Effect of Molecular Modification on the Inhibition of Lymphocyte Blastogenesis by Cardiac Glycosides

By Bruce R. Gordon, George B. Segel, James K. Brennan, and Marshall A. Lichtman

Cardiac glycosides are potent inhibitors of cell division. They may be useful as antitumor agents if the antimitotic effect can be dissociated from their cardiotoxic effect. We studied congeners of cardiac glycosides to determine whether the saccharide or the genin portion of the molecule contributes to its antimitotic effect. The concentration of glycoside required to inhibit PHA-stimulated ³H-thymidine incorporation into DNA at 72 hr in human lymphocytes was studied. The inhibiting concentration varied by over tenfold if either the genin or the saccharide portion of the molecule was altered. Therefore either the genin or the saccharide influences independently the antimitotic effect of the glycosides. The relative inhibitory dose of specific genins or glycosides on lymphocyte blastogenesis was similar to the relative arrhythmogenic effects. Therefore development of congeners with antimitotic action but without arrhythmogenicity may be difficult. The arrhythmogenic effect of cardiac glycosides has been shown to be related to their ability to inhibit the plasma membrane Na-K ATPase of myocardial cells. The antimitotic effects parallel closely the arrhythmogenicity of individual glycosides. This relationship provides added evidence to support the concept that the antimitotic effect of glycosides is related to inhibition of Na-K ATPase. Moreover, these findings support the critical role of the transport ATPase in cell division.

QUASTEL AND KAPLAN reported in 1968 that ouabain, a cardiac glycoside, inhibited phytohemagglutinin (PHA)-stimulated lymphocyte blastogenesis. Subsequent studies in the same laboratory demonstrated that ouabain inhibited lectin-stimulated RNA, DNA, and protein synthesis by human lymphocytes and inhibited nascent DNA synthesis in leukemic blasts. Ouabain also inhibited the proliferation of spontaneously dividing cells in culture. Mayhew showed reversible inhibition of cell division in Ehrlich ascites cells, and subsequently ouabain inhibition of other mammalian cell lines was reported.

Since cardiac glycosides inhibit the mitosis of Ehrlich ascites tumor cells as well as normal and malignant lymphocytes in culture, they may be useful as antitumor agents. Unfortunately, the concentration of ouabain that inhibits human cells in culture is toxic to the human myocardium. Myocardial toxicity of cardiac glycosides is linked to their ability to inhibit the sodium-potassium pump. Molecular modification may permit inhibition of mitosis with less disturbance of myocardial function if the two functions can be dissociated. There-
fore we examined the effect of various congeners of cardiac glycosides on lectin-induced lymphoblast transformation and $^3$H-thymidine incorporation into lymphocyte DNA and compared this dose-dependent function to their effects on cardiac rhythmogenicity.

**MATERIALS AND METHODS**

*Lymphocyte Preparation*

Blood was obtained from healthy donors by venipuncture, and white cell concentrates were obtained by leukoplateletapheresis using the Haemonetics Model 30 Cell Separator.$^{10}$ Blood or leukoplateletapheresis residue was diluted to 300 ml with tissue culture medium 199 containing Earles salts (TC-199) and heparin (5 U/ml). The cell suspension was sedimented at 160 g for 15 min and the platelet-rich supernatant fraction discarded. The sedimented cells were resuspended in TC-199, and the mononuclear cells were isolated using a step gradient.$^{11}$ After sedimentation at 500 g for 30 min at 20°C the mononuclear cell layer was harvested and washed three times with TC-199 at 4°C. Cells were resuspended in TC-199 plus 20%, V/V human AB serum or plasma at a concentration of $10^6$ cells/ml. The cell suspensions consisted of more than 75% lymphocytes as judged by morphologic appearance and cell size distribution. The remaining cells were monocytes. The proportion of lymphocytes excluding trypan blue dye was greater than 95%.

