British Journal of Pharmacology (2000) 131, 342–348
www.nature.com/bjp

Effect of MEN 10755, a new disaccharide analogue of doxorubicin, on sarcoplasmic reticulum Ca^{2+} handling and contractile function in rat heart

*,[1]Riccardo Zucchi, [1]Gongyuan Yu, [1]Sandra Ghelardoni, [1]Francesca Ronca & [1]Simonetta Ronca-Testoni

[1]Dipartimento di Scienze dell'Uomo e dell'Ambiente, Sezione di Chimica e Biochimica Medica, University of Pisa, Pisa, Italy

1 The use of anthraquinone antineoplastic agents is limited by their cardiac toxicity, which is largely due to activation of the sarcoplasmic reticulum (SR) Ca^{2+} release channel (ryanodine receptor). MEN 10755 is a new disaccharide analogue of doxorubicin. We have evaluated its effects on SR function and its toxicity in isolated working rat hearts.

2 In rat SR vesicles, doxorubicin stimulated [^{3}H]-ryanodine binding by increasing its Ca^{2+}-sensitivity. At 1 μM Ca^{2+}, ryanodine binding increased by 15.3 ± 2.5 fold, with $EC_{50} = 20.6$ μM. Epirubicin produced a similar effect, i.e. 9.7 ± 0.6 fold stimulation with $EC_{50} = 11.1$ μM. MEN 10755 increased ryanodine binding by 1.9 ± 0.3 fold ($P < 0.01$ vs doxorubicin and epirubicin), with $EC_{50} = 38.9$ μM.

3 Ca^{2+}-induced Ca^{2+} release experiments were performed by quick filtration technique, after SR loading with $^{45}Ca^{2+}$. At 2 μM Ca^{2+}, doxorubicin (50 μM) increased the rate constant of Ca^{2+} release to 82 ± 5 s^{-1} vs a control value of 22 ± 2 s^{-1} ($P < 0.01$), whereas 50 μM MEN 10755 did not produce any significant effect (24 ± 3 s^{-1}).

4 Ca^{2+}-ATPase activity and $^{45}Ca^{2+}$-uptake were not significantly affected by doxorubicin, its 13-dihydro-derivative, epirubicin, MEN 10755 and the 13-dihydro-derivative of MEN 10755, at concentrations $\leqslant 100$ μM.

5 In isolated heart experiments, administration of 30 μM doxorubicin or epirubicin caused serious contractile impairment, whereas 30 μM MEN 10755 produced only minor effects.

6 In conclusion, in acute experiments MEN 10755 was much less cardiotoxic than equimolar doxorubicin or epirubicin. This result might be accounted for by reduced activation of SR Ca^{2+} release.

British Journal of Pharmacology (2000) **131,** 342–348

Keywords: MEN 10755; doxorubicin; epirubicin; anthraquinones; sarcoplasmic reticulum; calcium; ryanodine receptor; Ca^{2+}-ATPase; heart

Abbreviations: MEN 10755, 7-*O*-[2,6-dideoxy-4-*O*-(2,3,6-trideoxy-3-amino-α-L-*lyxo*-hexopyranosyl)-α-L-*lyxo*-hexopyranosyl]-4-demethoxy-14-hydroxydaunomycinone hydrochloride; MEN 11383, 7-*O*-[2,6-dideoxy-4-*O*-(2,3,6-trideoxy-3-amino-α-L-*lyxo*-hexopyranosyl)-α-L-*lyxo*-hexopyranosyl]-4-demethoxy-13-dihydro-14-hydroxydaunomycinone hydrochloride; SR, sarcoplasmic reticulum

Introduction

Anthraquinones such as doxorubicin and epirubicin are widely used as antineoplastic agents, but their clinical administration is often limited by side effects, and particularly by cardiac toxicity. Acute cardiac effects include a transient positive inotropic response followed by sustained negative inotropic action, prolonged time to peak twitch tension, and decreased relaxation rate, whereas chronic doxorubicin toxicity is characterized by the progressive development of cardiomyopathy, leading to congestive heart failure (Buzdar *et al*., 1985; Jensen, 1986; Hagane *et al*., 1988; Doroshow, 1991; Rhoden *et al*., 1993; Wang & Korth, 1995; Nysom *et al*., 1998).

Several molecular mechanisms might contribute to these toxic effects. Doxorubicin stimulates free radical production, interferes with mitochondrial respiration, and affects sarcoplasmic reticulum (SR) function. According to a widely held opinion, cardiac toxicity is largely due to interference with intracellular Ca^{2+} homeostasis (Jiang *et al*., 1994; Temma *et al*., 1994; Halili-Rutman *et al*., 1997; Maeda *et al*., 1998; Feng *et al*., 1999), and particularly to stimulation of SR Ca^{2+} release. Doxorubicin binds to the SR Ca^{2+} channel (also known as ryanodine receptor) and favours channel opening, leading to increased cytosolic Ca^{2+} (Zorzato *et al*., 1985; Abramson *et al*., 1988; Kim *et al*., 1989; Ondrias *et al*., 1990; Pessah *et al*., 1990; Tian *et al*., 1991; Boucek *et al*., 1993). Cytosolic Ca^{2+} overload transiently stimulates muscle contraction, but triggers several injurious mechanisms, such as activation of Ca^{2+}-dependent proteases and phospholipases, finally leading to contractile dysfunction and cellular necrosis (Nayler, 1988).

Strong efforts have been performed to produce doxorubicin derivatives that retain antineoplastic efficacy while showing reduced cardiac toxicity. Recently, disaccharide analogues of doxorubicin have been synthesized, in which the amino sugar moiety appears as the second residue bound in axial orientation to the first residue, that in turn is characterized by the substitution of the amino group for a hydroxyl group (Arcamone *et al*., 1997). One of these analogues, MEN 10755 (7-*O*-[2,6-dideoxy-4-*O*-(2,3,6-trideoxy-3-amino-α-L-*lyxo*-hexo-

*Author for correspondence; E-mail: r.zucchi@med.unipi.it

pyranosyl)-α- L-*lyxo*-hexopyranosyl]-4-demethoxy-14-hydroxydaunomycinone hydrochloride), has proved to be superior to doxorubicin in the treatment of human tumour xenografts (Pratesi *et al*., 1998). Before MEN 10755 can be proposed for clinical use, it is necessary to assess its side effects, and particularly its cardiac effects. In this work we have compared the effects of MEN 10755, doxorubicin, and epirubicin (the 3′-epimer of doxorubicin) on cardiac ryanodine receptors. The 13-dihydro derivatives of doxorubicin and MEN 10755, which are formed *in vivo* during anthraquinone catabolism, have also been tested. Finally, we have compared the acute toxicity of MEN 10755, doxorubicin, and epirubicin in the isolated perfused rat heart.

