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In Vitro Rat Myocyte Cardiotoxicity Model for Antitumor Antibiotics Using Adenosine Triphosphate/Protein Ratios[1]

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ABSTRACT

Cumulative cardiotoxicity consistently limits the use of antitumor anthracyclines such as doxorubicin (DOX). Several *in vivo* and *in vitro* model systems have been developed for screening cardiotoxic agents. Problems with these models include excessive time and nonquantitative toxicity end points. We describe an *in vitro* system for culturing cardiac myocytes which overcomes these problems. Optimal myocyte cultures were obtained using serial 0.2% crude trypsin digestions of hearts from 1–2-day-old rats. Three-day-old myocyte cultures were treated with DOX for 6 h at concentrations of 0.1 to 10 μg/ml (0.18 to 18 μM). Electron microscopy performed on control and DOX-treated cultures showed characteristic histopathological signs of anthracycline damage. These changes included mitochondrial swelling, nuclear pleomorphism, chromatin clumping, and a diffuse loss of membrane integrity. Intracellular ATP, quantitated by the luciferase bioluminescence method, was shown to provide a simple and consistent quantitative biochemical marker of myocyte viability over the range of DOX concentrations used. The results showed both time- and dose-dependent decrements in ATP/protein ratios 72 h following exposure to DOX at concentrations > 0.1 μg/ml. Leakage of lactate dehydrogenase activity, trypan blue uptake, and myocyte beating rates were variable and not as sensitive as ATP levels for evaluation of myocyte viability. Other cytotoxic agents which are not known to be cardiotoxic (dactinomycin, 1-β-D-arabinofuranosylcytosine, fluorouracil, melphalan, and vincristine), required extremely high concentrations to produce myocyte damage *in vitro*. Tests with anthracycline analogues also demonstrated the ability of the assay to rank-order cardiotoxic agents on a weight basis: idarubicin > DOX > daunomycin > aclarubicin. When the *in vitro* drug concentrations required to lower ATP/protein ratios to 50% of controls were related to clinically achievable concentration × time products, DOX and daunomycin proved to be the most cardiotoxic in this series. These results suggest that comparative cardiotoxic screening studies may be performed *in vitro* using ATP levels in beating neonatal myocytes.

INTRODUCTION

DOX[2] is a potent anticancer agent which intercalates into DNA (1, 2) and also produces strand scission (3) ostensibly via oxygen free radical formation (4) or by inhibition of DNA topoisomerase II activity (5). The drug has a broad spectrum of activity, but clinical utility is limited due to cumulative cardiotoxicity (6). Because of this complication, a lifetime dose limit of 550 mg/m^2 is empirically applied in the clinical use of this drug (7). However, when total drug doses are limited to 550 mg/m^2, a small percentage of patients will nonetheless develop cardiotoxicity (8). Thus, numerous investigators have sought new anthracyclines with similar anticancer properties but without the associated cardiotoxicity. The preclinical screening of numerous DOX analogues synthesized in laboratories worldwide has now assumed major proportions (9).

Routine preclinical cardiotoxicity screening currently utilizes rodents which are chronically dosed for up to 6 months (10). End points include serial electrocardiographic assessments for QRS widening, (11, 12) or histopathological scoring of endomyocardial biopsies (13, 14). Due to the cost, time, and labor-intensive procedures needed in such studies, several *in vitro* cardiac myocyte systems have been developed (15–19). A major problem in these *in vitro* assays is that the myocyte viability end points often rely on semiquantitative toxicity indices. These have included simple pH changes in the culture medium (19), visually determined beating rates (17), or delayed and often unreliable indicators of cell damage such as impaired membrane integrity (*e.g.*, enzyme leakage or trypan blue dye exclusion) (20, 21). In addition, mammalian heart cells have very limited clonogenic potential (22, 23); thus, routine cytotoxicity assays based on myocyte proliferation or colony formation are useless. Therefore, a need still existed for a quantitative and reliable end point to measure cardiotoxicity *in vitro*.

In this report we describe a myocyte culture system for evaluation of cardiotoxic agents based on the role of intracellular ATP in myocyte metabolism (24–26). Our results show that intracellular ATP content in myocytes provides a broad dynamic index of DOX-induced damage to neonatal rat heart myocytes.

