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DEVELOPMENT OF A SPECIFIC ASSAY FOR CARDIAC GLYCOSIDE-LIKE COMPOUNDS BASED ON CROSS-RESISTANCE OF HUMAN CELL MUTANTS

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Abstract—The cross-resistance patterns of single- and two-step mutants of HeLa cells resistant to SC4453 (a digoxin analog) and digoxin, which involve specific alteration in Na^+, K^+-ATPase towards numerous other compounds, have been examined. The mutants exhibited increased resistance to *all* of the steroidal compounds known to elicit a digitalis-like positive inotropic response (viz. various cardiac glycosides and their genins, erythrophleum alkaloid cassaine), but they showed no cross-resistance to any of a large number of other compounds which do not show cardiac glycoside (CG)-like biological activity. Based on the above characteristics of the mutants, a new cross-resistance assay for identifying compounds that show CG-like activity has been developed. In this assay, a sample is considered to possess CG-like activity if, in comparison to the parental HeLa cells, the CG^R mutants exhibit increased resistance to it. From the known D_{10} value (drug concentration which reduces cloning efficiency of cells to 10%) of the drug for HeLa cells and the sample dilution necessary to produce equivalent cytotoxicity, the concentration of CGs in a given sample can be estimated. In blind studies the assay correctly identified *all* of the samples containing CGs; none of the other samples which lacked such activity tested positive. In the blind studies the assay also provided a good estimate of the concentration of CGs (±50% of the actual concentration) that was not affected by the presence of either serum components or a 20-fold excess of various steroidal compounds known to interfere in other assays. In view of the high specificity of the present assay for CG-like compounds, it should prove very useful in establishing/characterizing the presence of such activity in various biological (namely endogenous digitalis-like substances) and other samples.

Cardiac glycosides (CGs) such as digoxin and ouabain, which produce a marked inotropic effect on the failing heart, are among the most extensively used drugs in clinical medicine [1–3]. These compounds, however, also constitute one of the most toxic groups of substances known, and plant extracts containing CGs have been used for centuries as arrow poisons (see Ref. 2). Due to the narrow differences (≃2-fold) in the therapeutic and toxic concentrations of these drugs, the incidence of toxicity among patients receiving such drugs is generally very high. This has led to increased emphasis on monitoring the serum concentration of these drugs so that an optimal therapeutic effect could be achieved without development of toxicity [3–7]. The predominant technique that is employed for such determination is the radioimmunoassay (RIA) based on the use of a digoxin-specific antibody [4–6]. In addition, a number of other assays based on either the chemical characteristics of CGs (e.g. gas chromatographic or HPLC separation) or their biological effects (namely inhibition of ^{86}Rb-uptake, inhibition of sodium and potassium activated adenosine triphosphatase (Na^+,K^+-ATPase), inhibition of [^{3}H]ouabain or [^{3}H]digoxin binding to Na^+, K^+-ATPase) have also been developed [5]. Although some of these assay systems (namely RIA) can detect very low concentrations of CGs (sensitivity range for digoxin 0.1 to 0.2 ng/ml), their sensitivity and specificity for accurately detecting/estimating CGs are questionable [5–7], especially when the identity of the compound that a patient has been receiving is unknown. A number of endogeneous substances (digitalis-like substances: DLS), whose concentrations appear to be elevated in specific groups of human subjects, also interfere in the above assays [6–15].

For the past few years we have been investigating the mechanism of action of CGs with their cellular receptors in cultured human and rodent cells using a combination of genetic and biochemical approaches [16–22]. In HeLa (a human cell line) cells, a number of different types of mutants resistant to ouabain (Oua^R), digoxin (Dig^R) and the digoxin analog SC4453 ([23]; SC^R mutants), which involve specific alterations in the plasma membrane Na^+, K^+-ATPase, have been isolated [17, 18, 20]. The cross-resistance studies with these mutants show that they exhibit increased resistance to *all* of the compounds known to possess CG-like activity, but no cross-resistance is observed to any of the other compounds which lack such activity. Based on the highly specific cross-resistance patterns of these mutants, a new assay system for identification of compounds which show CG-like activity and for quantification of such activity has been developed. The behaviour of this assay system in identifying and measuring CG-like activity in blind reconstruction experiments using different CG derivatives as well as other interfering substances has been examined. Results of these studies presented here show that the present assay is capable of accurately identifying/estimating the pres-

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Table 1. Cross-resistance pattern of the CG^R-mutants towards different cardiac steroids