Methods

Preparation of cellular fractions

Male Wistar rats (275–300 g body weight), fed with standard diet, were anaesthetized with a mixture of ether and air. After injection of 1000 u sodium heparin in the femoral vein, the heart was quickly excised and the ventricles were finely minced and homogenized in five volumes of 300 mM sucrose and 10 mM imidazole (pH 7.0 at 4°C) by 15 + 15 passes in a Potter-Elvejheim homogenizer set at 800 r.p.m. and kept in a cold room at 4°C. The homogenate was then used to prepare a microsomal fraction enriched in SR, as described in detail elsewhere (Zucchi *et al*., 1995a). The protein content of each fraction was determined by the Lowry method, using bovine serum albumin as a standard.

Assay of [^{3}H]-ryanodine binding

High affinity ryanodine binding was assayed at different free Ca^{2+} concentrations, as described previously (Zucchi *et al*., 1994; 1995a). Briefly, vesicles were incubated at 37°C in a buffer containing imidazole 25 mM (pH 7.4 at 37 °C), 1 M KCl, 0.2 to 50 nM [^{3}H]-ryanodine (6 Ci mmol^{-1}), EGTA 0.95 mM, and variable amounts of $CaCl_2$, in order to obtain the desired free Ca^{2+} concentration. After 60 min, the binding reaction was stopped by filtration through cellulose nitrate filters with pores of 0.45 μM (Sartorius, Göttingen, Germany), presoaked in 25 mM imidazole and 1 M KCl (washing buffer). The filters were then washed with 2 × 5 ml aliquots of washing buffer and shaken overnight in 8 ml of scintillation fluid (Optiphase II, LKB, Turku, Finland). Radioactivity was counted at 50% efficiency in an LKB Wallac 1214 scintillation counter (Turku, Finland). Incubations were performed in duplicate, and non-specific binding was measured in the presence of 10 μM unlabelled ryanodine. The difference between the counts of duplicate samples was <10% in all cases.

Assay of SR Ca^{2+} release

SR Ca^{2+}-induced Ca^{2+} release was determined as described previously (Zucchi *et al*., 1995b), with the only difference that vesicle loading was performed in a medium containing 100 μM $^{45}CaCl_2$, as described by Chu *et al*. (1988). ^{45}Ca release was induced by washing the loaded vesicles with a release buffer containing HEPES-potassium 20 mM (pH 6.8), KCl 100 mM, and various amounts of $CaCl_2$, buffered with EGTA (1 mM) to obtain the desired free Ca^{2+} concentration. A rapid filtration system with time resolution on the order of 10 ms was used (Dupont, 1984; Moutin & Dupont, 1988), and the rate constant of quick Ca^{2+} release was calculated over the first 100 ms by exponential fitting (Zucchi *et al*., 1995b). In order to confirm that the quick phase of Ca^{2+} release represented SR Ca^{2+} release, we tested the inhibition of Ca^{2+} release by a 'non-release' buffer, containing (in mM) HEPES-potassium 20 (pH 6.8), KCl 100, $MgCl_2$ 10 and 10 μM ruthenium red.

Assay of SR Ca^{2+}-ATPase activity and SR Ca^{2+}-uptake

ATPase activity was determined in the absence of Ca^{2+} gradient by a coupled enzyme reaction, as described by Feher & Lipford (1985), with minor changes. Briefly, the reaction mixture contained KCl 100 mM, imidazole buffer 20 mM (pH 7.0 at 37°C), phosphoenolpyruvate 1 mM, NADH 0.3 mM, 9 IU ml^{-1} lactate dehydrogenase, 6 IU ml^{-1} pyruvate kinase, NaN_3 5 mM, A23187 2 μM, ATP 1 mM, EGTA 0.2 mM and $CaCl_2$ 0.25 mM where appropriate. Ca^{2+}-dependent ATPase activity was defined as the difference between the activity measured in the presence and in the absence of 0.25 mM $CaCl_2$.

Oxalate-supported Ca^{2+}-uptake was determined in the crude homogenate in the presence of a concentration of ryanodine able to block the Ca^{2+} release channel, as described previously (Zucchi *et al*., 1994). As discussed elsewhere, oxalate is accumulated into the SR, and therefore such an assay provides a reliable estimate of SR Ca^{2+}-uptake, even in crude preparations (Feher & Lipford, 1985). Unless otherwise specified, free Ca^{2+} concentration was 1 μM.

Perfusion experiments

Rats were anaesthetized as described above. After injection of 1000 u sodium heparin in the femoral vein, the heart was quickly excised and perfused according to the working heart technique, as described previously (Zucchi *et al*., 1994). The preload (height of the atrial chamber) and the afterload (height of the aortic chamber) were set at 20 and 100 cm, respectively. The standard perfusion buffer included (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.5, glucose 11. Perfusions were carried out using 200 ml of recirculating buffer, which was equilibrated with a mixture of O_2 (95%) and CO_2 (5%). Temperature was kept between 36.8 and 37°C, and the pH was 7.4. After 5 min of equilibration, doxorubicin, epirubicin or MEN 10755 were added to the perfusion buffer, and the haemodynamic variables were monitored for 60 min.

Chemical and radionuclides

MEN 10755, MEN 11383 (7-*O*-[2,6-dideoxy-4-*O*-(2,3,6-trideoxy-3-amino-α-L-*lyxo*-hexopyranosyl)-α-L-*lyxo*-hexopyranosyl]-4-demethoxy-13-dihydro-14-hydroxydaunomycinone hydrochloride, i.e. the 13-dihydro derivative of MEN 10755) and doxorubicinol (13-dihydro-doxorubicin) were provided by Menarini Ricerchi S.p.A (Pomezia, Italy). Doxorubicin (Adriblastina) and epirubicin (Epirubicina) were obtained from commercial preparations. Ryanodine was purchased from Calbiochem (San Diego, CA, U.S.A.). EGTA was obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). All other reagents were of analytical grade. Free Ca^{2+} concentration was calculated according to Fabiato & Fabiato (1979). Free Ca^{2+} was also measured with the antipyrylazo III technique (Scarpa, 1979), and the results of the assay were generally in accordance with the theoretical values. [^{3}H]-ryanodine and $^{45}CaCl_2$ were obtained from New England Nuclear – DuPont (Milan, Italy).

