J. Enzyme Inhibition, 1994, Vol. 8, pp. 197–205
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STRUCTURE-ACTIVITY RELATIONSHIP AT THE GLYCOSIDIC MOIETY OF DIGITALIS COMPOUNDS AS FOUND IN TESTS WITH NA/K-ATPASE ISOFORMS FROM CARDIAC MUSCLE OF GUINEA-PIG AND MAN

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(Received 12 April 1994)

KEY WORDS: Na/K-ATPase test, digitalis compounds, glycosidic moiety, structure-activity relationship, kinetic parameters, evaluation

INTRODUCTION

The glycosidic moiety plays an important role in the pharmacokinetic and pharmacodynamic behaviour of cardiac glycosides like digitoxin and digoxin. Their tridigitoxoside side chain becomes slowly removed in the animal body and hence delays the inactivation of the drugs by epimerization and conjugation of the C3β-hydroxy group to which the glycosidic side chain is attached.[1,2] In addition, the glycosidic component affects the kinetics of the glycoside interaction with the receptor enzyme, i.e., the Na/K-ATPase, by influencing both the lag time for the onset of action and the half-life time for the length of action.[3] The determination of the association and dissociation rate constants has shown that these parameters of receptor kinetics are modulated by the structure of the monoside bound proximately to the steroid moiety.[4] Although

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Abbreviations and definitions: Na/K-ATPase, Na^+/K^+-transporting adenosine triphosphate phosphohydrolase (EC 3.6.1.37); isoform, congeneric name for Na/K-ATPase preparations from different species which may or may not consist of more than one isoenzyme entity; digitalis, congeneric name for cardenolides with A/B-trans or A/B-cis ring junction; SAR, structure-activity relationship; k_{on} and k_{off}, association or dissociation rate constant, respectively.

repeatedly analysed, the attempts of modelling the structure-activity relationships (SAR) have not reached general significance and predictive capacity[5] as required for the safe utilization in synthetic work. The lack of such information appears to be due to the conformational flexibility of the glycoside side chain.[6,7]

Attempts have been made to overcome this difficulty by calculation of the conformational preference of the proximate sugar side chain and by application of the corresponding structural-conformational information to the interpretation of SAR findings.[6,7] Unfortunately, the results of conformation energy calculations are heavily dependent on the type of the potential energy programme applied.[7] Moreover, there is no universally accepted method to satisfactorily account for the effect of the hydration of the molecules in solution which therefore has been neglected.[6] However, the difficulty of conformational flexibility has appeared to be surmountable by the use of a cardiac glycoside like gomphoside 9 in which the glycosidic moiety is bilinked through the oxygen atoms at C2α and C3β of the aglycone gomphogenin 5 and thus is conformationally rigid[8] (cf. Figure 1). The non-rotating glycosidic linkages apparently accounted for the high potency of gomphoside such that this glycoside seemed to be a reference compound for the interpretation of SAR studies.[9,10] As stated by Watson *et al.*,[11] a complicating factor in the acceptability of gomphoside as reference glycoside is the presence of the C2α-OH in gomphogenin which strongly reduces the potency of the parent aglycone, i.e., uzarigenin (cf. Figure 1). An additional complicating factor of acceptability of gomphoside as reference tool is the trans-junction of the steroid rings A and B, whereas cis-junction of these rings is common in most cardiac glycosides. This change of ring linkage much affects the spatial disposition of the sugar side chain in the familiar cardiac glycosides with a flexible chain[12] (cf. Figure 1).

The properties of gomphoside and related compounds have hitherto been evaluated by determining the inotropic activity in isolated guinea-pig left atria.[9,11] Since the diffusion characteristics in guinea-pig myocardium can dissociate the inotropic effect from receptor binding,[13] we thought it necessary to eliminate this superimposition by using digitalis receptor enzyme preparations from that source.[14] As even more important for the utility of any information to the targeted design of glycosides of novel digitalis-like acting steroids potentially suitable as drugs (cf.[15,16]), the general validity of conclusions had further to be probed by parallel studies with enzyme preparations from cardiac muscle of man in the elaborated test.[17,18] The sugar binding subsite within the digitalis binding matrix has namely been found to differ for various Na/K-ATPase isoforms.[19]

The primary purpose of the present study has been to evaluate the usefulness of gomphoside as reference compound for the educated interpretation of SAR studies. The superordinate goal has been to establish those carbohydrate substitutions which produce: first, potency gains as high as contributed by the glycosidic moiety of gomphoside, and second, medicinally required receptor kinetics, i.e., high association and low dissociation rates, as favourable as those found with digoxin.

