Biochemical Pharmacology, Vol. 42, No. 12, pp. 2283–2287, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

COMPARATIVE CARDIOTOXICITY OF DOXORUBICIN AND A MORPHOLINO ANTHRACYCLINE DERIVATIVE (KRN8602)

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(*Received* 20 *February* 1991; *accepted* 14 *August* 1991)

Abstract—The toxicities of doxorubicin (DXR) and 3′-deamino-3′-morpholino-13-deoxy-10-hydroxycarminomycin (KRN8602) were compared in perfused rat heart preparations. In addition, their toxicities in isolated myocytes as well as their abilities to cause oxidation of intracellular reduced glutahione (GSH) were evaluated. Finally, their capacities to initiate oxygen consumption and lipid peroxidation in isolated microsomes were determined. DXR caused a dramatic decrease in left ventricular pressure in the perfused hearts and a leak of lactic acid dehydrogenase (LDH) from isolated myocytes. These effects were not observed with KRN8602 at comparable concentrations although KRN8602 caused oxidation of GSH to oxidized glutathione (GSSG) in the myocytes. Both anthracyclines caused an increase in oxygen consumption and lipid peroxidation in isolated microsomes although these effects were more pronounced with KRN8602. The results of these experiments demonstrate that, despite the high capacity to redox cycle and to generate oxygen-derived oxidants, KRN8602 exhibited no measurable cardiotoxicity. The findings are consistent with a view that, if the formation of such oxidants is central to the cardiotoxicity of DXR, these substances must be formed in cellular compartments, e.g. plasma membranes, where the morpholino derivative (KRN8602) does not accumulate.

Reactive species of oxygen have been implicated in the chemotherapeutic actions of numerous naturally occurring and synthetic substances. Among the latter are the group of anthracycline antibiotics whose interactions with molecular oxygen depend on the presence of a quininoid moiety [1, 2].

The anthracycline antibiotic, doxorubicin (DXR‡), is among the most useful antineoplastic agents known. It has significant therapeutic utility in the treatment of hematogenous malignancies as well as solid tumors of the breast, long bone and thyroid. In addition to its ability to intercalate with DNA, this quininoid antibiotic has a wide variety of biochemical effects including the formation of oxygen-derived oxidants. Whereas the role of such substances in antineoplastic activity is as yet uncertain, there is a considerable amount of evidence that the cardiotoxicity of anthracycline agents is related to their interactions with oxygen. This and other potential mechanisms of anthracycline cardiotoxicity have been reviewed recently [3]. DXR is one of the most effective anthracycline antineoplastic agents and yet its use has been restricted because of its dose-limiting histopathological and clinical cardiotoxicity.

A number of DXR analogues of potential therapeutic superiority and lower cardiotoxicity are currently under investigation. One of these is 3′-deamino-3′-morpholino-13-deoxy-10-hydroxycarminomycin, KRN8602 [4]. In this paper, we report experiments designed to examine the comparative cytotoxic responses of DXR and KRN8602 in isolated myocytes and perfused heart preparations from rats. Additionally, we investigated the comparative capacity of these agents to undergo redox cycling and affect the reduced glutathione (GSH) status of cells. Finally, we studied their effects on isolated microsomes in causing the uptake of oxygen and the initiation of lipid peroxidation.

MATERIALS AND METHODS

Male Sprague–Dawley rats (300–400 g) were heparinized (500 units) and anesthetized with pentobarbital (30 mg/kg). Under anesthesia, the hearts were removed quickly and immediately immersed in ice-cold buffer solution. Within 60 sec, the ascending aorta was cannulated distal to the coronary arteries and perfusion established by the method of Langendorff [5]. The perfusing buffer contained the following in millimolar concentrations: 118.0 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 0.5 calcium-EDTA, 25.0 $NaHCO_3$, and 10.0 glucose, pH 7.4. The perfusate was bubbled continuously with a 95% O_2–5% CO_2 gas mixture. After removing the blood, the perfusate was recirculated at a constant rate of 10 mL/min by a peristaltic pump which consistently yielded a perfusion pressure of about 65 mm Hg. End diastolic pressure was not controlled through the experiment. The total volume of the perfusate was 100 mL. A catheter was inserted into the left ventricular cavity through the left atrium and held in place with a

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‡ Abbreviations: DXR, dixorubicin; KRN8602, 3′-deamino-3′-morpholino-13-deoxy-10-hydroxy-carminomycin; GSH, reduced glutathione; GSSG, oxidized glutathione; LDH, lactic acid dehydrogenase; CPK, creatine phosphokinase; and TBARS, thiobarbituric acid reactive substances.

purse-string ligature [6]. The catheter was connected to a pressure transducer by a three-way stopcock to allow recording of developed left ventricular and end diastolic pressures. The temperature was maintained at 37° in an enclosed chamber. After a 30-min period of stabilization, either isotonic saline or the appropriate anthracycline was added to the perfusate.

