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Comparison of cytotoxicity in heart cells and tumor cells exposed to DNA intercalating agents *in vitro*

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A new approach to antitumor analog selection was evaluated using *in vitro* cytotoxicity assays in tumor cells and heart cells. Eight anthracycline antibiotics and five non-anthracycline DNA intercalating agents were separately exposed to human 8226 myeloma cells and neonatal rat heart myocytes *in vitro*. Survival was measured after six days of culture by the MTT dye method for tumor cells and by ATP content for heart cells. Inhibitory drug concentrations in 50% of cells (IC_{50}) were determined from log-linear dose–response curves for each agent. The IC_{50} values in the tumor cells ranged from 0.002 μg/ml for idarubicin to 3.5 μg/ml for the primary metabolite of doxorubicin, doxorubicinol. In contrast, IC_{50} values for anthracyclines in rat heart cells averaged approximately 357-fold higher than in the tumor cells. The heart cell/tumor IC_{50} ratio was 114.4 for the parent anthracycline doxorubicin. Compounds with poor cytotoxic selectivity for tumor cells included doxorubicinol, amonafide, amsacrine and bisantrene. Compounds with reduced cardiotoxicity included the anthracyclines daunorubicin (IC_{50} ratio of 550), esorubicin (IC_{50} ratio of 1500) and the anthracene derivative mitoxantrone (IC_{50} ratio of 500). These results show that simultaneous comparisons of cytotoxicity in heart cells and tumor cells can identify agents such as daunorubicin and mitoxantrone which are known to produce less cardiac toxicity *in vivo*. With further testing, this methodology may be applicable to preclinical screening programs to select active DNA intercalating agents with low cardiotoxic potential.

Key words: Anthracyclines, cardiotoxicity, doxorubicin, mitoxantrone, toxicity.

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Introduction

Cumulative damage to the myocardium is a serious toxicity of a number of DNA intercalating antitumor agents. Quantitative dosing limits to prevent cardiac damage have been established for the anthracyclines doxorubicin (DOX),[1] and daunomycin (DAUN),[2] and for the synthetic anthracene, mitoxantrone (MITOX).[3] A variety of mechanisms have also been forwarded to explain the selective cumulative damage to myocardial cells by such DNA intercalators. For doxorubicin, the mechanism is believed to involve membrane lipid oxidation mediated by drug-induced oxygen free radicals.[4,5] Alternatively, there may be inhibition of oxidative mitochondrial function leading to ATP depletion and cell death.[6,7] Unfortunately, none of the postulated mechanisms adequately explains the cardiac toxicity of DNA intercalating drugs such as MITOX which does not produce oxygen free radicals and can actually inhibit oxidative drug metabolism in defined microsomal enzyme systems *in vitro*.[8] This has made it difficult to predict whether a new DNA intercalating agent will produce cardiotoxicity and whether the effect will be more or less potent than with existing agents.

To overcome this problem, preclinical studies with new intercalators in rodents have sought to develop preclinical 'therapeutic ratios' relating antitumor activity and cardiotoxicity *in vivo*.[9] This is complicated by the fact that the endpoints for cardiotoxicity studies *in vivo*, such as QRS widening,[10] or histologic changes in myocardial cells,[11–13] are difficult to quantitate precisely.

Biologic variation *in vivo* also engenders the use of large numbers of animals which must be closely followed for months.[14]

In contrast, *in vitro* assays for cardiotoxicity require significantly fewer animals and can yield quantitative data within days.[15,16] In the past, these assays have been primarily applied to simple comparisons of cardiotoxic potency for different intercalating agents.[13,15–17] Thus, no previous *in vitro* cardiotoxicity studies with anthracyclines involved concurrent comparisons with tumor cell cytotoxicity. Most often, intercalating agents were compared solely in cardiac myocytes using endpoints such as the pH change in the medium,[18] myocyte contraction rates, morphologic changes,[19] enzyme leakage, typically lactic dehydrogenase (LDH),[15] or ATP content.[7,15,16] This type of cardiotoxicity evaluation does not provide quantitative estimates of the relative therapeutic efficacy for a new analog.