Compound	D_{10} value for HeLa cells (μM)	Relative degree of resistance of the mutants	
		SC^R-1	$ADig^R$-31
Digitoxin	0.3	50	>1000
Digitoxigenin	0.15	160	>1000
Dihydrodigitoxin	6.5	>5	>10
Digitoxigenin monodigitoxoside	0.02	200	>1000
Digitoxigenin bisdigitoxoside	0.015	250	>1000
Nerifolin	0.01	120	>5000
Digoxin	0.05	200	>500
Digoxigenin	0.60	100	>100
Dihydrodigoxin	0.10	>5	>5
α-Methyldigoxin	0.10	150	>150
β-Methyldigoxin	0.14	120	>100
α-Acetyldigoxin	0.05	140	>200
β-Acetyldigoxin	0.06	125	>250
α,β-Diacetyldigoxin	0.05	150	>250
Lanatoside C	0.10	120	>200
SC4453	0.20	200	>200
12-Acetyldigoxin	2.5	>15	>15
Ouabain	0.05	120	>5000
Ouabagenin	3.0	15	>20
Dihydroouabain	1.2	40	>100
Gitoxin	0.3	200	>200
Gitoxigenin	2.0	25	>40
16-Acetylgitoxin	0.06	60	>200
Lanatoside A	0.04	>200	>500
Lanatoside B	0.3	>50	>100
Convallatoxin	0.01	200	>2000
Strophanthidin	0.30	>100	>1000
Oleandrin	0.02	4	>200
Bufalin	0.005	500	>5000
Actodigin	2.0	50	>50
Cassaine	5.0	>10	>50

The relative resistance of different cell lines in comparison to the parental HeLa cells was determined from their plating efficiencies in medium containing different concentrations of the above compounds, as described in Materials and Methods. Assuming the D_{10} value (drug concentration which reduces cloning efficiency of cells to 10% of control) of the drugs for HeLa cells to be 1, the relative degree of resistance of the mutant cell was calculated.

ence of CG-like activity without interference from other compounds. The high specificity of this assay for CG-like compounds indicates that it could prove particularly useful in identifying other substances (including endogeneous DLS) which show this type of activity.

MATERIALS AND METHODS

Drugs and chemicals. 14-Acetoxypregnenolone, chlormadinone acetate, cortisone, corticosterone, 11α-hydroxyprogesterone, prednisolone, megesterol acetate and progesterone were purchased from the Sigma Chemical Co. (St Louis, MO). The sources of all other drugs and chemicals have been described in earlier studies [17–20].

Cell lines and culture growth conditions. SC^R-1 is a single-step HeLa cell mutant selected in the presence of the digoxin analog SC4453 [18]. $ADig^R$-31 is a second-step CG resistant mutant of HeLa cells which has been derived from the Oua^R-1 mutant (a group A mutant; [17]) after a second-step selection in the presence of toxic concentrations (5 μM) of digoxin (unpublished results). The biochemical and other characteristics of these mutants have been described elsewhere; however, both these mutants involve specific alterations in Na^+,K^+-ATPase ([17, 18] and manuscript in preparation). The parental HeLa cells as well as the mutant cell lines were routinely grown as monolayer cultures in Minimum Essential Alpha Medium (Grand Island Biological Co., Grand Island, NY) supplemented with 5% fetal calf serum, in the absence of any CGs, at 37° in a 95% air–5% CO_2 atmosphere. The drug-resistant phenotype of all the mutant cell lines is completely stable under these conditions. Cell count measurements were made using a Coulter electronic counter (model Zf).

Measurements of relative plating efficiency and cellular toxicity of the drugs. The relative cellular toxicity of different drugs towards various cell lines was determined from their plating efficiencies in the presence of different drug concentrations, as described earlier [17–19]. Briefly, in these studies 200 and 500 cells from the parental and the mutant cell lines were seeded in duplicate in 0.5 ml of growth

Table 2. Specificity of the cross-resistance pattern of the CG^R mutants

Compound	D_{10} value for HeLa cells (μM)	Relative degree of resistance of the mutant lines*	
		SC^R-1	$ADig^R$-31
Veratridine	70	1	1
Sanguanarine nitrate	3.0	1	1
Penicillic acid	10	1	1
Vanadium pentoxide	1.8	1	1
Harmaline-HCl	90	1	1
5,5′-Diphenylhydantoin	250	1	1
Quindonium bromide	250	1	1
Methyl quinolizinum bromide	40	1	1
Estradiol 17β-acetate	30	1	1
Testosterone propionate	800	1	1
21-Acetoxypregnenolone	40	1	1
β-Estradiol	30	1	1
Digitonin	15	1	1
Tigogenin	>20	1	1
Tomatine	60	1	1
Ethacrynic acid	10	1	1
Megestrol acetate	20	1	1
Chlormadinone acetate	15	1	1
Prednisolone	>200	1	1
Prednisone	>250	1	1
Deoxycorticosterone	100	1	1
Cortisone	>50	1	1
Progesterone	>50	1	1
11α-Hydroxyprogesterone	100	1	1
5β-Pregnane	150	1	1
Allotetrahydrocortisol	>70	1	1
3β-Allotetrahydrocortisol	>100	1	1
11-Deoxytetrahydrocortisol	>100	1	1
Allopregnane 3β,17α,21-triol-20-one	>100	1	1
Deoxycorticosterone 21-hemisuccinate	>100	1	1
Adriamycin®	0.015	1	1

