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Relative cardiotoxicity and cytotoxicity of anthraquinonyl glucosaminosides

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Summary. The cardiotoxicity and cytotoxicity for tumor cells of four new synthetic anthraquinonyl glucosaminosides were compared in vitro. The nonhydroxylated anthraquinone was not cardiotoxic, and its cytotoxic activity was the weakest of the compounds in the series. Increasing the number of hydroxyl groups on the anthraquinone moiety increased the inhibition of growth of L-1210 leukemia cells and pancreatic or colonic adenocarcinomas in a soft agar colony formation assay. However, cardiotoxicity was also increased in proportion to the number of hydroxyl groups present. The adenocarcinomas were slightly more sensitive than the leukemias to the inhibitory action of the dihydroxylated anthraquinonyl glucosaminosides on cell growth.

Introduction

Daunorubicin (Daunomycin) and doxorubicin (Adriamycin) are listed among the most effective antineoplastic agents available for the treatment of acute myelocytic and lymphocytic types of leukemia. They are also used alone or together with surgical resection and/or radiotherapy in the treatment of many other cancers such as Ewing's sarcoma, osteogenic sarcoma, small cell carcinoma of the lung, diffuse histiocytic lymphoma, and carcinoma of the breast, to name just a few [1]. However, a serious limitation to the use of these antineoplastic agents is the risk of acute or chronic cardiotoxicity. Acute cardiotoxicity appears as arrhythmias, which can be avoided by using reduced dose levels per injection. Signs of acute cardiotoxicity usually do not warrant withdrawal of the drug [1, 3]. Chronic cardiotoxicity, which occurs in about 30% of patients who receive cumulative doses that exceed 550 mg/m^2 body surface area, appears as a severe congestive heart failure that may become irreversible [3].

The B, C, and D rings in daunorubicin (equivalent to the anthraquinone nucleus) and the oxygenated functionalities contained therein may be viewed as playing key roles in determining the biological properties of the anthracyclines. Among other things, this portion of the molecule is responsible for intercalation into DNA and for the redox characteristics associated with this class of antitumor agents [14]. In an effort to assess the relative contributions played by the oxygen functionalities on the B and D rings, we have prepared a series of anthraquinonyl glucosaminosides (Fig. 1, compounds I–IV) for use as water-soluble model compounds to study the effect of hydroxyl substitution on relative cardiotoxicity and cytotoxicity for tumor cells in vitro. The daunosamine analogue of compound I has been studied by Henry [11] for its ability to inhibit nucleic acid synthesis in cultured L1210 cells and for its effect on thermal denaturation of calf thymus DNA. However, no attempt was made to assess the role of sequential hydroxyl substitution on the chromophore in influencing these biological properties.

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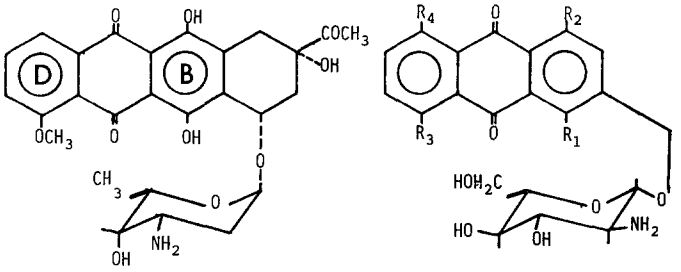
Materials and methods

Cardiotoxicity in vitro. Numerous small-animal models for the evalutaion of anthracycline-induced cardiotoxicity have been described in a recent literature review [9]. Recently, Perkins et al. [15, 16] developed a sensitive functional type of screening method using the guinea pig right atrium in vitro. We have adapted Perkin's method for use in our laboratory to evaluate the relative cardiotoxicity of new anthraquinones compared with daunomycin. We constructed complete dose-effect curves for positive chronotropes such as histamine before and after a 3-h incubation with an anthraquinone. This permitted a complete pharmacological evaluation of the parameters of the dose-effect relationship, namely the maximal response, pD_2 and slope of the log dose-effect curve. The pD_2 was the negative log of the histamine concentration that produced one-half the maximal increase in atrial contraction rate. The pD_2 was obtained by computer analysis of each dose-effect curve using the Pharmacological Calculation System (Version 3.3).

