(both K_m and K_i values) increased only slightly when the templates used were changed from RNA to DNA, indicating that the inhibition of both steps of reverse transcriptase activity by the triphosphates of 2′,3′-dideoxynucleosides was equally effective. This has great significance in terms of inhibition of HIV replication.

Mitsuya and Broder [3] ranked the inhibitory activities of 2′,3′-dideoxynucleosides against the cytopathic effect of HIV as 2′,3′-dideoxycytidine > 2′,3′-dideoxyadenosine > 2′,3′-dideoxyguanosine > 2′,3′-dideoxythymidine. However, as shown in Table 1, the order of inhibitory activities against HIV reverse transcriptase was ddTTP ≃ ddGTP > ddCTP > ddATP. This lack of correlation between anti-HIV activities of the 2′,3′-dideoxynucleosides and the ability of their triphosphate derivatives to inhibit HIV reverse transcriptase may result from several factors:

(1) The efficiency of the phosphorylation of 2′,3′-dideoxynucleosides to their triphosphate derivatives and the affinities of the triphosphate derivatives to HIV reverse transcriptase may both vary independently. Two compounds, 2′,3′-dideoxycytidine and 3′-azidothymidine, have been shown to be phosphorylated to their triphosphate derivatives in HIV infected and mock infected cell cultures [9–11]. Also the artificial templates/primers used in these assays may not truly reflect the affinities of these triphosphates to HIV reverse transcriptase inside the infected cells.
(2) Metabolic inactivation by deamination (e.g. 2′,3′-dideoxycytidine and its monophosphate derivative are potential substrates for enzymic deamination, 2′,3′-dideoxyadenosine was deaminated by adenosine deaminase at 26% of the rate of deamination of adenosine) and phosphorolysis of the nucleosides could be different.
(3) In addition to their triphosphate derivatives, unknown metabolites of 2′,3′-dideoxynucleosides may also contribute to their anti-HIV activities. One of the anabolites of 2′,3′-dideoxycytidine was reported to be 2′,3′-dideoxycytidine-choline [9, 10].

In summary, the triphosphates of 2′,3′-dideoxythymidine and 2′,3′-dideoxyguanosine were the most potent inhibitors of RNA to DNA synthesis with the other compounds in the following order: ddTTP ≃ ddGTP > IdUTP > ddATP. All the inhibitors showed a slightly lower degree of activity against DNA to DNA synthesis catalyzed by HIV reverse transcriptase.

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REFERENCES

1. S. Broder and R. C. Gallo, *New Engl. J. Med.* **311**, 1292 (1984).
2. H. Mitsuya, K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. N. Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry and S. Broder, *Proc. natn. Acad. Sci. U.S.A.* **82**, 7096 (1985).
3. H. Mitsuya and S. Broder, *Proc. natn. Acad. Sci. U.S.A.* **83**, 1911 (1986).
4. H. Mitsuya, M. Popovic, R. Yarchoan, H. Matsushita, R. C. Gallo and S. Broder, *Science* **226**, 172 (1984).
5. D. Dormont, B. Spire, F. Barre-Sinoussi, L. Montagnier and J. C. Cherman, *Annls Inst. Pasteur, Paris* **136E**, 75 (1985).
6. P. S. Sarin, Y. Taguchi, D. Sun, A. Thornton, R. C. Gallo and B. Oberg, *Biochem. Pharmac.* **34**, 4075 (1985).
7. P. Chandra, A. Vogel and T. Gerber, *Cancer Res.* **45**, 4677s (1985).
8. M. S. Chen, K. L. Woods and W. H. Prusoff, *Molec. Pharmac.* **25**, 441 (1984).
9. D. A. Cooney, M. Dalal, H. Mitsuya, J. B. McMahon, M. Nadkarni, J. Balzarini, S. Broder and D. G. Johns, *Biochem. Pharmac.* **35**, 2965 (1986).
10. M. C. Starnes and Y. Cheng, *J. biol. Chem.* **262**, 988 (1987).
11. P. A. Furman, J. A. Fyfe, M. H. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrmann, D. P. Bolozemsi, S. Broder, H. Mitsuya and D. O. W. Barry, *Proc. natn. Acad. Sci. U.S.A.* **83**, 8333 (1986).