In the current study, a series of DNA intercalating agents was compared based on relative cardiotoxicity and tumor cell cytotoxicity *in vitro*. The results show that the potency for cardiotoxicity and antitumor cytotoxicity can differ substantially for different DNA intercalating agents.

Materials and methods

Drugs and chemicals

Doxorubicin (Adriamycin®, Adria Laboratories, Columbus, OH), daunorubicin (Cerubidine®, Ives Laboratories), and dactinomycin (Cosmegen®, Merck, Sharpe and Dohme, West Point, PA) were obtained commercially as vials of lyophilized drug. Plicamycin (Mithracin®) was obtained as a 2.5 mg lyophilized powder (Miles Laboratories, Inc., West Haven, CT). These drugs were reconstituted in sterile phosphate-buffered saline (PBS) (pH 7.4) prior to use in the cell culture studies. The anthracenes, bisantrene (CL 216,942) and mitoxantrone (Novantrone®), were obtained from Lederle Laboratories, Wayne, NJ, as a lyophilized powder reconstituted in water and diluted into 5% Dextrose for Injection USP (bisantrene) or as a sterile aqueous 1 mg/ml solution (mitoxantrone). Doxoubicinol, epirubicin (4′-epidoxorubicin), esorubicin (4′-deoxydoxorubicin), and idarubicin (4-demethoxydaunorubicin) were kindly provided by Farmitalia Carlo Erba, Milano, Italy. They were supplied as lyophilized powders which were diluted directly into PBS. The anthracyclines menogaril (NSC 269148) and aclarubicin hydrochloride (formerly aclacinomycin NSC 208734) were kindly provided from J. Paul Davignon, RPh, of the National Cancer Institute (NCI). They were supplied in vials containing sterile lyophilized powder. Each agent was reconstituted in sterile water followed by dilution in PBS. The benzisoquinolinedione derivative amonafide (formerly nafidimide, NSC 308847) and the acridine dye derivative amsacrine (mAMSA, NSC 249992) were also supplied by the NCI. Amsacrine was initially dissolved in a lactic acid–*N,N*-dimethylacetamide diluent provided with the drug. Amonafide was dissolved in water and diluted into sterile PBS. The plant alkaloid Emetine (Sigma Laboratories, St. Louis, MO) was tested as a non-antitumor agent, positive cytotoxic control. The tetrazolium MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium used in the growth inhibition assays was also obtained from Sigma Chemical.

Tumor cell growth inhibition assays

Human 8226 multiple myeloma cells[20] (CCL 155) were chosen as representative of rapidly growing hematologic tumor cells (doubling time 40 h). The cells were obtained from American Type Tissue Culture, Rockville, MD, and were grown in suspension culture in RPMI 1640 medium (Grand Island Biologicals, NY) supplemented with (1) 10% (by volume) heat-inactivated fetal bovine serum, and with (2) 1% (by volume) each of penicillin (100 unit/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) (all from Flow Laboratories, Bethesda, MD). Cells (2×10^5) in exponential growth were pipetted into 96-well plastic plates in culture medium. Drugs (0.02 ml of 10 × concentration) were added to achieve final concentrations of 0.01–10.0 μg/ml in a final volume of 0.2 ml per well. Each drug concentration was tested in six wells and each assay was repeated. The plates were then incubated for six days at 37°C in a humidified atmosphere of 5% CO_2, 95% air. At the end of the six days, 0.05 ml of MTT solution (1 mg/ml in media) was added to each well. After 4 h the plates were centrifuged (1000*g* × 10 min), the medium was aspirated and the cells were washed twice with fresh medium. Dimethylsulfoxide, 0.1 ml, was then added to solubilize the blue–black formazan product of mitochondrial reductase activity which is used to indicate tumor cell viability.[21] Dye intensity (viability) was quantitated spectrophotometrically at 540 nM on an automated microplate

spectrophotometer (Biomek®, Becton Dickinson, Inc., Rutherford, NJ).

