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ORIGIN OF DIFFERENCES OF INHIBITORY POTENCY OF CARDIAC GLYCOSIDES IN Na^+/K^+-TRANSPORTING ATPase FROM HUMAN CARDIAC MUSCLE, HUMAN BRAIN CORTEX AND GUINEA-PIG CARDIAC MUSCLE

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Abstract—The inhibitory potency of altogether 95 steroidal compounds (including cardenolides, bufadienolides and their glycosides) on the Na/K-ATPases (Na^+/K^+-transporting ATPases, EC 3.6.1.37) from human cardiac muscle, human brain cortex and guinea-pig cardiac muscle was compared to probe the complementary chemotopology of the inhibitor binding site areas on the three enzyme variants. The changes of potency, resulting from systematic variations of the geometry of steroid skeleton and the character as well as the structure of side chains at C3 or/and C17 of steroid backbone, allowed the following major conclusions.

With the human cardiac and cerebral enzyme forms, the paired $K_{0.5}$ (K_D') values for 77 steroid derivatives, covering seven orders of ten, were highly correlated. On an average, the total of compounds showed a 1.5-fold higher affinity to the cardiac enzyme. This tiny differentiation did not appear to be connected with an important difference in the chemotopology of the complementary subsites for steroid nucleus binding on the two enzyme forms. With the human and guinea-pig cardiac enzyme variants, the $K_{0.5}$ values for 69 steroid derivatives, covering six orders of ten, were determined. For 41 5β,14β-androstane derivatives only, the paired $K_{0.5}$ values showed a close correlation. Here, the human enzyme variant exhibited 27-fold higher affinity. However, the paired $K_{0.5}$ values determined on both enzymes for 28 steroid derivatives of differing structural features were but poorly correlated. Essentially, the geometries of the steroid nucleus determined the differential contributions of the side chains at C3 and C17 to the integral inhibitory potency on the two enzyme variants. Thus, the species differences in the potency of cardiac glycosides were traced to species differences in the complementarity of the steroid binding subsites. Hence, estimates of the potency of new steroidal compounds obtained on the guinea-pig cardiac enzyme can be neither quantitatively nor qualitatively easily extrapolated to the human cardiac enzyme.

The extrathermodynamic analysis of the data opened major new insights in the structure–activity relationships concerning the role of C14β-OH, the character of the lead structure in cardioactive steroid lactones, and the significance of the configuration of A/B ring junction.

The Na/K-ATPase (Na^+/K^+-transporting ATPase, EC 3.6.1.37), the enzymatic machinery of the Na^+/K^+ pump, is now widely accepted as the digitalis receptor (for recent reviews see [1, 2]). The hypothesis on the receptor function of this enzyme advanced in the early sixties [3–5] was partially derived from the discovery [3, 6] that the large species differences in digitalis sensitivity are based on corresponding differences in the sensitivity of the Na/K-ATPases to the inhibitory action of digitalis compounds. This finding, confirmed and extended by numerous laboratories (cf. e.g. [7, 8]), was followed by the discovery that the digitalis sensitivity of the Na/K-ATPases from cardiac muscle and brain can differ in one and the same species [9–11].

Between the two components of biological specificity, thermodynamic equilibrium binding and kinetic control, the major determinant is known to be thermodynamic and to arise through the fit of complementary structures [12]. The early notion [6] that the species differences in the susceptibility of Na/K-ATPases to inhibition by digitalis compounds might be caused by differences in the degree of complementarity between the interacting surfaces of the binding site and the steroid moiety of the inhibitors, will be examined in the present paper by comparing the Na/K-ATPases from human cardiac muscle, human brain cortex and guinea-pig cardiac muscle with respect to the inhibitory potency of 95 steroidal inhibitors differing in the structure of the steroid nucleus and the side chains at C3 and C17. The practical aim underlying the comparison of the Na/K-ATPases just from these tissues and species has been to assess whether or how far the structure–activity relationships determined with any of these enzyme variants can be extrapolated to any of the two other enzyme forms.

METHODS

Preliminaries. The potency of all steroidal compounds analysed was assessed by determining their

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Table 1. Inhibitory potency of C3- or C17-substituted 5β-14β-androstan-14-ol derivatives expressed by the apparent dissociation constants (K'_D) and apparent Gibbs interaction energies ($\Delta G^{0'}$) of their complexes with the Na/K-ATPases of human heart (h.h.), human brain (h.b.), and guinea-pig heart (g.-p.h.)