* The relative degree of resistance of the mutants to various compounds was determined as described in Table 1 and in Materials and Methods.

medium into wells of 24-well tissue culture dishes containing 0.5 ml of a drug solution made up in growth medium at twice the required concentration. Controls containing no drug were included in all experiments. The cells were allowed to grow for 8–10 days at 37° after which the medium was carefully aspirated and the cells were stained with 0.5% methylene blue made up in 50% methanol. The numbers of stained colonies in each well were scored, and their averages were determined. From the average number of colonies observed in the presence of various drug concentrations, the relative plating efficiencies were calculated as the ratios of the number of colonies at a particular drug concentration to those observed in the absence of any drug. The D_{10} value of a drug for a cell line refers to the drug concentration which reduces cloning efficiency of a cell line to 10% of that obtained in the absence of any drug [17–19].

Cross-resistance assay. To determine whether a given sample possesses CG-like activity, 2-fold serial dilutions of the sample in the growth medium were prepared. Eleven dilutions of the sample were generally made; 0.5 ml of the different dilutions, as well as a control lacking any drug, were added (in duplicate for each of the cells lines) into the wells of 24-well tissue culture dishes. Generally, it was adequate to employ only HeLa cells and one of the resistant cell lines (i.e. SC^R-1 or $ADig^R$-31). However, in most of the experiments described here, both of the mutant cell lines were employed. A single cell suspension of the cell lines was prepared in the growth medium, and 0.5-ml volumes containing 250 cells were added to the dishes containing different sample dilutions. In parallel with the test sample, growth of the above cell lines was also examined in the presence of different dilutions of a known stock of CGs (e.g. digoxin or ouabain for unknown samples, or a known concentration of the same CGs whose concentrations was to be determined). The dishes were incubated for 8–10 days at 37°, after which they were stained and the average number of colonies at different drug dilutions was determined. In this test, a sample was considered to possess CG-like activity if, in comparison to HeLa cells, the mutant cell lines exhibited increased resistance to it. To estimate the concentration of CG in a given sample, the drug dilution which was necessary to reduce cloning efficiency of HeLa cells to 10% of control value was determined from the plating efficiency data. Knowing the D_{10} value of the particular drug for HeLa cells (from the parallel control

experiment), its concentration in the given sample could be determined as follows:

$$\text{Concentration of the drug in test sample} = \text{D}_{10} \text{ value of the drug for HeLa cells} \times \text{Sample dilution necessary to reduce HeLa cell survival to 10\% of control}$$

In cases where the identity of the CGs is not known, the drug concentrations can be calculated in terms of a reference CG (e.g. digoxin or ouabain).

RESULTS

Table 1 presents data on the cross-resistance pattern of two of the CG-resistant mutants (namely SC^R-1 and $ADig^R$-31) towards various CGs and related compounds. As can be seen, both of these mutants exhibited increased resistance to all of the CG derivatives examined. In comparison to the SC^R-1 mutant, the $ADig^R$-31 line, obtained after two selection steps, showed a higher degree of resistance to most CGs. In addition to CGs, the mutants also exhibited increased resistance to the erythrophleum alkaloid cassaine, which shows biological activity similar to that of CGs and has been shown to contain structural determinants similar to those present in CGs [16, 24–26]. It should be noted that these two mutants showed increased resistance to each and every one of the compounds known to exhibit CG-like activity and that no exception was observed.

In contrast to the above compounds, the mutants exhibited no cross-resistance to a large number of other compounds (Table 2) which do not show CG-like activity. This group of compounds includes numerous steroidal hormones, a number of which have been reported to interfere with the RIA of digoxin [16], several compounds which either inhibit or interact with Na^+,K^+-ATPase but do not show positive inotropic response [27–34], as well as various other drugs and inhibitors. The complete lack of resistance of these mutants to the latter group of compounds provides strong evidence that their cross-resistance pattern is highly specific and is restricted to only those compounds which possess CG-like activity.

