pancreas. Its inhibition of glucagon release encourages the search for related compounds devoid of effect on the B-cell as a therapeutical means to reduce the hyperglucagonism associated with diabetes mellitus [14].

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Reduction of doxorubicin toxicity by methylene blue in cultured rat myocardial cells

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The antitumor anthracycline doxorubicin (DOX) is a widely used antineoplastic agent effective in the treatment of a variety of human cancers [1]. However, its clinical use is associated with dose-limiting acute and chronic cardiotoxic effects [2]. The mechanism of DOX cardiotoxicity, though not completely understood, is thought to involve the generation of reactive oxygen species resulting from NAD(P)H-dependent reduction of DOX to its semiquinone free radical form [3, 4]. Doroshow [5] has shown that rat cardiac sarcosomal, mitochondrial, and cytosolic fractions generate superoxide in the presence of a variety of anthracyclines in an NAD(P)H-dependent manner even in the presence of oxygen radical detoxifying enzymes. He proposed that the generation of reactive oxygen species in excess of the detoxification capabilities of the myocardium is the mechanism of importance in anthracycline cardiotoxicity.

Methylene blue (MB) is a redox dye which is capable of oxidizing NAD(P)H in biological systems [6, 7]. Hrushesky *et al.* [7] postulated that MB administered concurrently with DOX would affect the concentration of intracellular reducing agents, prevent the reduction of DOX, and thus protect the myocardium from DOX-mediated damage. MB reduced cardiotoxicity in the mouse yet the antitumor activity of DOX was not affected in the tumor model studied [7].

In the present study, beating rat myocardial cells in culture were used to examine further the protective effects

of MB with regard to DOX toxicity. Results of this study demonstrated that MB reduces DOX-mediated cardiotoxicity in a concentration-dependent manner as measured by cytosolic enzyme leakage, that MB reduces the effect of DOX on myocyte beating frequency, and that the protective effects of MB are not attributable to an inhibition by MB of DOX accumulation in the myocytes.

Materials and methods

Doxorubicin hydrochloride and [^{14}C]doxorubicin (purified by HPLC prior to use) were the gifts of Dr. William J. M. Hrushesky, Department of Medicine, University of Minnesota Medical School, Minneapolis, MN. Sperm-positive female Sprague–Dawley rats were purchased from Holtzman Laboratories, Madison, WI. Eagle's Minimum Essential Medium (MEM), methylene blue chloride, trypsin, NADH, pyruvate, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and TRIZMA base were obtained from the Sigma Chemical Co. (St Louis, MO). Horse serum and penicillin/streptomycin solution were purchased from Gibco Laboratories (New York, NY). Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT).

Isolation and culturing of myocytes. Myocardial cells were isolated and cultured essentially according to the method of Bollon *et al.* [8] with several modifications aimed at increasing the yield of beating myocytes over that of non-muscle cells [9–11]. Cells were plated in separate 35-mm culture dishes, incubated in Eagle's MEM buffered with 20 mM HEPES, pH 7.4, and containing 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 unit/ml insulin at 37° and 95% humidity and used in the described studies 7–8 days after initial plating. Homogeneity of the cultures was enhanced further by replenishing only part of the culture medium every 4 days, a practice which inhibits proliferation of non-muscle cells resulting in cultures containing > 90% muscle cells of which up to 98% are beating.

Incubations with DOX and methylene blue. Just prior to starting the incubations, the conditioned HEPES-buffered MEM was removed from the myocyte cultures, DOX and/or MB in 20 mM HEPES buffer were added to this medium, and the volume was adjusted with fresh medium to achieve final drug concentrations. Incubations were initiated by returning this modified conditioned medium to the cultures and were performed at 37°. Controls for each experiment were treated in the same manner with 20 mM HEPES substituted for DOX and MB where appropriate.

Determination of lactic acid dehydrogenase (LDH) leakage. The effect of various incubation conditions on myocyte viability was determined based on the leakage of a cytosolic enzyme, LDH. Aliquots (0.1 ml) for LDH measurements were removed from the culture medium at appropriate time points. In the case of the concentration-dependence experiments (Table 1), aliquots were removed from each culture at zero time and 14 hr. LDH determinations during time-course experiments (Fig. 1) were made using separate cultures for each time point. Total cellular LDH activity was then measured after lysing cells with Triton X-100 (final concn. 0.5%). LDH leakage was determined as LDH activity present in the medium expressed as the percentage of total LDH activity in lysed cells plus culture medium. LDH activity was measured spectrophotometrically by the method of Lindstrom *et al.* [12].