MATERIALS AND METHODS

Isolation, Culture Growth, and Identification of Cardiac Myocytes. Hearts from 1–2-day-old neonatal Sprague-Dawley rats were isolated under sterile conditions, minced into 1-mm^2 fragments and dissociated with 0.2% crude trypsin (Difco Laboratories, Detroit, MI) dissolved in calcium and magnesium free Hank's balanced salt solution (Irvine Scientific, Santa Ana, CA).

Seven 15-min digestions were performed with fresh 0.2% trypsin added at the onset of each digestion. The first two of these digestions were discarded. The last five serial digestions were collected, pooled, washed twice in Liebovitz's M3 medium (27), counted, and then plated at 3–4 × 10^7 cells/150-cm^2 culture flask for rapid fibroblast attachment (28, 29). After 2 h, the resultant myocyte-enriched supernatant was poured off, counted, and plated in 24-well *Primaria* plates (Falcon, Oxnard, CA) at a density of 1.1 × 10^6 cells/mm^2. The medium was supplemented daily beginning on day 2 of culture with total medium volume never exceeding 0.65 ml/well. A one step trichrome stain (30) was applied to intact myocytes to differentiate myocardial fibroblasts from muscle cells microscopically.

Electron Microscopy. Cell cultures grown in 35-mm Costar plates were fixed *in situ* on the plastic culture dishes. A solution of Karnovsky's fixative (31), diluted 1:1 with phosphate buffered saline, pH 7.4, was added to the cultures. After 50 min, the fixative was removed and replaced with full strength Karnovsky's fixative for an additional 50 min. The cells were gently scraped with a rubber policeman and pelleted in a microfuge at 15,000 × *g* for 5 min. This was followed by rinsing in 0.1 M phosphate buffer. Cells were postfixed in 1% osmium tetroxide for 1 h on ice in the dark, stained with 1% uranyl acetate *en bloc*, dehydrated in ascending grades of ethanol, and embedded in Spurr resin. Ultrathin sections (80–90 nm) were stained with lead citrate and

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[2] The abbreviations used are: DOX, doxorubicin; IC_{50}, drug concentration which lowers ATP/protein ratios to 50% of controls; LDH, lactate dehydrogenase; DOXOL, doxorubicinol.

examined with a Philips 300 transmission electron microscope, operating at an accelerating voltage of 60 kV.

Labeling Indices. At days 1, 2, 3, 4, and 6 in culture, the DNA labeling index of cells was performed. The procedure consists of a 1-h "pulse labeling" of cultures with [^{3}H]thymidine (5 μCi/ml) with a specific activity of 80 Ci/mmol. Quantification of grains/cell/field by autoradiography was assumed to represent the percentage of cells undergoing DNA synthesis during that hour (32).

Viability Indices. *In situ* myocyte cultures were evaluated for beating rates and trypan blue dye exclusion. Beating rate averages (per minute) were composed of the rates from 10 random microscopic fields. Trypan blue (0.15%) was added directly to the viable cultures and observations of the dye uptake were made on a Nikon TMS inverted phase contrast microscope.

Samples for quantification of ATP and protein were obtained from each well. Cells were rinsed with phosphate-buffered saline and 5% trichloroacetic acid was added to each well to lyse the cells and extract the ATP. Precipitated protein was solubilized with 0.1% Triton X-100 in 0.5 N NaOH. All samples were frozen at −80°C until assayed.

ATP levels were determined photometrically using a standard firefly luciferin-luciferase bioluminescent assay (1251 luminometer; LKB-Wallac, Finland; Turner Instruments and Reagents, Mountain View, CA). Acid extracts were diluted 200-fold with 0.025 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.75, prior to analysis.

Protein content was determined using the Bio-Rad method (Sigma Diagnostics, Sigma Chemical Co., St. Louis, MO) with bovine serum albumin dissolved in the cell solubilization solution as a standard.

Quantitation of LDH activity was performed on the incubation medium using a standard spectrophotometric analysis (Sigma Diagnostics). Assay sensitivity for the LDH determination is 3.3 mU of activity.