Cross-resistance assay for CG-like activity. In our earlier studies with mutants of mammalian cells resistant to the protein synthesis inhibitors emetine and cryptopleurine [35], microtubule inhibitors podophyllotoxin and nocodazole [36, 37], and DNA topoisomerase II inhibitors etoposide and teniposide [38], it was shown that the cross-resistance studies with the mutants provide a highly sensitive and specific assay for identifying other compounds which possess similar activity. The rationale of the cross-resistance assay could briefly be described as follows. If the biological activity (leading to cellular toxicity) of a particular group of compounds is manifested due to interaction with a specific cellular target, and if that particular target is specifically altered as a result of genetic mutation in mutants selected for resistance to a given drug, then the mutant would also show increased resistance to all other compounds which interact in a similar manner with that target. Earlier biochemical investigations on the two CG^R mutants (SC^R-1 and $ADig^R$-1) examined in the present study showed that they involve specific alterations in the plasma membrane Na^+,K^+-ATPase ([18] and manuscript in preparation), which is believed to be the cellular receptor for these drugs. In comparison to the parental cells, the mutants show reduced binding of the labeled drugs (namely ouabain and digoxin), and a fraction of Na^+,K^+-ATPase activity in the mutants was also found to be highly resistant to inhibition by CGs. The increased resistance of these mutants (bearing alteration in the Na^+,K^+-ATPase) to all CG derivatives provides strong evidence that cellular toxicity of CGs results from their interaction with Na^+,K^+-ATPase. Further, the fact that these mutants exhibit increased resistance to only those inhibitors of Na^+,K^+-ATPase which show CG-like activity is indicative that the affected site on the enzyme may also be involved in the biological response of these drugs.

Based on the nature of the genetic alteration in these mutants and the above premise, it is expected that, if a substance possessed CG-like activity (and if its cellular toxicity was due to this), then in comprison to the parental HeLa cells, the CG^R mutants should show increased resistance to the compound. The experiment is typically carried out by making a number of serial dilutions of the test substance in growth medium and examining the cloning efficiency of HeLa cells and the CG^R mutants in medium containing different concentrations of the test substance. There are three possible types of results that one could obtain in these experiments. In case I, where the test sample does not show any toxicity to either of the cell lines at the highest concentration examined, it is generally indicative of the absence of CG-like activity. However, if a sample contains a very low concentration of CG-like material (i.e. less than the cytotoxic concentration), such a result could also be obtained. In case II, where the test sample shows similar toxicity to both the parental and the mutant cell lines, the cellular toxicity is manifested due to an unrelated mechanism. Case III is the typical result that one obtains with CG-like substances. In this case, although the degree of resistance of the mutant line could vary depending upon the test substances, as long as the mutant line shows higher resistance in comparison to the parental line (tested in parallel) it is indicative of the presence of CG-like activity.

To test the performance of this system, the solutions of a number of different drugs which included CG derivatives, other steroidal hormones, as well as unrelated cytotoxic drugs (e.g. colchicine, Adriamycin® and tubercidin) were prepared and *coded*, and their response in the cross-resistance assay was *blindly* examined. The results of these studies are presented in Table 3. As can be seen, the drugs which are not known to show CG-like activity (namely colchicine, β-estradiol, Adriamycin®, quindonium bromide, emetine, verapamil, progesterone, sanguinarine nitrate, vanadium pentoxide and tubercidin) proved equitoxic to both the parental and the mutant cell lines, indicating a lack of CG-like response. The compound testosterone propionate

Table 3. Performance of the cross-resistance assay in identification of cardiac glycosides in blind studies

Sample*	Compound	Concentration [M]	Cellular toxicity of the sample†			Interference‡	Cardiac glycoside concentration§ [M]
			HeLa	SC^R-1	$ADig^R$-31		
1	Colchicine	1×10^{-5}	1:64	1:64	1.64	Toxic; non CG-like	
2	Digoxin	1×10^{-6}	1:32	Non-toxic	Non-toxic	CG-like	1.6×10^{-6}
3	β-Estradiol	1×10^{-3}	1:32	1:32	1:32	Toxic; non CG-like	—
4	Adriamycin®	1×10^{-6}	1:16	1:16	1:16	Toxic; non CG-like	—
5	Ouabain	1×10^{-6}	1:16	Non-toxic	Non-toxic	CG-like	8.0×10^{-7}
6	Quindonium bromide	1×10^{-3}	1:4	1:4	1:4	Toxic; non CG-like	—
7	Emetine	1×10^{-5}	1:256	1:256	1:256	Toxic; non CG-like	—
8	Testosterone propionate	1×10^{-4}	Non-toxic	Non-toxic	Non-toxic	Non-toxic	—
9	Verapamil	1×10^{-3}	1:8	1:8	1:8	Toxic; non CG-like	—
10	Digitoxin	1×10^{-5}	1:256	1:4	Non-toxic	CG-like	7.7×10^{-6}
11	Cassaine	5×10^{-5}	1:8	Non-toxic	Non-toxic	CG-like	4.0×10^{-5}
12	Progesterone	1×10^{-3}	1:16	1:16	1:16	Toxic; non CG-like	—
13	Digitoxigenin	1×10^{-5}	1:12	Non-toxic	Non-toxic	CG-like	7.2×10^{-6}
14	Sanguinarine nitrate	1×10^{-4}	1:32	1:32	1:32	Toxic; non CG-like	—
15	Vanadium pentoxide	1×10^{-4}	1:50	1:50	1:50	Toxic; non CG-like	—
16	Ouabain	2×10^{-5}	1:256	1:2	Non-toxic	CG-like	1.3×10^{-5}
17	Tubercidin	1×10^{-6}	1:256	1:256	1:256	Toxic; non CG-like	—

* The samples of the compounds at the indicated concentrations were coded and blindly analyzed in the cross-resistance assay.