Statistical analysis

Results are expressed as mean ± s.e.mean. Data analysis was performed using GraphPad Prism version 3.00 for Windows (GraphPad Sofware, San Diego, CA, U.S.A.). Binding experiments were analysed using the models mentioned in the description of the specific experiments. Release experiments were analysed by least squares linear regression, after logarithmic transformation (see also Zucchi *et al.*, 1995b). Differences between groups were evaluated as follows. One-way analysis of variance was used a global test for differences between means. If between-groups variance was significantly ($P<0.05$) higher than within-groups variance, individual groups were compared by Student-Neuman-Keuls test.

Results

[^{3}H]-ryanodine binding experiments

Ryanodine binding is strongly Ca^{2+} -dependent. Ca^{2+} affected both the K_D for ryanodine and the B_{max}. In our SR preparation, at 1 μM Ca^{2+} ryanodine binding was characterized by $K_D = 14.3$ nM and $B_{max} = 450$ fmol mg^{-1} protein, whereas at 30 μM Ca^{2+} the K_D decreased to 2.1 nM and the B_{max} increased to 4.1 pmol mg^{-1}. The EC_{50} for Ca^{2+} (determined at 2 nM [^{3}H]-ryanodine; see Figure 1, triangles) averaged 5.1 μM ($pEC_{50} = 5.15 \pm 0.09$).

The chief effect of doxorubicin was to increase the Ca^{2+}-sensitivity of ryanodine binding (Figure 1), by reducing the EC_{50} to 1.0 μM ($pEC_{50} = 5.98 \pm 0.03$). A dose-response curve obtained at fixed concentrations of ryanodine and free Ca^{2+} (2 nM and 1 μM, respectively) is shown in Figure 2. Doxorubicin increased ryanodine binding by up to 15.3 ± 2.5 fold, with $EC_{50} = 20.6$ μM ($pEC_{50} = 4.69 \pm 0.14$). Epirubicin, the 3′-epimer of doxorubicin, produced similar effects, namely 9.7 ± 0.6-fold maximum stimulation with $EC_{50} = 11.1$ μM ($pEC_{50} = 4.95 \pm 0.06$). Doxorubicinol caused 3.1 ± 0.1-fold stimulation with $EC_{50} = 3.8$ μM ($pEC_{50} = 5.42 \pm 0.08$). These effects were due both to increased B_{max}, and to decreased K_D (data not shown). On the other hand, at 30 μM Ca^{2+} concentration, doxorubicin increased ryanodine binding by less than 50% (Figure 3).

MEN 10755 produced much lower effects than doxorubicin or epirubicin (Figures 1 and 2). In particular, at 1 μM free Ca^{2+} concentration, MEN increased ryanodine binding by up to 1.9 ± 0.3 fold ($P<0.01$ vs doxorubicin and epirubicin), with $EC_{50} = 38.9$ μM ($pEC_{50} = 4.41 \pm 0.19$). This effect was basically due to increased B_{max}, whereas the K_D was unchanged (data not shown). MEN 11383, the 13-dihydroderivative of MEN10755, did not affect ryanodine binding (data not shown). At 30 μM Ca^{2+} concentration, no significant increase in ryanodine binding was produced by MEN 10755, even at 100 μM concentration (Figure 3).

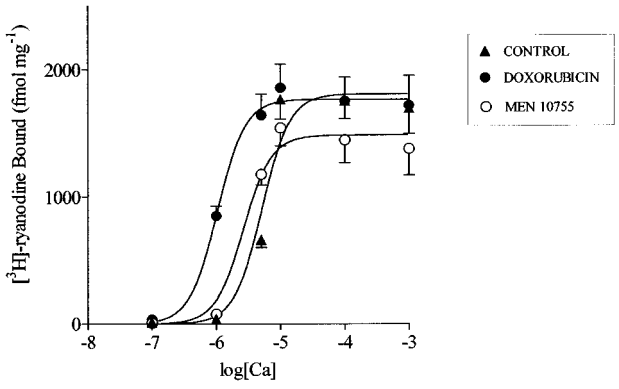

Figure 1 [^{3}H]-Ryanodine binding was determined at 2 nM [^{3}H]-ryanodine concentration and different free Ca^{2+} concentrations, under control conditions and in the presence of 50 μM doxorubicin or 50 μM MEN 10755. Data points represent mean ± s.e.mean of three different experiments. Data was analysed by GraphPad Prism 3.00 software, using a sigmoidal dose-response model. EC_{50} values averaged 5.1, 1.0 and 2.6 μM in the three groups, respectively ($pEC_{50} = 5.15 \pm 0.09$, 5.98 ± 0.03 and 5.58 ± 0.09), and the difference between any pair of groups was statistically significant ($P<0.01$ by ANOVA and Student-Neuman-Keuls test). Maximum binding averaged 1810 ± 144, 1768 ± 34 and 1487 ± 69 fmol mg^{-1} protein, respectively. The Hill coefficient was close to 2 in all groups.

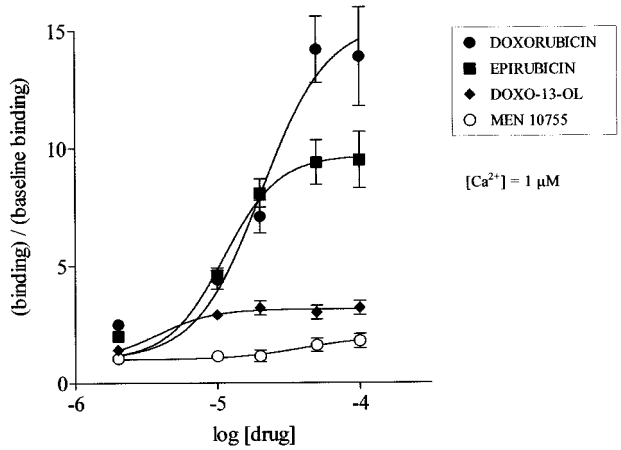

Figure 2 [^{3}H]-Ryanodine binding was determined at 2 nM [^{3}H]-ryanodine concentration, in the presence of 1 μM free Ca^{2+}, and of 0 – 100 μM doxorubicin, epirubicin, doxorubicinol or MEN 10755. Data points represent mean ± s.e.mean of three different experiments and are expressed as ratio to baseline binding. Data was analysed by GraphPad Prism 3.00 software, using a sigmoidal dose-response model. EC_{50} values averaged 20.6, 11.1, 3.8 and 38.9 μM, respectively ($pEC_{50} = 4.69 \pm 0.14$, 4.95 ± 0.06, 5.42 ± 0.08 and 4.41 ± 0.19). Maximum binding stimulation averaged 15.3 ± 2.5, 9.7 ± 0.6, 3.1 ± 0.1 and 1.9 ± 0.3. The difference between the MEN 10755 curve and either the doxorubicin or the epirubicin curve was statistically significant ($P<0.01$ by ANOVA and Student-Neuman-Keuls test).