No.	Systematic (trivial) name	K'_D (μM)			$-\Delta G^{0'}$ (kJ/mole)		
		h.h.	h.b.	g.-p.h.	h.h	h.b.	g.-p.h.
1	5β,14β-Androstane-3β,14-diol	150[b]	210	700[a]	22.7	21.8	18.7
2	5β,14β-Androstane-3β,14,17β-triol	180[a]	180[a]	2400[a]	22.2	22.2	15.5
3	5β,14β-Androstane-3β,14,17β-triol 17-acetate	230[b]	322[a]	1700[a]	21.6	20.7	16.5
4	3β-Rhamnosyloxy-5β,14β-androstan-14-ol	6.5[b]	12	290[a]	30.8	29.2	21.0
5	3β-Rhamnosyloxy-5β,14β-androstane-14,17β-diol	2.4[b]	4.9	170[a]	33.3	31.5	22.3
6	3β-Rhamnosyloxy-5β,14β-androstane-14,17β-diol 17-acetate	4.9[b]	n.d.	n.d.	31.5	—	—
7	3β,14-Dihydroxy-5β,14β-androstan-17-one	1100[a]	n.d.	n.d.	17.6	—	—
8	3β,14-Dihydroxy-5β,14β-androstane-17-guanylhydrazone	17	37	100[a]	28.4	26.3	22.9
9	17β-(Fur-3′-yl)-5β-14β-androstane-3β,14-diol 3-acetate	0.16[b]	0.12	3.5[a]	40.4	41.1	32.4
10	(2O*R*)-3β,14-Dihydroxy-21-nor-5β,14β-chol-22-ene-24,20-lactone	15[b]	11	190[a]	28.6	29.5	22.2
11	(2O*S*)-3β,14-Dihydroxy-21-nor-5β,14β-chol-22-ene-24,20-lactone	93[a,b]	120	n.d.	24.0	23.3	—
12	3β-Glucosyloxy-14-hydroxy-24-nor-5β,14β-chol-20(22)-ene-21,23-lactone (actodigin)	1.8[b]	1.9	160[a]	34.2	34.0	22.5
13	(23*R*)-23-methyl-actodigin	0.35[b]	0.75	40[a]	38.4	36.4	26.1
14	(23*S*)-23-methyl-actodigin	1.5[b]	3.6	130[a]	34.6	32.3	23.2
15	17β-Amino-3β-tridigitoxosyloxy-5β-14β-androstan-14-ol	n.d.	0.80	18	—	36.2	28.2
16	3β-14-Dihydroxy-5β-14β-card-20(22)-enolide (digitoxigenin)	0.053[b]	0.086	1.9	43.2	42.0	34.0
17	Digitoxigenin 3-acetate	0.070	0.089	1.6	42.5	41.9	34.4
18	3β-Deoxy-3β-(*n*-propylsulfonamido)-digitoxigenin	0.017	0.036	0.19	46.2	44.2	40.0
19	Digitoxigenin 3-sulfuric acid ester	0.65[b]	0.45	25	36.8	37.7	27.3
20	3β-*O*-arabinofuranosyl-digitoxigenin	0.017[b]	0.040	0.73	46.1	44.0	36.4
21	3β-*O*-digitoxosyl-digitoxigenin (evatromonoside)	0.0051[b]	0.0082	0.12	49.2	48.0	41.1
22	3β-*O*-glucosyl-digitoxigenin	0.0080[b]	0.017	1.0	48.1	46.2	35.6
23	3β-*O*-rhamnosyl-digitoxigenin (evomonoside)	0.0033[b]	0.0068	0.12	50.4	48.5	41.1
24	3β-*O*-tridigitoxosyl-digitoxigenin (digitoxin)	0.0079[b]	0.013	0.19	48.1	46.8	40.0
25	3β-*O*-(3′-*O*-methyl-rhamnosyl)-digitoxigenin	0.0072	0.0043	0.14	48.3	49.7	40.8
26	3β-*O*-(4′-deoxy-4′ξ-amino-rhamnosyl)-digitoxigenin	0.0016	0.0031	0.019	52.3	50.5	45.9
27	3β-*O*-(2′,3′-*O*-isopropylidene-rhamnosyl)-digitoxigenin	0.0079	0.0098	0.092	48.1	47.6	41.8
28	3β-*O*-(2′,3′-*O*-isopropylidene-4′-dehydro-rhamnosyl)-digitoxigenin	0.012	0.017	0.097	47.1	46.2	41.6
29	3α-Methyl-digitoxigenin	0.70[b]	1.0	18	36.6	35.5	28.1
30	3β-*O*-glucosyl-3α-methyl-digitoxigenin	0.66[b]	0.78	13	36.7	36.3	29.0
31	3α-Methyl-3β-*O*-rhamnosyl-digitoxigenin	0.55[b]	1.3	25[a]	37.2	35.1	27.3
32	(20*R*)-20,22-dihydro-digitoxigenin	5.4[b]	7.5	140[a]	31.3	30.4	23.0
33	(20*S*)-20,22-dihydro-digitoxigenin	2.3[b]	3.7	85[a]	33.5	32.3	24.2
34	22-Hydroxy-digitoxigenin 3-acetate	0.34	0.45	3.5	38.4	37.7	32.4
35	22-Hydroxy-digitoxigenin 3,22-diacetate	0.16	0.24	4.1	40.4	39.3	32.0
36	22-Hydroxy-digitoxigenin 3-acetate 22-propionate	0.19	0.30	6.0	40.0	38.7	31.0
37	22-Methyl-digitoxigenin 3-acetate	0.25[b]	0.80	7.2	39.3	36.2	30.5
38	22-Methoxy-digitoxigenin 3-acetate	4.6	9.0	66	31.7	30.0	24.8
39	22-Allyl-digitoxigenin	19	24	n.d.	28.0	27.5	—

40	22-Propyl-digitoxigenin	46[a]	63[a]	n.d.	25.8	25.0	—
41	22-Methyl-digitoxin	0.025[b]	n.d.	0.99	45.2	—	35.6
42	(21*R*)-21-methyl-digitoxigenin 3-acetate	3.1[b]	2.7	28[a]	32.7	33.1	27.0
43	(21*S*)-21-methyl-digitoxigenin 3-acetate	21[a,b]	18	170[a]	27.8	28.2	22.4
44	(21*R*)-3β-*O*-glucosyl-21-methyl-digitoxigenin	0.56[b]	0.68	21	37.1	36.6	27.8
45	(21*S*)-3β-*O*-glucosyl-21-methyl-digitoxigenin	2.1[b]	2.6	120[a]	33.7	33.2	23.3
46	3β,14-Dihydroxy-5β,14β-bufa-20,22-dienolide (bufalin)	0.0034	0.0059	0.057	50.3	48.8	43.0
47	3β-*O*-rhamnosyl-bufalin	0.00043	0.00039	0.0068	55.6	55.9	48.5
48	3β-Deoxy-3β-amino-bufalin	n.d.	0.0086	0.055	—	47.9	43.1

[a] Half-maximum inhibition not reached mostly owing to limited solubility. [b] Experimental data from reference [25], but calculated by a different mathematical procedure (see Methods). n.d., not determined.

inhibitory effect on the activity of the enzyme variants, but not by measuring their capability for binding to the enzymes that may occur preferentially to non-cycling intermediates (cf. [1]). The proper reason for this decision was to mimic as much as possible the condition for the development of the *in vivo* digitalis actions which are evoked through the inhibition of the Na^+/K^+ pump [1, 2].

The activity of the three enzymes compared was promoted by using the same concentrations of Na^+, K^+, Mg^{2+} and ATP, since the $K_{0.5}$ values and Hill coefficients for these ligands are remarkably similar in Na/K-ATPases of widely different digitalis sensitivity [8, 13, 14], meaning that the structural differences responsible for different digitalis sensitivities are not extended to the binding domains of the activating cations and ATP [8].

As the attainment of the equilibrium stage of the inhibitory action of certain glycosides required extended incubation times, the incubation medium was supplemented by an ATP-regenerating enzyme system to exclude a possible interference of ADP in the enzyme–inhibitor interaction. An interference of orthophosphate arising from ATP hydrolysis could be taken as absent in the presence of the high Na^+ concentrations applied [15]. The inhibitory potency of the compounds was measured under maximized turnover conditions to favour the presence of a single type of receptive intermediary Na/K-ATPase state. Under the conditions chosen the concentration of the inhibitors was greatly in excess of the concentration of the enzymes such that the concentration of free inhibitor was approximately equal to the concentration applied.