DOX and the primary alcohol metabolite, doxorubicinol, was quantitated in detached myocyte culture lysates using reverse-phase high-performance liquid chromatography with fluorescence detection (excitation at 480 nm and emission at 550 nm) (33).

Dosing Regimen. Drugs in M3 medium were added to 3-day-old myocyte cultures for 6 h. The cells were then rinsed three times with M3 media to remove free drug. Fresh media was added to the cells which were then incubated at 37°C in a 5% CO_2 incubator for an additional 72 h. Drug concentrations for 6-h exposures were chosen in a standard fashion by using clinically achievable concentration × time products seen in patients given one course of the drug (34).

Doxorubicin was obtained from Adria Laboratories (Columbus, OH) and used as the model cardiotoxic agent. It was reconstituted in sterile phosphate-buffered saline (1 mg/ml) and frozen at −80°C for up to 1 month. Thawed stock solution was diluted in M3 medium to the appropriate concentration and used immediately. Doxorubicin was shown by high-performance liquid chromatography to be stable in M3 media for 6 h at 37°C.

Mercuric chloride (35), digoxin (36), and emetine (37) (Sigma) were each evaluated as positive cytocidal control compounds.

Several congeners of doxorubicin were also evaluated. These included the nonhydroxylated DOX congener daunomycin (38), 4-demethoxydaunorubicin or idarubicin (39), the DOX metabolite doxorubicinol (40), and the polysaccharide analogue aclarubicin, which is reportedly less cardiotoxic than DOX (41). Daunomycin and aclarubicin were reconstituted from 20-mg vials obtained from Ives Laboratories and the National Cancer Institute, respectively. Idarubicin (5-mg vials; Farmitalia, Milan, Italy) was reconstituted in sterile water to yield a concentration of 1 mg/ml.

Several noncardiotoxic anticancer agents were also evaluated at concentrations well above tumoricidal plasma levels. These included the alkylating agent melphalan (Sigma), the *Vinca* alkaloid vincristine (Eli Lilly and Co., Indianapolis, IN), the intercalating agent dactinomycin and the antimetabolites fluorouracil (Roche Laboratories, Nutley, NJ); and 1-β-D-arabinofuranosylcytosine (Cytosar; Upjohn Laboratories, Kalamazoo, MI). Melphalan, a bifunctional DNA cross-linking agent (42), was used at 100 μg/ml which is well above the antineoplastic concentration range of 1–5 μg/ml for this drug *in vitro* (34). Vincristine was tested at 1.0 μg/ml, or 100 times the antileukemic concentration of 0.01 μg/ml (43), and 1-β-D-arabinofuranosylcytosine was tested at 100 μg/ml, which is 100 times the antileukemic concentration of 0.1 μg/ml (44). Fluorouracil was tested at a concentration of 1000 μg/ml which is well above a peak human plasma level following an i.v. bolus dose (45). Dactinomycin (Cosmegen; Merck Sharpe and Dohme) was tested *in vitro* at a cytotoxic concentration of 0.5 μg/ml (34).

RESULTS

Methods Development. Cumulative cell yields from the seven serial trypsin digestions after preplating averaged 5–7 × 10^6 cells/heart with 81 ± 2% (SE) viability by trypan blue dye exclusion.

Fibroblasts were initially reduced to 8% of the culture population by plating the digested heart cell suspension in large surface area plastic flasks for 2 h. The myocyte-enriched supernatant was then plated densely at 1.1 × 10^6 cells/mm^2 using Liebovitz's M3 medium for culture maintenance (27). Fibroblast contamination increased slowly over the 7-day assay period: 8% on day 1; and increasing to a 30% maximum on day 7. Myocytes attached and began beating within 24 h of plating. Synchronous beating consistently began 1–2 days after plating.

In a separate experiment, cardiac fibroblasts were grown to confluence and exposed to 0.1, 1.0, and 5 μg/ml DOX for 6 h.

Electron Microscopy. Transmission electron microscopy was performed on 6-day-old myocyte cultures with and without a prior 6-h doxorubicin treatment of 1.0 or 10.0 μg/ml. A transmission electron microscopy micrograph from untreated myocytes (Fig. 1*a*) shows characteristic myofibrils, numerous mitochondria, continuous plasma membrane integrity, and no apparent cellular vacuolization. In contrast, the DOX-treated myocytes (Fig. 1, *b* and *c*) show several histopathological changes characteristic of this agent (46). These alterations include myofilament degeneration and dropout, discontinuous plasma membranes, chromatin clumping, nuclear pleomorphism, extensive cytoplasmic vacuolization, and swelling of the mitochondria.