*Cell Concentration and Cell Volume*

The number of cells in suspension was determined in quadruplicate with a Coulter model ZBI Particle Counter standardized with Leucotrol. The cell volume was calculated from the median channel of cell volume distribution generated on a particle volume analyzer (Channelyzer) attachment. The relative percentage of lymphocytes and monocytes was determined by integration of the area under the distribution curves of the cell volume distribution.$^{10}$ Differential cell counts were also determined on Wright-stained cytocentrifuge preparations.

*Cell Incubations*

The lymphocytes were resuspended at a cell concentration of $10^6$/ml in TC-199 plus 20%, human AB plasma or serum containing penicillin-G (100 U/ml), streptomycin (100 μg/ml), and amphotericin-B (25 μg/ml). This concentration of amphotericin did not alter thymidine incorporation. Cell suspensions of 1 ml were incubated in polystyrene culture tubes at 37°C under 7% CO$_2$ in air. The tubes were not agitated during incubation, and pH was maintained at 7.4 ± 0.1. Additives were dissolved in TC-199, whenever possible, and added in volumes equal to or less than 50 μl. Some cardiac glycosides were insoluble in TC-199 at stock concentrations and were dissolved in 95% ethanol. Identical concentrations of ethanol were added to control incubations.

*Lymphoblast Transformation*

In order to quantify blastogenesis, cell suspensions were sampled after 72 hr of incubation, applied to slides with a cytocentrifuge, and treated with Wright stain. The lymphoblast was defined as a cell with a diameter greater than 10 μm and a nucleus with fine chromatin and the presence of nucleoli. Two hundred cells were counted on each slide, and the percentage of lymphoblasts was determined. The slides were evaluated by an observer unaware of the experimental conditions from which the cell suspension was obtained.

$^3$H-Thymidine Incorporation Into Acid-precipitable Material

Lymphocytes were incubated for 48 hr. Cardiac glycosides were added at the beginning of the cell incubations unless otherwise noted. After 48 hr 2.0 μCi $^3$H-thymidine (specific activity 2 Ci/mmmole) were added to each culture tube containing 1.0 ml cell suspension. The tubes were agitated, gased with 7% CO$_2$ in air, and sealed. After another 24 hr (72 hr total) of incubation, the cells were dispersed and 100-μl samples were removed in duplicate and placed on filter paper discs suspended on straight pins. The discs were air dried, and the nucleic acids were precipitated at 4°C with 10% trichloroacetic acid (TCA) and washed with solvent.$^{12,13}$ Each filter disc was
placed in a liquid scintillation vial with 5 ml of Bray’s solution, and the β radioactivity was determined.

The phytohemagglutinin (PHA) concentration used in these experiments was 1.0 μg/ml. This concentration of PHA caused maximal stimulation of thymidine incorporation under the culture conditions used. 14

Continuous Culture of Human Lymphoid Cells

RPMI 1788, a lymphoblastoid cell line derived from normal human blood, was grown in continuous culture. The cells were suspended in McCoy’s 5A medium supplemented with 10% V/V fetal bovine serum (FBS), hepes buffer (25 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). The mean cell doubling time under these conditions was 24 hr. For individual experiments, exponentially growing cells were washed in 50 volumes of phosphate-buffered saline (PBS), pH 7.4, and resuspended at a concentration of 10^6 cells/ml. One-tenth milliliter of cell suspension was added to 9.9 ml of hepes-buffered McCoy’s 5A medium with 10%, FBS to produce a final concentration of 10^4 cells/ml.

Ouabain was dissolved in PBS and gitoxin in absolute ethanol. Solutions containing one hundred times the experimental concentrations were prepared by dilution with PBS. For experiments, 0.1 ml was added to each 10-ml culture. Appropriate dilutions of ethanol were added to control cultures.

Lymphoblasts were cultured in 25 sq cm tissue culture flasks at 37°C in a humidified atmosphere of 8% CO2 in air. These conditions maintained the pH at 7.2 ± 0.1. Cell concentration was determined with a Coulter particle counter during the period of observation. The initial rate of cell doubling time (DT) was measured and the percentage inhibition of cell growth was calculated by the formula

\[
1 - \frac{\text{DT}_{\text{con}}}{\text{DT}_{\text{exp}}} \times 100,
\]

where DT_con is the doubling time in control cultures and DT_exp the doubling time in experimental cultures. The percentage inhibition was plotted against the drug concentration, and the 10%, 50%, and 90% inhibitory concentrations (ID) were derived by interpolation.


