Biochemical Pharmacology, Vol. 36, No. 22, pp. 3829–3833, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

HUMAN CELL MUTANTS AFFECTED IN THE INTERACTION OF THE 12β-OH GROUP OF CARDIAC GLYCOSIDES WITH THE DIGITALIS RECEPTOR

RADHEY S. GUPTA* and ARVIND CHOPRA
Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

(*Received* 4 *December* 1986; *accepted* 3 *April* 1987)

Abstract—The cross-resistance patterns of two different types of mutants of HeLa cells selected for resistance to the digoxin analog SC4453 (SC^R mutants) in which the Na^+/K^+-ATPase is affected [A. Chopra and R. S. Gupta, *J. biol. Chem.* **261**, 2034 (1986)], and towards numerous cardiac glycosides (CGs) and genins, were examined. One type of SC^R mutant (designated as group C) was highly resistant to all CGs and genins investigated. In contrast, the other type of SC^R mutant (group D) showed a high degree of cross-resistance towards selected CG derivatives (viz. digoxin, SC4453, digoxigenin, lanatoside C, α- and β-methyldigoxin, dihydrodigoxin, α- and β-acetyldigoxin, α,β-diacetyldigoxin), all of which contained a free 12β-OH group in the steroid structure. Slight cross-resistance of the group D mutants was also observed for other compounds (viz. ouabain, ouabagenin, dihydroouabain) that contain a free 11α-OH group in the molecule. However, these mutants exhibited no cross-resistance to other CG derivatives, which either lacked the above groups (viz. digitoxin, digitoxigenin, dihydrodigitoxin, digitoxigenin mono- and bisdigitoside, nerifolin, gitoxigenin, gitoxin, 16-acetylgitoxin, lanatosides A and B, cymarin, convallatoxin, oleandrin, strophanthidin, actodigin and bufalin) or in which the 12β-OH group was acetylated (viz. as in the case of 12-acetyldigoxin). Since the 12β-OH group is not required for CG-like activity, to account for these observations it is suggested that the genetic lesion in the group D mutant leads to the creation of a new binding site in the digitalis receptor, which specifically interacts with the 12β-OH group (the site presumably also interacts weakly with the 11α-OH group) and either prevents or distorts the binding of the compounds to the drug binding site on the receptor. Further investigations with the different classes of CG-resistant mutants at the molecular level should prove very useful in identifying the drug receptor site and in understanding how these drugs interact with it.

Cardiac glycosides (CGs) such as ouabain and digoxin are specific inhibitors of the plasma membrane sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase), which is believed to be the sole or major receptor for these drugs [see Refs. 1–4]. To understand the mechanism of interaction of CGs with their cellular receptors, we have been employing a combined genetic and biochemical approach using cultured human cells [5–9]. We described previously the isolation of two different types of HeLa cell mutants resistant to ouabain (Oua^R mutants) [6]. One type of Oua^R mutants (group A), in which Na^+/K^+-ATPase is affected, exhibits a high degree of cross-resistance to various CGs and their genins (e.g. ouabain, digitoxin, bufalin, digoxin and digitoxigenin) but exhibits no cross-resistance to the digoxin analog SC4453, which contains a pyridazine ring at the C-17 position in place of the lactone ring found in most other CGs [10, 11]. These studies provide evidence that the mechanism of interaction of SC4453 with Na^+/K^+-ATPase differs from that of other CGs and that in the group A mutants the enzyme is affected at a site that interacts with the lactone ring of the CGs [6]. In contrast to the group A mutants, the group B Oua^R mutants exhibit comparatively lower levels of cross-resistance to all CGs and their genins, including SC4453, but they showed no apparent change in Na^+/K^+-ATPase in the initial studies [6]. However, recent studies with these mutants indicate that they also involve a structural alteration in Na^+/K^+-ATPase which, due to the low level of resistance of the mutants, was not detected originally (Ref. 8 and unpublished results).