† The cellular toxicity of the samples is given in terms of the drug dilutions (i.e. fold-dilution) which reduce cloning efficiencies of the cell lines to approximately 10% of that observed in the absence of any drug.

‡ A sample is considered to possess CG-like activity if, in comparison to HeLa cells, the mutant cell lines exhibit increased resistance to it. The toxicity of other compounds, for which both HeLa and the mutant cell lines show similar sensitivity, is presumed to result from other unrelated mechanisms.

§ The CG concentrations were estimated based on the D_{10} value of the drugs for the HeLa cell line and the drug dilution factor which reduces cell survival to a similar extent. Even if the identity of the CG-like drug is not known, its concentration can be estimated in terms of digoxin (or any other CG) equivalents.

Table 4. Effect of interfering substances on measurement of CGs concentration

Drug	Medium and additions*	Behavior in the cross-resistance assay†	Concentration calculated‡ [M]
Digoxin	H_2O (growth medium)	CG-like	1.6×10^{-6}
(2×10^{-6} M)	Bovine serum (BS)	CG-like	1.5×10^{-6}
	BS + megesterol acetate	CG-like	1.2×10^{-6}
	BS + 11α-hydroxyprogesterone	CG-like	1.9×10^{-6}
	BS + estradiol 17β-acetate	CG-like	1.9×10^{-6}
	BS + cortisone	CG-like	1.9×10^{-6}
	BS + progesterone	CG-like	2.6×10^{-6}
	BS + 14-acetoxypregnenolone	CG-like	1.9×10^{-6}
	BS + corticosterone	CG-like	1.9×10^{-6}
	Human serum (HS)	CG-like	1.8×10^{-6}
	HS + megesterol acetate	CG-like	1.3×10^{-6}
	HS + progesterone	CG-like	1.3×10^{-6}
	HS + cortisone	CG-like	1.3×10^{-6}
	HS + chlormadinone acetate	CG-like	1.6×10^{-6}
	HS + corticosterone	CG-like	1.2×10^{-6}
Ouabain	H_2O (growth medium)	CG-like	1.8×10^{-6}
(2×10^{-6} M)	BS	CG-like	1.8×10^{-6}
	BS + 11α-hydroxyprogesterone	CG-like	2.4×10^{-6}
	BS + cortisone	CG-like	1.6×10^{-6}
	BS + chlormadinone acetate	CG-like	3.0×10^{-6}
	BS + megesterol acetate	CG-like	2.4×10^{-6}
	BS + 14-acetoxypregnenolone	CG-like	1.3×10^{-6}
	HS	CG-like	1.4×10^{-6}
	HS + 11α-hydroxyprogesterone	CG-like	1.6×10^{-6}
	HS + cortisone	CG-like	2.4×10^{-6}
	HS + chlormadinone acetate	CG-like	1.2×10^{-6}
	HS + progesterone	CG-like	1.9×10^{-6}
	HS + corticosterone	CG-like	1.5×10^{-6}
	HS + megasterol acetate	CG-like	2.6×10^{-6}

* A series of coded samples of digoxin or ouabain containing 4×10^{-5} M of the indicated interfering substances were prepared in either bovine serum or human serum. The samples were blindly analyzed in the cross-resistance assay employing HeLa and the SC^R-1 cell lines. A control of known concentrations of digoxin and ouabain was run in parallel. For a number of samples in Tables 3 and 4, for which repeat independent measurements were carried out, the day-to-day variation in the drug estimates was found to be in the range of 10–30%.

† All of the samples examined showed CG-like behavior, i.e. the SC^R-1 line exhibited increased resistance in comparison to HeLa cells.

‡ The drug concentrations were calculated from the dilution factor necessary to reduce cell survival to 10% of control and the known D_{10} value of the drug for HeLa cells.

was non-toxic to all the cell lines at the concentration examined. In contrast to these substances, all of the CG derivatives examined showed the expected CG-like response (i.e. the mutant cells were more resistant in comparison to the parental HeLa cells) in the assay system.