RESULTS

Survival and Viability of Cultured Lymphocytes

When blood lymphocytes were cultured at a concentration of 10^6 cells/ml, lymphocyte survival was 73% at 72 hr. Survival was determined by counting the number of cells remaining in the culture tubes. The percent viability of the surviving cells was greater than 95% as judged by trypan blue dye rejection.

Ouabain Inhibition of 3H-Thymidine Incorporation Into DNA

The effect of increasing ouabain concentrations on 3H-thymidine incorporation into DNA by unstimulated lymphocytes is shown in Fig. 1A. The data
represent the mean values of duplicate measurements in five populations of human blood lymphocytes. Unstimulated lymphocytes incorporated 1200 cpm/10^5 lymphocytes. Ouabain concentrations above 10 nM caused a progressive decrease in ^3H-thymidine incorporation into DNA. The ouabain concentration producing 50% inhibition in unstimulated cells was 40 nM.

When human lymphocytes were stimulated with an optimal dose of PHA (1.0 µg/ml), ^3H-thymidine incorporation increased to 37,000 cpm/10^5 lymphocytes (Fig. 1B). Increasing the ouabain concentration above 10 nM caused a progressive inhibition of thymidine incorporation into DNA. The ID-50 for ouabain inhibition was 30 nM, a value very similar to that which inhibited unstimulated cells. This inhibitory concentration of ouabain was similar to that observed previously.2

**Ouabain Inhibition of Blastogenesis**

The proportion of lymphoblasts present in unstimulated lymphocyte preparations at 72 hr was less than 1%. When PHA was added to the cell culture 73% of the cells were lymphoblasts after 72 hr. The effect of increasing ouabain concentrations on blastogenesis was nearly identical to the effect on ^3H-thymidine incorporation. The percentage of lymphoblasts decreased progressively as the ouabain concentration exceeded 10 nM. The concentration of ouabain that inhibited blastogenesis 50% (ID-50) was 40 nM.
Table 1. Inhibition of Growth of RPMI 1788 Cells (μmol/liter)

<table>
<thead>
<tr>
<th>Compound</th>
<th>ID-10 (10% Inhibition)</th>
<th>ID-50 (50% Inhibition)</th>
<th>ID-90 (90% Inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain</td>
<td>0.005 ± 0.002</td>
<td>0.09 ± 0.01</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Gitoxin</td>
<td>0.04 ± 0.02</td>
<td>11 ± 4</td>
<td>80 ± 30</td>
</tr>
</tbody>
</table>

Data represent mean ± SE of duplicate measurements from three experiments.

Inhibitory Potency of Cardiac Glycosides on Spontaneously Dividing Cells in Tissue Culture

Inhibition of ³H-thymidine incorporation into DNA and morphologic blastogenesis is strong presumptive evidence that lymphocyte mitosis is inhibited by cardiac glycosides. Moreover, mitosis of animal lymphoblasts has been shown conclusively to be inhibited by ouabain. To confirm the inhibitory effect of cardiac glycosides on human lymphocyte mitosis, we assayed the proliferation of a spontaneously dividing lymphoblast cell line in the presence of ouabain and gitoxin. Concentrations of these agents producing 10⁰, 50⁰, and 90⁰ inhibition of cell growth are shown in Table 1. Both drugs inhibited lymphoblast mitosis. The ID-50 of ouabain in this system, 90 nM, was three times the ID-50 for thymidine incorporation by PHA-stimulated blood lymphocytes (30 nM), and the ID-50 of gitoxin, 11 μM, was seven times the 1.5 μM necessary for 50⁰ inhibition of thymidine incorporation.

