values are means of two experiments.

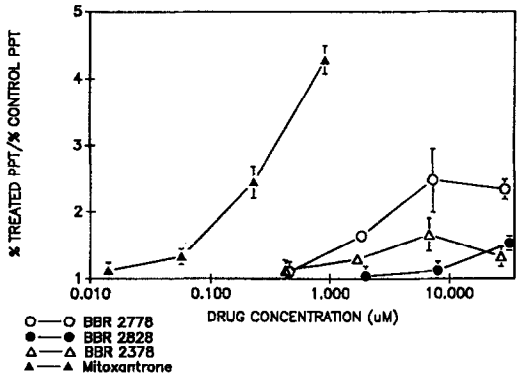

Fig. 6. KCl-SDS precipitation of DNA from drug-treated L1210 cells. Cells were treated with various concentrations of drug for 2 hr. The cells were then lysed and precipitated with KCl-SDS. Only DNA associated with protein will precipitate under these conditions. % Treated PPT/% Control PPT = percent of total radioactivity precipitated from drug-treated cells/percent of total radioactivity precipitated from control cells. Values are means ± SD, N = 3.

2828. Again, the difference in potency between BBR 2778 and mitoxantrone was approximately 50-fold, while the difference between BBR 2778 and BBR 2828 was approximately 10-fold. Perhaps the most interesting observation made was that BBR 2378 produced no measurable double-strand breaks (Fig. 5). This was indeed surprising given the fact that mitoxantrone, BBR 2778 and BBR 2828 all produced double-strand breaks.

KCl–SDS precipitation

To test if these compounds are capable of inducing SDS-resistant DNA–protein cross-links, we utilized the KCl–SDS method. As depicted in Fig. 6, the profile of drug-induced protein cross-links in L1210 cells was similar to what was observed for single-strand breaks as measured by alkaline elution. There was an approximately 50-fold difference in potency of DNA–protein cross-link formation between mitoxantrone and BBR 2778, and a 5- to 10-fold difference between BBR 2778 and BBR 2828 in L1210 cells. In contrast, at only the 6.75 μM concentration were DNA–protein cross-links observed for BBR 2378.

In S180 cells, there was an approximately 5- to 8-fold

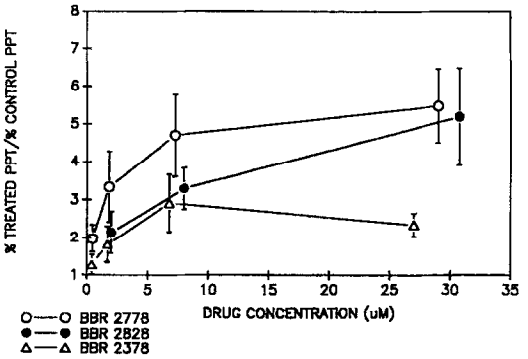

Fig. 7. KCl–SDS precipitation of DNA from drug-treated S180 cells. The conditions and procedures were identical to those discussed in Fig. 6. Values are means ± SD, N = 3.

Table 3. Cytotoxicity of BBR 2778, BBR 2378 and mitoxantrone against altered topoisomerase II expressing MDR cells

Drug	IC_{50} (μg/mL) CEM	CEM/VM-1	RF
BBR 2778	0.225	2.8	12.44
BBR 2378	0.24	0.25	1.04
Mitoxantrone	0.005	0.055	11

Cells were incubated in various concentrations of drug for 72 hr. The IC_{50} values were calculated as the concentration required to inhibit cell growth by 50% with respect to control cells. The resistance factor (RF) was calculated as the IC_{50} in CEM/VM-1 cells divided by the IC_{50} value obtained in CEM cells. The data shown are averages of two experiments.

difference in potency between BBR 2778 and BBR 2828. Again, drug-induced protein–DNA cross-links initiated by BBR 2378 did not increase with increasing concentrations above 6.75 μM (Fig. 7).

Cytotoxicity against cells expressing altered topoisomerase II activity

The unexpected differences in the amount and type of DNA damage induced by BBR 2378 compared with the other drugs tested suggested that BBR 2378 may kill cells through a mechanism unrelated to topoisomerase II inhibition. We reasoned that if this were true, then BBR 2378, but neither BBR 2778 nor mitoxantrone would be active in the CEM/VM-1 cells. As shown in Table 3, the resistance factors for BBR 2778 and mitoxantrone were approximately 10, whereas it was near 1 for BBR 2378.

DISCUSSION

We have developed a promising class of anti-cancer agents that exhibit a wide range of biological activities [1]. We are currently attempting to elucidate the mechanism of action of this novel class of compounds. We have determined previously that the aza-anthracenediones are capable of intercalation; however, the DNA binding affinity constants do not correlate well with cytotoxicity [23]. In an earlier study, we determined that differences in cytotoxicity in the HL-60 cells were related to differences in their ability to inhibit topoisomerase II [25]. In this paper, we addressed whether DNA breaks and/or DNA–protein interactions correlate with cytotoxic activity of three aza-anthracenediones.

Our data demonstrated a lack of direct correlation between DNA damage and cytotoxicity for the aza-anthracenediones. For example, while over a 30-fold difference in cytotoxicity between BBR 2778 and BBR 2828 in L1210 and S180 cells was observed, there was only a 5- to 10-fold difference in potency of single-strand breaks. This lack of correlation between potency of single-strand breaks and potency of cytotoxicity is further substantiated when one compares the number of strand breaks induced at 2 hr by equitoxic concentrations of the drugs. For example, it is interesting to note that at the 2-hr IC_{50} concentration, the least cytotoxic aza-anthracenedione (BBR 2828) produced at least an equivalent number of single-strand breaks in L1210 cells as did BBR 2778 (Table 4). Others have reported a similar dissociation between DNA damage and cytotoxicity for other DNA-damaging drugs [5, 30].