In addition to ascertaining whether a given test sample has CG-like activity, the assay also provides a quantitative estimate of the concentration of such substances either in terms of a known compound (i.e. if the identity of the test substance is known) or a reference CG (namely digoxin). For example, knowing the D_{10} value of the compound for HeLa cells (Table 1) and the sample (which shows CG-like activity) dilution factor which reduces the survival of HeLa cells to a similar extent, the concentration of CG in the test sample could be readily estimated (see Materials and Methods and Table 3). For the test samples which showed positive response in the blind testing, the drug concentrations in the sample provided were estimated and the values obtained are given in Table 3. As can be seen, for the different CG derivatives examined, the estimated drug concentrations were very close to the actual drug concentrations and they differed from it by a factor of less than 50%. Two different samples of ouabain (Samples 5 and 16 in Table 3) included in the above study were correctly identified with regard to the differences in the drug concentrations.

We next investigated whether the ability of the cross-resistance assay to correctly identify CGs and to provide an estimate of their concentration was affected by the presence of serum components and other steroid hormones, some of which are known to interfere in the radioimmunoassay and other assays. To determine this, a known concentration of either digoxin or ouabain was added to either H_2O, bovine serum (BS), human serum (HS), or bovine and human serum supplemented with a 40 μM concentration of different interfering substances. The samples were again coded and blindly analyzed in the cross-resistance assay. A summary of the results

Table 5. Comparison of the relative potencies of cardiac glycosides based on the cross-resistance assay, equilibrium dissociation constant (K_d values) for binding to Na^+,K^+-ATPase and positive inotropic response (F_{75} values)

Compound	D_{10} value for HeLa cells* ($\times 10^{-8}$ M)	K_d ($\times 10^{-9}$ M)† (±SE)	Relative potency‡		
			D_{10} values	K_d values	ΔF_{75} values§
Digoxin	5.1 ± 1.0	3.6 ± 0.40	1.0	1.0	1.0
Ouabain	4.8 ± 0.9	2.9 ± 0.15	1.1	1.2	2.3
Digitoxin	2.8 ± 0.5	1.1 ± 0.2	1.8	3.3	4.2
Digitoxigenin	15.0 ± 2.5	10.3 ± 0.43	0.34	0.35	0.48
Digitoxigenin monodigitoxoside	2.1 ± 0.3	1.6	2.4	2.3	7.3
β-Methyldigoxin	14.4 ± 2.6	3.7	0.35	0.97	
Dihydroouabain	120 ± 10	89	0.04	0.04	0.048
Gitoxin	30.6 ± 4.8	15.0 ± 3.0	0.17	0.24	0.67
Gitaloxin	4.3 ± 0.7	2.5 ± 0.5	1.2	1.4	
Convallatoxin	1.2 ± 0.16	0.86	4.2	4.2	1.8
Nerifolin	1.1 ± 0.15	0.59 ± 0.08	4.6	6.1	12.9

* Mean ± SE from three or more experiments.

† The K_d values (i.e. equilibrium dissociation constants) for the binding of CGs to beef heart Na^+,K^+-ATPase, as determined by [^{3}H]ouabain displacement, were taken from the work of Brown *et al.* [40].

‡ Assuming the potency of digoxin in various assays to be 1, the relative potencies of other compounds were calculated.

§ The ΔF_{75} values are the drug potencies causing a 75% increase in force of contraction in isolated guinea pig left atria. These values were taken from Refs 39 and 40.

of these experiments is presented in Table 4. All of the samples examined showed a CG-like response in the assay system, i.e. the mutant cell line (SC^R-1) was more resistant in comparison to the parental HeLa cells. More importantly, the presence of serum components or other interfering steroidal substances (which were present in 20-fold molar excess) had no apparent effect on the ability of the assay to estimate the concentrations of CGs in the test samples.

In our earlier studies with mutants resistant to other drugs [35–38] it has been shown that, for different compounds to which the mutants exhibit specific resistance, the information regarding relative activities/potencies of the compounds could be obtained from a comparison of the molar D_{10} values (or any other convenient measure of equivalent cytotoxicity) of the compounds for the parental sensitive (namely HeLa) cells. Table 5 gives the D_{10} values of a number of CGs, whose relative potencies have been determined previously in other systems [39, 40]. Assuming the molar D_{10} value of digoxin for HeLa cells to be 1, the relative activities of other compounds can be calculated from the ratios of their D_{10} values for HeLa cells. Table 5 also gives the relative potencies of the compounds based on equilibrium dissociation constants (K_d values) for binding of steroid to beef heart Na^+,K^+-ATPase (determined by displacement of [^{3}H]ouabain binding) and from their inotropic effect on isolated guinea pig left atria (given by ΔF_{75} values; Table 5) [39, 40]. In all cases, the activity of digoxin was assumed to be 1 and relative potencies in comparison to it were calculated. Figure 1 shows the relationship between the relative activities of the compounds calculated from their D_{10} values (i.e. cross-resistance assay) and those obtained from either relative binding affinities for Na^+,K^+-ATPase or from positive inotropic response in guinea pig atria. As can be seen, the relative activities of the compounds in the cross-resistance assay showed a very good correlation to the potencies of the compounds in the other two systems (correlation coefficient $r = 0.96$ for D_{10} vs K_d, and $r = 0.86$ for D_{10} vs ΔF_{75} values). The observed correlation between the D_{10} and the K_d values of the various CGs indicates that cellular toxicity of the drugs in the cross-resistance assay results from their interaction with Na^+,K^+-ATPase, an inference which is supported by other genetic and biochemical studies [17, 18].