Myocyte Culture Development. Characterization of control myocyte cultures was performed on days 1–6 after plating. Cell density of attached myocytes measured by total cellular protein ranged from 115 ± 12 μg protein/well (24 h), 182 ± 14 μg protein/well (48 h), up to 248 ± 18 μg protein/well (72 h). Protein values of untreated myocytes remained constant for the remainder of the experimental period.

Serial assessments of ATP/protein ratios, the [^{3}H]thymidine labeling index, and beating rates of control myocytes are shown in Fig. 2. ATP levels normalized to cellular protein remained constant throughout the week of culture growth. Myocyte and fibroblast cell division are indicated by the labeling index peaks on day 3 of culture which correlates with visual confluence of the monolayer. Beating rate measurements, while useful as a gross assessment of cell viability, were variable in our experiments. This is most likely due to changes in medium temperature and pH which occur while making observations without an environmentally controlled microscope stage. Therefore, quantitative results of beating rate data are not presented except for control cultures (Fig. 2). Drug treatment was begun on day 3 at which time myocyte cultures had fully established their cell to cell communications and were beating synchronously.

Viability Assessments of Myocyte Cultures. Trypan blue tests of myocytes produced similar myocyte staining patterns regardless of the beating status, intracellular ATP content, or degree of LDH leakage. Other anomalies with the trypan blue viability method included observations that cytoplasmic extensions from