Right atria were removed from guinea pigs of either sex (Hartley strain, 400–500 g, supplied by Murphy Labs., Plainfield, Ind, USA) and mounted on glass tissue supports in double-walled 10-ml glass tissue baths (Metroware, Inc.) that contained Kreb's solution of the following composition (mol/l): NaCl, 118; KCl, 4.6; $CaCl_2$, ($2H_2O$), 2.5; $MgSO_4$ ($6H_2O$), 0.5; $NaHCO_3$, 24.9; NaH_2PO_4, 7; and *d*-glucose, 11.1. The solution was aerated with a mixture of 95% oxygen and 5% carbon dioxide and warmed to 37 °C by water pumped through the outer jacket by a Haake pump. The initial atrial tension was 0.5 g, and the contrac-



DAUNORUBICIN

	R_1	R_2	R_3	R_4
I	H	H	H	H
II	OH	H	H	H
III	OH	OH	H	H
IV	OH	H	OH	H

Fig. 1. Comparison of the chemical structures of daunorubicin and the anthraquinonyl glucosaminosides. The chemical structure of 4-demethoxydaunorubicin (not shown) differs from daunorubicin where the methoxy group on the D ring is replaced by $-H$

tions were recorded with a Grass isometric transducer (FTo3C) and displayed by a Grass polygraph (model 79D). Contraction rate was measured by a Grass tachograph (model 7P4F) triggered by the interval between atrial contractions. When atrial rate was stabilized in the baths, a cumulative dose–effect curve to histamine dihydrochloride (10 n*M* to 0.3 m*M*) was performed. The tissues were then washed (5 × 10 ml), and when a baseline atrial rate was re-established an anthraquinone (13–130 μ*M*) was incubated with the atria for a 3-h period. At the end of the incubation period, the cumulative histamine dose–effect curve was repeated with freshly prepared histamine solutions. The responses were expressed graphically as the percentage change in atrial rate from the baseline rate and recorded as the mean ± SEM, for each dose of histamine.

The paired *t*-test was used for comparison of tissue responses to histamine before and after incubation with an anthraquinone antibiotic and for statistical analysis of any change in baseline atrial rate produced by the anthraquinones. Comparisons between atrial preparations were performed with the aid of the unpaired *t*-test. A statistically significant difference between means was indicated by $P < 0.05$. All statistical tests were performed on an IBM PC microcomputer using the Pharmacological Calculation System (Version 3.3) written by Tallarida and Murray [18] and supplied on a diskette by Microcomputer Specialists, Inc. (Elkins Park, Pa, USA).

Anthraquinonyl glucosaminosides used in these studies were synthesized in the medicinal chemistry section of the Dept. of Pharmaceutical Sciences. Details of the syntheses have been described elsewhere [2]. Daunorubicin HCl and histamine 2HCl were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). 4-Demethoxydaunorubicin HCl (idarubicin, NSC 256439) was generously provided by the USPHS NIH NCI Natural Products Branch, Division of Cancer Treatment.

Cytotoxicity for tumor cells in vitro. The test system was a soft-agar colony-formation assay in which cytotoxicity was determined for a leukemia (L-1210) and one or more solid tumor cell lines (colon adenocarcinoma ≠38, pancreatic ductal adenocarcinoma ≠03). The assay procedure described previously [5–8] was a simple variation of the Kirby-Bauer disk diffusion antibiotic test used in microbiology.

Table 1. Right atrial contraction rate before and 3 h after incubation with various anthraquinones

Compound	Concentration (μ*M*)	Atrial contraction rate[a] Mean (± SE)	
		Before	After
Daunorubicin	13	213 (8)	210 (15)
	130	204 (8)	168[b] (4)
4-Demethoxy-daunorubicin	1.3	265 (7)	245 (7)
	13	215 (16)	224 (14)
	130	218 (7)	173[b] (8)
I	130	246 (2)	270[c] (4)
II	130	209 (5)	136[b] (5)
III	13	199 (8)	198 (9)
	130	198 (11)	155[b] (6)
IV	13	198 (2)	186 (4)
	130	218 (14)	156[b] (2)