Rat heart myocyte assays

Hearts were obtained by surgical excision from 1- to 2-day-old neonatal Sprague-Dawley rat pups of mixed gender. The hearts were minced with scalpels and digested in 0.24% trypsin. Isolated myocytes were suspended in Liebovitz' M-3 culture medium.[22] Fibroblast contamination was reduced by short-term plating in plastic flasks, which allows for rapid fibroblast attachment. The myocyte-enriched supernatant was then plated onto sterile 24-well plastic plates (Falcon Plastics) at a density of 1×10^6 cells/well and incubated at 37°C under the same conditions as the 8226 cells. Spontaneous myocyte beating began in isolated cells on day 1 and was synchronous at 40–60 beats/min by day 3 at which time drugs were added for short-term (3–6 h) exposures.[16] The drug-containing medium was then aspirated off and the myocytes were washed twice with fresh medium and returned to the incubator.

Because the myocytes do not divide, myocyte viability was quantitated photometrically (model 1251 Luminometer, LKB Wallac, Finland) after 72 h by determining the intracellular ATP levels using the firefly luciferin–luciferase assay.[23] ATP levels were normalized to total cellular protein using Coomassie Brilliant Blue dye staining in precipitated cell lysates.[24]

Results

The rat heart cells responded to the antitumor agents with significant decreases in ATP and the simultaneous loss of LDH and diminution in spontaneous contractions (data not shown). These effects usually occurred sequentially over the 72 h following drug exposure as has been previously described in detail for doxorubicin.[16] Each of the DNA intercalating agents was found to produce dose-dependent decrements in intracellular ATP concentrations but at widely varying drug concentrations.

Cytotoxic log(dose) responses were achieved for each DNA intercalating agent added to the 8226 myeloma cells. Inhibitory concentrations in 50% of the cells (IC_{50}) ranged from 0.002 μg/ml for idarubicin to 3.5 μg/ml for doxorubicinol (Table 1). This metabolite of DOX was the least potent anthracycline in the tumor cells. Conversely, the myeloma cells were sensitive to most of the other anthracyclines with IC_{50} values of 0.01–0.08 μg/ml. Exceptions include menogaril and aclarubicin which had a relatively high IC_{50} values in the myeloma tumor cells.

The results in the third column of Table 1 show that significantly higher concentrations of the anthracyclines were required to produce cytotoxicity in the rat heart myocytes. These concentrations, listed in μg/(ml h) to normalize the result for a 3 or 6 h drug exposure, ranged from 0.84 μg/(ml h) for idarubicin to 90 μg/(ml h) for doxorubicinol.

The last column in Table 1 directly compares the ratio of cytotoxic IC_{50} values for heart cells and tumor cells. On average, the IC_{50} values in myocytes were over 350-fold greater than those in the myeloma cells. In comparing these ratios, it is apparent that there is a wide range of heart cell/tumor cell cytotoxic ratios for the different anthracyclines tested. Thus, daunorubicin (IC_{50} ratio of 550), idarubicin (IC_{50} ratio of 420), and esorubicin (IC_{50} ratio of 1500) each have cytotoxic potency in the tumor cells. In contrast, three other anthracyclines (doxorubicin, epirubicin, and menogaril) had similar heart cell/tumor cell cytotoxic ratios with a mean value of 101 ± 21.2 (mean $\pm$SD). The compound with the weakest cytotoxic potency against heart cells, doxorubicinol, also had the poorest cytotoxic selectivity for tumor cells. In other words, while doxorubicinol had relatively low cytotoxic potency overall it did not discriminate well between tumor cells and normal heart cells in its inhibitory effects. Aclarubicin and plicamycin also had relatively poor selectivity for the tumor cell line.