Effect of Glycoside Structure on Inhibition of DNA Synthesis

The cardiac glycoside molecule has two major components, a genin moiety with a steroid structure and a saccharide moiety. In order to determine whether the saccharide or the genin portion of the molecule contributes to the antimitotic effect of the drug, we varied each independently. We first studied five cardiac genins which have no saccharide moiety. In Fig. 2 the percentage of ³H-thymidine incorporation into DNA is plotted against increasing genin concentration. There was an order of magnitude difference between the ID-50 of bufogenin (4 μM) and digoxigenin (0.5 μM). Gitoxigenin (2 μM), digitoxigenin (0.9 μM), and strophanthidin (0.55 μM) were intermediate in inhibitory potency.

Fig. 2. Cardiac genin inhibition of thymidine incorporation into DNA. Ordinate, percentage of control ³H-thymidine incorporation into DNA by PHA-stimulated lymphocytes; abscissa, genin concentration. Data represent the mean values of duplicate measurements in four populations of human blood lymphocytes. ●, digoxigenin; •, strophanthidin; ○, digitoxigenin; Δ, gitoxigenin; ○, bufogenin.
Effect of Genin Alteration in the Presence of a Constant Saccharide on DNA Synthesis

Figure 3 shows the potency of various glycoside molecules composed of a trisaccharide of digitoxose linked with various genins. In this series, gitoxin was the least potent, with an ID-50 of 1.5 $\mu M$. The most potent glycoside was digoxin, with an ID-50 of 75 nM. There was an order of magnitude difference in ID-50 between gitoxin and digoxin. Gitaloxin, with an ID-50 of 0.35 $\mu M$, and digitoxin, with an ID-50 of 0.2 $\mu M$, were intermediate in potency. A similar alteration in glycoside potency was observed when the constant saccharide was ramnose and ouabain was compared to convallatoxin. The ID-50 for ouabain was 30 nM and convallatoxin 7 nM. Variation of the genin without alteration of the saccharide therefore markedly altered the antimitotic potency of a cardiac glycoside.

Inhibitory Effect on DNA Synthesis of Alteration in the Saccharide in the Presence of a Constant Genin

Figure 4 shows the effect on DNA synthesis of several cardiac glycosides when the genin strophanthin was held constant and the saccharide moiety varied. The percentage of control $^3$H-thymidine incorporation into DNA was
The previously published toxic concentrations of digoxin and digitoxin in man have been summarized by Smith and Haber.86

Fig. 5. Correlation of the antimitotic effect of cardiac glycosides with cardiac toxicity. Ordinate, mean lethal dose of glycoside (nmole/kg body weight) for the cat; abscissa, ID-50 for $^3$H-thymidine incorporation into DNA of human blood lymphocytes (nM). Data for cardiac glycoside toxicity in the cat have been published previously.15

measured at various glycoside concentrations. The least potent drug in this series was strophanthidin (no saccharide), with an ID-50 of 0.55 $\mu$M. The most potent glycoside was convallatoxin containing the saccharide ramnose, with an ID-50 of 7 nM. In this series there was nearly two orders of magnitude difference in ID-50 between strophanthidin and convallatoxin. Intermediate in value were K-strophanthin (ID-50, 60 nM), Cymarin (ID-50, 50 nM), and Helveticoside (ID-50, 30 nM). Alteration of the saccharide therefore also altered markedly the antimitotic potency of the glycoside.

Relationship of Inhibition of DNA Synthesis to Cardiac Toxicity

Figure 5 shows the correlation of the ID-50 of cardiac glycosides with their cardiac toxicity expressed as the mean lethal dose when assayed in cats.15 Death resulted from cardiac rhythm disturbance. All of the glycosides studied for which there were data on cardiac toxicity were plotted, and nearly perfect correlation was found ($r = 0.99$). Inhibition of blastogenesis of human lymphocytes therefore paralleled the arrhythmogenicity of the cardiac glycosides as assayed in the cat. For example, convallatoxin was the most potent inhibitor of lymphocyte mitosis (ID-50, 7 nM) and was likewise the most toxic drug (mean lethal dose, 145 nmole/kg). Alternatively, digitoxigenin was the least potent (ID-50, 900 nM) and the least toxic drug (mean lethal dose, 1230 nmole/kg).