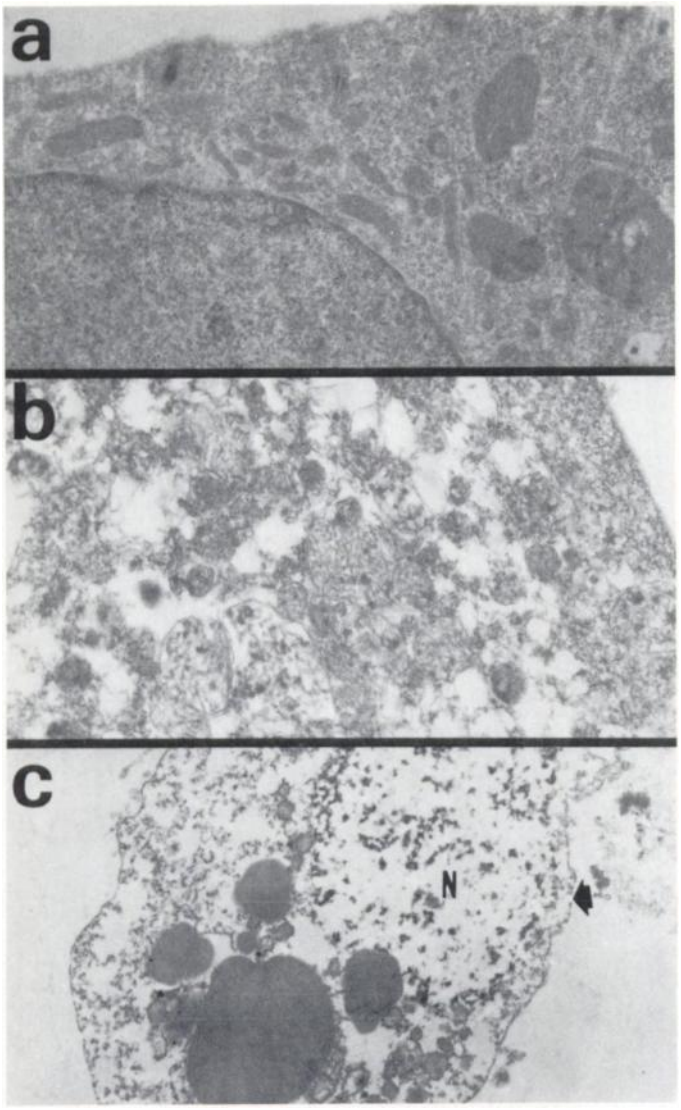

Fig. 1. Transmission electron micrographs of neonatal rat heart myocytes with and without DOX treatment. In *a*, untreated myocyte after 6 days in culture contains a cytoplasm rich in cellular organelles. The integrity of the nuclear and plasma membranes has been maintained. In *b*, a myocyte from a culture treated with 1 μg/ml DOX for 6 h reveals mitochondrial swelling and diffuse vacuolization. In *c*, a representative myocyte from a culture treated with 10 μg/ml DOX for 6 h shows a discontinuous plasma membrane (*arrow*), a nucleus (*N*) with extensive chromatin clumping, and a complete loss of cytoplasmic organization and myofibrils. *a*, × 10,900; *b*, × 13,300; *c*, × 7,100.

nonbeating, dead cell bodies consistently excluded the dye. Similar inconsistencies with the trypan blue dye test have been described in tumor cells by other researchers (21).

As stated previously, control cultures showed variability in beating rates and this increased markedly following drug exposure. A transient chronotropic response was noted 24–48 h following DOX exposure with a concomitant reduction in the number of cells beating. Beating synchrony declined slowly over the first 48 h following exposure. By 72 h post-exposure, there was a complete cessation of beating in cells treated with > 1.0 μg/ml DOX. These observations are in close agreement with those of Lampidis *et al.* (16).

A general correlation between LDH leakage (Fig. 3) and ATP levels (Fig. 4) was observed. However, LDH leakage varied more following DOX exposure and was relatively nonquantitative at high DOX concentrations. This was especially evident at DOX concentrations producing a ≥ 50% decrease in ATP levels (*i.e.*, ≥ 1.0 μg/ml).

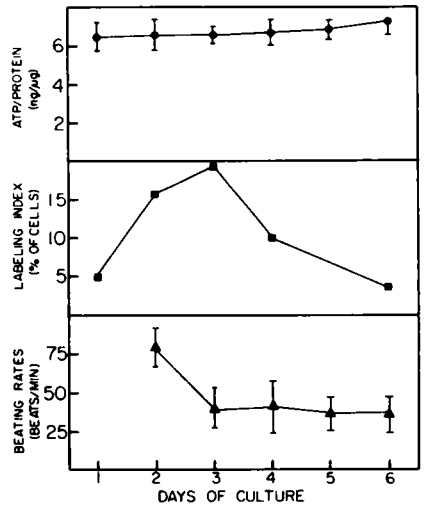

Fig. 2. Myocyte maturation profile. Maturation profile of cultured neonatal rat cardiac myocytes. The labeling index is determined by the degree of incorporation of [^{3}H]thymidine activity into cellular DNA (n = 4–8).

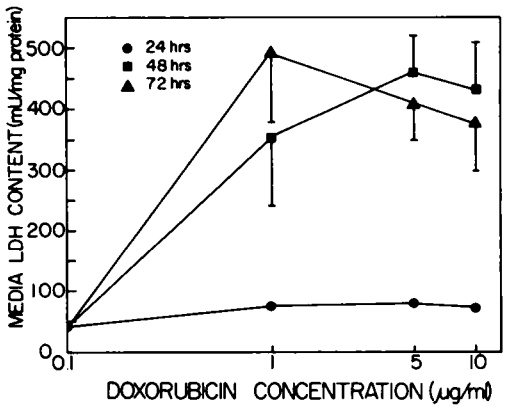

Fig. 3. LDH release from DOX-treated myocytes with varying postincubation times. Dose-response patterns for doxorubicin-induced release of LDH activity into media from cardiac myocytes. On day 3 myocytes were incubated with 0.1, 1, 5, and 10 μg/ml DOX for 6 h. Cultures were then postincubated in drug-free M3 medium for 24, 48, and 72 h. Each *point* represents mU LDH released/well normalized to cellular protein content/well. Control values ranged from 30 to 45 mU/mg protein. *Bars*, SEM (n = 3–4).