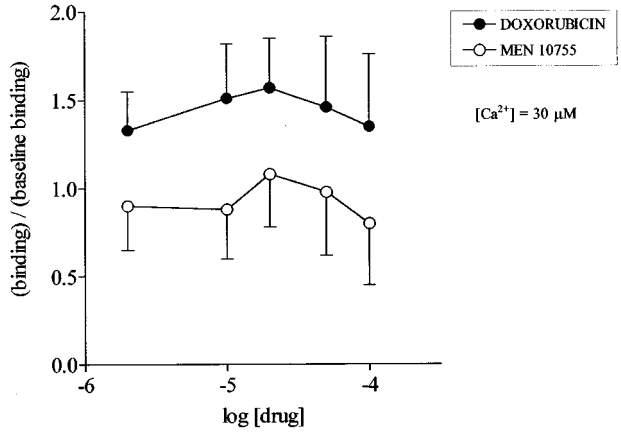

Figure 3 [^{3}H]-Ryanodine binding was determined at 2 nM [^{3}H]-ryanodine concentration in the presence of 30 μM free Ca^{2+}, and of 0 – 100 μM doxorubicin, or MEN 10755. Data points represent mean ± s.e.mean of two different experiments and are expressed as ratio to baseline binding.

^{45}Ca release experiments

To confirm the functional implications of the ryanodine binding experiments, we assayed SR Ca^{2+}-induced Ca^{2+} release. After *in vitro* loading, the $^{45}Ca^{2+}$ content of our SR preparation was of the order of 20–25 nmol mg^{-1} protein. Exposure to the release buffer determined the quick release of 8–12 nmol mg^{-1}, which was completed over 100–120 ms. Thereafter the rate of Ca^{2+} release decreased considerably. The quick component of Ca^{2+} release was inhibited by 10 mM Mg^{2+} plus 10 μM ruthenium red, confirming that it represented Ca^{2+} efflux through the SR release channels (release curves were quite similar to those shown in Zucchi *et al*., 1995b).

The effects of doxorubicin and MEN 10755 were tested using a release buffer that included 2 μM free Ca^{2+}. The extent of $^{45}Ca^{2+}$ loading and of the $^{45}Ca^{2+}$ pool subjected to quick release were similar after treatment with either 50 μM doxorubicin or 50 μM MEN 10755 (releasable Ca^{2+} averaged 10.3 ± 1.5 nmol mg^{-1} protein, without any significant difference between groups). The rate constant of Ca^{2+} release averaged 22 ± 2 s^{-1} under control conditions, and it increased by about 4 fold (82 ± 5 s^{-1}, $P<0.01$ vs control) in the presence of doxorubicin (Figure 4). On the other hand, in the presence of MEN 10755, SR Ca^{2+} release was not significantly different from the baseline (rate constant 24 ± 3 s^{-1}, $P<0.01$ vs doxorubicin).

Assay of SR Ca^{2+} -ATPase activity and of oxalate-supported Ca^{2+} -uptake

In our microsomal preparation, Ca^{2+}-ATPase activity averaged 1.24 ± 0.10 μmol min^{-1} mg^{-1} protein. As shown in Table 1, none of the compounds tested (i.e. doxorubicin, doxorubicinol, MEN 10755 and MEN 11383) produced any significant effect at concentrations $\leqslant 100$ μM. At higher concentrations, some inhibition occurred with doxorubicinol (42% inhibition at 200 μM concentration, $P<0.05$). The assay of Ca^{2+}-uptake confirmed that no compound was able to produce significant effects at 50 μM concentration, whereas a slight inhibition (14%) was observed with 200 μM doxorubicinol, although it did not reach the threshold of statistical significance.

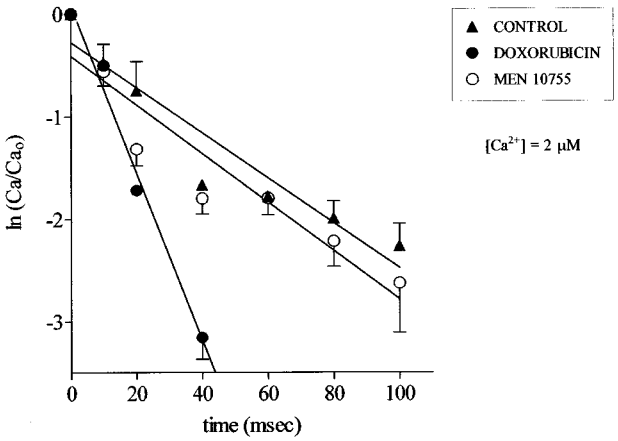

Figure 4 SR Ca^{2+}-induced Ca^{2+} release curves were determined with a release buffer containing 2 μM free Ca^{2+}, under control conditions and in the presence of 50 μM doxorubicin or 50 μM MEN 10755. The vertical axis shows the logarithm of the ratio of $^{45}Ca^{2+}$ to $^{45}Ca^{2+}$ at time zero, after subtraction of the amount of $^{45}Ca^{2+}$ not involved in quick Ca^{2+} release. Since the release follows exponential kinetics, the slope of the regression lines represents the rate constant of Ca^{2+}-induced Ca^{2+} release (for further details see Zucchi *et al*., 1995b). Data points represent mean±s.e.mean of three different experiments. Data was analysed by GraphPad Prism 3.00 software, using a linear regression model. The rate constant averaged 82 ± 5 s^{-1} in the presence of doxorubicin and 24 ± 3 s^{-1} in the presence of MEN 10755, vs a control value of 22 ± 2 s^{-1}. The doxorubicin curve was significantly different from the control and MEN 10755 curves ($P<0.01$ by ANOVA and Student-Neuman-Keuls test), whereas the difference between the control and MEN 10755 curves did not achieve statistical significance. The amount of Ca^{2+} subjected to quick release averaged 10.3 ± 1.5 nmol mg^{-1} protein, without any significant difference between groups.