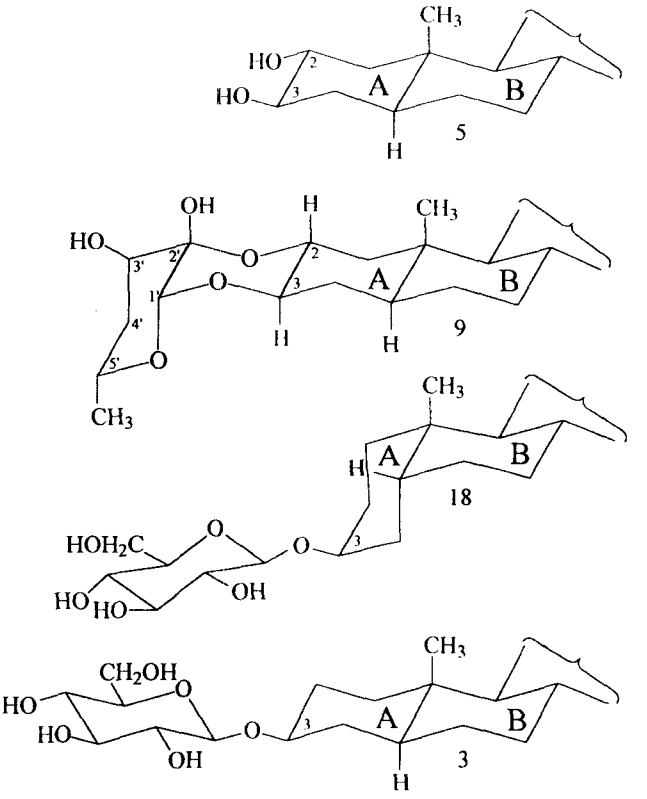

FIGURE 1 Partial structures of gomphogenin **5**, gomphoside **9**, 3β-O-(β-D-glucosyl)-digitoxigenin **18** and 3β-O-(β-D-glucosyl)-uzarigenin **3**. Note the large distinction in the spatial disposition of the sugar side chain in **3** and **18** with trans- or cis-junction of the rings A and B, respectively.

MATERIALS AND METHODS

Digitalis derivatives used

The compounds **2, 5** and **9** were synthesized or isolated as published.[6,8,9,11] The monoacetates **6** and **7** were prepared from **5** by treatment with triethyl orthoacetate in tetrahydrofuran followed by partial hydrolysis of the obtained cyclic 2,3-orthoester and chromatographic separation. The derivatives **8, 10** and **11** were synthesized by acetylation of **5** or **9**, respectively, with acetic anhydride in pyridine. The glycosidation of **1** to give **3** or **4** was performed by a method described earlier.[22] The sources of the compounds **12...23** listed in Table I were specified in our earlier publications.[14,20,21] The compounds were dissolved in pure dimethyl sulfoxide and transferred in this solution into the assay medium.

Preparation of Na/K-ATPase and determination of activity

The procedure described by Matsui and Schwartz[23] was applied for the preparation of the isoforms from cardiac muscle of man and guinea-pig. Enzyme activity was monitored at 37°C in the optical test system[14,20] using a recording Beckman DU-7500 diode-array spectrophotometer.

Determination and expression of inhibitory digitalis potency

Inhibition was studied under conditions of maximum enzyme activity to mimic the circumstances for the development of the *in vivo* digitalis action which is evoked through the inhibition of the Na^+/K^+ pump[3,15] and to favour the presence of a single type of digitalis-receptive intermediary state of the enzyme. All the curves representing Na/K-ATPase inhibition as a function of digitalis concentration showed a monophasic, purely sigmoidal shape. This clearly indicated the absence of enzyme conformers or isoenzymes in the enzyme preparations differing in digitalis affinity by more than two orders of ten (cf.[24]). On the basis of this finding, a pseudomonomolecular interaction scheme was applied for the expression of the kinetics of inhibition. Specifically, the association and dissociation rate constants for the inhibitory interaction of the digitalis derivatives with the enzyme preparations were estimated from the time course of inhibition as reasoned.[25] The registered absorbance vs time curves were subjected to nonlinear regression using the Marquardt algorithm.[26] The curves fitted well to the simple exponential, $I_t = \{[I]/([I] + K'_D)\} \cdot (1 - e^{-(k_{on}[I]+k_{off})t})$, where I_t is the percentage of inhibition of enzyme activity at time $t_1[I]$ the inhibitor concentration and k_{on} and k_{off} association and dissociation rate constants.