The above studies prompted us to isolate HeLa cell mutants resistant to SC4453 (SC^R mutants) and, based upon their cross-resistance pattern towards CGs, two additional types of mutants in which Na^+/K^+-ATPase is affected were identified [9]. One type of SC^R mutant (group C) exhibited cross-resistance to all CGs and their genins but they showed much higher levels of resistance to different drugs in comparison to the group B Oua^R mutants. The other type of SC^R mutant (group D) was unique in showing cross-resistance only to a selected group of CGs (viz. digoxin, digoxigenin and SC4453), and not to others (e.g. digitoxin, convallatoxin and gitaloxin). To understand the structural basis for the observed behaviour of the group D mutants, in the present studies cross-resistance of the Oua^R and the SC^R mutants towards a large number of additional CG derivatives was examined. Results of these studies showed that the group D mutants exhibit a high degree of cross-resistance toward only those CG derivatives that contain a free 12β-OH group in their structures (viz. various digoxin derivatives, SC4453, lanatoside C, etc.). When the 12β-OH group was not free, as in the case of 12-acetyldigoxin, then

* To whom all correspondence should be addressed.

cross-resistance to the group D mutant was not observed. These observations can be understood if the genetic lesion in the group D mutant were to create a "sticky site" in the Na^+/K^+-ATPase, which would interact with the 12β-OH group and either interfere or distort the binding of the CGs to the active (i.e. drug binding) site.

MATERIALS AND METHODS

Cell lines and culture growth conditions. SC^R-1 and SC^R-4 are two HeLa cell mutants, selected for resistance to SC4453, whose biochemical and other characteristics have been described in our earlier studies [9]. Oua^R-1 and Oua^R-17 are two single-step ouabain-resistant HeLa cell mutants which have been designated previously as group A and B mutants respectively [6, 9]. The parental HeLa cells as well as the mutant cell lines were routinely grown as monolayer cultures in MEM 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 of the mutant cell lines is completely stable under these conditions. Cell count measurements were made using a Coulter electronic counter (model Zf).

Measurement of relative plating efficiency and cellular toxicity of the drugs. The relative cellular toxicities of different drugs towards various cell lines were determined from their plating efficiencies in the presence of different drug concentrations, as described earlier [6–9]. Briefly, in these studies 200 and 500 cells from the parental and the mutant cell lines were seeded in duplicate in 0.5 ml growth 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 10–12 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 plating efficiency of a cell line to 10% of that obtained in the absence of any drug [6, 9].

Drugs and chemicals. Dihydrodigoxin, α- and β-methyldigoxin, α- and β-acetyldigoxin, α,β-diacetyldigoxin, 12-acetyldigoxin, 16-acetylgitoxin, dihydro-β-methyldigoxin, lanatosides A, B and C, cymarin, dihydrodigitoxin, digitoxigenin monodigitoside, digitoxigenin bisdigitoside and nerifolin were obtained from Serva Biochemicals, Heidelberg, Federal Republic of Germany. All other cardiac glycosides and chemicals were obtained from sources described earlier [6, 7, 9].

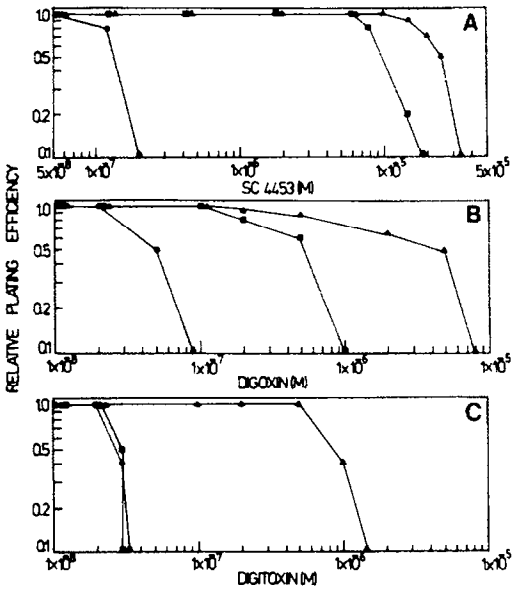

Fig. 1. Dose–response curves of the SC^R mutants towards SC4453, digoxin and digitoxin. Key: (●) HeLa; (▲) SC^R-1 and (■) SC^R-4.