DISCUSSION

Results presented in this paper show that the cross-resistance studies with the set of HeLa cell mutants resistant to CGs (which involve alterations in the Na^+,K^+-ATPase) provide a highly specific assay for identification and estimation of CGs. Of the large number of compounds examined, the mutants showed increased resistance to all of the CG derivatives as well as to the erythrophleum alkaloid cassaine which shows CG-like cardiotonic and biological properties [24, 25]. The mutants, however, showed no cross-resistance to any of the other compounds, which included various steroidal hormones as well as a number of other inhibitors of Na^+,K^+-ATPase, which do not show CG-like biological response. In the blind studies, the assay correctly identified all of the samples containing different CGs; none of the other samples containing various other substances showed a positive response. Further, the ability of the assay to correctly identify CGs in a sample, or to provide an estimate of their concentrations, was not affected by the presence of either serum component (bovine or human), or by a 20-fold molar excess of a number of different compounds that either show certain degrees of structural similarity to CGs or have been reported to exhibit one or more CG-like (e.g. inhibition of Na^+,K^+-ATPase, competition for [^{3}H]ouabain binding) properties, but which do not show a positive inotropic response [27–

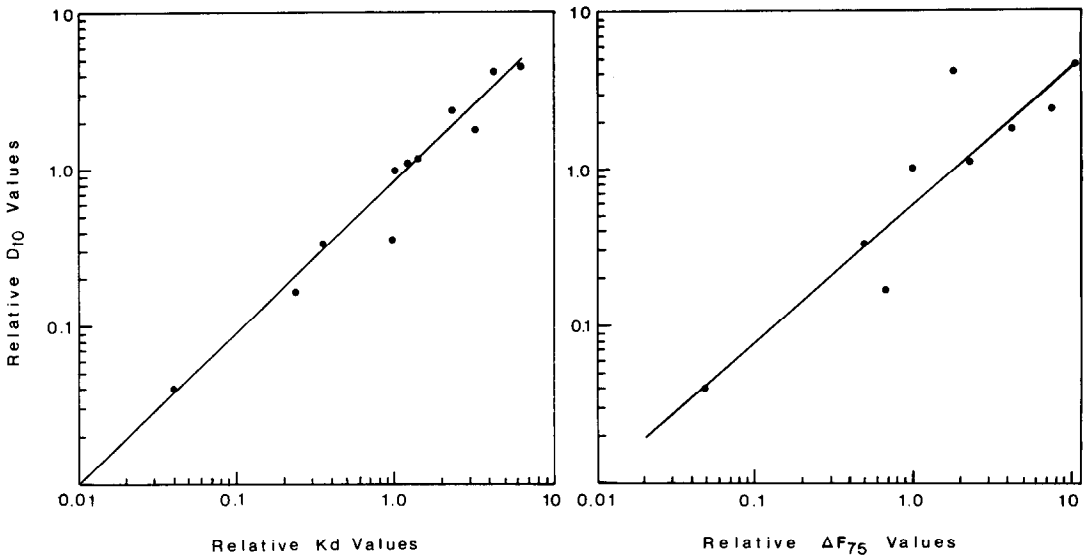

Fig. 1. Relationship between the relative potencies of various CGs based on different biological activities. Left panel: Correlation between relative cellular toxicity towards HeLa cells (D_{10} values) and relative K_d values (equilibrium dissociation constant) for binding to beef heart Na^+,K^+-ATPase. Right panel: Correlation between D_{10} values for HeLa cells and relative inotropic potencies in guinea pig left atria.

30]. All of the above observations strongly indicate that the cross-resistance assay is completely specific for recognition of compounds that show CG-like inotropic response.