The equilibrium value of inhibition, I_∞, was either determined directly or calculated according to

$$I_t = I_\infty(1 - e^{-kt}) \tag{1}$$

in which t, k and I_t denote the exposure time, the time constant, and the inhibitory degree at the time t, respectively.

Initially, the concentration of a compound C producing half-maximum inhibition, $K_{0.5}$, was calculated according to

$$I_\infty = \frac{[C]^h}{[C]^h + (K_{0.5})^h} \tag{2}$$

The Hill coefficient, h, was found to be not significantly different from unity for all compounds and enzyme variants (not shown), except the interaction of progesterone-3,20-bis-guanylhydrazone with human brain enzyme ($h = 1.5$). Since else $h \approx 1$, the inhibitory potency expressed by the K_D' value was calculated according to the simpler Eqn (5). The time course of inhibition followed Eqn (1) corresponding to the model

$$E_{rec} + C \rightleftharpoons E_{rec} \cdot C \tag{3}$$

in which E_{rec} denotes the (unknown) receptive intermediary state of the enzyme. This time course and $h = 1$ indicate that the interaction sites were all identical and independent as simply follows from mass law considerations [16]. In the equilibrium, the concentration at which the Na/K-ATPase activity is inhibited half-maximally is known to be equivalent to

Table 2. Inhibitory potency of steroidal compounds differing from 5β,14β-androstan-14-ol by diverse ring junctions, steroid nuclei, or ring substituents. The potencies are expressed by the apparent dissociation constants (K'_D) and apparent Gibbs interaction energies ($\Delta G^{0'}$) of their complexes with the Na/K-ATPases of human heart (h.h.), human brain (h.b.), and guinea-pig heart (g.-p.h.)

No.	Systematic (trivial) name	K'_D (μM)			$-\Delta G^{0'}$ (kJ/mole)		
		h.h.	h.b.	g.-p.h.	h.h.	h.b.	g.-p.h.
49	5α-14β-Androstane-3β,14,17β-triol	130[a,b]	200[a]	120[a]	23.1	22.0	23.3
50	8α,14β-Estra-1,3,5(10)-triene-3,14,17β-triol	710[a,b]	770[a]	n.d.	18.7	18.5	—
51	11β,17α,21-Trihydroxy-pregna-1,4-diene-3,20-dione (prednisolone)	2200[a,b]	1000[a]	n.d.	15.8	17.8	—
52	Pregn-4-ene-3,20-dione (progesterone)	120[b]	160	n.d.	23.2	22.5	—
53	Prednisolone-3,20-bis-guanylhydrazone	14[b]	22	0.27	28.7	27.7	39.0
54	Progesterone-3,20-bis-guanylhydrazone	1.3[b]	1.9	0.69	34.9	34.0	36.6
55	Butan-4'-olide-4'-spiro-17β-14α-androst-4,6-dien-3-one (canrenone)	120[a,b]	85[a]	590[a]	23.2	24.2	19.2
56	Cassiac acid dimethylaminoethyl ester (cassaine)	0.65[b]	1.4	1.6	36.7	34.8	34.4
57	3β-Rhamnosyloxy-5β-androst-14-ene	43[a,b]	32	190[a]	25.9	26.7	22.0
58	3β-(2',3'-*O*-isopropylidene-4'-dehydro-rhamnosyloxy)-5β-androst-14-ene	37[a]	56[a]	n.d.	26.3	25.2	—
59	6-Chloro-3β,17α-dihydroxy-pregna-4,6-dien-20-one 17-acetate (chlormadinol acetate)	4.1[b]	7.2	160[a]	32.0	30.5	22.5
60	3β-*O*-arabinofuranosyl-chlormadinol acetate	26[a,b]	87[a]	n.d.	27.2	24.1	—
61	3β-*O*-rhamnosyl-chlormadinol acetate	32[a,b]	51[a]	38[a]	26.7	25.5	26.3
62	14-Hydroxy-3β-rhamnosyloxy-19-nor-5β,14β-pregnan-20-one	n.d.	2.8	160[a]	—	33.0	22.6
63	(20*R*)-3β-rhamnosyloxy-19-nor-5β,14β-pregnane-14,20-diol	n.d.	3.2	85[a]	—	32.6	24.2
64	(20*R*)-14-amino-3β-rhamnosyloxy-5β,14β-pregnan-20-ol	0.024[b]	0.025	0.51	45.2	45.1	37.4
65	17β-(Pyrid-3'-yl)-14β-androst-4-ene-3β,14-diol	0.13[b]	0.15	1.4	40.9	40.5	34.7
66	Δ^4-digitoxigenin (canarigenin)	0.30[b]	0.21	4.7	38.7	39.6	31.6
67	Δ^4-bufalin (scillarenin)	0.0057[b]	n.d.	0.17	49.0	—	40.2
68	3β-*O*-rhamnosyl-scillarenin (proscillaridin A)	0.0023[b]	n.d.	0.013	51.3	—	46.8
69	3β-Rhamnosyloxy-14-hydroxy-5β,14β-chola-4,20,22-triene-24,21-lactam	0.18[b]	n.d.	4.9	40.1	—	31.6
70	3β,14-Dihydroxy-5α-14β-card-20(22)-enolide (uzarigenin)	0.38	0.66	9.2	38.1	36.7	29.9
71	3β-Deoxy-3β-amino-uzarigenin	n.d.	0.40	2.5	—	38.0	33.3
72	(21*R*)-3β-acetoxy-14,21-epoxy-5β,14β-card-20(22)-enolide	44[a]	97[a]	n.d.	25.9	23.8	—
73	3β,12β,14-Trihydroxy-5β,14β-card-20(22)-enolide (digoxigenin)	0.21[b]	0.41	13	39.7	37.9	29.0
74	3β-*O*-tridigitoxosyl-digoxigenin (digoxin)	0.014[b]	0.057	0.55	46.6	43.0	37.2
75	22-Methyl-digoxin	n.d.	0.13	2.5	—	40.8	33.3
76	3β,14,16β-trihydroxy-5β,14β-card-20(22)-enolide (gitoxigenin)	0.50[b]	0.98	22	37.4	35.7	27.6
77	3β-*O*-arabinofuranosyl-gitoxigenin	0.12[b]	0.31	5.6	41.6	38.6	31.2
78	3β-*O*-tridigitoxosyl-gitoxigenin (gitoxin)	0.035[b]	0.11	1.1	44.3	41.2	35.5
79	Gitoxigenin 16-nitric acid ester	0.0092	0.0064	0.11	47.7	48.7	41.3
80	Gitoxigenin 3,16-dinitric acid ester	n.d.	0.026	n.d.	—	45.0	—
81	Gitoxigenin 3,16-bis-trimethylsilyl ether	0.73	n.d.	8.5	36.4	—	30.1
82	Gitoxigenin 3-acetate 16-chloroacetate	n.d.	0.34	3.0	—	38.4	32.8
83	Gitoxin 16-trimethylsilyl ether	n.d.	0.11	n.d.	—	41.4	—
84	(21*R*)-21-fluoro-gitoxigenin	n.d.	0.98	n.d.	—	35.7	—
85	(21*S*)-21-fluoro-gitoxigenin	n.d.	4.7	n.d.	—	31.6	—
86	16α-Gitoxigenin (16-epi-gitoxigenin)	6.0[b]	11	n.d.	31.0	29.4	—
87	16α-Gitoxigenin 16-nitric acid ester	0.0087	0.014	0.21	47.9	46.6	39.6