RESULTS AND DISCUSSION

In earlier studies, cross-resistance of the SC^R mutants towards a limited number of CG derivatives was examined [9]. Based on these studies, two different types of SC^R mutants were identified. The two types of mutants could be readily distinguished from each other based on their cross-resistance for digoxin and digitoxin (Fig. 1). While the group C mutants (viz. SC^R-1) were highly resistant to both these drugs, the group D mutant (viz. SC^R-4) exhibited no resistance to digitoxin but was highly resistant to digoxin. To investigate the structural basis of the observed specificity in cross-resistance patterns of these mutants, further cross-resistance studies with the above mutants, as well as with a set of Oua^R mutants, towards a large number of additional CG derivatives were undertaken. The chemical structures of the CG derivatives that were investigated in these studies are shown in Fig. 2 and Table 1. The derivatives examined differed from each other in terms of various structural features, viz. substitutions on the

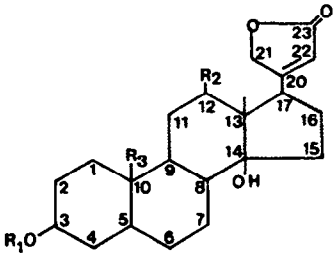

Fig. 2. Chemical structures of cardiac glycosides and their genins. The substituents in different positions are indicated in Table 1.

Table 1. Structural substitutions in different cardiac glycoside derivatives

Derivative	R_1 (C-3)	R_2 (C-12)	R_3 (C-10)	Other substitutions
Ouabain	L-Rhamnose	H	CH_2OH	1β-OH; 5β-OH; 11α-OH
Ouabagenin	H	H	CH_2OH	1β-OH; 5β-OH; 11α-OH
Dihydroouabain	L-Rhamnose	H	CH_2OH	1β-OH; 5β-OH; 11α-OH; 20-H and 22-H
Digitoxin	(DGTose)	H	CH_3	—
Digitoxigenin	H	H	CH_3	—
Dihydrodigitoxin	$(DGTose)_3$	H	CH_3	20-H and 22-H
Digitoxigenin monodigitoside	DGTose	H	CH_3	—
Digitoxigenin bisdigitoside	$(DGTose)_3$	H	CH_3	—
Nerifolin	L-Thevetose	H	CH_3	—
Digoxin	$(DGTose)_3$	OH	CH_3	—
Digoxigenin	H	OH	CH_3	—
Dihydrodigoxin	$(DGTose)_3$	OH	CH_3	20-H and 22-H
α-Methyldigoxin	(α-MeDGTose)$(DGTose)_2$	OH	CH_3	—
β-Methyldigoxin	(β-MeDGTose)$(DGTose)_2$	OH	CH_3	—
α-Acetyldigoxin	(α-AcDGTose)$(DGTose)_2$	OH	CH_3	—
β-Acetyldigoxin	(β-AcDGTose)$(DGTose)_2$	OH	CH_3	—
α,β-Diacetyldioxgin	(α,β-DiAcDGTose) $(DGTose)_2$	OH	CH_3	—
Lanatoside C	$(D\text{-}DGTose)_2$ AcDGTose-D-glucose	OH	CH_3	—
SC4453	$(D\text{-}DGTose)_3$	OH	CH_3	Pyridazine ring at C-17
12-Acetyldigoxin	$(D\text{-}DGTose)_3$	$OCOCH_3$	CH_3	—
Gitoxin	$(D\text{-}DGTose)_3$	H	CH_3	16β-OH
Gitoxigenin	H	H	CH_3	16β-OH
16-Acetylgitoxin	$(D\text{-}DGTose)_3$	H	CH_3	16β-$OCOCH_3$
Lanatoside A	$(D\text{-}DGTose)_2$ AcDGTose-D-glucose	H	CH_3	—
Lanatoside B	$(D\text{-}DGTose)_2$ AcDGTose-D-glucose	H	CH_3	16β-OH
Cymarin	D-Cymarose	H	CHO	5β-OH
Convallatoxin	L-Rhamnose	H	CHO	5β-OH
Strophanthidin	H	H	CHO	5β-OH
Oleandrin	3-*O*-Methyl arabinose	H	CH_3	16β-$OCOCH_3$
Bufalin	H	H	CH_3	Pentadienolide ring at C-17
Acetodigin	D-Glucose	H	CH_3	Isomeric lactone ring at C-17

