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The Effect of *in Vivo* Doxorubicin (Adriamycin) and Aclacinomycin Administration on Guanylate Cyclase Activity in Rat Tissues[1] (41197)

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Abstract. Doxorubicin is an important antitumor agent whose long-term administration results in severe cardiotoxic reactions. In a recent publication, we reported that doxorubicin inhibited guanylate cyclase activity in human and rat heart *in vitro*, and also lowered cyclic GMP levels in tissue slices. These effects were specific for cardiac tissue. In the present study we administered doxorubicin *in vivo* to male Sprague–Dawley rats and measured guanylate cyclase activity at various times after treatment in several tissues. We found that enzyme activity remained unchanged up to 4 hr after injection of the drug. By 24 hr, however, guanylate cyclase activity in rat heart was depressed and remained so for at least 72 hr, the longest time measured. Inhibition was dose dependent, and significant reduction in enzyme activity was observed in kidney, liver, and lung, as well as in heart with a 10 mg/kg dose. In contrast, the injection of aclacinomycin, a structurally related analog of doxorubicin, had no effect on guanylate cyclase activity in any tissue, even at 40 mg/kg. Other cardiotoxic analogs such as 4-epi-doxorubicin were also inhibitory, whereas 7-OMEN and Quelamycin, each at 20 mg/kg after 24 hr, had no effect. The mechanism of the inhibition of guanylate cyclase by doxorubicin and its relationship to cardiotoxicity remains to be elucidated.

The anthracycline antibiotic doxorubicin (Adriamycin) is an effective chemotherapeutic agent in the treatment of a variety of solid tumors. Its clinical usefulness has unfortunately been limited by the appearance of a variety of cardiotoxic effects including arrhythmias and heart block, as well as a late onset, dose-dependent cardiomyopathy which may result in severe, sometimes irreversible congestive heart failure (1–3).

We have recently demonstrated that doxorubicin causes a selective inhibition of rat and human cardiac guanylate cyclase activity *in vitro* (4). In contrast, the structural analog aclacinomycin and 7-OMEN, which have 1/10 to 1/15 the cardiotoxicity of doxorubicin, did not decrease rat cardiac guanylate cyclase activity *in vitro* at concentrations at which doxorubicin produced a 40–50% decrease in guanylate cyclase activity (5).

These findings gave support to our hypothesis that cardiac guanylate cyclase activity plays a role in anthracycline-induced cardiotoxicity. Since all our earlier observations were obtained *in vitro*, the effect of *in vivo* injections of doxorubicin on guanylate cyclase activity in heart and other tissues was determined. The present report describes the effect of doxorubicin, aclacinomycin 4-epi-doxorubicin, 7-OMEN, and quelamycin on guanylate cyclase activities of several rat tissues after *in vivo* administration.

Methods. Doxorubicin, aclacinomycin, 4-epi-doxorubicin, 7-OMEN, and quelamycin were dissolved in dimethyl sulfoxide (DMSO) and diluted with saline. Sprague–Dawley, 150-g male rats were injected with a single dose of either the DMSO–saline control, or one of the drugs intraperitoneally in a total volume of 0.5 to 1.0 ml in the doses indicated in the results. Doses higher than 40 mg/kg of doxorubicin could not be administered because at such doses many animals died before the end of the experiment. At the times indicated, the animals were sacrificed by cervical dislocation, various tissues removed, and homogenized in cold 30 m*M* Tris–HCl,

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pH 7.6, buffer. Guanylate cyclase activity was measured as described previously (6) in the supernatant obtained after the tissue homogenate was centrifuged at 37,000*g* in a Sorval refrigerated centrifuge at 4° for 15 min.

The supernatant was assayed at 37° for 10 min for guanylate cyclase activity, using a reaction mixture consisting of 20 m*M* Tris–HCl, pH 7.6; 5 m*M* $MnCl_2$; 2.67 m*M* cyclic GMP; a GTP regenerating system (5 m*M* creatine phosphate, 11.25 U creatine phosphokinase); 100 μg serum albumin; 1 m*M* 3-isobutyl-1-methylxanthine; and 1.2 m*M* [α-^{32}P]GTP (International Chemical and Nuclear Corporation, Irvine, Calif.), approximately 5×10^5 cpm. The enzyme preparation contained about 50 to 200 μg protein. The reaction was terminated by the addition of 10 μl of 30 m*M* EDTA, pH 7.6, containing about 30,000 cpm of cyclic-[^{3}H]GMP (to estimate recovery in subsequent steps) and boiling for 3 min. After cooling in an ice bath, the cyclic-[^{32}P]GMP was isolated by sequential chromatography on Dowex 50-H^+ and alumina using the modification of Krishna and Krishnan (6) and counted in a Packard Tri-Carb Liquid Scintillation spectrometer. The overall recovery of cyclic GMP after the two-stage chromatographic procedure was 70–80%. Blank ^{32}P ± counting rates averaged 30–40 cpm.