[a] Mean of four or five experiments
[b] Statistically significant decrease in baseline atrial contraction rate from control ($P < 0.05$, *t*-test)
[c] Statistically significant increase in baseline atrial contraction rate from control ($P < 0.05$, *t*-test)

Results

Effects of anthraquinones on baseline atrial contraction rate

The effects of a 3-h incubation with various anthraquinones on baseline atrial contraction rates are shown in Table 1. At a concentration of 13 μ*M* or less these anthraquinones did not change the baseline atrial contraction rate. At a higher concentration (130 μ*M*), all the hydroxyl-substituted anthraquinones reduced the atrial contraction rate. In contrast, compound I (the nonhydroxylated anthraquinone) increased the atrial contraction rate.

Inhibition of histamine-induced increases in atrial contraction rate by hydroxyl-substituted anthraquinones

The responsiveness of the right atrial preparation to histamine was relatively stable over a 3-h period between cumulative dose–effect curves, as depicted in Fig. 2 for a single experiment.

Daunorubicin (130 μ*M*, 3 h) reduced the maximal right atrial positive chronotropic response to histamine (Fig. 3A). However, the pD_2 for histamine (5.848 ± 0.044) was not significantly different (6.211 ± 0.225) after this dose of daunorubicin. With a lower dose of daunorubicin (13 μ*M*) the mean maximal contraction rate produced by histamine was slightly less than in controls, but the differences were not statistically significant ($P < 0.05$, paired *t*-test). These results were similar to data reported by Perkins et al. [15, 16], in which doxorubicin (130 μ*M*, 3 h) reduced the right atrial responsiveness to a single dose of histamine (3.6 μ*M*) by 36%, but 43.1 μ*M* of this anthracycline did not significantly reduce right atrial responses to histamine. Therefore, we initially examined the cardiotoxicity of all the anthraquinonyl glucosaminosides at a concentration of 130 μ*M*.

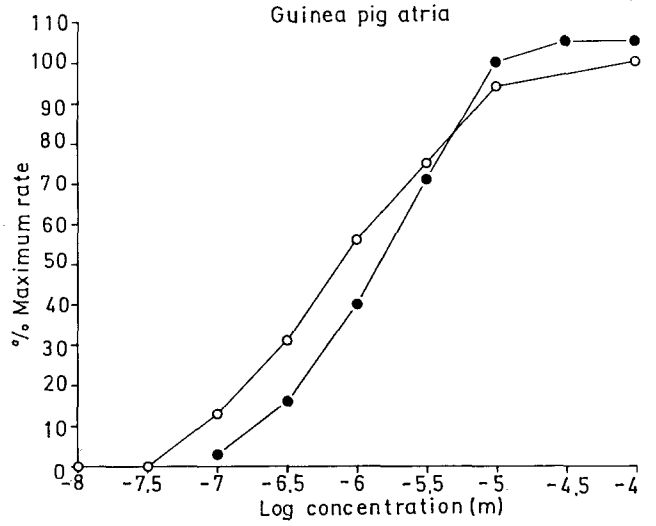


Fig. 2. Positive chronotropic response of an isolated guinea pig right atrium to histamine dihydrochloride: *Second curve* (●) was produced 3 h after *first* (control) *curve* (○) and demonstrates the stability of this preparation over a typical 3-h incubation period

Idarubicin is more closely related to the anthraquinonyl glucosaminosides than is daunorubicin, because of demethoxylation of the anthraquinone moiety. Therefore, we also compared the cardiotoxicity of this anthraquinone to the cardiotoxicity of the anthraquinonyl glucosaminosides. Idarubicin (13 or 130 μM, 3 h) reduced the maximal positive chronotropic effect of histamine (Fig. 3B). The pD_2 for histamine (5.903 ± 0.034) was virtually unchanged by either concentration of idarubicin (13 μM: 5.909 ± 0.365; 130 μM: 5.696 ± 0.148). The lowest concentration of idarubicin (1.3 μM) did not decrease the maximal atrial response to histamine, but it did produce about a 4-fold parallel shift to the right in the dose–effect relationship for histamine ($pD_2 = 5.282 \pm 0.054$). Thus, the lowest dose of idarubicin (1.3 μM) appeared to competitively inhibit the action of histamine on the atria, but higher concentrations were noncomepetitively inhibitory to histamine.