The majority of the single-strand breaks induced by all drugs tested were protein concealed. The persistence

Table 4. Comparison of drug-induced DNA breaks to drug cytotoxicity in L1210 cells

Drug	IC_{50} (nM)	Rad equivalents (at IC_{50})
BBR 2778	337	140
BBR 2378	23	0
BBR 2828	10,520	260
Mitoxantrone	8.2	<20

Two-hour IC_{50} values were obtained as previously described in Table 1. The rad equivalents shown above are extrapolated from Fig. 2 at the IC_{50} concentration for each drug in L1210 cells. The data shown are the averages of three experiments; the *SD* was less than 10% of the mean.

of single-strand breaks was similar for mitoxantrone, BBR 2378, BBR 2828 and BBR 2778, while amsacrine induced readily reversible single-strand breaks. A possible explanation for this is differential efflux rates between the compounds. Zwelling *et al.* [31, 32] have reported previously that within 5 min after drug removal, at drug concentrations lower than 7.5 μM, only residual amounts of amsacrine are detectable in L1210 cells at 37°. We have reported previously that the efflux rates of BBR 2373, BBR 2828, BBR 2778 and mitoxantrone are similar in L1210 cells at 37° [33]. The efflux rates that we observed are significantly slower than the literature efflux rates for amsacrine, and thus differential efflux rates may explain the differences in repair between amsacrine and aza-anthracenediones.

Another possibility is that the nature of the break is significantly different, and thus the repair machinery of the cell reacts differently to the break. Several investigators have suggested that the lack of correlation between single-strand breaks and potency of cytotoxicity may be a reflection of where in the genome this damage occurs [5, 30]. This could be a plausible explanation for the difference in persistence observed between amsacrine and mitoxantrone. However, Zunino and co-workers [34] did not observe a difference in the pattern of drug-induced cutting by topoisomerase II and anthracenedione derivatives. They also demonstrated that the efficiency of cutting correlated with DNA binding affinity at low drug concentration. We also observed a correlation between DNA binding and single-strand breaks. We had reported previously an approximately 5-fold difference in DNA affinity between BBR 2778 and BBR 2828 [23], which correlates well with the 5- to 10-fold difference in potency of single-strand breaks between BBR 2778 and BBR 2828.

Perhaps the most interesting observation was that BBR 2378 demonstrated no double-strand breaks. Topoisomerase II is characterized as being transiently covalently bound to double-stranded DNA. The DNA–topoisomerase II intermediate (cleavable complex) is stabilized by topoisomerase II inhibitors. Digestion of these complexes with proteinase K results in double-stranded breaks within the DNA. This can be detected by neutral elution, and thus topoisomerase II inhibitors can be characterized as forming double-strand breaks. It must be stated, however, that the ratios of single- to double-strand DNA lesions can vary significantly among topoisomerase II inhibitors. This difference may be related to the fact that some topoisomerase II drugs trap the complex after one of the DNA strands has been resealed. Thus, these lesions would appear to be single-stranded in spite of the fact that the drug is inhibiting topoisomerase II.

The observation that BBR 2378 did not cause double-strand breaks is important because it questions the role of topoisomerase II as a target for this compound. That BBR 2378 is a very potent drug and yet causes no double-strand breaks suggests that cell death is mediated via an alternative mechanism. This hypothesis is strengthened by the observation that only BBR 2378 was active in the CEM/VM-1 cells. These cells have been shown to be cross-resistant to a variety of topoisomerase II inhibitors, as a result of altered topoisomerase II activity [35], and were cross-resistant to BBR 2778 and BBR 2828, both known to inhibit topoisomerase II. Taken together our results strongly suggest that BBR 2378 is killing cells through a mechanism unrelated to topoisomerase II inhibition.

Considering the structural similarities between BBR 2778 and BBR 2378, this, as of yet, undiscovered target of BBR 2378 may also be a putative target for BBR 2778. The possibility exists that BBR 2378 is a topoisomerase I inhibition as single-strand breaks but not double-strand breaks were detected. Kapuscinski and Darzynkiewicz [36] have demonstrated that the cytotoxicity of ametantrone and mitoxantrone correlates well with their ability to condense single-stranded RNA. Thus, it is possible that a similar mechanism could exist for the aza-anthracenediones. Investigations probing these possibilities are currently ongoing in our laboratory.

We have determined previously that DNA reactivity is a necessary component for activity of the aza-anthracenediones, but is not sufficient for predicting potency of cytotoxicity [23]. Although we did not observe a tight correlation between single-strand breaks and potency of cytotoxicity of the aza-anthracenediones, it is possible that protein-concealed DNA breaks are a critical component of mediating cell death. The assays employed to measure DNA breaks only measure total protein-concealed DNA breaks and do not probe the identity or phosphorylation of the proteins. Darzynkiewicz and his co-workers [37] have demonstrated that the treatment of nuclei with ametantrone or mitoxantrone results in differential displacement of proteins. Thus, it is plausible that the majority of the protein-concealed DNA breaks induced by BBR 2828 results in non-lethal lesions. We are currently developing a technique that will enable us to identify the actual proteins that are tightly associated with DNA upon drug treatment, as well as the phosphorylation state of these proteins.

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