Statistical analysis was performed using the unpaired Student *t* test. The results are presented as the mean ± standard deviation of *n* observations. The mean, standard deviation, the number of replicates, and significant differences are given in the results section.

Results. *In vivo* administration of doxorubicin was followed by measurement of guanylate cyclase activity from rat heart at various times after injection. Table I shows that up to 4 hr after the drug injection, guanylate cyclase activity remains unchanged. By 24 hr, however, the activity of the enzyme is significantly depressed, and this reduced activity is observed for at least 72 hr after treatment, the longest time measured. Longer time periods were not studied systematically because too many animals died as a result of the drug treatment. In the animals that survived for longer periods, there was a tendency for a reduction of the inhibition; this effect was not evaluated statistically due to the insufficient number of animals.

TABLE I. RAT HEART GUANYLATE CYCLASE AT DIFFERENT TIMES AFTER DOXORUBICIN TREATMENT

	pmole cyclic GMP/mg protein/ 10 min
Control	187 ± 15
Doxorubicin (20 mg/kg)	
4 hr	180 ± 20
24 hr	140 ± 17*
48 hr	140 ± 12*
72 hr	128 ± 18*

Note. $n = 6 \pm$ SD, * $P < 0.05$.

As an additional control to test the specificity of the effect reported here, we measured adenylate cyclase and phosphodiesterase activities in heart and liver in controls and animals treated with 20 mg/kg doxorubicin for 24 hr. There was no difference between the controls and the treated group with any of the enzyme tested.

The inhibition of the guanylate cyclase enzyme was dose dependent as shown in Table II. The highest dose used (40 mg/kg) was more effective than the lowest dose. Enzyme inhibition after a 10 mg/kg dose is significantly different from the value obtained after 40 mg/kg. Both are also significantly below the control. Since the *in vitro* effects reported previously were present only in the heart, we were interested to see if this specificity is retained after *in vivo* administration. Table III indicates that 24 hr after doxorubicin treatment, guanylate cyclase activity is significantly below con-

TABLE II. RAT HEART GUANYLATE CYCLASE 24 hr AFTER DOXORUBICIN TREATMENT

	pmole cyclic GMP/mg protein/10 min
Control	198 ± 20
Doxorubicin 10 mg/kg	158 ± 14*
Doxorubicin 20 mg/kg	148 ± 17*
Doxorubicin 40 mg/kg	132 ± 17*

Note. $n = 6 \pm$ SD, *$P < 0.05$.

TABLE III. RAT TISSUE GUANYLATE CYCLASE IN DIFFERENT TISSUES 24 hr AFTER DOXORUBICIN TREATMENT

Tissue	Control	Doxorubicin (20 mg/kg)
Heart	185 ± 26[a]	120 ± 21*
Liver	220 ± 29	139 ± 17*
Kidney	650 ± 81	494 ± 64*
Spleen	700 ± 78	630 ± 58
Lung	3780 ± 320	3062 ± 215*
Uterus	526 ± 67	368 ± 42*

[a] pmole cyclic GMP/mg protein/10 min.
* $P < 0.05$, $n = 6 \pm$ SD.

trol activities in a variety of tissues. This is in contrast to the *in vitro* effects, where inhibition is observed in homogenates from heart tissue only. The development of inhibition in tissues other than heart is shown in Table IV. It is evident that similar to heart, inhibition is absent at 4 hr, but is fully developed by 24 hr, and is maintained for at least 72 hr.

In order to evaluate the specificity of the *in vivo* effect of doxorubicin, we measured guanylate cyclase activity following *in vivo* treatment of the experimental animals with aclacinomycin, a structurally related analog of doxorubicin (7, 8). Enzyme activity was measured 24 and 48 hr after injection of the drug and the doses were from 0 to 40 mg/kg. Guanylate cyclase activities remained unchanged, when compared to the controls, at all times and doses tested both in heart and in liver. For representative data, see Table V. Additional analogs, namely, 4-epi-doxorubicin, 7-OMEN, and quelamycin were also tested. Due to the very limited

TABLE IV. RAT LIVER GUANYLATE CYCLASE AT DIFFERENT TIMES AFTER DOXORUBICIN TREATMENT

	pmole cyclic GMP/mg protein/10 min
Control	230 ± 32
Doxorubicin (20 mg/kg)	
4 hr	220 ± 42
24 hr	139 ± 26*
48 hr	112 ± 28*
72 hr	150 ± 35*

Note. $n = 6 \pm$ SD. *$P < 0.05$.