To prepare myocytes [7], hearts were removed from animals treated as described above and quickly placed in 100 mL of a modified Krebs–Ringer buffer of the following composition in millimolar amounts: 110.0 NaCl, 2.6 KCl, 1.2 Na_2HPO_4, 1.2 KH_2PO_4, 25.0 NaH_2PO_4, 0.025 $CaCl_2$, 11.0 glucose, 0.1 octanoate and 5.0 creatine. The hearts were mounted on a Langendorff perfusion system and perfused with calcium-free buffer which was equilibrated with 95% O_2 and 5% CO_2. The temperature and the pH of the buffer were maintained at 37° and pH 7.4, respectively. Noncirculating flow of the buffer was continued for about 5 min until 30–40 mL of the perfusate had washed through the hearts to remove blood and extracellular calcium ions. At this point, a new buffer solution containing 0.1% Class 2 collagenase (Worthington Biochemical Corp., Freehold, NJ) was recirculated until the hearts became flaccid. The hearts were then removed from the perfusion system, placed in dishes containing 20 mL of buffer solution and teased apart with forceps until no cells were present in the heart matrix. The cell dispersion was placed in hydrophobic tubes on their side for 5 min and then upright for an additional 3 min. The supernatants were then removed and the settled cells resuspended in fresh buffer solution. This procedure was repeated three times.

Myocytes prepared as above were incubated in a total volume of 7.0 mL (containing about 10^6 cells/mL) in siliconized flasks at 37° under 95% O_2–5% CO_2 in order to measure enzyme release in the presence and absence of the anthracyclines. To measure such release, aliquots of cell suspensions were removed at appropriate time intervals and the activity of lactic acid dehydrogenase (LDH) was determined on the supernatant fraction obtained after sedimentation of the cells by centrifugation. Enzyme activity was determined by following the decrease in absorption of NADH at 340 nm after the addition of pyruvate [8]. Total LDH activity of the cells was determined by treatment of a similar aliquot of cells with a solution of 1% Triton X-100 (Bio-Rad Laboratories) and removal of cell debris by centrifugation.

For the measurement of intracellular glutathione in myocytes, the cells were sedimented and washed with ice-cold 50 mM phosphate buffer, pH 7.4. The cells were finally resuspended in this buffer containing 0.1% Triton X-100 and the cellular debris was removed by a final centrifugation. Both reduced (GSH) and oxidized glutathione (GSSG) were determined by the method described by Adams *et al.* [9].

The preparation of hepatic microsomes and the measurement of oxygen consumption and lipid peroxidation were carried out by previously described methods [10].

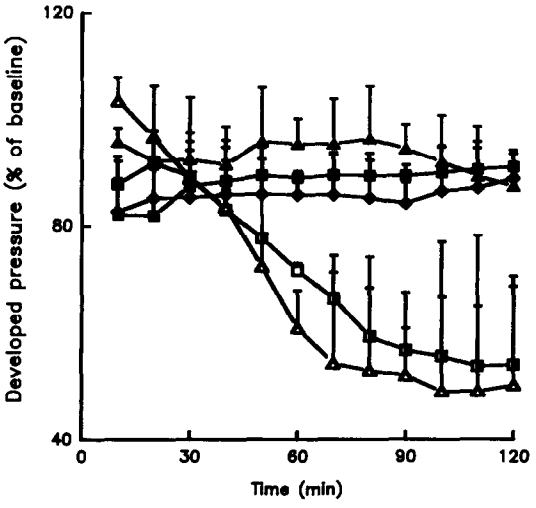

Fig. 1. Effects of DXR and KRN8602 on left ventricular developed pressure in isolated (Langendorff) rat hearts. Points and lines represent means ± SD for 3–5 hearts in each group. Key: untreated hearts (◆); DXR; 10 μM (□) and 20 μM (△); KRN8602; 10 μM (■) and 20 μM (▲). Statistical analysis was performed between the data of the control and treated groups using an analysis of variance, followed by the Bonferroni test for multiple comparisons [11]. Beginning at 60 min, the DXR-treated groups showed significant depression of ventricular pressure ($P < 0.001$), compared to both the control and the KRN8602-treated groups.

Collagenase was obtained from the Worthington Biochemical Corp. All other biochemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). KRN8602 was obtained from the Kirin Brewery Co. (Maebashi-shi Gunma, Japan).