R_1, R_2 and R_3 and other substituents are with reference to Fig. 2. Abbreviations: DGTose, digitoxose; Ac, acetyl; Mc, methyl.

steroid nucleus, nature of the sugar residue, alteration in the lactone ring structure, etc.

The cross-resistance of one representative of group C and D mutants (i.e. SC^R-1 and SC^R-4) as well as two different Oua^R mutants (Oua^R-1 and Oua^R-17 representing groups A and B respectively) towards various CG derivatives was determined from their relative plating efficiencies in medium containing different concentrations of the compounds. From the dose–response curves for the parental and the mutant cell lines, such as those shown in Fig. 1, the degree of resistance of the mutants towards various CG derivatives was calculated, and these values are shown in Table 2. From the results presented in Table 2, it is evident that the Oua^R-1 mutant (group A) showed cross-resistance to all CGs and genins except the novel CG SC4453, which contains a pyridazine ring in place of the lactone ring at the C-17 position [6, 10]. Further, the Oua^R-17 (group B) and the SC^R-1 lines (i.e. group C) were resistant to all CG derivatives examined. However, in comparison to the Oua^R-17, the SC^R-1 line showed 5- to 10-fold higher resistance to most of these compounds. In contrast to these mutants, the SC^R-4 line (group D mutant) exhibited a high level of cross-resistance towards only digoxin, digoxigenin, α- and β-methyldigoxin, α- and β-acetyldigoxin, α,β-diacetyldigoxin, dihydrodigoxin, SC4453 and lanatoside C. Slight cross-resistance (≈2-fold) of the SC^R-4 line was also observed for ouabain and its derivatives, viz. ouabagenin and dihydroouabain. However, for a large number of other CG derivatives, which included digitoxin, dihydrodigitoxin, digitoxigenin, digitoxigenin mono- and bisdigitosides, convallatoxin, bufa-

Table 2. Cross-resistance pattern of the SC^R and Oua^R mutants towards cardiac glycoside derivatives