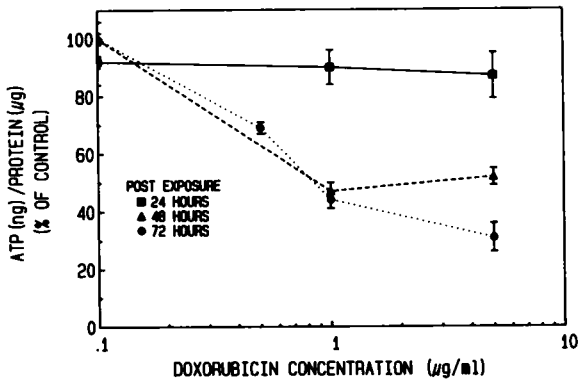

Fig. 4. ATP depression in DOX-treated myocytes with varying postincubation times. Dose-response patterns for doxorubicin-induced ATP/protein ratio depression in rat cardiac myocytes. On day 3 myocytes were incubated with 0.1, 0.5, 1.0, and 5.0 μg/ml DOX for 6 h. Cultures were then postincubated in drug-free M3 medium for 24, 48, and 72 h. Each *point* represents ATP/protein ratio averages. *Bars*, SEM (n = 4–8).

Doxorubicin Pharmacology. The effects of 6-h DOX treatments (0.1 to 5 μg/ml) on the ATP/protein ratio of myocyte cultures are shown in Fig. 4. For a given DOX exposure, ATP/

protein ratios decreased in proportion to the myocyte incubation time in drug-free medium. Thus, no myocyte damage was evident within 24 h after a 6-h DOX treatment. However, this increased significantly with 48 h of incubation. Incubation for 72 h in drug-free medium proved to be a critical time for detecting maximal dose-dependent DOX damage (Fig. 4). Treatment of myocytes with 10 μg/ml DOX for 6 h did not produce further decreases in ATP levels (data not shown), or further increases in LDH release (Fig. 3). This is most likely due to DOX self-association in the media as well as DOX association with certain amino acids and nucleotides (47).

The dose-response evaluation for DOX-treated myocytes using the 72-h postdrug incubation time shows proportional decreases in ATP/protein ratios. These data show that the IC_{50} of DOX in this system was 0.8 μg/ml for 6 h. Cardiac fibroblasts were less sensitive to DOX with an ATP IC_{50} greater than 5 μg/ml. However, a confounding variable was the high rate of detachment of DOX-treated fibroblasts. This resulted in a loss of up to 90% of the cardiac fibroblasts depending on the dose of DOX (data not shown).

Table 1 lists the results of intracellular DOX recovery studies from myocytes incubated in drug-free medium for 72 and 96 h following a 6-h DOX exposure. These results show a proportional dose-dependent retention of DOX at both the 72- and 96-h time points. Thus, substantial DOX is still present in cardiac myocytes 96 h after a 6-h DOX exposure *in vitro*.

DOXOL levels were barely detectable (≤ 1 ng) in the myocytes examined at both the 72- and 96-h time points. A control dose-response experiment using pure DOXOL showed that DOXOL was significantly less potent than DOX at depressing myocyte ATP/protein ratios. The IC_{50} for DOXOL in this system is 15 μg/ml compared to 0.8 μg/ml for DOX.

Specificity of the Cardiotoxicity Model. Characterization of the specificity of our assay system required testing tumoricidal and noncardiotoxic agents, along with other known cardiotoxic antineoplastic agents (Table 2). Initially, a positive cardiotoxic control with specificity to myocytes was sought. The ideal cardiotoxin would be a non-antitumor agent which would produce characteristic delayed suppression of ATP/protein ratios of pharmacological *in vitro* concentrations. Mercuric chloride (0.1 μg/ml for 1 h) produced an immediate, nonspecific depression of ATP/protein ratios to less than 5% of control myocyte values. Interestingly, digoxin, even at high concentrations, did not significantly depress myocyte ATP levels. Emetine produced a dose- and duration-dependent suppression of ATP levels in the myocytes. This agent is a well-known clinical cardiotoxin (48) and may comprise a valid nononcological positive control for this *in vitro* cardiotoxicity assay.