All further methodical details were comprehensively specified in earlier publications from our group.[14,20]

RESULTS

Table 1 presents both the equilibrium and kinetic parameters characterizing the inhibitory interaction of 22 digitalis derivatives with enzyme preparations from cardiac muscle of guinea-pig and man. The compounds are grouped according to related structure. The evaluation will initially be confined to the data obtained with the guinea-pig enzyme preparation.

1. A/B-trans cardenolides

Gomphoside **9**, when compared to two glycosides of uzarigenin though with flexible glycosidic chain (**3,4**), shows actually a much higher potency measured by the dissociation constant. This results from more favourable association and dissociation rate constants. The impact of acetylation at the glycosidic component of gomphoside on potency (**9**→**10** and **9**→**11**) suggests that 2′-OH contributes more than 3'-OH to binding in the receptor cleft, possibly by involvement in hydrogen bonding. Remarkably, the greatest part of increase in activity by transformation of gomphogenin 5 into gomphoside **9** results from the 'occlusion' of C2α-OH and scarcely from the occlusion of

TABLE 1
Association and dissociation rate constants (k_{on} and k_{off}) and equilibrium dissociation constant (K'_D) characterizing the inhibitory interaction of A/B-trans cardenolides **1...11** and A/B-cis cardenolides **12...23** with Na/K-ATPase isoforms from guinea-pig heart (g.-p.h.) or human heart (h.h.) estimated under optimum turnover conditions.

No.	Systematic (trivial) name of inhibitor	Enzyme origin	k_{on} [$\mu M^{-1} \cdot min^{-1}$]	k_{off} [min^{-1}]	K'_D[μ M]
1	3β, 14-Dihydroxy-5α, 14β-card-20 (22)-enolide (uzarigenin)	g.-p.h.	0.084	0.53	6.3
		h.h.	1.3	0.39	0.29
2	Uzarigenin 3-acetate	g.-p.h.	0.024	0.43	18.1
3	3β-*O*-(β-D-Glucosyl)-uzarigenin	g.-p.h.	0.099	13	131
		h.h.	0.023	0.21	8.7
4	3β-*O*-(α-L-Rhamnosyl)-uzarigenin	g.-p.h.	1.01	1.32	1.31
		h.h.	1.09	0.12	0.11
5	2α-Hydroxy-uzarigenin (gomphogenin)	g.-p.h.	0.014	0.32	23.4
		h.h.	0.18	0.33	1.8
6	Gomphogenin 2-acetate	g.-p.h.			0.89
		h.h.	9.9	0.64	0.064
7	Gomphogenin 3-acetate	g.-p.h.	0.30	3.1	10.3
		h.h.	0.63	0.35	0.56
8	Gomphogenin 2,3-diacetate	g.-p.h.	0.96	0.65	0.66
9	(1'S,2'S,3'R,5'R)-3,2-Di-*O*-(2',3'-dihydroxy-5'-methyl-tetrahydropyran-1',2'-diyl)-gomphogenin (gomphoside)	g.-p.h.	7.4	1.05	0.14
		h.h.	5.8	0.054	0.0093
10	Gomphoside 3'-acetate	g.-p.h.	3.7	1.05	0.28
11	Gomphoside 2',3'-diacetate	g.-p.h.	0.35	1.8	5.9
12	3β, 14-Dihydroxy-5β, 14β-card-20 (22)-enolide (digitoxigenin)	g.-p.h.	0.56	1.1	1.9
		h.h.	9.9	0.52	0.053
13	Digitoxigenin 3-acetate	g.-p.h.	0.61	0.64	0.95
		h.h.	5.2	0.21	0.041
14	3α, 14-Dihydroxy-5β, 14β-card-20 (22)-enolide (3-epi-digitoxigenin)	g.-p.h.	0.145	13	90
		h.h.	0.80	3.9	4.9
15	3-Epi-digitoxigenin 3-acetate	g.-p.h.	1.33	0.63	0.47
		h.h.	8.2	0.18	0.024
16	3β-*O*-(β-D-Digitoxosyl)-digitoxigenin	g.-p.h.	1.4	0.17	0.12
		h.h.	12	0.061	0.0051