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Effect of daunorubicin on dihydropyridine-sensitive binding sites in cardiac sarcolemma

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Calcium ion movement through voltage-activated channels in the cardiac cell membrane in response to voltage changes is largely responsible for control of the contractile mechanisms in the heart [1]. Any agent that alters the movement of calcium across the cardiac cell membrane can be expected to influence cardiac contractile force and rate. Agents that are positive inotropes and chronotropes and increase the movement of calcium through calcium channels in the cell membrane include the beta-receptor stimulating catecholamines, cyclic AMP, and histamine [1]. The anthracycline antitumor agents, e.g. doxorubicin or daunorubicin, have been shown to inhibit many aspects of cardiac function. They noncompetitively block the positive inotropic or chronotropic action of isoproterenol and histamine [2, 3]. Cardiac sarcolemmal membrane systems are affected adversely by anthracyclines. For example, doxorubicin decreases the activity of adenyl cyclase [4] and calmodulin [5] *in vitro*. Attempts to reverse the acute cardiotoxicity of carminomycin with isoproterenol, digoxin or calcium infusion in the isolated rat heart showed that it can be performed best by an increase in the extracellular calcium ion concentration [6]. In a recent report, Villani *et al.* [7] showed that doxorubicin can acutely inhibit the increase in contractile force produced by increasing con-

centrations of calcium in the isolated guinea pig atrial preparation. The calcium contractile–response curves in the isolated atria were noncompetitively blocked by doxorubicin but competitively blocked by verapamil. Thus, it was suggested that doxorubicin does not block contractile activity through a specific interaction with calcium-channel regulatory receptors in the sarcolemmal membrane.

In this paper, we tested the hypothesis that anthracyclines (represented by daunorubicin) can reduce the number of calcium regulatory binding sites which are represented in part by dihydropyridine-sensitive receptors in the sarcolemmal membrane.

Methods

Hartley guinea pigs (400–500 g) of either sex were obtained from Murphy Laboratories (Plainfield, IN), housed two to four per cage, and allowed food and water *ad lib*.

Acute cardiotoxocity in vitro. Right atria were removed from guinea pigs of either sex and mounted in pairs on glass tissue supports in double-walled 10-ml glass tissue baths (Metroware, Inc.) that contained a modified Tyrode's solution of the following composition (mmol/l): NaCl, 136.8; KCl, 5.37; $CaCl_2$ $(2H_2O)$, 0.05; $MgCl_2$, 0.51; $NaHCO_3$, 11.9; NaH_2PO_4, 0.46; and *d*-glucose, 11.1. The low calcium concentration was the minimal amount that would produce consistent atrial contractions [7]. The solution was aerated with a mixture of 95% oxygen and 5% carbon dioxide and warmed to 37° by water pumped through the outer jacket by a Haake pump. The initial atrial tension was 1 g, and the contractions were recorded with a Grass isometric transducer (FT03C) and displayed by a Grass polygraph (model 79D). After a 1-hr equilibration period in the bath, a cumulative calcium concentration–effect experiment (calcium dihydrochloride, 50 μM to 25.7 mM) was done. The tissues were then washed (5 × 10 ml) and, when the baseline atrial force was re-established, daunorubicin (100 μM) was incubated with the atria for a 1-hr period. At the end of the incubation period, the cumulative calcium dihydrochloride concentration–effect experiment was repeated. The tissues were then washed (5 × 10 ml) and, when the baseline atrial force was re-established, a third calcium concentration–effect experiment was performed in the absence of daunorubicin.

Preparation of cardiac tissue homogenates. The ventricles were removed, weighed and placed in an ice-cold (4°) buffer solution (50 mM Tris–HCl, pH 6.8). Preparation of the partially-purified sarcolemmal membrane fraction followed the procedures described by Karliner *et al.* [8].

Assay of dihydropyridine receptors.