Table 2 describes similar cytotoxic ratios for five non-anthracycline DNA intercalating agents. Of interest, the most potent antitumor agent in this group, mitoxantrone, was the least toxic in the heart cells (IC_{50} ratio of 500). In contrast, two of the other non-anthracycline intercalating agents exhibited extremely poor cytotoxic selectivity for the tumor cells. These more potent cardiotoxins include amonafide and amsacrine. Bisantrene and dactinomycin had intermediate ratios of about 100 similar to doxorubicin. The very low cytotoxic ratio of 5.5 for the benzisoquinoline derivative, amonafide, was the lowest value obtained with any of the DNA intercalating agents tested in this series.

Conclusion

The current results show that there are wide differences in cytotoxic potency for DNA intercala-

Table 1. Comparison of cytotoxicity with anthracycline-based DNA intercalating agents *in vitro*

DNA intercalator	Mean IC_{50} values (SD)		IC_{50} ratio (heart/tumor)
	8226 tumor cells (μg/ml continuous exposure)	Heart cells (μg/(ml h))	
Aclarubicin	0.20 (0.05)	11.4 (6.3)	57.0
Daunorubicin	0.03 (0.019)	16.5 (3.0)	550.0
Doxorubicin	0.05 (0.017)	5.72 (3.2)	114.4
Doxorubicinol	3.5 (1.4)	90.0 (22.1)	25.7
Epirubicin	0.08 (0.029)	5.7	71.3
Esorubicin	0.01 (0.002)	15.0 (4.1)	1500.0
Idarubicin	0.002 (0.002)	0.84	420.0
Menogaril	0.19 (0.02)	22.5	118.0
Plicamycin	2.0 (0.8)	110.0	55.0

ting agents simultaneously evaluated in tumor cells and heart cells. There were agents which had low cardiotoxic potency. These included mitoxantrone, idarubicin, daunorubicin and esorubicin. Conversely, three compounds displayed high cardiotoxic potency including amonafide, amsacrine and doxorubicinol. Importantly, the weak cardiotoxins identified in this analysis are known similarly to produce significantly less cardiotoxicity *in vivo*. For example, daunorubicin requires higher drug exposures to produce congestive heart failure than does doxorubicin both in rats[10] and in cancer patients.[2] In humans the cumulative cardiotoxic dose limit for daunorubicin is approximately twice that of doxorubicin for a similar incidence of congestive heart failure.[1] Similarly, esorubicin and idarubicin have also been reported to produce less cardiotoxicity than doxorubicin in animal models[25] and in cancer patients.[26] Another relatively weak cardiotoxin in the current study was mitoxantrone. This anthracene-based agent is known to be significantly less cardiotoxic than doxorubicin in patients with solid tumor.[27,28]

Structure–activity relationships for the different *in vitro* cytotoxic potency ratios uncovered in this study are not clear. For example, daunorubicin is structurally quite similar to doxorubicin, differing by the lack of a hydroxyl on the side chain of the A ring, and generates reactive oxygen species equivalent to doxorubicin *in vitro*.[29] However, equimolar dosing of daunomycin and doxorubicin in mice leads to significantly less daunomycin accumulation in the heart.[30] This may be due to the decreased polarity of daunorubicin.[31] Daunomycin is also more readily reduced to C-13 alcohol metabolite, daunorubicinol, by ubiquitous cytosolic aldoketoreductase enzymes. This may be important for daunorubicin's lower cardiac toxicity since *in vitro* daunorubicinol has been shown to be much less active than daunorubicin. This has been demonstrated in normal bone marrow cells[32] and in various tumor cell lines.[31–35] It would be of interest to study daunorubicinol and cardiotoxicity in the current assay system.