Measurement of myocyte beating frequency. The effect of DOX or DOX and MB on myocyte beating frequency was evaluated at appropriate time points by counting cultures at six randomly selected areas and expressing frequency as beats per minute (bpm). Counts were initially taken as seconds per 20 beats using a stopwatch and then converted to bpm. Cells were viewed with an inverted tissue culture microscope (phase contrast, 200 x). Measurement of one 35-mm plate (6 counts) typically was completed within 2 min of removal from the 37° incubator, and decreased beating frequency due to temperature change did not occur during this time.

Table 1. Concentration-dependent reduction of doxorubicin toxicity in cultured myocardial cells

Incubation condition	Percent LDH leakage*		Percent protection
	Zero time	14 hr	
Control	10 ± 1	12 ± 1	
DOX (150 μM)	12 ± 2	94 ± 2	
MB (10 μM)	9 ± 2	12 ± 1	
MB (10 μM) + DOX (150 μM)	12 ± 2	74 ± 5†	24
MB (20 μM)	8 ± 1	14 ± 1	
MB (20 μM) + DOX (150 μM)	12 ± 2	42 ± 6†	66
MB (30 μM)	10 ± 2	35 ± 5‡	
MB (30 μM) + DOX (150 μM)	12 ± 1	45 ± 5†	88

Culture dishes (35 mm) containing beating myocardial cells were incubated with either 150 μM doxorubicin (DOX) in the presence of 10, 20, or 30 μM Methylene blue (MB) or with 10, 20, or 30 μM MB alone. Control cultures were treated in the same manner as under other conditions with 20 mM HEPES buffer substituted for MB and DOX in equivolume amounts. Equivolume HEPES was also substituted for DOX in cultures treated with MB alone and for MB in cultures treated with DOX alone. Final incubation volume was 2 ml. Aliquots (0.1 ml) were taken at zero time and at 14 h and assayed for LDH activity. The percent LDH leakage and percent protection by MB were then calculated as described in the text. Data represent duplicate determinations from three experiments, each using a separate myocardial cell preparation.

* Mean ± SE.

† $P < 0.001$ compared to DOX.

‡ $P < 0.01$ compared to control.

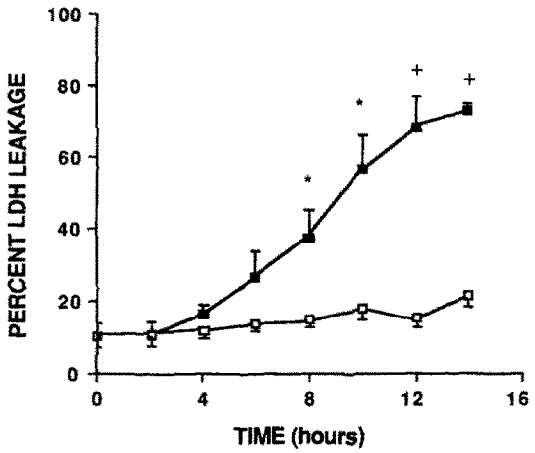

Fig. 1. Reduction of DOX toxicity by MB. Cultured myocardial cells were incubated with 150 μM DOX in the presence (□) and absence (■) of 20 μM MB. At each time point, the percent LDH activity was determined in both the culture medium and cells, and the percent leakage was calculated as described in Materials and Methods. Individual cultures were used for each time point. Data are the means ± SE from three experiments, each using a separate myocardial cell preparation. Key: (*) $P < 0.05$; and (+) $P < 0.01$, unpaired Student's *t*-test.

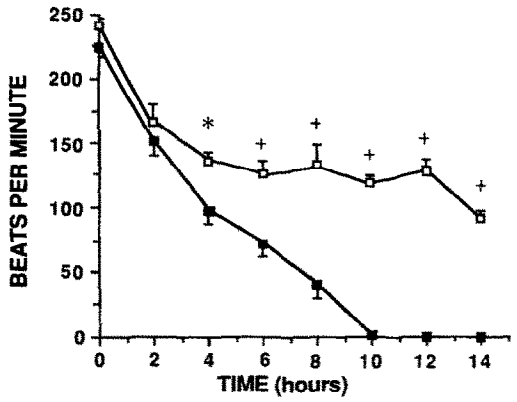

Fig. 2. Effects of DOX and DOX + MB on myocyte beating frequency. Cultured myocardial cells were incubated with 150 μM DOX in the presence (□) and absence (■) of 20 μM MB. At each time point myocyte beating frequency was measured by viewing the cells under an inverted tissue culture microscope, measuring seconds per 20 beats in six randomly selected fields and converting to beats per minute. Data are means ± SE from three experiments, each using a separate myocardial cell preparation. Key: (*)$P < 0.01$; (+) $P < 0.001$, unpaired Student's *t*-test.