RESULTS

Functional alterations in the isolated perfused rat heart. Figure 1 illustrates that, although there was a small increase in left ventricular developed pressure in control hearts during the first 10 min of perfusion (baseline = 100%), perfusion pressure remained relatively stable thereafter for periods of up to 120 min. On the other hand, hearts exposed to DXR at concentrations of 10 and 20 μM exhibited an increase in perfusion pressure over the baseline in the first 10 min of the experiment. Thereafter, they displayed a progressive decline in developed pressure for about 60–70 min when these pressures reached about 50% of the initial ones. These values were maintained for the remainder of the incubation periods. Further analysis of these data demonstrated that, although systolic pressure remained stable, there was an increase in diastolic pressure. Initial perfusion pressure in these experiments was about 65 mm Hg and remained stable for all groups. Heart rate was about 250 beats/min and remained stable for the control and KRN8602-treated hearts. Although the data are not shown, there was a small (10–15%) depression of heart rate during exposure to DXR. There were no significant differences

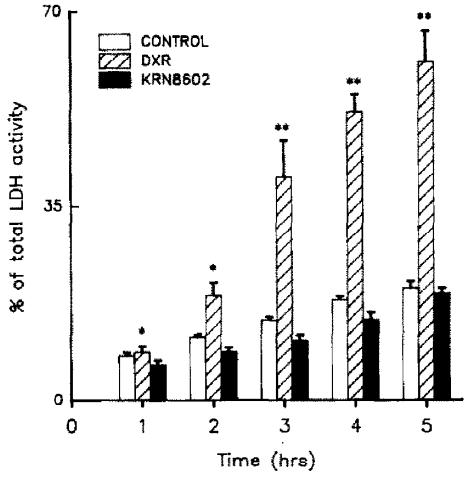

Fig. 2. Effects of DXR (500 μM) and KRN8602 (500 μM) on the leak of lactic acid dehydrogenase (LDH) from isolated myocytes. The bars and lines represent means ± SEM from data obtained from 4–8 experiments in each group. Statistical analysis was performed between the data of the control and treated groups using an analysis of variance, followed by the Bonferroni test for multiple comparisons. LDH release in the presence of DXR was significantly greater than that from both control and KRN8602-treated myocytes (* $P < 0.05$; ** $P < 0.01$).

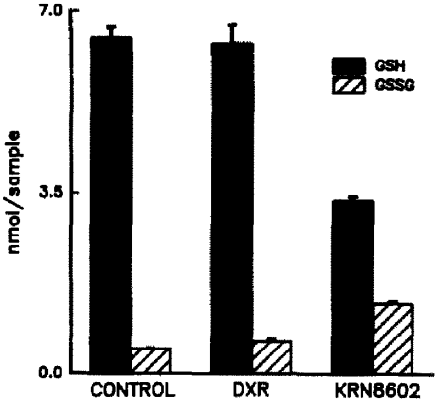

Fig. 3. Effects of DXR (100 μM) and KRN8602 (100 μM) on the GSH and GSSG levels of isolated myocytes. Bars and lines represent means ± SEM from data obtained from 12–24 experiments per group. A one-way analysis of variance was used to test for inequalties of means, followed by the Bonferroni method for multiple comparisons. KRN8602 significantly decreased the levels of GSH ($P < 0.001$) and increased the levels of GSSG ($P < 0.05$) compared to control values after 60 min of incubation.

between the effects observed at 10 and 20 μM DXR. As may also be seen in Fig. 1, the depression of developed pressure was not observed when KRN8602 was substituted for DXR in the perfusion medium. The small increases that may be noted in Fig. 1 at 20 μM KRN8602 were not significantly different from the values obtained with control hearts. There were also no significant alterations in heart rate at either concentration of the morpholino derivative.

Cellular damage in isolated myocytes. The apparent lack of dramatic toxicity caused by KRN8602 in the experiments described above was confirmed in experiments with isolated myocytes. These experiments are illustrated in Fig. 2. It may be seen in this figure that, even at a concentration as high as 500 μM, KRN8602 had little effect on the integrity of myocytes in suspension over a period of 5 hr as measured by the leak of LDH from treated cells. In contrast, it should be noted that at the same concentration DXR causes a leak of LDH of about 60% of total cellular LDH. Untreated, control cells and those exposed to KRN8602 lost about 20% of the enzyme during these extended incubations. Parenthetically, it should be mentioned that it was not possible to measure creatine phosphokinase (CPK) leak in the presence of either DXR or KRN8602 since both agents acted as inhibitors of the enzyme. Such an effect is in keeping with previously reported inhibition of CPK by superoxide anions [12].