(A) *Displacement studies.* The affinity of nifedipine for PN200-110-labeled binding sites in sarcolemmal membranes was obtained by incubating a fixed concentration of PN200-110 (0.1 nM) with increasing concentrations of unlabeled nifedipine. The concentration at which 50% displacement of PN200-110 occured (IC_{50}) was calculated for nifedipine, and the dissociation constant (K_i) of nifedipine at the PN200-110-labeled receptor was obtained from the equation [9]:

$$K_i = \frac{IC_{50}}{1 + (L)/K_d}$$

where (L) and K_d are the fixed concentrations of PN200-110 and its dissociation constant respectively. By this method we were able to determine the affinity of nifedipine for receptors radiolabeled by PN200-110 and the percentage of specific and nonspecific binding of PN200-110 to the 1,4-dihydropyridine-sensitive receptors in the cardiac sarcolemma.

(B) *Receptor saturation experiments.* The calcium channel ligand (+)-[^{3}N]PN200-110 (sp. act. 80 Ci/mmol) was purchased from the Amersham Corp. All assays were performed in duplicate. The sarcolemmal membranes and PN200-110 (0.1 to 1 nM) were incubated in the dark at 23° for 1 hr in a volume of 1 ml. The amount of PN200-110 specifically bound to the dihydropyridine-sensitive calcium channels in the sarcolemmal membranes was determined by the concurrent incubation of sarcolemmal membranes with nifedipine (1 μM) which served as a "blank". The "blank" for each concentration of PN200-110 was subtracted from total PN200-110 binding to obtain the specific calcium channel binding. The incubations were terminated by rapid, vacuum filtration of each sarcolemmal membrane suspension over a glass fibre filter (Whatman GF/A). Each incubation vial was washed twice with 2-ml aliquots of ice-cold buffer (4°), and the washes were poured over the filter. Filters with adhering sarcolemmal membranes were placed in scintillation vials, and 10 ml of scintillation fluid (ACS, Amersham) was added prior to placement of the vials into a Beckmann model LS133 liquid scintillation system to determine PN200-110 binding at 35–40% efficiency. Counting efficiency was established by the automatic external standard system and a standardized chloroform quench curve prepared with sealed samples purchased from the Amersham Corp. Specifically bound PN200-110 (fmol/mg protein as determined by the Bradford [10] protein-dye binding assay) was recorded, and Scatchard analysis followed by nonlinear regression analysis was used to calculate the dissociation constant (K_d) and the number of binding sites (B_{max}) for radiolabeled PN200-110. Hill coefficients were determined from the B_{max} value obtained in the Scatchard analysis. All calculations were performed on a microcomputer (IBM PC or Olivetti M-24) with the Pharmacological Calculation System (Version 3.3) [11] obtained from Microcomputer Specialists, Inc., Elkins Park, PA (U.S.A.) or with the EBDA program by McPherson [12] supplied by Elsevier-BIOSOFT. A nonlinear least-squares curve-fitting program [13] was used for graphic presentation of the data.

Exposure to daunorubicin in vivo. To determine the effects of exposure to daunorubicin *in vivo* on the dihydropyridine binding sites in cardiac sarcolemma, we performed an experiment according to the methods described by Perkins *et al.* [14, 15]. Guinea pigs were divided into two groups, weighed, and injected i.p. with equal volumes of either saline (control) or a solution of daunorubicin (2 mg/kg). Injections were performed on days 1 and 7 of the experiment, and the animals were killed on day 14. The ventricles were removed and prepared for receptor binding studies as described above.

Results

The effect of daunorubicin on calcium-induced increases in atrial contractile force for two atria are shown in Fig. 1. Daunorubicin noncompetitively inhibited the contractile response to calcium. The inhibitory effect of daunorubicin on contractile force was easily reversed by washing the tissue with Tyrode's solution.