Derivative	D_{10} value for HeLa cells (μM)	Relative degree of resistance of the mutants			
		Oua^R-1(A)	Oua^R-17(B)	SC^R-1(C)	SC^R-4(D)
Digitoxin	0.03	600	10	50	1.0
Digitoxigenin	0.15	1000	12	160	1.0
Dihydrodigitoxin	6.5	ND*	ND	>5	1.0
Digitoxigenin monodigitoside	0.02	800	12	200	1.0
Digitoxigenin bisdigitoside	0.015	1000	15	250	1.0
Nerifolin	0.01	400	6	120	1.0
Digoxin	0.04	25	25	200	20
Digoxigenin	0.50	30	15	100	120
Dihydrodigoxin	0.10	ND	ND	>5	>5
α-Methyldigoxin	0.10	12	15	150	300
β-Methyldigoxin	0.14	10	16	120	200
α-Acetyldigoxin	0.05	20	25	140	200
β-Acetyldigoxin	0.06	20	28	125	220
α,β-Diacetyldigoxin	0.05	15	25	150	300
Lanatoside C	0.10	15	25	120	100
SC4453	0.20	1	20	200	300
12-Acetyldigoxin	2.5	6	8	>15	1.1
Ouabain	0.05	150	40	120	2.8
Ouabagenin	3.0	20	10	15	2.2
Dihydroouabain	1.2	20	10	40	2.5
Gitoxin	0.3	50	5	200	1.2
Gitoxigenin	0.25	>50	50	25	1.0
16-Acetylgitoxin	0.06	100	5	60	1.0
Lanatoside A	0.04	500	30	>200	1.0
Lanatoside B	0.3	>50	10	>50	1.0
Cymarin	0.015	1000	200	300	1.0
Convallatoxin	0.01	400	20	200	0.9
Strophanthidin	0.30	600	40	>100	1.0
Oleandrin	0.02	100	3	4	1.0
Bufalin	0.005	>1000	15	500	1.0
Actodigin	2.0	5	4	50	1.0

* Not determined.

lin, strophanthidin, oleandrin, gitoxin, gitoxigenin, lanatosides A and B, 16-acetylgitoxin, nerifolin, cymarin and actodigin, no cross-resistance was seen with the SC^R-4 line. The cross-resistance of all of the above mutants towards CG derivatives was highly specific, as these mutants exhibited no cross-resistance to any of a large number of other compounds which either show limited structural resemblance to CGs (but lack this kind of activity), or are known to interact in a different manner with Na^+/K^+-ATPase ([6, 7, 9] and other unpublished results).

From an examination of the structures of the CG derivatives to which the group D mutants exhibited increased resistance, it became evident that all of these compounds contained one unique structural feature that was absent in other CG derivatives, to which the mutants did not show increased resistance. This structural feature is the presence of a free hydroxyl (—OH) group at the 12-β position in the steroid nucleus, which is a characteristic of digoxin derivatives. In contrast to the 12-OH group, the presence of a free —OH group at some of the other positions in the steroid nucleus (viz. 16-OH as in gitoxin and lanatoside B; or 5-OH as in cymarin and convallatoxin) did not have any effect on the resistance of the group D mutants. The good correlation that was observed in these studies between the presence of a 12-OH group in the CG molecule and increased resistance of the group D mutant to the compounds strongly indicated that the genetic lesion in this mutant was affecting the interaction with the 12-OH group. Since our earlier biochemical studies with the SC^R-4 mutant show it contained a biochemicaly-altered form of Na^+/K^+-ATPase that was resistant to inhibition by specific CGs to which the mutant exhibited increased resistance [9], the genetic lesion in this class of mutant involved an alteration in the receptor site of the drug which led to altered interaction with the CGs containing a free 12β-OH group.

Although resistance of the group D mutant to CGs correlated with the presence of a 12-OH group, this group is not essential for CG-like activity. This is evident from the structures of other CGs such as digitoxin, gitoxin, convallatoxin, strophanthidin and bufalin which lack the 12-OH group, but are similar to digoxin in biochemical, physiological and clinical activities [12–15]. However, the 12β-OH group which is located on the "upper" side of the digitalis molecule (binding of CGs to the receptor molecule is thought to occur through the "under" side) is capable of hydrogen binding with a suitable group in the receptor molecule [15–16]. In view of this, specific cross-resistance of the group D mutants to the CGs containing a 12β-OH group would be understood if the genetic lesion in this mutant were to

create a "novel binding site" in the receptor molecule that would specifically bind to the 12β-OH group. The binding/interaction of this "new sticky site" with the 12β-OH group would then either prevent or interfere with the normal binding of the drug to the active site in the receptor molecule. Such a change could conceivably be produced by mutational conversion of a neutral amino acid in the receptor molecule to another amino acid residue (e.g. histidine) that would be capable of hydrogen bond formation with the 12-OH group. Such a change should affect the binding of only those CGs that contain a free 12β-OH group, but other CGs that lack this group would be expected to interact normally with the active site, thus accounting for the cross-resistance pattern of the mutants. Based on the behaviour of the mutant, such a change would appear not to have any apparent effect on the normal functioning of the enzyme.