TABLE 1
Continued

No.	Systematic (trivial) name of inhibitor	Enzyme origin	k_{on} [$\mu M^{-1} \cdot min^{-1}$]	k_{off} [min^{-1}]	K'_D[μ M]
17	3β-*O*-(α-L-Thevetosyl)-digitoxigenin	g.-p.h.			0.20
18	3β-*O*-(β-D-Glucosyl)-digitoxigenin	g.-p.h.	2.1	0.82	0.39
		h.h.	9.1	0.073	0.0080
19	3β-*O*-(α-L-Rhamnosyl)-digitoxigenin	g.-p.h.	1.8	0.22	0.12
		h.h.	9.7	0.032	0.0033
20	3β-*O*-(3'-*O*-Methyl-α-L-rhamnosyl)-digitoxigenin	g.-p.h.			0.14
		h.h.			0.0073
21	3β-*O*-(2',3'-*O*-Isopropylidene-α-L-rhamnosyl)--digitoxigenin	g.-p.h.			0.091
		h.h.			0.0079
22	3β-*O*-(4'ξ-Amino-4'-deoxy-α-L-rhamnosyl)-digitoxigenin	g.-p.h.	9.8	0.22	0.021
		h.h.	22	0.035	0.0016
23	3β-*O*-(β-D-Tridigitoxosyl)-digitoxigenin (digitoxin)	g.-p.h.	1.2	0.23	0.19
		h.h.	2.8	0.022	0.0079

C3β-OH. This emerges from the findings that acetylation of C2α-OH (**5**→**6**) but not that of C3β-OH (**5**→**7**, **6**→**8**) effects a great increase in potency. Hence, concerning the interaction with the sugar binding subsite in the receptor cleft, C3β-OH is relatively inert, whereas C2α-OH appears to be an unpaired hydrogen bonding candidate which is generally known to effect polar repulsion.[27] The favourable outcome of C2α-OH acetylation may then be based on formation of a derivative with a carbonyl oxygen able to accept two hydrogen bonds.[28]

The comparison of the kinetic parameters of the inhibitors **1...11** found with the enzyme preparations from guinea-pig and human cardiac muscle shows some parallelisms, but also interesting distinctions. The higher potency of gomphoside **9** compared with gomphogenin **5** is based with the guinea-pig isoform on a strong increase of k_{on}, whereas with the human isoform also a strong decrease of k_{off} is involved. The intensification of the inhibitory activity of uzarigenin by rhamnosylation (**1**→**4**) stems for the guinea-pig isoform from a strong elevation of k_{on}, but with the human isoform solely from a decrease of k_{off}. The drop in potency of uzarigenin by glucosylation (**1**→**3**) found with both enzyme preparations results for the guinea-pig isoform from a strong increase of k_{off}, whereas with the human isoform it is based on a great decrease of k_{on}.

In conclusion, the data, obtained with both Na/K-ATPase isoforms, in principle confirm the postulate, derived from pharmacological evaluations with cardiac muscle preparations of guinea-pig, that the conformational distribution of the glycosidic moiety in cardenolides with A/B-trans ring junction (uzarigenin derivatives) is the

major determinant of their activity,[9,11,12] though the exceptionally high potency of gomphoside is concomitantly due to the 'occlusion' of the repulsive C2α-OH group. Most importantly, the above postulate does but partially apply to cardenolides with A/B-cis ring junction (digitoxigenin derivatives) as will be shown in the following.

2. A/B-cis cardenolides

At first it may be mentioned that, similar to the detrimental effect of C2α-OH in the A/B-trans cardenolide (**1**→**5**), also C3α-OH in the A/B-cis cardenolide (**12**→**14**) effects a large drop in potency, which becomes by acetylation even more than compensated (**14**→**15**). As detailed above, this appears likewise to be caused by transformation of a repulsively acting unpaired polar hydrogen bonding candidate into a two hydrogen bonds accepting derivative. Taken together, these findings lead to the conclusion that there is an adequate 'receptor excluded volume' (for a general definition see[29]) in the proximity of both C2α-OH and C3α-OH to accomodate an acetyl residue for forming hydrogen bonds. Interestingly enough, the detrimental effect of the conversion of digitoxigenin into 3-epi-digitoxigenin and its annulment by C3α-OH acetylation was observed also in pharmacological studies measuring the inotropy potencies in isolated guinea-pig atria.[30]

Scrutiny of Table I reveals that the glycosides of A/B-cis cardenolides with flexible glycosidic chain (**16...19**) exert a similarly high potency as gomphoside **9** with a rigid glycosidic moiety. The occlusion of 2′-OH, 3′-OH or 2′,3′-OH in the glycosidic moiety as given in **17, 20** and **21**, respectively, drops the potency of the comparative glycosides only a little. On the other hand, conversion of 4'-OH into 4′ξ-NH_2 (**19**→**22**) elevates the potency due to increase of k_{on} with both isoforms. Almost generally, with the guinea-pig isoform and both A/B-cis and A/B-trans cardenolides, the k_{on} values are lower and the k_{off} values higher thus explaining the lower digitalis susceptibility of the guinea-pig.[14]