Effect of MB on myocyte accumulation of [^{14}C]DOX. The HPLC conditions of Quattrone and Ranney [13] were used to isolate [14-^{14}C]DOX (17 mCi/mmol) to a radiochemical purity of > 95%. [14-^{14}C]DOX was diluted with unlabeled DOX to a final specific activity of 89 μCi/mmol and added to myocyte cultures to a final concentration of 150 μM. At appropriate time points the medium was removed, the cells were rinsed three times (1.0 ml each) with saline and lysed with Triton X-100 (final concn. 1.0%), and aliquots of the lysate were analyzed for radioactivity by liquid scintillation counting.

Results

The effects of three different concentrations of MB on DOX toxicity in cultured myocardial cells were examined, and the results are summarized in Table 1. DOX at a concentration of 150 μM resulted in 94 ± 2% LDH leakage at 14 hr, indicating extensive cell damage as assessed by loss of membrane integrity. MB concentrations of 10, 20, and 30 μM afforded 24, 66, and 88% protection, respectively, against a 150 μM DOX challenge at 14 hr. While 30 μM MB afforded significant protection against the DOX challenge, this concentration of MB was toxic to the cells, resulting in 35 ± 5% LDH leakage at 14 hr. To calculate percent protection (Table 1), the percent LDH leakage for each concentration of MB-treated cells at 14 hr was subtracted from that of the corresponding MB and DOX treated incubations. Each of these amounts was then subtracted from a value of 82, that equals the difference between the leakage of 14-hr DOX-treated cells and 14-hr control cells. The resulting values are presented as a percentage of 82. These data demonstrate that MB effected a concentration-dependent reduction of DOX toxicity in cultured myocardial cells and that MB was toxic to the cells at a concentration of 30 μM. This MB-induced toxicity also exhibited a concentration-dependence, with 60 μM MB causing approximately a 2-fold increase in LDH leakage (62 ± 3%) compared to values for 30 ±M MB (35 ± 5%) during 14-hr incubations.

From Table 1 it appears that 20 μM MB afforded optimal protection of the cultured myocardial cells. While the percent protection observed for 30 μM MB was higher, this concentration of MB alone was toxic to the myocytes, as measured by LDH leakage. In contrast, 20 μM MB significantly reduced DOX-induced damage, yet produced no toxicity in the cultured cells. In experiments summarized graphically in Fig. 1, the time-course of the effects of 20 μM MB against DOX toxicity in cultured myocardial cells is described. After 4 hr of incubation, LDH leakage increased rapidly in cultures treated with 150 μM DOX to approximately 73 ± 1% leakage at 14 hr, indicating extensive cellular damage. In cultures treated with 150 μM DOX and 20 μM MB, this rapid increase in LDH leakage was suppressed dramatically and at 14 hr only 20% leakage had occurred. It is of interest to note that, in the experiments summarized in Table 1, 94 ± 2% leakage of LDH from cells treated with 150 μM DOX was observed, while in the experiments summarized in Fig. 1 only 73 ± 1% leakage occurred under the same incubation conditions. In both sets of experiments, cultures were fed on day 5, but concentration-dependent experiments (Table 1) were performed exclusively with day 7 cells while time-course data (Fig. 1) was generated using only day 8 cells. An age-related decrease in sensitivity toward DOX has been observed by Shirhatti *et al.* [14]. It is clear from the time-course studies that MB protects the cultured myocardial cells from the rapid loss of cellular membrane integrity resulting from DOX treatment.