Effects of DXR and KRN8602 on glutathione levels in myocytes. Although the experiments described above suggest that KRN8602 does not cause damage to isolated myocytes which result in enzyme (LDH) leak from these cells, this anthracycline derivative is not without its effects on these cells. For example, Fig. 3 shows that after 60 min of incubation the level of intracellular GSH had decreased by about 50% in cells exposed to this agent. Figure 3 also shows that a comparable effect was not observed with DXR. It should be noted in this figure that most of the loss of GSH may be attributed to its oxidation to GSSG which accumulates within the cells. Not shown is that some GSSG appeared in the medium. Again, there was no marked increase in GSSG when DXR was included in the incubation medium.

Effects of DXR and KRN8602 in isolated microsomes. The enhancement of GSH loss in myocytes suggests that, despite the apparently paradoxical lack of myocyte damage, KRN8602 undergoes redox cycling presumably with the ultimate formation of hydrogen peroxide as well as other oxygen-derived oxidants. This is apparently the case since, as illustrated in Table 1, while DXR caused some increase in oxygen consumption in microsomes prepared from rat liver (from 11.3 to 15.6 nmol/min), KRN8602 caused an increase in oygen consumption to 34.0 nmol/min. Also shown in this table is that oxygen uptake in the presence of both anthracyclines was accompanied by the formation of lipid peroxidation products as determined by the accumulation of TBARS (thiobarbituric acid reactive substances). The formation of TBARS with KRN8602 was almost double that observed with DXR.

DISCUSSION

It is likely that anthracyclines such as DXR perturb cardiac function by a mechanism(s) which ultimately might involve disturbances in calcium homeostasis [13, 14]. The basis for such an effect is in the well-known propensity of quininoid substances to undergo redox cycling, interactions with oxygen and the

Table 1. Effects of DXR and KRN8602 on oxygen consumption and lipid peroxidation (TBARS formation) in hepatic microsomes*

Additions	O_2 (nmol/min/mg protein)	TBARS A_{532}) × 10
None	11.32 ± 2.2	2.26 ± 0.04
DXR (100 μM)	15.60 ± 1.3	4.43 ± 0.04
KRN8602 (100 μM)	34.02 ± 5.3	7.84 ± 0.13

* Incubation tubes contained 0.1 mM Tris–HCl buffer, pH 7.4, to which an NADPH-generating system (NADP, 1.9 mM; glucose-6-phosphate, 20 mM; glucose-6-phosphate dehydrogenase, 1.1 units; $MgCl_2$, 8.6 mM) was added. The reactions were started by the addition of microsomes (1.0 mg protein) and the temperature was maintained at 37°. Values are means ± SEM, N = 10.

formation of oxygen-derived oxidants [9, 15–17]. It has also been demonstrated that quininoid-derived oxidants have profound effects on trans-membrane signaling mechanisms [18, 19] which may lead to alterations in calcium transport. Thus, it is not unreasonable to presume that the formation of free radicals or other oxidants is the basis for the cardiotoxicity of this group of anti-tumor compounds.

It is apparent from our experiments with isolated myocytes that KRN8602 enters the myocyte as evidenced by its effects on intracellular glutathione (Fig. 3). The loss of GSH is mostly accounted for by the appearance of GSSG, suggesting that hydrogen peroxide generated by the drug is adequately detoxified. If this is the case, it is not surprising that no toxicity based on oxidant damage was observed in these cellular preparations (Fig. 2). The limitations of the oxidant theory of anthracycline cardiotoxicity have been described thoroughly by Olson and Mushlin [3]. While it is possible that concepts of redox cycling are not relevant to DXR toxicity, it is also possible that DXR redox activity is localized to areas of the cell, e.g. the plasma membrane, not accessible to detoxifying mechanisms. It should be noted that, in fact, there is ample evidence for the interaction of anthracyclines with the cell surface [20, 21]. Such interactions and the generation of oxidants might well affect signal transduction, alter calcium homeostasis, and trigger myocardial damage.

The cardiotoxicity of anthracyclines might also be divorced from their capacity to affect cancer cells. The mechanism(s) of the latter effects is thought to involve intercalation into DNA. However, in this connection it is of interest that, despite the fact that KRN8602 intercalates calf thymus DNA about 10-fold less than DXR, it is more toxic to cancer cell lines than the latter compound [4]. It is, therefore, possible that the greater potential for redox cycling of KRN8602, once intercalated into DNA, may play a role in cancer cell death if not in cardiotoxicity. In any event, it is clear that anthracycline derivatives such as KRN8602 warrant continued mechanistic and clinical investigation.

Acknowledgements—The authors thank Dr. Enrique Cadenas for helpful discussions during the course of this work which was supported, in part, by grants from the Los Angeles Affiliate of the American Heart Association and the National Institutes of Health (PO1ES03816).

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