Esorubicin, which was also less cardiotoxic than doxorubicin, lacks an oxygen atom at the 4′ position of the amino sugar. It has greater basicity and lipophilicity than doxorubicin.[9] However, it also has reduced or absent reductive enzymatic conversion to a semiquinone free radical.[36] Since

Table 2. Comparison of cytotoxicity for non-anthracycline-based DNA intercalating agents *in vitro*

DNA intercalating agent	Mean IC_{50} values (SD)		IC_{50} ratio (heart/tumor)
	8226 tumor cells (μg/ml continuous exposure)	Heart cells (μg/(ml h))	
Amonafide	2.0 (1.1)	11.0	5.5
Amsacrine	1.3 (0.5)	30.0	23.1
Bisantrene	0.05 (0.019)	5.0	100.0
Dactinomycin	0.08 (0.031)	10.0	125.0
Mitoxantrone	0.008 (0.003)	4.0 (0.65)	500.0

oxygen radicals are associated with anthracycline heart toxicity,[37] the lack of activation with esorubicin may explain its reduced cardiotoxicity. A similar biochemical difference may explain the reduced cardiotoxic potency of mitoxantrone. This anthracene antitumor agent readily intercalates into DNA[38,39] but does not produce oxygen free radicals and can even act as a microsomal enzyme antioxidant *in vitro*.[40]

Conversely, the enhanced cardiotoxic potency of some other non-anthracycline DNA intercalators is not explainable by oxygen free radical mechanisms. Amonafide,[41] amsacrine,[42] and bisantrene[39] are believed to act primarily as DNA intercalators which, like the anthracyclines, may also inhibit DNA topoisomerase II activity.[43–45] None of these compounds has been shown to produce oxygen free radicals.

Another explanation for the low tumor cell/heart cell ratios with some of the agents in this study is that an individual drug may have poor cytotoxic activity against the 8226 myeloma tumor cell line. This would reduce the apparent heart cell/tumor cell IC_{50} ratio and thereby accentuate cardiotoxicity relative to tumor cell cytotoxicity. The relatively high IC_{50} values in the 8226 cells for amonafide, amsacrine, and bisantrene (Table 2) suggest that this may be a possibility.

Overall, doxorubicinol had the lowest cytotoxic selectivity for tumor cells (heart cell/tumor cell IC_{50} ratio of 25.7). This suggests that doxorubicinol would be a poor candidate for development as a clinical anticancer agent. Indeed, increased cardiotoxicity for doxorubicinol has already been described *in vitro* by others. For example, Olson *et al.* have reported increased potency for doxorubicinol at inhibiting cardiac function in isolated rabbit heart ventricular papillary muscles. Their study also described significantly less antitumor cytotoxicity for doxorubicinol in several human and hamster cell lines *in vitro*.[46] This directly corroborates the current findings.

Unfortunately, data to support the potent *in vitro* cardiotoxicity of amonafide, amsacrine and bisantrene are not readily available. Bisantrene has been dropped from clinical trials and amonafide is just undergoing early clinical evaluation.[47] However, amonafide has shown poor cytotoxic specificity for primary human tumors compared with human bone marrow cells evaluated *in vitro*.[48] This is similar to the current results with rat heart cells.

Amsacrine has previously produced severe cardiac toxicities in leukemic patients.[49–52] Most cases involve acute cardiac rhythm disturbances which are exacerbated by concurrent hypokalemia. However, one prospective clinical toxicity study with amsacrine could not demonstrate cardiac dysfunction (decreased left ventricular ejection fraction) in six patients who received cumulative doses of 325–510 mg/m^2.[53]

Overall, the current findings support a reduced cardiotoxic potential for several DNA intercalating agents including daunorubicin, idarubicin, esorubicin, and mitoxantrone. Clearly, cytotoxicity studies in other cell lines will be needed to see whether these ratios are consistently obtained with other tumor types. Nonetheless, the current findings suggest that simultaneous *in vitro* toxicity tests in heart cells and tumor cells may yield important data for identifying less cardiotoxic DNA intercalators for further development.

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