Since the cat is relatively resistant to the effects and toxicity of cardiac glycosides, the antimitotic potency of the drugs must be compared to their toxicity in man. Most of these congeners have not been studied adequately in man. However, mean toxic concentrations for digoxin and digitoxin have been re-

<table>
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<tr>
<th>Cardiac Glycosides</th>
<th>$^3$H-Thymidine Incorporation</th>
<th>Mean Toxic Concentration</th>
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<tbody>
<tr>
<td></td>
<td>ID-10</td>
<td>ID-50</td>
</tr>
<tr>
<td>Digoxin</td>
<td>40</td>
<td>75</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>120</td>
<td>200</td>
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The previously published toxic concentrations of digoxin and digitoxin in man have been summarized by Smith and Haber.16
ported from many laboratories and have been summarized by Smith and Haber. These data are compared to the ID-50 for 3H-thymidine incorporation in Table 2. The concentration of digoxin that inhibits 3H-thymidine by 50\% (75 nM) was 17 times the mean toxic concentration (4.4 nM); the ID-50 of digitoxin (200 nM) was 3 times the mean toxic concentration (63 nM).

**DISCUSSION**

Cardiac glycosides have been found to be potent inhibitors of cell proliferation. This effect was studied initially by Quastel and Kaplan using PHA-stimulated human blood lymphocytes treated with ouabain. They found a progressive decrease in incorporation of 3H-thymidine into DNA with increasing concentrations of ouabain. Ouabain also inhibited the high rate of nascent thymidine incorporation into DNA in leukemic blast cells. Subsequently, other laboratories showed that cardiac glycosides inhibited the proliferation of tumor cells in vitro.

Quastel et al. suggested that glycosides might be of use in the treatment of human tumors. The concentration of glycoside necessary to inhibit lymphocyte blastogenesis and leukemic cell mitosis, however, was at least several times greater than could be tolerated in vivo because of the cardiotoxicity at that concentration. Since cardiac glycosides have a complicated molecular structure that includes a genin and a saccharide moiety, we considered the possibility that alteration of one portion of the molecule might allow dissociation of their inhibitory effect on cell proliferation from their cardiac arrhythmogenicity. Our data indicate that inhibition of spontaneous and PHA-stimulated 3H-thymidine incorporation into DNA, lymphocyte blastogenesis, and lymphoid cell mitosis in tissue culture depend independently on the genin and the saccharide side chains. When either was held constant and the other was varied, marked variation in antimitotic potency was observed.

The pharmacology of cardiac glycosides has been studied extensively because of their importance in the treatment of the failing myocardium. There are extensive data on the concentrations of these agents leading to cardiac arrhythmias. The mechanism of the cardiac glycoside inotropic effect remains controversial. The cardiotoxicity of these drugs is related to inhibition of cardiac muscle Na-K ATPase. Like their cardiotoxicity, the antimitotic effect of cardiac glycosides is linked closely to their inhibition of Na-K ATPase activity. The extent of ouabain inhibition of K\(^+\) influx and the resultant decrease in K\(^+\) content have been correlated with the extent of inhibition of growth in murine lymphoblasts. Furthermore, adaptation of tumor cells, continually exposed to ouabain, results in an increase in K\(^+\) influx, a recovery of K\(^+\) content, and restoration of growth. An association between ouabain-induced fall in cell K\(^+\) and growth inhibition has been demonstrated in Ehrlich ascites cells, in Girardi and HeLa cells, and in human fibroblasts.

We compared the potency of glycoside congeners in inhibiting lectin-stimulated 3H-thymidine incorporation with their potency in producing cardiac arrhythmias, since both appeared to be related to the effects of glycosides on membrane Na-K ATPase activity. The data indicate that the most potent antimitotic glycosides are also the most arrhythmogenic. The data in man are limited to digoxin and digitoxin. The glycoside concentrations necessary to
inhibit mitosis are at least several times greater than the mean toxic concentrations in man.

Our findings strongly support the concept that the glycoside antimitotic effect is dependent on inhibition of Na-K ATPase and suggest that it may be impossible to dissociate the antimitotic effect from the cardiotoxic effect by molecular alteration. These studies provide further support, however, for the concept that cell division requires an intact monovalent cation transport system.

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