88	16α-Gitoxin (16-epi-gitoxin)	0.65[b]	1.2	13	36.7	35.1	29.1
89	3β-*O*-oleandrosyl-16-anhydro-gitoxigenin	50[a]	63[a]	n.d.	25.5	24.9	—
90	16-Anhydro-gitoxin	53[a]	180[a]	n.d.	25.4	22.3	—
91	16-Anhydro-gitoxin 3′,3″,3‴,4‴-tetraacetate	35[a]	190[a]	n.d.	26.5	22.1	—
92	(21*R*)-21-fluoro-16-anhydro-gitoxin 3′,3″,3‴,4‴-tetraacetate[c]	3.6	4.7	40	32.3	31.6	26.1
93	14-Anhydro-gitoxigenin 3,16-diacetate	2.3	3.1	31[a]	33.5	32.7	26.8
94	1β,3β,5,11α,14,19-Hexahydroxy-5β,14β-card-20(22)-enolide (ouabagenin)	0.47[b]	1.6	3.9	37.6	34.5	32.1
95	3β-*O*-rhamnosyl-ouabagenin (ouabain)	0.027[b]	0.033	0.80	45.0	44.4	36.2

[a] Half-maximum inhibition not reached mostly owing to limited solubility. [b] Experimental data from reference [25], but calculated by a different mathematical procedure (see Methods). [c] Tentative assignment of configuration of fluorine substituent.

the apparent dissociation constant of the inhibitor–enzyme complex determined in binding experiments under similar conditions [11, 17–19]. Taken together, the described relationships justified to use the concentration producing half-maximum inhibition as equal to the apparent dissociation constant, K'_D, of the enzyme–inhibitor complex and as a suitable measure for the potency of the inhibitor. For quantitative structure–activity analysis the K'_D value was converted into the apparent Gibbs energy change, $\Delta G^{0\prime}$, by means of the familiar equation

$$\Delta G^{0\prime} = RT \ln K'_D \qquad (4)$$

Both values are shown in Tables 1 and 2.

Preparation and determination of Na/K-*ATPases.* The human tissue sources were cardiac ventricle muscle and brain cortex from undigitalized men taken within 12 hr *post mortem*. These plentiful sources were chosen as rapid deterioration of the enzyme *post mortem* does not occur judged from the finding that the activity changes in response to variations of pH and of concentrations of Na^+, K^+ and Mg^{2+} in the assay medium is similar in Na/K-ATPase preparations from biopsy and necropsy specimens of human cardiac muscle [20]. Moreover, the digitalis (digoxin) sensitivity of cardiac enzyme preparations from biopsy specimen [10] and necropsy samples (this paper) is similar. The guinea-pig tissue source was cardiac ventricle muscle from mature animals of both sexes.

The enzyme variants of the different sources were little purified as tissue-dependent differences in digitalis sensitivity can be an artifact of more intensive purification procedures [21]. The Na/K-ATPase from guinea-pig cardiac muscle was prepared according to the procedure described by Matsui and Schwartz [22] (occasionally omitting the deoxycholate treatment) or by Samaha [23] (omitting the LiCl treatment). The variant procedures had no influence on the ouabain sensitivity of the enzyme preparations. The Na/K-ATPases from human cardiac muscle and cerebral cortex were prepared as described by Matsui and Schwartz [22] and Samaha [23] (omitting the LiCl treatment), respectively. Protein was determined by the procedure of Bensadoun and Weinstein [24].

The medium for estimating Na/K-ATPase activity contained 2 mM ATP, 4 mM $MgCl_2$, 80 mM NaCl, 5 mM KCl, 80 mM imidazole–HCl buffer (pH 7.4 at 37°), 0.1 mM EDTA, 0.3 mM phosphoenolpyruvate, 0.2 mM NADH, 8 I.U. pyruvate kinase, 8 I.U. lactate dehydrogenase, 11 mM $(NH_4)_2SO_4$ and the membraneous enzyme preparation, all in a volume of 2 ml. Substrates and auxiliary enzymes were from Boehringer (Mannheim, F.R.G.). All other chemicals were of analytical grade.

The reaction was started by addition of 20 μl of the membrane suspensions containing 28–64 μg, 5–66 μg, and 1.7–6.8 μg protein of the enzyme preparations from human or guinea-pig cardiac muscle, and human cerebral cortex, respectively. ATPase activity was measured at 37° by the decrease of extinction at 334 nm. The portion of activity suppressed by 0.1 mM ouabain was taken as Na/K-ATPase activity amounting to 2.2–11.5, 1.0–38.8, and 39–109 $\mu mole \cdot mg^{-1} \cdot hr^{-1}$ for the enzymes from

human or guinea-pig heart, and human brain, respectively. This portion was mostly more than 90% of total ATPase activity. The Na/K-ATPase activity remained essentially unchanged during incubation lasting maximally 2 hr.