The above hypothesis regarding the nature of genetic alteration in the group D mutant has received strong support from the behaviour of the compound 12-acetyldigoxin, in which the OH-group at the 12-position is acetylated. In contrast to digoxin and its other derivatives to which the group D mutants are highly resistant, these mutants did not show any cross-resistance to 12-acetyldigoxin (see Table 2). If the hydroxyl group at the 12-position were involved in hydrogen bonding with a "sticky group", then this behaviour would indeed be expected, as acetylation of the OH—would abolish its hydrogen-bonding ability. These results thus strongly support our contention that the genetic lesion in the group D mutants has led to the creation of a new binding site that interacts with the 12-OH group in CGs and either prevents or distorts the interaction of the drug with the active site on the receptor molecule. The presence of a new binding site in the group D mutants could also account for their low levels of resistance to ouabain derivatives (viz. ouabain, ouabagenin, dihydroouabain) that contain an 11α-OH group. Because of the proximity of the 11α-OH group to the 12β-OH group, it appears that a new binding site in the group D mutant would also be able to interact weakly with this group.

From our studies on CG-resistant mutants, it is evident that a number of different types of mutants of human cells, which uniquely affect the interaction of Na^+/K^+-ATPase with specific structural groups of CG derivatives, have been isolated. Further studies on molecular characterization of the genetic alteration in these mutants at the nucleotide and amino acid sequence levels should prove extremely useful in identifying the specific regions of the receptor molecule which are involved in interaction with diffent structural parts of the CG derivatives.

REFERENCES

1. A. Schwartz, G. E. Lindenmayer and J. C. Allen, *Pharmac. Rev.* **27**, 3 (1975).
2. T. Akera and T. M. Brody, *Pharmac. Rev.* **29**, 187 (1978).
3. D. Noble, *Cardiovas. Res.* **14**, 495 (1980).
4. O. Hansen, *Pharmac. Rev.* **36**, 143 (1984).
5. R. S. Gupta, *Biochem. Pharmac.* **30**, 3039 (1981).
6. R. S. Gupta and A. Chopra, *J. biol. Chem.* **260**, 6843 (1985).
7. R. S. Gupta, A. Chopra and D. K. Stetsko, *J. cell. Physiol.* **127**, 197 (1986).
8. A. Chopra and R. S. Gupta, *Somat. Cell. molec. Genet.* **12**, 367 (1986).
9. A. Chopra and R. S. Gupta, *J. biol. Chem.* **261**, 2034 (1986).
10. T. Godfraind and D. Lutete, *Eur. J. Pharmac.* **60**, 329 (1979).
11. T. Godfraind and J. Ghysel-Burton, *Eur. J. Pharmac.* **60**, 337 (1979).
12. C. Tamm, in *Proceedings of the First International Pharmacological Meeting, Stockholm* (Eds. W. Wilbrandt and P. Lindgren), Vol. 3, pp. 11–26. Macmillan, New York (1963).
13. A. Yoda, *Molec. Pharmac.* **9**, 51 (1973).
14. L. Brown, E. Erdmann and R. Thomas, *Biochem. Pharmac.* **32**, 2767 (1983).
15. D. H. Fullerton, in *Wilson and Gisvold's Textbook of Organic, Medicinal and Pharmaceutical Chemistry* (Ed. R. F. Doerge), pp. 657–732. J. B. Lippincott, Toronto (1982).
16. R. Thomas, L. Brown, J. Boutagy and A. Gelbart, *Circulation Res.* **46** (Suppl. I), I–167 (1980)