In contrast, dactinomycin, melphalan, vincristine, 1-β-D-arabinofuranosylcytosine, and fluorouracil did not depress ATP/protein ratios in the myocytes even at suprapharmacological concentrations. The lowest concentrations of these drugs were selected from studies showing marked cytotoxicity in different tumor cell lines (40–43), but no cardiotoxicity in the clinic (49). Table 2 shows that even extremely high concentrations of the non-anthracycline anticancer agents did not depress myocyte ATP levels to 50% of control *in vitro*. When compared to clinically achievable concentration × time products, these drugs required an acute *in vitro* exposure to over 40 clinical concentration × time product equivalents (Table 2). In contrast, the anthracycline antitumor agents required acute exposures to only 1.2–8 clinical concentration × time product equivalents for 50% cytotoxicity *in vitro*.

Of the three anthracycline analogues which were evaluated, daunorubicin was slightly less toxic than DOX on a weight basis. It suppressed the ATP/protein ratio to 50% of control levels at a concentration of 2 μg/ml. Aclarubicin also produced less cardiotoxicity than DOX with an IC_{50} of 4.2 μg/ml. In contrast, idarubicin was more toxic than DOX on a weight basis with an IC_{50} of 0.4 μg/ml. For all analogues, maximal ATP suppression was noted after 72 h of drug-free incubation. In terms of clinical concentration × time product equivalents, both DOX and daunomycin were the most cardiotoxic agents whereas aclarubicin and idarubicin were significantly less toxic (Table 2, bottom section). In this analysis, doxorubicinol was far less toxic and did not produce 50% cytotoxicity until over 90 clinical course equivalents were used *in vitro*.

Table 1 *Doxorubicin levels in myocyte cell culture lysates*[a]

DOX exposure (μg/ml for 6 h)	Intracellular DOX levels (ng/μg protein) after different incubation periods in DOX-free medium	
	72 h	96 h
0.1	0.5 ± 0.12	0.4 ± 0.04
1.0	1.4 ± 0.36[b]	1.2 ± 0.44
10	6.5 ± 1.96[b]	4.5 ± 1.48[b]

[a] Mean of 3 determinations ± SD.
[b] Indicates time points wherein trace doxorubicinol peaks (< 1 ng) were observed on HPLC chromatograms.

Table 2 *Activity of various agents on ATP/protein ratios in myocyte cultures*

Compound	IC_{50} ATP/protein (μg/ml)[a]	Clinical comparison ratio[b]	Ref.
Non-anthracycline anticancer agents			
Digoxin	> 1.0		
Emetine	1.2		
Mercuric chloride	< 0.1[c]		
Non-anthracycline anticancer agents			
1-β-D-Arabinofuranosylcytosine	> 10	> 218	34
Dactinomycin	> 0.5	> 40	34
Fluorouracil	> 1000	> 368	34
Melphalan	> 100	> 243	34
Vincristine	> 1.0	> 86	34
Anthracycline anticancer agents			
Aclarubicin	4.2	7.9	50
Daunomycin	2.0	1.24	34
DOX	0.8	1.25	34
Doxorubicinol	15	91	51
Idarubicin	0.4	7.3	52

[a] 6-h concentration of drug which reduces ATP/protein ratios to 50% of control measured 72 h after exposure.
[b] The *in vitro* exposure (μg/ml/h) required to reduce ATP/protein ratios, divided by one clinical exposure concentration × time product in μg/ml/h from a standard therapeutic dose.
[c] 1-h exposure, ATP/protein measured immediately following treatment (see text).

DISCUSSION

The choice of intracellular ATP standardized to total protein as a cardiotoxicity index is based on the obvious importance of energy-dependent processes in viable cardiac cells (53). The use of ATP as an index of cardiotoxicity is further strengthened by the observation that some effects of DOX may be directly targeted to this biochemical system. For example, DOX is known to interfere with oxidative-phosphorylation reactions (54) and has previously been shown to reduce cellular respiratory activity (10) and intracellular ATP levels (25). DOX is also reported to inhibit the sodium-potassium ATPase pump (55).

We have shown that intracellular ATP, as measured by the luciferase bioluminescence reaction and standardized to pro-

tein, is a reliable, reproducible marker of such myocyte damage and has advantages over beating rate or LDH leakage as cardiotoxicity end points. The contribution of fibroblasts to the ATP/protein ratios was minimized by reporting results as a percentage of control and by the observation of relative DOX resistance by cardiac fibroblasts. We have also confirmed morphologically that DOX-induced damage *in vitro* is similar to that previously reported *in vivo*. Transmission electron micrographs of DOX-treated myocytes in culture displayed classic morphological hallmarks of anthracycline damage as seen in human patients (8), in animals (56), and in other mammalian myocyte culture systems (16, 46, 57).