Determination and expression of inhibitory steroid potency. The compounds were dissolved and transferred in dimethylsulfoxide into the assay medium so that the concentration of the solvent was always 1.5% (v/v). Parallel to the determination of total and ouabain-suppressible ATPase activity, the inhibitory effect of a given steroid on Na/K-ATPase activity was assessed by testing at least four concentrations (covering two orders of ten) around the concentration producing half-maximum inhibition. From the equilibrium values of inhibition, I_∞, found for several concentrations of a compound, the K'_D value was calculated by non-linear regression according to

$$I_\infty = \frac{[C]}{[C] + K'_D}. \quad (5)$$

For all compounds and enzymes the standard deviation of K'_D values amounted to 11% in the mean. When half-maximum inhibition could not be covered due to limited solubility of the compound in question, the SD of the calculated K'_D value was in the mean 19%, 22% and 15% for the human or guinea-pig cardiac enzyme, and the human brain enzyme, respectively. In multiple determinations of a K'_D value and different batches of an enzyme preparation (not checked in each case) the standard error amounted in the mean to 30%, 25%, and 18% for the human or guinea-pig cardiac enzyme, and the human brain enzyme, respectively. Accordingly, the mean error of the $\Delta G^{0'}$ values was less than ±1 kJ/mole.

Steroids used in this study. The sources of most of the compounds (Tables 1 and 2) were given in a foregoing paper [25] but note that the numbering of the compounds is not identical. The following compounds were generous gifts: **7** from Prof. Wicha, Warsaw, Poland; **18** from VEB Jenapharm, Jena, G.D.R.; **46, 48, 71** from Prof. Meyer, Basle, Switzerland; **50** from Prof. Ananchenko, Moscow, U.S.S.R.; **70** from Prof. Tschesche, Bonn, F.R.G.; **87** from VEB AWD, Dresden, G.D.R.; **93** from Dr. Voigtländer, Boehringer, Mannhein, F.R.G. The compounds **2, 25, 26, 27, 28, 47, 58, 61, 62, 63** were prepared by one of us (J.W.); **8, 17, 34, 35, 36, 38, 72, 89, 90** by Dr Lindig; **79, 80, 81, 83, 84, 85, 92** by Dr Megges; and **15, 39, 40, 75, 82, 91** by Dr Streckenbach (all Central Institute of Molecular Biology, Berlin, G.D.R.). The preparations of the compounds were carried out by established procedures or will be shortly published elsewhere.

RESULTS

The general plan of present study was to compare the Na/K-ATPases of human cardiac muscle, human brain cortex and guinea-pig cardiac muscle with regard to the inhibitory potency of steroidal effectors of very different structure. The underlying aim was to probe similarities or differences in the complementary chemotopology of the inhibitor binding site areas of the three enzymes. As these areas could differ in the subsites interacting with the steroid nucleus and any side chains at C3 or C17, a series of steroid derivatives differing in the three substructures were assessed as to the influence of the variant substructures on the value of the apparent dissociation constant, K'_D, of the complexes of the derivatives with the enzymes. In Tables 1 and 2, the measured K'_D values are also expressed in terms of the apparent standard Gibbs energy changes, $\Delta G^{0'}$, to allow the extrathermodynamic approach as a rational way to correlate in a quantitative manner potencies and substructural variables [26, 27]. Thus, the energetic contributions of individual substituents to the integral inhibitor–enzyme interaction energies were calculated from the differences of the $\Delta G^{0'}$ values of the 85 pairs of derivatives which differed in only one substituent. The comprehensive list of $\delta\Delta G^{0'}$ values in hand (not shown) revealed that certain substructural variables contributed similarly to the integral interaction energies of diverse inhibitors with the human enzymes from cardiac muscle and brain cortex (epitomized in section 1), whereas other substructural variables contributed differentially to the overall interaction energies with the enzyme forms of human and guinea-pig cardiac muscle (specified in section 2). When appropriate, the pairs evaluated are indicated by the numbers of the derivatives the systematic names of which are given in Tables 1 and 2. The arrow between the numbers identifies then the direction of the structural modification in question.

1. *Comparison of the inhibitor-responsiveness of Na/K-ATPases from human cardiac muscle and human brain cortex*

The inhibitory potency of 77 steroid derivatives, expressed in terms of the K'_D values of their complexes with the two enzymes, covered 7 orders of ten (Tables 1 and 2). The K'_D values measured with the two enzyme forms were nevertheless highly correlated as shown in Fig. 1. On the other hand, the regression equation calculated for the correlation between the K'_D values of the two enzymes (Fig. 1) revealed that the total of derivatives showed on an average a 1.5-fold higher affinity to the cardiac than to the cerebral enzyme. In any single determination, however, this affinity difference between the two enzyme forms was lying within experimental error which rendered as yet impossible its assignment to an individual substructural feature of the derivatives.

The comparative analysis included the impact of seven major structural modifications on the inhibitory potency in both enzyme forms. (I) Flattening of the A/B-ring plane angle through: conversion of A/B *cis* into *trans* junction, introduction of C4–C5 double bond or aromatization of ring A. (II) Flattening of C/D-ring plane angle through introduction of C14–C15 double bond. (III) Structural variations at C3 through: introduction of methyl in C3α-position, esterification of C3β-OH, glycosidation at C3β-OH with various sugars or sugar derivatives, and replacement of C3β-OH by C3β-(*n*-propylsulfonamide) or C3β-NH_2. (IV) Introduction of hydroxy groups at very different positions in steroid skeleton. (V) Esterification of C16β-OH with acetic

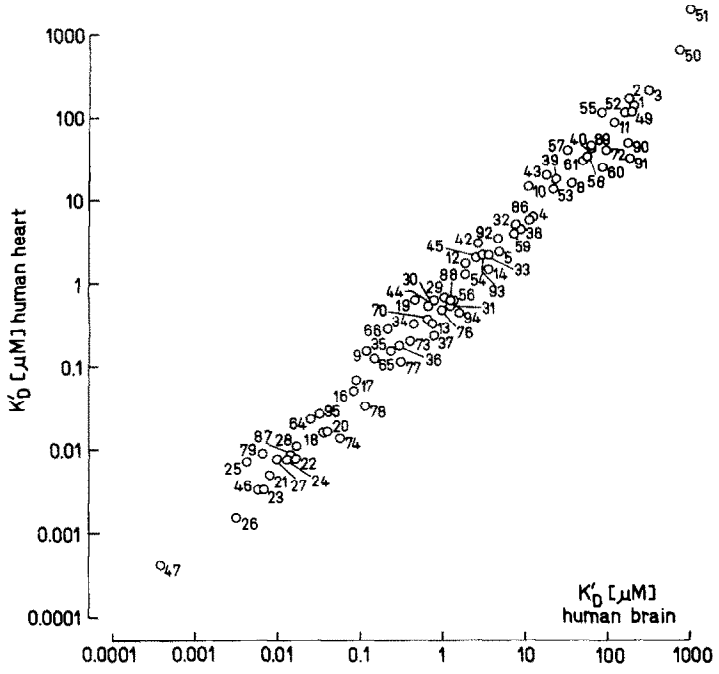

Fig. 1. Analysis of the correlation between the paired K'_D values for the inhibitory complexes of various steroidal compounds with Na/K-ATPases from human heart (h.h.) or human brain (h.b.), respectively. The numerals in the figure refer to Tables 1 and 2 where the structure of the compounds is shown. Linear regression of the 77 paired K'_D values gives the equation