TABLE V. HEART AND LIVER GUANYLATE CYCLASE ACTIVITY 24 hr AFTER ACLACINOMYCIN TREATMENT

Tissue	Control	Aclacinomycin (40 mg/kg)
Heart	168 ± 24*	158 ± 34
Liver	210 ± 37	244 ± 36

* pmole cyclic GMP/mg protein/10 min. $n = 6 \pm$ SD.

amount of these substances available, we measured only a 20 mg/kg dose. The animals were exposed to the drugs for 24 hr before enzyme activity was measured. 4-epi-doxorubicin was found to inhibit guanylate cyclase in heart, while 7-OMEN and quelamycin had no effect. These data are presented in Table VI.

Discussion We have previously demonstrated that inhibition of cardiac guanylate cyclase activity *in vitro* in rat and human is a specific effect (4). After *in vivo* administration there was no inhibition at times zero, or 4 hr after drug treatment. By 24 hr, however, guanylate cyclase activity was inhibited by doxorubicin and 4-epi-doxorubicin, not only in the heart but also in several other tissues as well. Aclacinomycin on the other hand had no effect at any dose tested at any time following *in vivo* treatment, and neither did quelamycin or 7-OMEN at 20 mg/kg. Although these agents are inhibitors of DNA and protein synthesis, the lack of effect on guanylate cyclase suggests that the inhibition of the enzyme is not due to reduced synthesis or turnover, although these have not been measured; rather, it must be due to some other effect, the mechanism of which is unclear at present.

TABLE VI. RAT HEART GUANYLATE CYCLASE 24 hr AFTER TREATMENT WITH SEVERAL ANALOGS OF DOXORUBICIN

Control	175 ± 18[a]
4-epi-doxorubicin (20 mg/kg)	125 ± 16[b]
7-OMEN (20 mg/kg)	156 ± 12
Quelamycin (20 mg/kg)	161 ± 16

Note. $n = 3 \pm$ SD.
[a] pmole cyclic GMP/mg protein/10 min.
[b] Significantly different from control, $P < 0.05$.

Although a 40 mg/kg dose of doxorubicin cannot be compared directly to a 40 mg/kg dose of aclacinomycin, it has been reported that aclacinomycin has about 1/10 to 1/15 the cardiotoxicity of doxorubicin (7). Our results indicate that both *in vitro* (4) and *in vivo* much smaller doses of doxorubicin than aclacinomycin are required to bring about inhibition of guanylate cyclase in rat tissues. These results are consistent with the findings of other investigators relating the relative cardiotoxicities of the two agents. Although 4-epi-doxorubicin, quelamycin, and 7-OMEN were tested at one dose only, the highly cardiotoxic 4-epi analog was inhibitory, while the less toxic analogs quelamycin and 7-OMEN had no effect on heart guanylate cyclase. These data give further support to the hypothesis that cardiotoxic analogs of doxorubicin are better inhibitors of guanylate cyclase than noncardiotoxic ones.

The inhibition of guanylate cyclase from several tissues after *in vivo* administration of doxorubicin indicates a lack of the issue specificity seen *in vitro*. The fact that the depression in enzyme activity is not apparent for several hours after administration of the drug suggests that cellular uptake, metabolism, and disposal may be important in determining the effect of these agents. The mechanisms by which doxorubicin inhibits guanylate cyclase *in vitro* and *in vivo* and how this relates to the cardiotoxicity seen after large doses are unclear and will require further experiments to elucidate.

1. Bristow, M. R., Billingham, M. E., Mason, J. W., and Daniels, J. R., Cancer Treat. Rep. **62**, 873 (1978).
2. Ferrans, V. J., Cancer Treat Rep. **61**, 955 (1978).
3. Minow, R. A., Benjamin, R. S., and Gottlieb, G. A. Cancer Chemother. Rep. **3**, 195 (1975).
4. Levey, G. S., Levey, B. A., Ruiz, E., and Lehotay, D. C., J. Mol. Cell. Cardiol. **11**, 591 (1979).
5. Levey, B. A., Ruiz, E., Lehotay, D. C., and Levey, G. S., Cancer Treat Rep., in press.
6. Krishna, G., and Krishnan, N., Anal. Biochem. **70**, 18 (1976).
7. Hori, S., Shirai, M., Hirano, S., Oki, T., Inui, T., Tsukagoshi, S., Ishizuka, M., Takeuchi, T., and Umezawa, H., Gann **68**, 685 (1977).
8. Ogawa, M., Inagaki, J., Horikoshi, N., Inoue, K., Chinen, T., Ueoka, H., and Nagura, E., Cancer Treat. Rep. **63**, 931 (1979).

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