Perfusion experiments

The results of perfusion experiments are summarized in Figure 5. Baseline values of the chief haemodynamic variables were similar in all experimental groups, and they averaged as follows: aortic flow 48.5 ± 1.9 ml min^{-1}, coronary flow 16.9 ± 0.9 ml min^{-1}, cardiac output 65.4 ± 2.7 ml min^{-1}, peak systolic aortic pressure 124 ± 3 mmHg, heart rate 265 ± 13 beats min^{-1}. In the control group, that was subjected to 60 min of aerobic perfusion, all haemodynamic variables were substantially stable (changes were <10% in all cases). The addition of 30 μM doxorubicin to the perfusion buffer produced a progressive decrease in aortic flow, coronary flow, cardiac output and peak aortic pressure. After 50–60 min, half of the hearts failed (i.e. they were no longer able to sustain a significant aortic flow). At the end of the perfusion, the mean values of all haemodynamic variables were <30% of the baseline. Epirubicin (30 μM) produced similar or even greater contractile impairment, since all the hearts of this group failed before 50 min. On the other hand, perfusion with 30 μM MEN 10755 produced only minor effects. After 60 min of perfusion, aortic flow and cardiac output averaged 85 ± 13 and 93 ± 8% of the baseline, respectively, whereas coronary flow, peak aortic pressure and heart rate were virtually unchanged. Statistical analysis showed that the effects of MEN 10755 were significantly different from those of doxorubicin or epirubicin ($P<0.01$ in both cases), while the difference between the control group and the MEN 10755 group was not significant.

Table 1 SR Ca^{2+}-ATPase and Ca^{2+}-uptake

	Ca^{2+}-ATPase activity (% of the baseline)	*Ca^{2+} -uptake* (% of the baseline)
Doxorubicin (50 μM)	112 ± 9	110 ± 13
Doxorubicin (100 μM)	85 ± 5	n.d.
Doxorubicin (200 μM)	83 ± 5	n.d.
Doxorubicinol (50 μM)	98 ± 4	94 ± 4
Doxorubicinol (100 μM)	80 ± 9	96 ± 5
Doxorubicinol (200 μM)	58 ± 14*	86 ± 8
MEN 10755 (50 μM)	92 ± 7	106 ± 3
MEN 10755 (100 μM)	92 ± 6	n.d.
MEN 10755 (200 μM)	73 ± 3	n.d.
MEN 11383 (200 μM)	86 ± 15	n.d.

Results were obtained in three different preperations and are expressed as percentage (mean±s.e.mean) of the baseline values, that averaged 1.24 µmol min^{-1} mg^{-1} protein for Ca^{2+}-ATPase activity and 12.9 nmol min^{-1} mg^{-1} protein for oxalate supported Ca^{2+}-uptake. Ca^{2+}-ATPase activity was determined in the microsomal fraction, whereas oxalate supported Ca^{2+}-uptake was determined in the crude homogenate. *$P<0.05$ vs 100%, by ANOVA for repeated measures and Student-Neuman-Keuls test