In addition to accurately identifying the presence of CG-like substances, the assay also provided an estimate of the concentration of such compounds. In a blind study, the assay provided a fairly close estimate of the concentrations of different CGs, which in most cases differed from the actual values by <50%. The performance of the assay in this regard, however, could be further improved by employing drug dilutions which are more closely spaced than those employed (differing by 2-fold) in the present work. In terms of its sensitivity for detection of CGs, the minimum concentration that this assay can detect is that which produces an observable cytotoxic effect. Using the D_{10} values of CGs as a measure of cellular cytotoxicity (Table 1), the lower limits of detection for digoxin and digitoxin for the assay would be approximately 40 and 25 ng/ml respectively. Since the serum concentrations of digoxin and digitoxin in patients receiving these drugs is generally in the range of 0.5–2.0 ng/ml and 5–30 ng/ml, respectively [3, 5], the assay will be of limited use in routine monitoring of CG concentrations in the patients. For such studies, other assays such as the RIA, which can detect CGs in the range of <0.1 ng/ml and rapidly provide the results, would remain the method of choice.

In contrast to the other assay systems (e.g. RIA and gas chromatographic) whose main application is in determination of the concentration of a *known* CG in a biological sample, the present assay is unique in its ability to provide an unambiguous (yes or no type) answer as to whether the CG-like activity is present or absent in a given sample. This is indicated by the fact that *all* of the compounds which show characteristic CG-like positive inotropic response (namely different CGs, their genins and the erythrophleum alkaloid cassaine) were clearly positive in the assay, whereas none of the other compounds examined (Table 2), which lacked cardiotonic activity, showed similar behaviour in the test. A number of compounds examined in this regard [namely chlormadinone acetate (CMA), megesterol acetate, ethacrynic acid, penicillic acid and quindonium bromide] have shown one or more CG-like properties (e.g. inhibition of Na^+,K^+-ATPase, inhibition of ^{86}Rb uptake, competition for [^{3}H]ouabain binding, etc.) in earlier studies, but unlike CGs, they do not elicit a positive inotropic response [27–34]. To account for the cardio-depressant activity of CMA, for which good evidence exists regarding binding to the same site as ouabain [29, 41–43], it has been suggested that this compound may have an additional cellular effect, in addition to inhibition of Na^+,K^+-ATPase [42, 43]. If this latter activity is exerted at a lower concentration than that required for inhibition of Na^+,K^+-ATPase activity, then this could account for its lack of positive response in the present assay (e.g. type II response mentioned above under "Cross-resistant assay for CG-like activity").

For the compounds that showed a positive response, the assay also provides a convenient measure of their relative potencies with respect to either each other or any other reference compound. For a number of CG derivatives, for which data on relative potencies were available in the present test system as well as on (i) equilibrium dissociation constants for binding to Na^+,K^+-ATPase (beef heart) and (ii) positive inotropic response in isolated heart muscle [39, 40], a good correlation in the relative potencies of the compounds in all three systems was observed. This observation strongly indicates that both (cellu-

lar) toxicity and positive inotropic response result from specific interaction of the drugs with Na^+,K^+-ATPase. The CG^R mutants which involve a specific alteration in the drug binding site on Na^+,K^+-ATPase are thus specifically altered in their (cytotoxic) response to these drugs.

In view of the above-mentioned characteristics, the cross-resistance assay should prove particularly useful in identifying other naturally occurring, as well as synthetic compounds which may possess CG-like positive inotropic activity. One area where this assay may prove specially useful is in the identification of endogenous DLS. The steroidal nature of CGs and the presence of high affinity, specific receptors (i.e. Na^+,K^+-ATPase) for these drugs in all animal species have led to the suggestion regarding existence of an endogenous ligand(s) for this enzyme, which could play an important role in the humoral regulation of Na^+,K^+-ATPase and in blood volume regulation [41–46]. Accordingly, there are many reports in the literature regarding the presence of endogenous DLS which show one or more of the following characteristics (namely interaction with digoxin antibody, inhibition of Na^+,K^+-ATPase, inhibition/displacement of [^{3}H]ouabain binding to Na^+,K^+-ATPase, inhibition of Na^+/K^+ fluxes, natriuretic activity) in human and animal studies [13–15, 41–46]. The concentration of endogenous DLS in blood has been reported to be elevated in new-born infants, pregnant women, hypertensive and normotensive subjects, patients with renal and hepatic deficiency, dialysis-dependent patients and cardiac-overloaded and volume-expanded subjects (see Refs 6–15 and 41–46). However, despite considerable circumstantial evidence regarding the existence of endogeneous DLS, the precise nature of such substances and whether they indeed show digitalis-like activity remain unclear at present [45]. An interesting observation in this regard has been made by LaBella and coworkers [43, 47], who have shown that one of the progesterone derivatives, 14β-hydroxyprogesterone, which competes for [^{3}H]ouabain binding to Na^+,K^+-ATPase, also shows positive inotropic activity in isolated cardiac tissue. This observation indicates that, similar to the CGs, the endogenous DLS may also be a steroid. An examination of the activity of the putative digitalis-like substances in the cross-resistance assay, which is highly specific for CG-like substances, should prove of great help in the identification and characterization of a true endogenous DLS.

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