The nonhydroxylated anthraquinonyl glucosaminoside (compound I, Fig. 4) did not exhibit cardiotoxicity in the isolated atrial preparation. However, the introduction of one hydroxyl group into the anthraquinone nucleus (compound II) significantly reduced the maximal response to histamine (Fig. 5) with virtually no change in the pD_2 (control: 5.98 ± 0.06; after compound II: 6.13 ± 0.1).

Compound III (130 μM) also reduced atrial responsiveness to histamine (Fig. 6). The pD_2 for histamine (5.76 ± 0.18) was not altered after compound III (5.65 ± 0.10). At a lower concentration (13 μM), compound III reduced the maximal response to histamine to $78\% \pm 2.5\%$ of control ($P < 0.05$, t-test) (data not shown) with virtually no change in the pD_2 for histamine (control: 5.93 ± 0.033; after compound III: 5.875 ± 0.053).

Compound IV (130 μM, 3 h) was the most effective inhibitor of histamine-induced increases in atrial contraction rate (Fig. 7). A 10-fold lower concentration of compound IV (13 μM) reduced the mean maximal response to 60% of control. Therefore, at about equally inhibitory concentrations (50%–60% of control) for either the anthraquinones or anthracyclines, compound IV was 10-fold more potent as an inhibitor of atrial reponsiveness to histamine than either daunorubicin or idarubicin. Compound IV did not, however, alter the apparent affinity of histamine for the atrial chronotropic receptors (Fig. 7), since there was no significant change in the pD_2 (control: 5.84 ± 0.04; after 13 μM: 5.64 ± 0.1; after 130 μM; 6.09 ± 0.9).

Cytotoxicity of the anthraquinonyl glucosaminosides for tumor cells in vitro

The cytotoxic activities of these compounds are compared in Table 2. Compound III was the most active anthraquinonyl agent against both leukemia and solid tumor cell lines. Compound III was about 10-fold more potent than compounds II or IV in the inhibition of leukemia cell