$$\log K'_D \text{ (h.h.)} = 1.001 \log K'_D \text{ (h.b.)} - 0.174$$

with the correlation coefficient $r = 0.991$.

or nitric acid. (VI) Substitution of C17β-H by various side chains differing in electron density distribution, van der Waals' geometry and conformational freedom. (VII) Change of the spatial orientation of the butenolide ring at C17β through introduction of C16-C17 double bond. Surprisingly enough, all above-mentioned structural modifications led to parallel decrements or increments of the energy of interaction of the derivatives with the cardiac and cerebral enzyme. This result suggested the conclusion that the tiny difference in the affinity of the two enzyme forms for the various inhibitors was not connected

Table 3. The integral energies of the interaction of C3β-*O*-rhamnosyl-digitoxigenin (**23**) and C3β-*O*-rhamnosyl-bufalin (**47**) with the Na/K-ATPases from human heart (h.h.) and human brain (h.b.), but less from guinea-pig heart (g.-p.h.) are the additive properties of the partial energetic contributions of their structural components

Assignment of interaction energy to structure		Apparent Gibbs energy (kJ/mole)		
		h.h.	h.b.	g.-p.h.
$-\Delta G^{0\prime}$ for steroid nucleus (**1**)		22.7	21.8	18.7
$-\delta\Delta G^{0\prime}$ for butenolide substituent (**1** → **16**)		20.5	20.8	15.3
$-\delta\Delta G^{0\prime}$ for rhamnose substituent (**1** → **4**)		8.1	7.4	2.3
	sum	51.3	50.0	36.3
$-\Delta G^{0\prime}$ for complete molecule (**23**)		50.4	48.5	41.1
$-\Delta G^{0\prime}$ for steroid nucleus (**1**)		22.7	21.8	18.7
$-\delta\Delta G^{0\prime}$ for pentadienolide substituent (**1** → **46**)		27.6	27.0	24.3
$-\delta\Delta G^{0\prime}$ for rhamnose substituent (**1** → **4**)		8.1	7.4	2.3
	sum	58.4	56.2	45.3
$-\Delta G^{0\prime}$ for complete molecule (**47**)		55.6	55.9	48.5

with an important difference in the chemotopology of the complementary subsites for steroid nucleus binding in the cardiac and cerebral enzyme.

Independent of the enzyme form studied, the magnitude of the interaction energy increments or decrements caused by one and the same structural modification varied in part with the structure of the precursor compound. The divergence was especially great when the substituent in question was surrounded by structural features of different space requirements. For instance, differences in steric hindrance accounted for the difference in the $\delta\Delta G^{0\prime}$ values resulting from rhamnosylation at C3β-OH in the absence (**16** → **23**) or in the presence (**29** → **31**) of C3α-methyl, and from introduction of methyl at C3α in the neighbourhood of C3β-OH (**16** → **29**) or C3β-O-rhamnose (**23** → **31**).

The above findings, however, did not generally invalidate the additivity concept [26, 27] which stood also here the test as the fundamental basis of quantitative structure–activity analyses. So, the integral interaction energy of C3β-O-rhamnosyl-digitoxigenin and C3β-O-rhamnosyl-bufalin with the two human enzymes proved to be the additive property of the partial energy contributions of their structural components, the steroid nucleus, the lactone substituent at C17β and the sugar substituent at C3β as shown in Table 3. This observation indicated a close fit between the interfaces of steroid nucleus and its binding subsites on both human enzymes that does not become altered through the attachment of the voluminous substituents at C3β or/and C17β. Note that the fulfilment of the additivity postulate was bound to the human enzyme forms and to 5β,14β-androstane-3β,14-diol as steroid nucleus (cf. section 2).

2. Comparison of the inhibitor-response of the Na/K-ATPases from human and guinea-pig cardiac muscle

Sixty-nine compounds specified in Tables 1 and 2 were compared as to their inhibitory potency in both enzymes. The range of the K'_D values of their complexes with either enzyme covered 6 orders of ten. As shown in Fig. 2, in general the paired K'_D values measured with the human and guinea-pig enzyme proved to be correlated, but the correlation coefficient was 0.909 only. The 41 paired K'_D values for 5β,14β-androstane derivatives carrying substituents on C3β or/and C17β as specified in Table 1, gave the regression equation:

$$\log K'_D \text{ (human)} = 0.988 \log K'_D \text{ (guinea-pig)} - 1.436$$

with $r = 0.970$. This close correlation, with the slope of the regression line near unity, means that the human enzyme showed 27-fold higher affinity for inhibitors of the structural type defined. The paired K'_D values measured with the human or guinea-pig enzyme and the 28 compounds specified in Table 2 yielded the regression equation:

$$\log K'_D \text{ (human)} = 0.965 \log K'_D \text{ (guinea-pig)} - 1.175$$

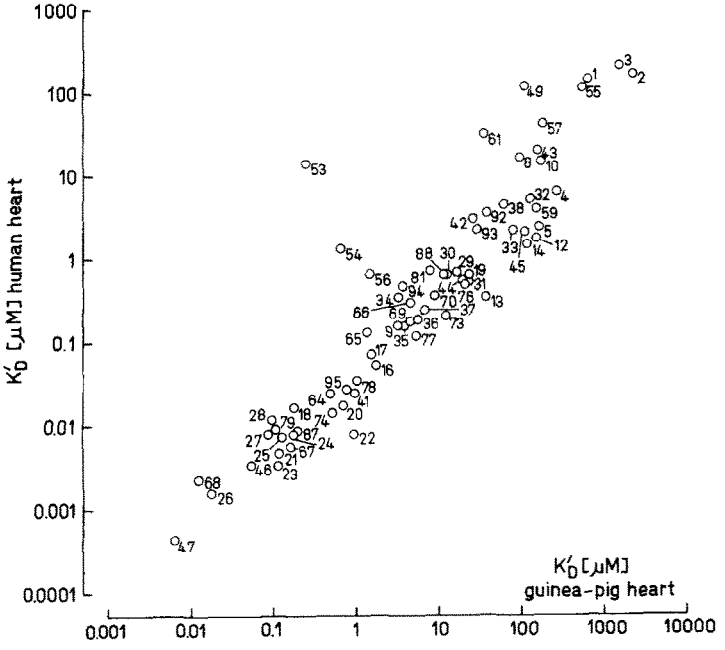

Fig. 2. Analysis of the correlation between the paired K'_D values for the inhibitory complexes of various steroidal compounds with Na/K-ATPases from human heart (h.h.) and guinea-pig heart (g.-p.h.), respectively. The numerals in the figure refer to Tables 1 and 2 where the structure of the compounds is shown. Linear regression of the 69 paired K'_D values gives the equation

$$\log K'_D \text{ (h.h.)} = 0.968 \log K'_D \text{ (g.-p.h.)} - 1.385$$

with the correlation coefficient $r = 0.909$.

with $r = 0.817$. This poor correlation was caused above all by the paired K'_D values for the compounds **49**, **53**, **54**, **56** and **61**. So, progesterone-bis-guanylhydrazone (**54**) or prednisolone-bis-guanylhydrazone (**53**) showed for the human enzyme 1.9-fold or 52-fold lower affinity than for the guinea-pig enzyme.

The chemotopology of the steroid and the sugar binding subsite appeared to differ in the two enzyme variants as the following pieces of evidence suggested.

The interaction energy of the steroid compounds with bent (*cis*) A/B ring junction (**1** . . .**5**) was considerably higher in the human enzyme, whereas that of the steroid compound with flat (*trans*) A/B ring junction (**49**) was similar in both enzymes. Flattening of the C/D ring junction through introduction of the double bond between C14 and C15 (**4** → **47**) led only in the human enzyme to reduction of the interaction energy. Incidentally, the steroid with A/B *trans* junction (**49**) was in the guinea-pig enzyme considerably more active than the A/B *cis* epimer (**2**).

The geometry of the steroid nucleus determined to a large extent the different effects of substituents of C3β-OH or C17β-H on the inhibitory potency in the two enzymes.

Steroids with bent (cis) *A/B ring junction.* Glucosylation at C3β-OH (**16** → **22**, **42** → **44**, **43** → **45**) increased the integral interaction energy considerably in the human enzyme, but little, if at all, in the guinea-pig enzyme. Apparently, the chemotopology of the sugar binding subsite is different in the two enzymes. However, the increment through rhamnosylation at C3β-OH was in both enzymes similar, if there was a substitution of C17β-H (**16** → **23**, **46** → **47**), but in the guinea-pig enzyme significantly lower in case of its absence (**1** → **4**). This is the reason for the poor additivity of the interaction energy contributions of substituents at C3β and C17β found in the guinea-pig enzyme (Table 3). Apparently, the C17β-substituent changed the spatial orientation of the glycosides **23** and **47** in their binding cleft of the guinea-pig enzyme and favoured in this way the interaction of the rhamnose residue with the sugar binding subsite. The presence of 5β-OH reduced the rhamnosylation increment in the guinea-pig, but not in the human enzyme (**94** → **95**). The replacement of C3β-OH by the *n*-propylsulfonamide residue (**16** → **18**) or of C4′-OH by C4′-NH_2 in the rhamnose side chain (**23** → **26**) increased the interaction energy in the guinea-pig enzyme more than in the human enzyme. At C17β, the attachment of the β-furane ring (**1** → **9**), the β-butenolide ring (**1** → **16**) or the γ-pentadienolide ring (**1** → **46**) increased the interaction energy in the human enzyme considerably more than in the guinea-pig enzyme.

Steroids with flat (trans) *A/B ring junction.* Rhamnosylation at C3β-OH effected in the guinea-pig enzyme a higher interaction energy increment (**67** → **68**) or even then an increment when in the human enzyme a considerable decrement resulted (**59** → **61**). The attachment of the β-butenolide ring at C17β (**49** → **70**) produced in the guinea-pig a much lower interaction energy increment than in the human enzyme, but in both enzymes the contribution of this substituent was in case of the precursor steroid with bent A/B ring junction (**1** → **16**) considerably higher.

In conclusion, the geometry of the steroid nucleus greatly determines the differential contributions of substituents at C3β and C17β to the overall interaction energy in the human and guinea-pig enzyme. Hence, the chemotopology of the complementary steroid binding subsite differs in the two enzymes. Thus, the species differences in the inhibitory power of cardiac glycosides with both bent and flat A/B ring junction are based on species differences in the complementarity of the steroid binding subsite.

DISCUSSION

General considerations on structure–activity relationships

The present findings confirm and extend earlier data on the inhibitory interaction between steroidal compounds (of comparatively small structural variation) with the Na/K-ATPases from guinea-pig cardiac muscle [10, 28–38] and from human cadiac muscle [10, 18, 30, 39–41]; the human cerebral enzyme has not been studied before. The following discussion will be confined to four major new conclusions derived from the present extended findings with the three enzymes compared (Tables 1 and 2).

(I) As a function of structural variation, the interaction energy of the inhibitors with the Na/K-ATPases of human or guinea-pig cardiac muscle may be varied by 34 kJ/mole and 20 kJ/mole, respectively, but the intrinsic inhibitory potency of the compounds with lowest $\Delta G^{0\prime}$ value remains unity.

(II) The replacement of C14β-OH by C14β-NH_2 (**63** → **64**) increases considerably the overall interaction energy (the absence or presence of a methyl group at C10 is neglected in this comparison because its contribution to interaction energy can be taken as negligibly small). The energy increment may result from the conversion of the negative potential well around C14β-OH into a positive region around C14β-NH_2, which is likely to exist as ammonium cation at physiological pH. This complements the positive potential shell which surrounds the steroid nucleus in the molecular electrostatic potential map of digitoxigenin [42]. Of course, ion-pair association can also contribute to the high interaction energy increment.