The receptor-specific binding of (+)-[^{3}H]PN200-110, a benzoxadiazol-substituted 1,4-dihydropyridine, to cardiac cell sarcolemma prepared from guinea pig ventricles is shown in Fig. 2. Specific radioligand binding was determined by the displacement of radiolabeled PN200-100 (0.1 nM) with increasing concentrations of nifedipine. Specific PN200-110 binding was 90% of total binding at a nifedipine concentration of 10 nM. The IC_{50} for nifedipine at the PN200-110-labeled calcium channel was 0.1 nM (K_i = 0.08 nM). The concentration of nifedipine added to the "blanks" used to determine nonspecific binding in the equilibrium saturation binding studies was 1 μM.

Results of the dihydropyridine receptor saturation studies with PN200-110 in the absence or presence of daunorubicin are shown in Fig. 3 and Table 1. Pretreatment of cardiac sarcolemmal membranes with daunorubicin (100 μM) for 1 hr reduced the concentration of PN200-110-sensitive binding sites to 55% of the control concentration.

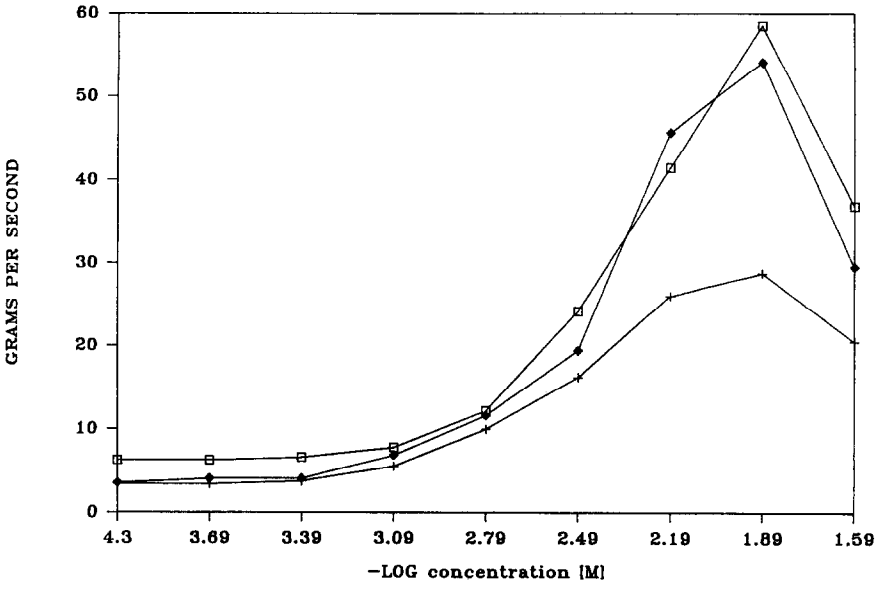

Fig. 1. Effect of daunorubicin on atrial contractile force. The force of contraction was measured in two spontaneously contracting isolated right atria. All measurements were taken when the effect of a particular concentration of calcium had reached a steady state. Concentrations of calcium ranged from 50 μM (baseline concentration Tyrode's solution) to 25 mM. After a 1-hr equilibration period for the atria in the tissue bath, the first concentration–response curve (□) was generated by cumulative addition of calcium to obtain the concentrations shown on the abscissae. The tissues were then washed with Tyrode's solution, and the force of contraction was allowed to return to the previous baseline levels (3–5 min). After a 1-hr incubation with daunorubicin (100 μM), a second calcium concentration–effect curve was run in the presence of daunorubicin (+). The tissues were then washed five times with 10 ml of Tyrode's solution and the final calcium concentration–reponse curve was run (◆).