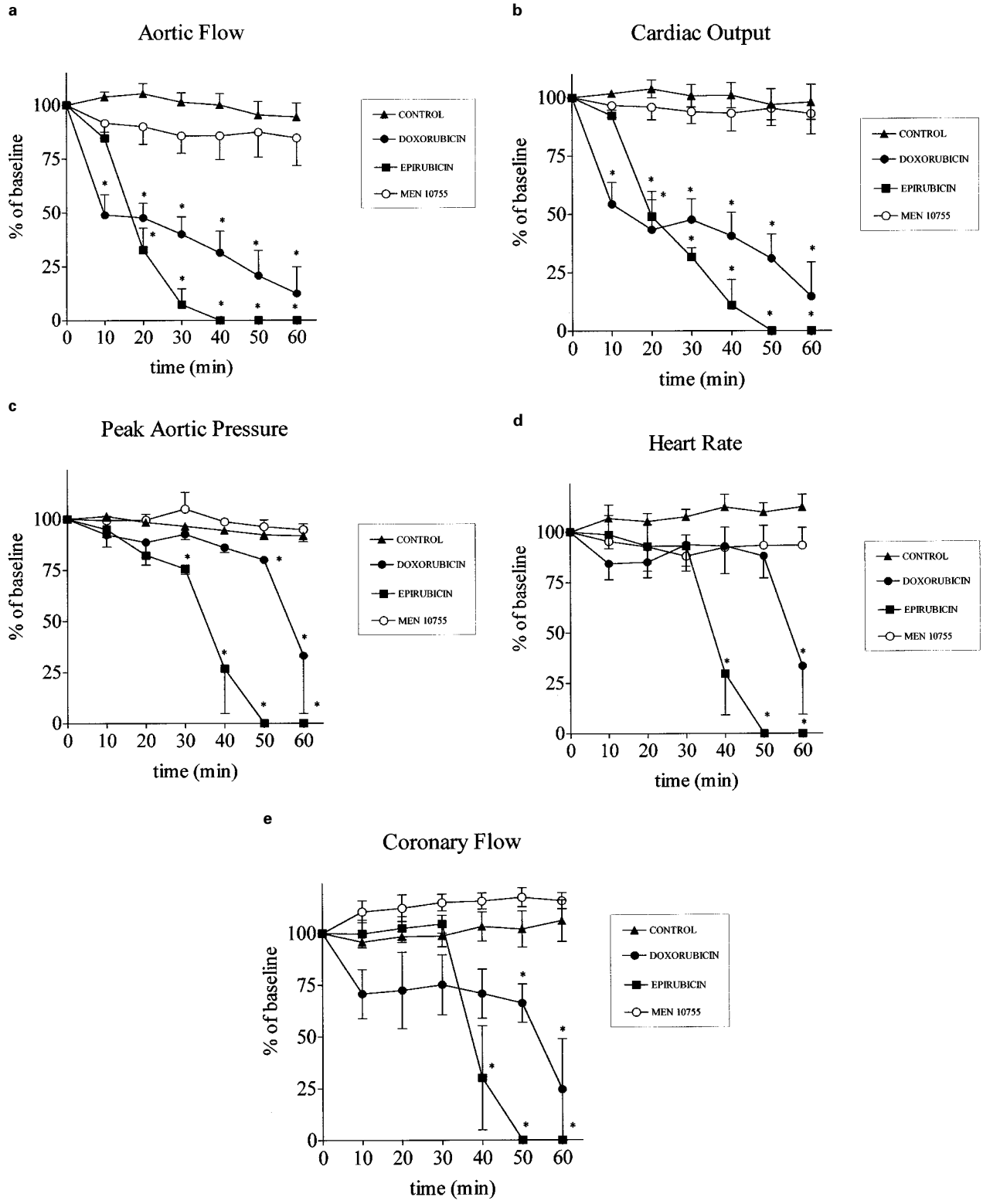

Figure 5 Time course of the chief haemodynamic variables in hearts perfused under control conditions or in the presence of 30 μM doxorubicin, 30 μM epirubicin, 30 μM MEN 10755. (a) aortic flow; (b) cardiac output; (c) peak aortic pressure; (d) heart rate; (e) coronary flow. Data points are expressed as percentage of the baseline values, and represent mean$\pm$s.e.mean of four hearts in each group. Actual baseline values are given in the text. *$P<0.01$ vs the control group and the MEN 10755 group, by ANOVA and Student-Neuman-Keuls test. Differences between the control group and the MEN 10755 group were not statistically significant for any variable, at any time.

Discussion

MEN 10755 is a disaccharide analogue of doxorubicin, which appears to be more effective than doxorubicin in the inhibition of topoisomerase II and in the treatment of human tumour xenografts (Arcamone *et al*., 1997; Pratesi *et al*., 1998). Since the clinical use of anthraquinone compounds is often limited by cardiac toxicity (Buzdar *et al*., 1985; Jensen, 1986; Hagane *et al*., 1998; Doroshow, 1991; Rhoden *et al*., 1993; Wang & Korth, 1995; Nysom *et al*., 1998), it seemed interesting to

evaluate the cardiac effects of MEN 10755, which were compared with those of doxorubicin and epirubicin.

The cardiac toxicity of anthraquinones does not seem to be related to topoisomerase II inhibition. There is strong evidence that a major role is played by impaired intracellular Ca^{2+} homeostasis (Kusuoka *et al*., 1991; Jiang *et al*., 1994; Temma *et al*., 1994; Halili-Rutman *et al*., 1997; Maeda *et al*., 1998). In particular, acute cardiac injury is likely to be due to stimulation of Ca^{2+} release through the SR Ca^{2+} channel. Doxorubicin acts by sensitizing the channel to the stimulatory action of extravescicular Ca^{2+}. As it is the case for many activators of the SR Ca^{2+} channel, doxorubicin increases the binding of ryanodine, a selective ligand that can only interact with the open state of the channel (Zorzato *et al*., 1985; Abramson *et al*., 1988; Kim *et al*., 1989; Ondrias *et al*., 1990; Pessah *et al*., 1990; Tian *et al*., 1991; Boucek *et al*., 1993; Zucchi & Ronca-Testoni, 1997). These effects were confirmed in the present investigation, since at micromolar Ca^{2+} concentration doxorubicin determined a remarkable increase in ryanodine binding and in the rate of Ca^{2+}-induced Ca^{2+} release. These actions were shared by epirubicin, the 3′-epimer of doxorubicin.

MEN 107551 produced only minor effects on the ryanodine receptor. At 30 μM free Ca^{2+} concentration, ryanodine binding was unaffected by up to 100 μM MEN 10755. At 1 μM free Ca^{2+}, the binding of 2 nM ryanodine was only slightly increased. The maximum stimulation was on the order of 1.9 fold (vs 15.3 fold in the case of doxorubicin). The results of release experiments were in accordance with these findings, since 50 μM MEN 10755 did not produce any significant change in the rate of SR Ca^{2+} release, at 2 μM free Ca^{2+} concentration.

The interaction between anthaquinones and the ryanodine receptor has not been characterized at molecular level. Anthraquinones are thought to bind to a site that overlaps at least in part with the caffeine binding site (Abramson *et al*., 1988; Pessah *et al*., 1990), and to modify hyperreactive cysteine residues (Feng *et al*., 1999). Our results suggest that the saccharidic moiety of the molecule and/or the 3′-methoxy group play a major role in the binding reaction, or in the modulation of channel function.

Some investigators have suggested that doxorubicin toxicity may be due to inhibition of the SR Ca^{2+}-ATPase. In particular, ATPase inhibition by doxorubicinol (13-dihydro-doxorubicin), a catabolite of doxorubicin, has been reported (Boucek *et al*., 1987; Olson *et al*., 1988). In our model, none of the compounds tested (doxorubicin, doxorubicinol, MEN 10755 and its 13-dihydro-derivative) significantly affected Ca^{2+}-ATPase activity, or Ca^{2+} uptake, at concentrations $\leqslant$100 μM. Some inhibition occurred only with higher concentrations of doxorubicinol. Therefore, it seems unlikely that interference with SR Ca^{2+} uptake may be relevant *in vivo*.

Since activation of SR Ca^{2+} release is believed to be the chief cause of acute anthraquinone toxicity, MEN 10755 should be much less toxic than doxorubicin or epirubicin. To test this prediction, we evaluated the acute effects of these compounds in an isolated heart model. The difference between 30 μM MEN 10755 and equimolar doxorubicin or epirubicin was dramatic. After 60 min of perfusion, serious contractile impairment occurred in every heart treated with doxorubicin or epirubicin, whereas in the MEN 10755 group all haemodynamic variables were close to the normal range. These findings are in good agreement with the results reported by Parlani *et al*. (1998) in rat right ventricular muscle, and induce to believe that in the clinical setting the acute administration of MEN 10755 should be much less cardiotoxic than observed with doxorubicin or epirubicin.