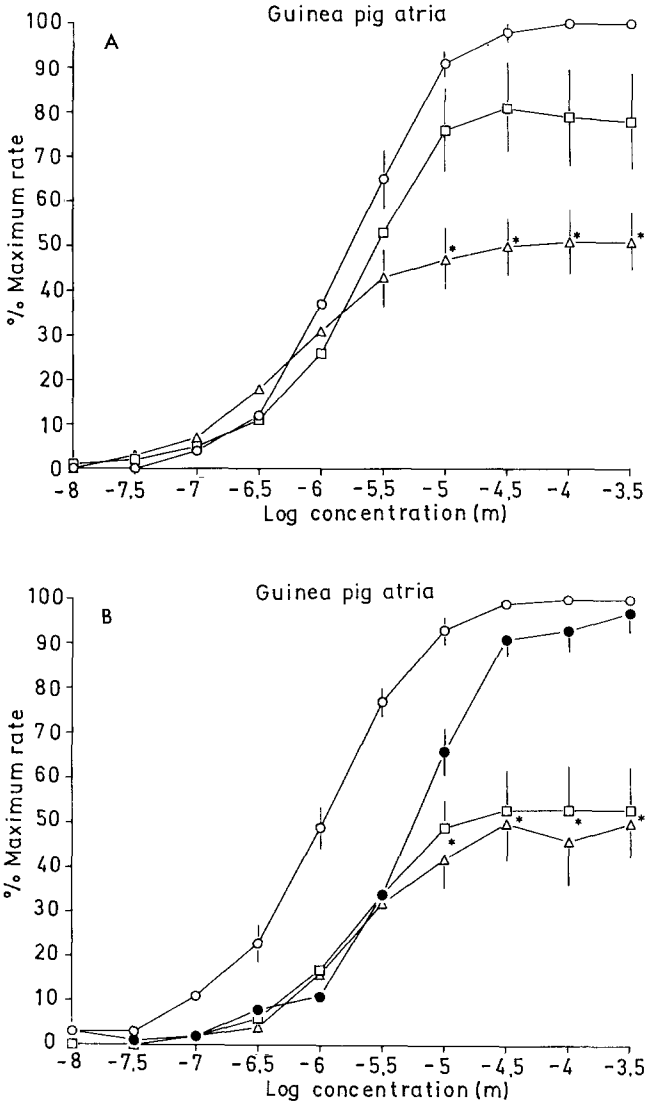


Fig. 3 A, B. Response to histamine dihydrochloride before and after exposure to either daunorubicin **(A)** or 4-demethoxydaunorubicin **(B)** for 3 h: controls (○), after 1.3 μM (●), after 13 μM (□), after 130 μM (△). *Symbols* represent means ± SE ($n = 3$–6). Statistically significant decreases in the maximal response to histamine are indicated by *asterisks*. **A** No rightward shift in the histamine curve was observed after daunorubicin treatment. **B** There was about a 4-fold rightward shift in the dose-response curve for histamine after incubation with the lowest concentration of 4-demethoxydaunorubicin (1.3 μM)

Table 2. Comparison of the antitumor activities of anthracyclines and anthraquinonyl glucosaminosides in vitro

Compound	Disk concentration (nmols/disk)	Disk assay zone units[a]	
		Leukemia[b]	Solid tumors
Doxorubicin	8.6	280–540	380[c]
Daunorubicin	8.9	300–460	330[c]
4-Demethoxy-daunorubicin	9.4	300–530	480[c]
I	375.6	100	0[c]
	751.1	200	300[d]
II	319.2	500	400[c]
			350[d]
III	33.7	400	700[d]
IV	333.7	300–400	800[c]
			700[d]

[a] 800 zone units = 1-in. zone of inhibition of tumor growth around the disk (1 zone unit = 25 μm)
[b] Leukemia cell line was L-1210
[c] Solid tumor was pancreatic ductal adenocarcinoma #03
[d] Solid tumor was colon adenocarcinoma #38

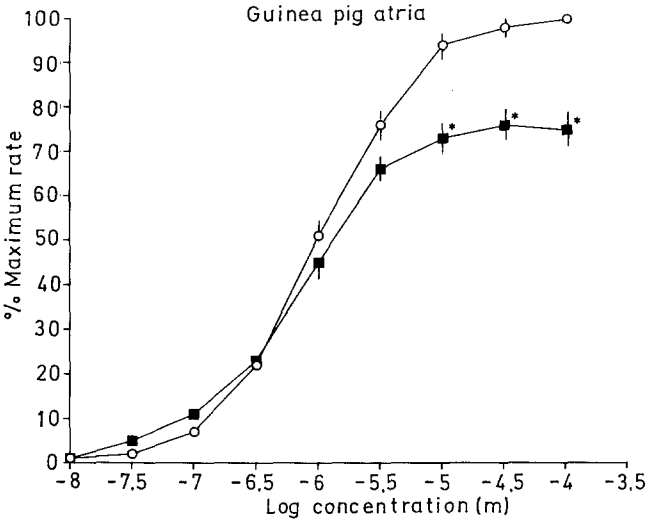


Fig. 5. Positive chronotropic response of the isolated guinea pig right atrium to histamine dihydrochloride before (○) and after (■) exposure to compound II (130 μ*M*). *Asterisks* denote the statistically significant decreases in maximal atrial chronotropic response. *Symbols* represent means of 4 experiments

growth. Hydroxylation at positions R_1 and R_2 (Fig. 1) produced greater inhibition of solid tumors than hydroxylation at R_1 and R_3. Compounds III and IV were slightly more cytotoxic for the solid tumors than I or II. The zones of inhibition of solid tumor growth were from 1.75- to 2-fold greater than the zones of inhibition of the leukemia for compounds III and IV. Compound II was about one-half as active as compound IV in the inhibition of solid tumor cell growth. Compound I was the least active agent in the series of anthraquinonyl glucosaminosides. This is probably due to the lack of hydroxyl substituents on the quinone ring system in compound I.