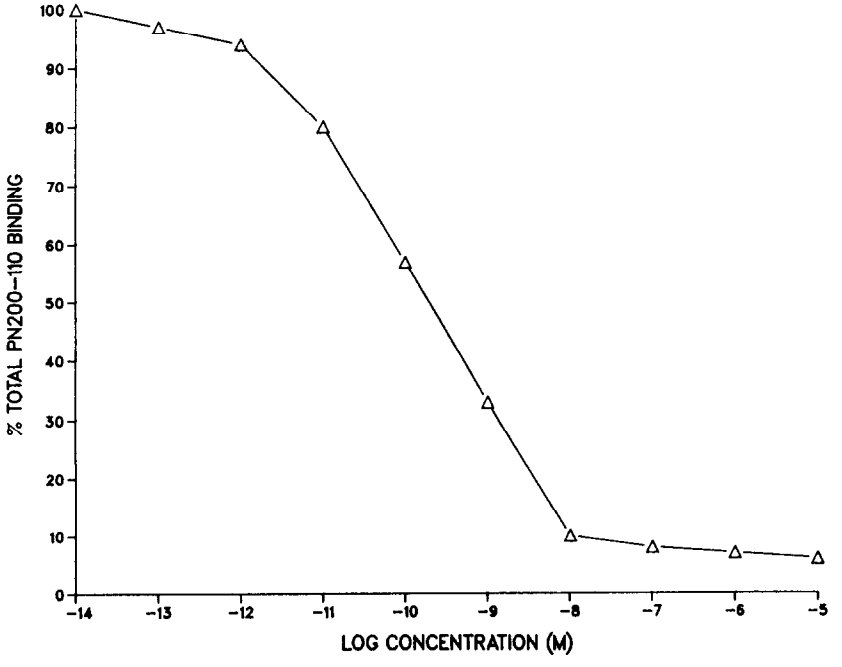

Fig. 2. Competition by nifedipine for binding sites in ventricular sarcolemma labeled by (+)-[^{3}H]PN200-110. Each symbol represents the mean effect in sarcolemma isolated from six ventricles.

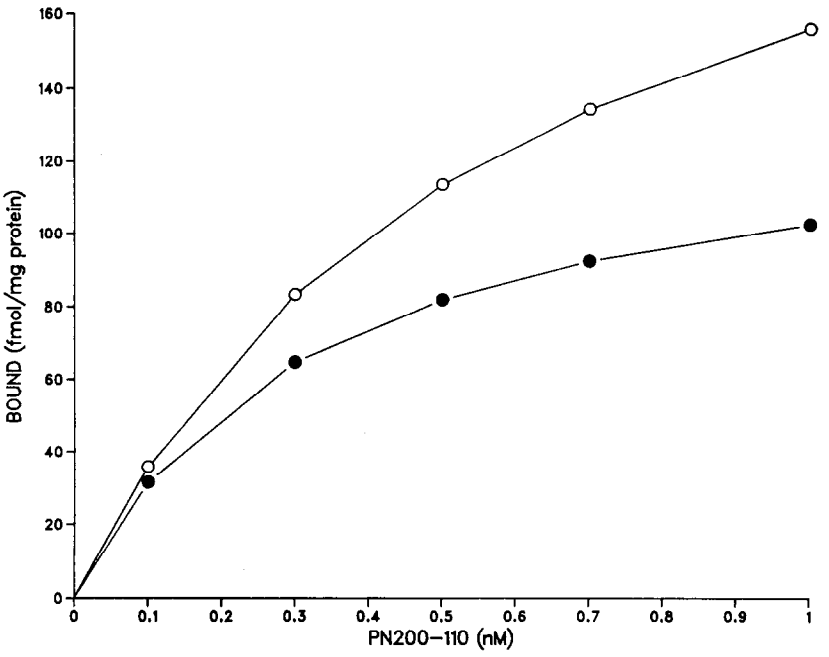

Fig. 3. Effect of daunorubicin on specific (+)-[^{3}H]PN200-110 binding to sarcolemma from guinea pig ventricles. Key: (○) controls; and (●) mean binding of PN200-110 to membranes pretreated with daunorubicin (100 μM, 1 hr). Specific PN200-110 binding ranged from 90 to 74% of total binding. Six ventricles were used for each curve.

Table 1. Specific (+)-[^{3}H]PN200-110 binding in sarcolemmal membranes of guinea pig ventricles in the absence or presence of daunorubicin

	K_d (nM)	B_{max} (fmol/mg protein)	Hill coefficient
Control	0.59 ± 0.02	247.2 ± 9.4	1.03 ± 0.03
Daunorubicin (1 μM)	0.31 ± 0.07	265.8 ± 24.8	1.01 ± 0.04
Daunorubicin (100 μM)	0.33 ± 0.05	136.34 ± 10.1*	0.99 ± 0.10

Data represent the mean ± SE. Six guinea pig ventricles were used for each treatment.
* Statistically significant decrease from control ($P < 0.05$, df = 15, two-tailed Dunnett's test).