The use of spontaneously beating cultured myocytes is advantageous in that the effects of various culture conditions on the physiological function of these cells can be determined. Since metabolic competence is requisite for myocardial cell contraction, the beating rate affords an early and sensitive indicator of toxic injury to the myocardial cell. In Fig. 2, the effects of 150 μM DOX and 150 μM DOX plus 20 μM MB on myocardial cell beating frequency are graphically shown. Initial beating rates of 225–240 bpm were reduced to 150–170 bpm during the first 2 hr under both incubation conditions. From 2 hr on, however, beating in DOX-treated cells decreased steadily until 10 hr when beating was abolished completely. In cells

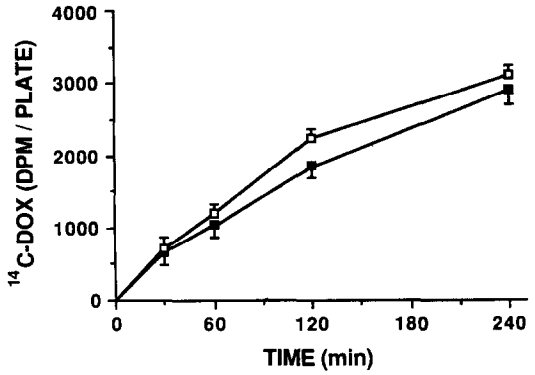

Fig. 3. Effect of MB on DOX content of cultured myocardial cells. Cells were incubated with 150 μM [^{14}C]DOX (sp. act. 89 μCi/mmole) in the presence (□) and absence (■) of 20 μM MB. At each time point culture plates were washed and then lysed with Triton X-100 (final concn. 1.0%). Aliquots from lysates were assayed for radioactivity by liquid scintillation counting. Values are means ± SE, N = 3.

treated with DOX and MB, beating continued throughout the 14-hr time-course and at 14 hr cells were still beating at approximately 100 bpm. These data, when considered in light of the other information presented here, suggest that MB reduced DOX-mediated damage to the heart cell, in terms of both cellular membrane integrity and physiological function.

The possibility existed that MB exerted its protective effect by preventing the accumulation of DOX in the myocytes. To address this issue cells were incubated with [^{14}C]DOX (150 μM) in the presence and absence of 20 μM MB, and the cellular content of [^{14}C]DOX was measured. The results, shown in Fig. 3, demonstrate that MB did not affect significantly myocyte accumulation of DOX.

Discussion

The results presented in this report clearly demonstrate a reduction of doxorubicin-treated toxicity in cultured myocardial cells by MB. This protective property of MB was manifest in the preservation of cellular membrane integrity as measured by leakage of cytosolic enzymes and by the preservation of physiological function as measured by myocyte beating frequency. It has been shown, *in vivo*, that MB ameliorates doxorubicin cardiotoxicity without compromising the antineoplastic activity of the drug [7]. It is clear from the literature that doxorubicin exerts its cardiotoxic effects in large part via the augmentation of electron flow from NAD(P)H to molecular oxygen resulting in the generation of cytotoxic superoxide ions [3–5]. The biochemical basis for the protective effect of MB is not known. MB can be reduced by NAD(P)H-dependent enzymes [6, 7] and thus may interfere with the NAD(P)H-dependent reduction of doxorubicin and the redox cycling of the drug which leads to oxygen radical formation. Recent work by Doroshow [15] suggests a cytotoxic electron shuttle involving the flow of electrons from cardiac myoglobin to DOX and the subsequent formation of oxygen radicals. Such a shuttle represents a possible site where redox active compounds such as MB may exert an effect. Other investigators have shown that treatment of animals with α-tocopherol, a free radical scavenger [16], or *N*-acetylcysteine, a sulfhydryl containing amino acid [17], results in prevention of doxorubicin-induced cardiotoxicity. This work indicates that modifying the intracellular environment of the heart cell with free radical scavengers, free sulfhydryl groups, or compounds which interfere with the reduction of doxorubicin to its semiquinone form could potentially improve the therapeutic index of this important broad-spectrum antitumor agent. Since the generation of reactive oxygen species by doxorubicin may not be the important toxic mechanism in the case of some tumors [18], it seems reasonable that ameliorating cardiotoxicity via these interventions could in fact improve the therapeutic index of this drug. MB seems a viable choice since it has been used therapeutically for various purposes [19, 20].

The data presented in this report indicate that the protective effects of methylene blue against doxorubicin toxicity can be demonstrated *in vitro* in a cultured myocardial cell culture system. Shirhatti *et al.* [14] have suggested that the use of an *in vitro* system consisting of beating myocardial cells offers a simple and highly useful method for the screening of anthracycline analogs that are potentially less cardiotoxic than the parent drugs. The results of our study demonstrate the utility of a beating myocardial cell culture system as a screen for therapeutic interventions aimed at decreasing and/or ablating the cardiotoxicity of doxorubicin, other anthracyclines, and their analogs.

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