The pathogenesis of chronic doxorubicin toxicity is poorly understood. The same mechanisms that are responsible for the acute effects might be involved. In particular, low doses of doxorubicin might produce marginal cellular injury (i.e. necrosis limited to a few cardiomyocytes), and doxorubicin cardiomyopathy might represent the cumulative result of these lesions. However, additional mechanisms are likely to play a role. After prolonged exposure to doxorubicin, ryanodine receptor activation is followed by a slowly-developing irreversible inactivation (reviewed in Zucchi & Ronca-Testoni, 1997). Reduced density of SR Ca^{2+} release channels has actually been observed in models of chronic doxorubicin toxicity (Pessah *et al*., 1992; Dodd *et al*., 1993), and the expression of several genes coding for SR proteins – ryanodine receptor, calsequestrin, SR Ca^{2+}-ATPase and phospholamban – is decreased (Arai *et al*., 1998). Such long-term changes in SR proteins might reduce the availability of Ca^{2+} for contractile protein activation, contributing to inotropic failure. Consistently, measurement of intracellular Ca^{2+} after prolonged exposure to doxorubicin has shown reduced amplitude and prolonged duration of Ca^{2+} transients (Jiang *et al*., 1994; Temma *et al*., 1994; Maeda *et al*., 1998), while the Ca^{2+}-accumulating capability of the SR was decreased (Halili-Rutman *et al*., 1997).

On the basis of the remarkable difference which we have observed between the SR effects of doxorubicin and MEN 10755, one could expect lower toxicity of the disaccharide analogue even after chronic administration. Recent experimental results appear to support this hypothesis (Cirillo *et al*., 2000), but specific studies on SR changes after prolonged exposure to MEN 10755 are still required for a thorough evaluation of this important issue.

This study was supported in part by Menarini Ricerchi S.p.A.

References

ABRAMSON, J.J., BUCK, E., SALAMA, G., CASIDA, J.E. & PESSAH, I.N. (1988). Mechanism of anthraquinone-induced calcium release from skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.,* **263,** 18750 – 18758.

ARAI, M., TOMARU, K., TAKIZAWA, T., SEKIGUCHI, K., YOKOYAMA, T., SUZUKI, T. & NAGAI, R. (1998). Sarcoplasmic reticulum genes are selectively down-regulated in cardiomyopathy produced by doxorubicin in rabbits. *J. Mol. Cell. Cardiol.,* **30,** 243 – 254.

ARCAMONE, F., ANIMATI, F., BERETTONI, M., BIGIONI, M., CAPRANICO, G., CASAZZA, A.M., CASERINI, C., CIPOLLONE, A., DE CESARE, M., FRANCIOTTI, M., LOMBARDI, P., MADANI, A., MANZINI, S., MONTEAGUDO, E., POLIZZI, D., PRATESI, G., RIGHETTI, S.G., SALVATORE, C., SUPINO, R. & ZUNINO, F. (1997). Doxorubicin disaccharide analogue: apoptosis-related improvement of efficacy in vivo. *J. Natl. Cancer. Inst.,* **89,** 1217 – 1223.

BOUCEK, R.J., BUCK, S.H., SCOTT, F., OQUIST, N.L., FLEISCHER, S. & OLSON, R.D. (1993). Anthracycline-induced tension in permeabilized cardiac fibres: evidence for the activation of the calcium release channel of the sarcoplasmic reticulum. *J. Mol. Cell. Cardiol.,* **25,** 249 – 259.

BOUCEK, R.J., OLSON, R.D., BRENNER, D.E., OGUNBUNMI, E.M., INUI, M. & FLEISCHER, S. (1987). The major metabolite of doxorubicin is a potent inhibitor of membrane-associated ion pumps. *J. Biol. Chem.,* **262,** 15851 – 15856.

BUZDAR, A.U., MARCUS, C., SMITH, T.L. & BLUMENSCHEIN, G.R. (1985). Early and delayed clinical cardiotoxicity of doxorubicin. *Cancer,* **55,** 2761 – 2765.

CHU, A., SUBMILLA, C., SCALES, D., PIAZZA, A. & INESI, G. (1988). Trypsin digestion of junctional sarcoplasmic reticlum vesicles. *Biochemistry,* **27,** 2827 – 2833.

CIRILLO, R., SACCO, G., VENTURELLA, S., BRIGHTWELL, J., GIACHETTI, A. & MANZINI, S. (2000). Comparison of doxorubicin- and MEN 10755-induced long-term progressive cardiotoxicity in the rat. *J. Cardiovasc. Pharmacol.,* **35,** 100 – 108.

DODD, D.A., ATKINSON, J.B., OLSON, R.D., BUCK, S., CUSACK, B.J., FLEISCHER, S. & BOUCEK, R.J. (1993). Doxorubicin cardiomyopathy is associated with a decrease in calcium release channel of the sarcoplasmic reticulum in a chronic rabbit model. *J. Clin. Invest.,* **91,** 1697 – 1705.

DOROSHOW, J.H. (1991). Doxorubicin-induced cardiac toxicity. *N. Engl. J. Med.,* **324,** 843 – 845.

DUPONT, Y. (1984). A rapid-filtration technique for membrane fragments or immobilized enzymes: measurements of substrate binding or ion fluxes with a few-millisecond time resolution. *Anal. Biochem.,* **142,** 504 – 510.

FABIATO, A. & FABIATO, F. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol., (Paris),* **75,** 463 – 505.

FEHER, J.J. & LIPFORD, G.B. (1985). Mechanism of action of ryanodine on cardiac sarcoplasmic reticulum. *Biochim. Biophys. Acta.,* **813,** 77 – 86.

FENG, W., LIU, G., XIA, R., ABRAMSON, J.J. & PESSAH, I.N. (1999). Site-selective modification of hyperreactive cysteines of ryanodine receptor complex by quinones. *Mol. Pharmacol.,* **55,** 821 – 831.

HAGANE, K., AKERA, T. & BERLIN, J.R. (1988). Doxorubicin: mechanisms of cardiodepressant actions in guinea pigs. *J. Pharmacol. Exp. Ther.,* **246,** 655 – 661.

HALILI-RUTMAN, I., HERSHKO, C., LINK, G., RUTMAN, A.J. & SHAINBERG, A. (1997). Inhibition of calcium accumulation by the sarcoplasmic reticulum: a putative mechanism for the cardiotoxicity of adriamycin. *Biochem. Pharmacol.,* **54,** 211 – 214.

JENSEN, R.A. (1986). Doxorubicin cardiotoxicity: contractile changes after long-term treatment in the rat. *J. Pharmacol. Exp. Ther.,* **236,** 197 – 203.