A lower concentration of daunorubicin (1 μM) did not change significantly the concentration of binding sites in the sarcolemmal membranes for PN200-110 (Table 1).

The injection of guinea pigs with a cumulative dose of 4 mg/kg daunorubicin did not cause a change in the concentration of dihydropyridine-sensitive binding sites in the cardiac sarcolemma. At the end of the 14-day study in saline-treated guinea pigs (N = 8), the B_{max} for PN200-110 was 316.2 ± 24.8 fmol/mg protein and the K_d was 0.54 ± 0.3 nM. In the daunorubicin-treated guinea pigs (N = 8), the B_{max} was 319.5 ± 4.9 fmol/mg protein and the K_d was 0.37 ± 0.2 nM. The general toxicity of the dose regimen was evident in the lack of weight gain for the daunorubicin-treated animals (day 1: 416.3 ± 8.8 g; day 14: 368.3 ± 18.8 g) as compared to the saline-treated animals (day 1: 430.5 ± 8.9 g; day 14: 515.4 ± 14.2 g). Mean ventricle weight in the daunorubicin-treated animals was significantly lower at 1.16 ± 0.04 g versus 1.49 ± 0.03 g in saline-treated animals ($P < 0.05$, df = 14, two-tailed t-test).

Discussion

We investigated the interaction of daunorubicin, an anthracycline antitumor agent, with dihydropyridine-sensitive binding sites in the cardiac sarcolemma. These binding sites are partly responsible for the regulation of calcium movement through the "slow calcium channels" found in the sarcolemma [1]. Treatment of the sarcolemmal preparation *in vitro* with daunorubicin decreases the concentration of dihydropyridine-sensitive binding sites and may thereby reduce the number of calcium channels. However, the reason for the increase in apparent affinity of radiolabeled PN200-110 for the remaining dihydropyridine-sensitive binding sites cannot be determined from these data. Thus, the only action of daunorubicin on dihydropyridine-sensitive sites appears to be noncompetitive, i.e. causing a nonreceptor specific decrease in dihydropyridine binding sites. The effect of daunorubicin on atrial contractile force *in vitro* was similar to the action of doxoru-

bicin reported by Villani *et al.* [7] in which doxorubicin (200 μM) acutely decreased the maximal contractile response to calcium with little change in the apparent affinity of the calcium receptive sites in the isolated atrial preparations for calcium. The low and high daunorubicin concentrations (1 and 100 μM) used in the present experiments were near the range of anthracycline concentrations observed in patients during infuson of these drugs (range = 7 to 70 μM, plasma concentrations reported for doxorubicin) [16]. The mechanisms behind these effects are not yet definable. It may be speculated that changes in membrane fluidity [17] induced as the anthracycline attaches to lipid or protein structures [18] in the cell membrane can lead to a decrease in the number of dihydropyridine binding sites and alter calcium channels. There is evidence that lipid peroxidation does not play an important role in the acute phase of doxorubicin-induced cardiotoxicity [19]. It is likely that the changes in dihydropyridine binding are only related to the "acute" phase of exposure to anthracyclines since the inhibitory effect of daunorubicin *in vitro* was rapidly reversed upon washout of the atrial tissue with fresh Tyrode's solution. In addition, there was no evidence for a long-lasting change in the PN200-110 binding to dihydropyridine-sensitive binding sites in cardiac sarcolemma exposed to cumulative doses of daunorubicin (4 mg/kg) *in vivo*.

In summary, the acute effects of an anthracycline antitumor agent, daunorubicin, on the slow calcium channels in cardiac sarcolemma were examined with the aid of a radiolabeled 1,4-dihydropyridine derivative, (+)-[^{3}H]PN200-110. Daunorubicin noncompetitively reduced the concentration of PN200-110-sensitive binding sites. Daunorubicin may inhibit the positive inotropic and chronotropic effects of calcium or neurotransmitters by the disruption of slow calcium channel function in cardiac tissues.