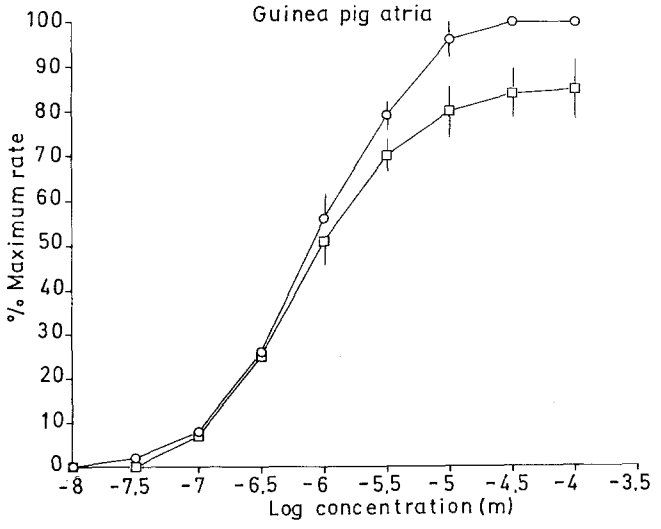


Fig. 4. Positive chronotropic response of the isolated guinea pig right atrium to histamine dihydrochloride before ○) and after (□) exposure to compound I (130 μ*M*). *Symbols* represent means of 4 experiments

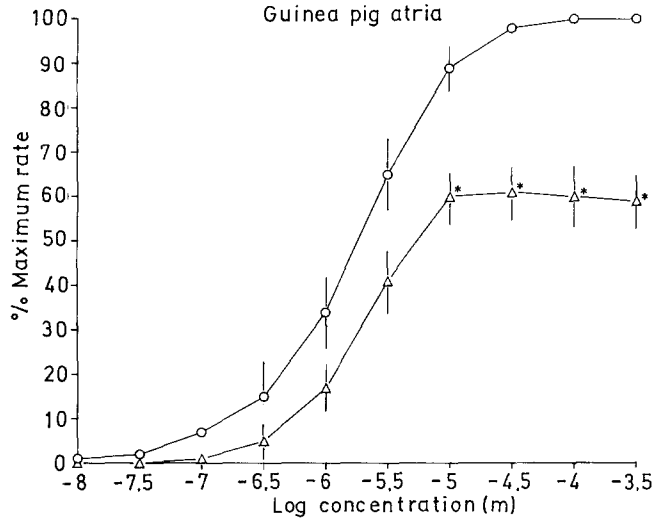


Fig. 6. Positive chronotropic response of the isolated guinea pig right atrium to histamine dihydrochloride before (○) and after (△) exposure to compound III (130 μ*M*). A 40% decrease in maximal atrial chronotropic response was observed. *Symbols* represent means of 4 experiments

In comparison to the anthraquinonyl glucosaminoside III, the anthracyclines (doxorubicin, daunorubicin, and idarubicin) were about 4-fold more potent inhibitors of the L-1210 leukemia cell line.

It should be noted that idarubicin is more toxic than doxorubicin (requiring approximately one-fourth the doxorubicin dose) in the conventional clonogenic assay (in which the drug is admixed with the tumor cells and then plated [12]. However, in the zone assay used here, the dose–response curve for these agents are shallow, requiring a 10-fold change in dose to produce less than a 3-fold change in the zone of inhibition.