JIANG, J., TEMMA, K. & AKERA, T. (1994). Doxorubicin-induced changes in intracellular Ca2+ transients observed in cardiac myocytes isolated from guinea-pig heart. *Can. J. Physiol. Pharmacol.,* **72,** 622 – 631.

KIM, D.H., LANDRY, A.B., LEE, Y.S. & KATZ, A.M. (1989). Doxorubicin-induced calcium release from cardiac sarcoplasmic reticulum vesicles. *J. Mol. Cell. Cardiol.,* **21,** 433 – 436.

KUSUOKA, H., FUTAKI, S., KORETSUNE, Y., KITABATAKE, A., SUGA, H., KAMADA, T. & INOUE, M. (1991). Alterations of intracellular calcium homeostasis and myocardial energetics in acute adriamycin-induced heart failure. *J. Cardiovasc. Pharmacol.,* **18,** 437 – 444.

MAEDA, A., HONDA, M., KURAMOCHI, T. & TAKABATAKE, T. (1998). Doxorubicin cardiotoxicity: diastolic cardiac myocyte dysfunction as a result of impaired calcium handling in isolated cardiac myocytes. *Jpn. Circ. J.,* **62,** 505 – 511.

MOUTIN, M.J. & DUPONT, Y. (1988). Rapid filtration studies of Ca^{2+}-induced Ca^{2+} release from skeletal sarcoplasmic reticulum. *J. Biol. Chem.,* **263,** 4228 – 4235.

NAYLER, W.G. (1988). *Calcium antagonists*. London: Academic Press, pp. 157 – 176.

NYSOM, K., COLAN, S.D. & LIPSCHULTZ, S.E. (1998). Late cardiotoxicity following anthracycline therapy for childhood cancer. *Prog. Pediatr. Cardiol.,* **8,** 121 – 138.

OLSON, R.D., MUSHLIN, P.S., BRENNER, D.E., FLEISCHER, S., CUSACK, B.J., CHANG, B.K. & BOUCEK, R.J. (1988). Doxorubicin cardiotoxicity may be caused by its metabolite, doxorubicinol. *Proc. Natl. Acad. Sci. U.S.A.,* **85,** 3585 – 3589.

ONDRIAS, K., BORGATTA, L., KIM, D.H. & EHRLICH, B.E. (1990). Biphasic effects of doxorubicin on the calcium release channel from sarcoplasmic reticulum of cardiac muscle. *Circ. Res.,* **67,** 1167 – 1174.

PARLANI, M., CIRILLO, R. & MANZINI, S. (1998). Analysis of negative inotropic actions of anthracyclins (doxorubicin, epirubicin and MEN 10755) in rat isolated rat ventricles [abstract]. Proceedings 6th Joint Meeting of the Italian Hungarian and Polish Pharmacological Societies, Pisa, 44.

PESSAH, I.N., DURIE, E.L., SCHIEDT, M.J. & ZIMANYI, I. (1990). Anthraquinone-sensitized Ca^{2+} release channel from rat cardiac sarcoplasmic reticulum: possible receptor-mediated mechanism of doxorubicin cardiomyopathy. *Mol. Pharmacol.,* **37,** 503 – 514.

PESSAH, I.N., SCHIEDT, M.J., SHALABY, M.A., MACK, M. & GIRI, S.N. (1992). Etiology of sarcoplasmic reticulum calcium release channel lesions in doxorubicin-induced cardiomyopathy. *Toxicology,* **72,** 189 – 206.

PRATESI, G., DE CESARE, M., CASERINI, C., PEREGO, P., DAL BO, L., POLIZZI, D., SUPINO, R., BIGIONI, M., MANZINI, S., IAFRATE, E., SALVATORE, C., CASAZZA, A., ARCAMONE, F. & ZUNINO, F. (1998). Improved efficacy and enlarged spectrum of activity of a novel anthracycline disaccharide analogue of doxorubicin against human tumor xenografts. *Clin. Cancer Res.,* **4,** 2833 – 2839.

RHODEN, W., HASLETON, P. & BROOKS, N. (1993). Anthracyclines and the heart. *Br. Heart J.,* **70,** 499 – 502.

SCARPA, A. (1979). Measurements of cation transport with metallochromic indicators. *Methods Enzymol.,* **56,** 301 – 338.

TEMMA, K., AKERA, T., AKIHITO, C., OZAWA, S. & KONDO, H. (1994). Cellular Ca^{2+} loading and inotropic effects of doxorubicin in atrial muscle preparations isolated from rat or giunea-pig hearts. *Eur. J. Pharmacol.,* **252,** 172 – 181.

TIAN, Q., KATZ, A.M. & KIM, D.H. (1991). Effects of azumolene on doxorubicin-induced Ca^{2+} release from skeletal and cardiac muscle sarcoplasmic reticulum. *Biochim. Biophys. Acta,* **1094,** 27 – 34.

WANG, Y.X. & KORTH, M. (1995). Effects of doxorubicin on excitation-contraction coupling in guinea pig ventricular myocardium. *Circ. Res.,* **76,** 645 – 653.

ZORZATO, F., SALVIATI, G., FACCHINETTI, T. & VOLPE, P. (1985). Doxorubicin-induced calcium release from terminal cisternae of skeletal muscle. *J. Biol. Chem.,* **260,** 7349 – 7355.

ZUCCHI, R. & RONCA-TESTONI, S. (1997). The sarcoplasmic reticulum Ca^{+} channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. *Pharmacol. Rev.,* **49,** 1 – 51.

ZUCCHI, R., RONCA-TESTONI, S., YU, G., GALBANI, P., RONCA, G. & MARIANI, M. (1994). Effect of ischemia and reperfusion on cardiac ryanodine receptors – sarcoplasmic reticulum Ca^{2+} channels. *Circ. Res.,* **74,** 271 – 280.

ZUCCHI, R., RONCA-TESTONI, S., YU, G., GALBANI, P., RONCA, G. & MARIANI, M. (1995a). Interaction between gallopamil and cardiac ryanodine receptors. *Br. J. Pharmacol.,* **114,** 85 – 92.

ZUCCHI, R., RONCA-TESTONI, S., YU, G., GALBANI, P., RONCA, G. & MARIANI, M. (1995b). Postischemic changes in cardiac sarcoplasmic reticulum Ca^{2+} channels. A possible mechanism of ischemic preconditioning. *Circ. Res.,* **76,** 1049 – 1056.

(Received April 18, 2000
Revised June 29, 2000
Accepted June 30, 2000)