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REFERENCES

1. W. Nayler and J. Horowitz, *Pharmac. Ther.* **20**, 203 (1983).
2. J. Less and J. Banning, *Pharmacologist* **27**, 150 (1985).
3. R. Jensen, *J. Pharmac. exp. Ther.* **236**, 197 (1986).
4. P. Singal and V. Panagia, *Res. Commun. Chem. Path. Pharmac.* **43**, 67 (1984).
5. R. Nwankwoala and W. West, *Cancer Chemother. Pharmac.* **16**, 133 (1986).
6. S. Saman, P. Jacobs and L. Opie, *Cancer Res.* **44**, 1316 (1984).
7. F. Villani, E. Monti, F. Piccinini, L. Favalli, A. Dionigi and E. Lanza, *Biochem. Pharmac.* **35**, 1203 (1986).
8. J. Karliner, P. Barnes, M. Brown and C. Dollery, *Eur. J. Pharmac.* **67**, 115 (1980).
9. Y. Cheng and W. Prusoff, *Biochem. Pharmac.* **22**, 3099 (1973).
10. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
11. R. Tallarida and B. Murray, *Manual of Pharmacologic Calculations with Computer Programs*. Springer, New York (1985).
12. G. McPherson, *J. pharmac. Meth.* **14**, 213 (1985).
13. R. Duggleby, *Analyt. Biochem.* **110**, 9 (1981).
14. W. Perkins, R. Schroeder, R. Carrano and A. Imondi, *Br. J. Cancer* **46**, 662 (1982).
15. W. Perkins, R. Schroeder, R. Carrano and A. Imondi, *Cancer Treat. Rep.* **68**, 841 (1984).
16. B. Maisch, O. Gregor, M. Zeuss and K. Kochsiek, *Basic Res. Cardiol.* **80**, 626 (1985).
17. J. Sigfried, K. Kennedy, A. Sartorelli and T. Tritton, *J. biol. Chem.* **258**, 339 (1983).
18. G. Yee, M. Carey and T. Tritton, *Cancer Res.* **44**, 1898 (1984).
19. E. Porta, N. Joun, L. Matsumura, B. Nakasone and H. Sablan, *Res. Commun. Chem. Path. Pharmac.* **41**, 125 (1983).

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Lack of inhibition of mouse catalase activity by cimetidine: an argument against a relevant general effect of cimetidine upon heme metabolic pathways

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Cimetidine, an H_2-histamine receptor antagonist, is used clinically in the treatment of a variety of conditions that respond to an increase in gastric pH, and is generally considered to have no serious side effects or contraindications. However, early studies demonstrated a weak, antiandrogenic effect in rodents that was also observed in humans treated for gastric hypersecretory states [1]. A number of clinical studies have shown that cimetidine alters the effective levels of a large number of widely prescribed pharmaceuticals including propanolol [2], diazepam [3], digitoxin and quinidine [4], and procainamide [5], several of which exhibit very narrow therapeutic indices and possess extreme potential for toxicity.

Cimetidine reduces both *in vitro* and *in vivo* hepatic mixed-function oxidase activity for a wide variety of substrates in the rat [6–8]. The appearance of a typical type II spectral change in rat liver microsomes incubated in the presence of cimetidine suggested a direct interaction of the compound with cytochrome P-450 [7]. Furthermore, several groups have demonstrated the presence of a high affinity binding site for cimetidine on cytochrome P-450 in liver microsomes obtained from both humans and rats [9, 10], with both the imidazole and cyano portions of cimetidine interacting with the hemin iron [11]. A binding site on cytochrome P-450 apparently does not exist for ranitidine, a structurally dissimilar histamine H_2 antagonist which does not inhibit hepatic mixed-function oxidases, thus prompting the suggestion that cimetidine alters the oxidative metabolism of other compounds by exerting a direct inhibitory effect on cytochrome P-450.

However, the results of yet other studies suggest that cimetidine may affect hepatic mixed-function oxidase