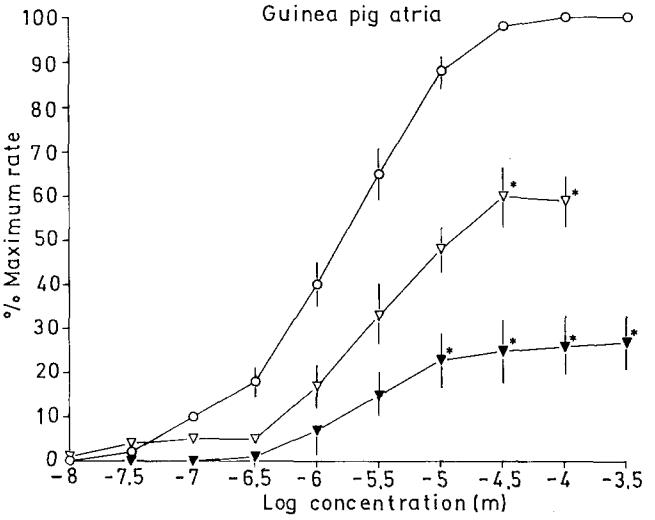


Fig. 7. Positive chronotropic response of the isolated guinea pig right atrium to histamine dihydrochloride before (○) and after exposure to compound IV at 13 (▽) or 130 μ*M* (▼). A 40% or 75% decrease, respectively, in maximal atrial chronotropic response was observed at these dose levels of compound IV. *Symbols* represent the mean of 4–8 experiments

Discussion

We have used two relatively recent methods for the evaluation of cardiotoxicity and cytotoxicity for tumor cells in vitro to assess the pharmacological activity of our new models of anthraquinone-containing antibiotics, the anthraquinonyl glucosaminosides. Their cardiotoxicity and cytotoxicity for tumor cells was compared to the activities of anthracyclines such as daunorubicin and idarubicin, a newer antitumor agent undergoing clinical investigation, which is active when administered orally.

Cardiotoxicity of the synthetic anthraquinonyl glucosaminosides was generally proportional to the number of hydroxyl groups present on the anthraquinone structure. This finding correlated with our earlier studies of these compounds: superoxide production increased with increasing hydroxyl substitution on the anthraquinone substituent [2]. The 1,8 dihydroxylated anthraquinonyl glucosaminoside was the most cardiotoxic agent in this series of synthetic anthraquinones. This agent was also more toxic than either daunorubicin or idarubicin in the isolated atrial preparation. Inhibition of the positive chronotropic effect of histamine by anthraquinonyl glucosaminosides can be classified generally as noncompetitive, because the decrease in maximal response to histamine was not accompanied by a rightward shift in the histamine dose–effect curve. This is similar to the inhibitory effect of daunorubicin on histamine-induced positive chronotropy. On the other hand, idarubicin exhibited both competitive inhibition (rightward parallel shift) of atrial responses to histamine at lower concentrations and noncompetitive inhibition of histamine-stimulated increases in atrial contraction rate at higher concentrations. A comparison of the concentrations of daunorubicin and idarubicin that produced 50% inhibition of atrial responses shows that idarubicin was 10-fold more potent (toxic) than daunorubicin. This is similar to comparisons of toxicity obtained in rats: idarubicin was about 7.4-fold more cardiotoxic than daunorubicin [4].

The mechanism underlying the noncompetitive inhibition of histamine by these anthraquinones is not apparent from the present study. However, it is known that daunorubicin (100 μ*M*) can reduce the rate of cyclic AMP formation by adenyl cyclase, a histamine-receptor coupled enzyme in the sarcolemmal membrane [17]. A lower cyclic AMP concentration relative to the concentration of histamine in vitro may contribute to the reduced positive chronotropic effect of histamine in the presence of the hydroxylated anthraquinones.

Cytotoxicity for tumor cells in a soft agar disk diffusion assay was generally proportional to the number of hydroxyl groups present on the anthraquinonyl glucosaminosides. In the present study, the 1,4-dihydroxy-substituted anthraquinonyl glucosaminoside (compound III) was the most potent in cytotoxic activity of the four synthetic analogues. An additional, unexpected finding was the slightly selective inhibition of solid tumors in vitro. We have used the following rationale to search for agents that selectively inhibit the growth of solid tumors. In the absence of a system in which one has a solid tumor cell line and its nonmalignant counterpart, we opted to test an agent simutaneously for selective cytotoxicity against two different tumor cell lines, a solid tumor and a leukemia. While cytotoxicity per se is an important consideration, an even more crucial consideration was the finding of evidence for preferential cytotoxicity for the solid tumor cell type over the leukemic cell type. Compound III or IV hold the most promise in this regard since the zone of inhibition for solid tumor cells in these experiments was somewhat larger than the zones for leukemia cells of the L-1210 type. Compound III or IV may serve as a prototypical agent from which more selectively cytotoxic agents for solid tumor cell lines may be developed.

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