In the past, the DOX concentrations used for *in vitro* testing have been variable and most studies have tested relatively high concentrations of DOX (10, 15, 17, 18). However, some dosing protocols similar to our exposure method have been used previously (25, 46). These latter studies consistently showed that DOX-induced cardiotoxicity *in vitro* required substantial drug-free incubation times to accumulate significant myocyte damage. For example, Seraydarian *et al.* dosed myocytes with 0.1–9 μg/ml/h of DOX and noted that 24–48 h of incubation following drug exposure were required for beating cessation (25). Similarly, Tobin and Abbot (46) reported that 9–21 h of incubation following drug exposure were needed for characteristic DOX-induced histopathological changes in mitochondria, nuclei, and the cytoplasm. Our own studies have shown the same time dependence for the expression of DOX-induced myocyte damage (*e.g.*, at least 48 h of drug-free incubation). These delayed effects may involve progressive uncoupling of oxidative phosphorylation in mitochondria (54) or perhaps the accumulation of oxygen free radical damage to membranes mediated by a slow cyclical bioreduction of DOX *in vitro* (58).

Our DOX concentration-response curves show that maximal damage is induced by a 5-μg/ml doxorubicin exposure for 6 h and that little damage is seen at 0.1 μg/ml exposure for 6 h (each with a 72-h post-drug exposure incubation). This represents a 50-fold concentration range for dynamic ATP sensitivity.

The utility of this assay also extended to other known cardiotoxic agents. A comparison of IC_{50} values for anthracycline analogues showed that a series of drugs could be ranked in order of decreasing cardiotoxicity on a weight basis as idarubicin > DOX > daunomycin > aclarubicin > doxorubicinol. Importantly, these results correlate well with cardiotoxicity patterns observed in chronic rodent toxicity studies and with results from clinical trials. Thus, animal studies have demonstrated that idarubicin is significantly more cardiotoxic than DOX on a weight basis (39), while cardiotoxicity from daunomycin is slightly less than DOX on a cumulative dose basis (38). Aclarubicin on the other hand is decidedly less cardiotoxic in animals (41), and this difference was seen in our *in vitro* assay. While doxorubicinol has been reported to be more cardiotoxic than DOX in isolated heart muscle preparations (40), in our assay, doxorubicinol was less toxic. Similarly, Danesi *et al.* (59) have described reduced cardiotoxicity for doxorubicinol in rats *in vivo*. Other researchers have suggested that the increased polarity of doxorubicinol over DOX may explain its limited cytotoxicity due to reduced cellular uptake *in vitro* (60).

None of the five non-anthracycline antitumor agents tested were cardiotoxic unless extremely high *in vitro* drug concentrations were tested. These relatively noncardiotoxic agents included a variety of mechanistically different cytotoxic agents including a DNA alkylator (melphalan), an intercalator (dactinomycin), a microtubule toxin (vincristine), and two antimetabolites (fluorouracil and 1-β-D-arabinofuranosylcytosine). Of interest, the cardiac glycoside digoxin was also not directly cardiotoxic to the myocytes, a result perhaps due to its predominant effects on cardiac conduction pathways. Likewise, the heavy metal poison, mercuric chloride, was also not specifically cytotoxic to the heart cells since it produced immediate cell death at concentrations which are universally toxic to other mammalian cell types (35).

In summary, DOX-induced cardiotoxicity in our 6-day myocyte culture system revealed that: (*a*) classic histological morphology is produced by DOX in mammalian myocyte cell cultures; (*b*) cardiotoxic antitumor analogues can be quantitatively compared *in vitro*; (*c*) myocyte damage appears to require long incubation times in drug-free medium; (*d*) myocytes retain DOX in proportion to different exposure levels; and (*e*) the level of cardiac myocyte damage can be correlated to clinically determined cumulative DOX concentration × time products. These findings suggest that the current method offers some important improvements over previous toxicity models for cardiotoxic antineoplastic agents.

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