DISCUSSION AND CONCLUSIONS

The conversion of an A/B-trans into an A/B-cis ring junction considerably increases the inhibitory potency of the aglycones as found with both the guinea-pig and human Na/K-ATPase preparation (**1** vs **12**). This epimerization heavily changes the spatial disposition of the sugar side chain bound to C3β-OH at ring A (cf. Figure 1, **3** vs **18**). The impact of the various glycosidic moieties on the dissociation constants of the complex with the human isoform suggests the presence of the following differences as to the accomodation of the side chains in the sugar binding subsite.

In the **A/B-trans epimer series**, the rhamnosyl chain effects only a small increase of potency (**1**→**4**), whereas the glucosyl chain brings about even a strong drop of potency (**1**→**3**). Apparently, these side chains do but poorly or not all fit into the sugar binding subsite volume thus hindering or blocking complex formation. The glycosidic part of gomphoside allows an immense increase of potency (**5**→**9**), but this is possible only because the glycosidic moiety is cramped up with ring A and it thus does not demand so much subsite volume. Its boundary appears to be marked by the moderately or

strongly detrimental effect of acetylation of 3′-OH and 2′,3′-OH, respectively (**9**→**10**, **9**→**11**). Most importantly, as derived in the results section, the major part of the enormous potency gain, which 2α-hydroxy-uzarigenin (gomphogenin **5**) undergoes through linkage of the glycosidic component to C2α-OH and C3β-OH (**5**→**9**), does not result from the inflexibility of the glycosidic moiety, but from the concomitant occlusion of the repulsive C2α-hydroxy group. — The differential impact of various modifications of gomphoside at carbons 2′, 3′ and 4′ on the inotropic effect of the derivatives on guinea-pig cardiac muscle has led to the conclusion that gomphoside binds to the receptor site through its 3′(axial)-hydroxy group.[6,9,11] In the highly potent glycosides of A/B-cis cardenolides with flexible sugar chain (cf. below), an axial 3′-hydroxy group of equivalent importance is not apparent. Otherwise, the 3′-O-methyl ether substituent added to β-D-digitoxose does but poorly decrease potency,[3] while the introduction of a 3′-O-acetyl substituent in β-D-digitoxose considerably decreases activity probably due to steric hindrance.[3] Taken together, the above findings do not appear to favour the suitability of gomphoside as a general reference compound for the interpretation of structure-activity relationships at the glycosidic moiety.

In the **A/B-cis epimer series**, on the other hand, both the rhamnosyl and glucosyl chain (**12**→**19**, **12**→**18**) as well as the digitoxosyl and 4′ξ-amino-4′-deoxy-rhamnosyl chains (**12**→**16**, **12**→**22**) appear to more or less perfectly circumscribe the sphere of the sugar binding subsite of both isoforms. Specifically, 4′-OH or 4′ξ-NH_2 in α-L-rhamnose have been deduced to provide acceptor groups for the hydrogen-bond-donating amino acid side chains in the subsite.[15] This interpretation is in line with earlier findings that the equatorial 4′-OH, not the 3′-OH, is common to all potent cardiac glycosides.[5,7,9,19]

In the human isoform, the potency of tridigitoxosyl-digitoxigenin **23**, judged from the dissociation constants, is inferior to the potency of various monosides of digitoxigenin, i.e., the digitoxoside **16**, the rhamnoside **19**, and the 4′ξ-amino-4′-deoxy-rhamnoside **22**. The half-life time of the inhibited complex (τ = ln 2/k_{off}), which is an important parameter for a convenient dose regimen in medicinal use, is 21.7 min with **19** and 19.8 min with **22**, thus not too much shorter than with **23** (31.5 min) and almost as long as with the tridigitoxoside of 12β-hydroxy-digitoxigenin, i.e., digoxin (τ = 15.1 min; to be published). The partial-synthetic preparation of rhamnosides of digitalis-like acting steroids appears to be most inviting with respect to rapid and cheap realization of glycosides with advantageous receptor kinetics.[15,16,31-33]

Acknowledgements

The work was supported by the Deutsche Forschungsgemeinschaft (Re 878/1–4) and the Fonds der Chemischen Industrie.

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