(III) The esterification of C16β-OH (**76** → **79**) or C16α-OH (**70** → **71**) with nitric acid increases considerably the integral interaction energy. This effect might be related to elimination of a repelling OH group and provision of oxygen-acceptors for hydrogen bonding to the steroid binding subsite.

(IV) The data presented in Tables 1–3 give an answer to the question of the lead structure in cardiotonic steroid lactones. The lead structure is here defined as the steroidal substructure that meets the minimal receptor recognition requirement and endows the lead, through attachment of the lactone ring, with highest potency in Na/K-ATPase inhibition. The earlier argumentation (not to be repeated here) concluded that the steroid nucleus in digitalis glycosides, i.e. 5β,14β-androstane-3β,14-diol (**1**), is the lead structure [25]. At first sight, this conclusion based on findings in the human cardiac enzyme, does not seem to apply in the guinea-pig cardiac enzyme. In this enzyme variant, 5α-14β-

androstane-3β,14,17β-triol (**49**) with A/B *trans* ring junction is, indeed, more potent than 5β,14β-androstane-3β,14,17β-triol (**2**) with A/B *cis* ring junction. However, the attachment of the butenolide ring at C17β increases the overall interaction energy in the A/B *trans* isomer by only −6.6 kJ/mol (**49** → **70**), but in the A/B *cis* isomer by as much as −15.3 kJ/mol (**2** → **16**). This reveals that, also on the guinea-pig enzyme, the A/B *cis* isomer 5β-14β-androstane-3β,14-diol (**1**) operates as the most potent lead structure.

On the other hand, the findings of present paper support the conclusions drawn from studies on the effects of some A/B *trans* cardenolides on guinea-pig atria, that the configuration of A/B ring junction does not play a critical role in the binding of the steroid nucleus to its receptor subsite in this species and that changes in activity associated with epimerization at C5 are secondary to changes in the orientation of substituent groups at C3β ([43] and present paper) and C17β (present paper).

Peculiarities of inhibitor-response by human cerebral cortex and cardiac muscle Na/K-*ATPases*

In the brain of rat, dog and calf, α and α(+) forms of the catalytic subunit of Na/K-ATPase occur which in these species appear to be related to low and high digitalis sensitivity, respectively [44]. In the cerebral cortex of man, α and α(+)forms have also been recently traced by means of specific antisera [14]. However, differences in digitalis sensitivity of Na/K-ATPases from various sources do not necessarily depend on whether they contain the α or/and α(+)forms of the catalytic subunit [8, 45]. Thus, it has been not too surprising that the concentration/inhibition curves for the interaction of the human brain cortex enzyme and the various steroidal compounds listed in Tables 1 and 2 show no such biphasic shape that had indicated in enzyme preparations from brain of rat [9, 44], mouse [9, 46], dog [44] and calf [44] the presence of digitalis-sensitive and -insensitive enzyme forms. Doubtless, monophasic curves as observed here do not exclude the involvement of two isoenzymes when, for example, they occur in a 1:1 ratio and differ in their digitalis affinity only by one order of ten or less (cf. [47]).

The Na/K-ATPase from brain was found to show higher digitalis sensitivity than the enzyme from cardiac muscle in rat [9, 11], mouse [9] and guinea-pig [10, 11]. However, in beef the cardiac enzyme proved to be more sensitive than the cerebral enzyme by factors of 1.5 for digitoxigenin, 3 for digitoxin, 4 for ouabain (all with A/B *cis* ring junction) and 40 for uzarigenin which is the A/B *trans* isomer of digitoxigenin (cf. the data in Tables 3 and 2 in references [17] and [38], respectively). These findings could let us expect that in the human cardiac and cerebral enzymes also larger divergencies in the relative inhibitor-sensitivity as a function of steroid structure would exist. Such divergences, however, are not found even on great modifications in structure of steroid nucleus and side chains at C3 and C17. However, the regression equation (cf. Fig. 1) calculated for the total of 77 derivatives indicates on an average a 1.5-fold higher sensitivity of the cardiac enzyme. This tendency (cf. Tables 1 and 2) appears to be most expressed in digitoxin (**24**), digoxin (**74**), gitoxin (**78**) and 16α-gitoxin (**88**), but less or not at all in actodigin (**12**), 3β-*O*-glucosyl-3α-methyl-digitoxigenin (**31**) and (21R)-3β-*O*-glucosyl-21-methyl-digitoxigenin (**44**), for which comparatively favourable relations between cardiotonic and toxic actions have been reported [48–50]. However, these claims have not been confirmed in other studies for **12** [51] and **31** [52] or await for **44** still full publication.

Peculiarities of inhibitor-response by human and guinea-pig cardiac muscle Na/K-*ATPases*

Under maximized turnover conditions as used here, the concentration/inhibition curves show for all compounds on both enzymes a monophasic shape and identical slope with a Hill coefficient near unity. This favours the assumption underlying the structure–activity interpretations given, that all the derivatives react with one and the same receptive intermediate of both enzymes. Under non-turnover conditions both enzymes have been reported to show on maximal effector-ligation one single class of binding sites [13, 39–41, 53, 54], but on submaximal ligation two classes of binding sites characterized by different digitalis affinities [13, 53, 54]. The latter findings suggest that the interaction of digitalis compounds and Na/K-ATPase of human and guinea-pig cardiac muscle is regulated by a ligation-controlled equilibrium between two limit enzyme conformers of different digitalis affinity as has been found to apply to pig cardiac and cerebral enzyme [55, 56].

On the other hand, the difference in digitalis sensitivity of human and guinea-pig cardiac Na/K-ATPase has been shown to be widely independent of ligation-induced enzyme conformation and so essentially determined by enzyme structure. The results of previous studies confined to a few compounds suggested that the human enzyme has for ouabain, digitoxin and digoxin about 10-fold higher affinity [10, 13, 30] and for progesterone-3,20-*bis*-guanylhydrazone about 10-fold lower affinity than the guinea-pig enzyme [30]. The present comparative estimates on 69 compounds show that the derivatives containing the lead structure develop on an average 27-fold higher inhibitory potency in the human enzyme, and that otherwise no constant potency ratio exists. The extreme divergences are represented by 3β-*O*-glucosyl-digitoxigenin (**22**) with 125-fold higher affinity and prednisolone-3,20-bis-guanylhydrazone (**53**) with 52-fold lower affinity to the human enzyme.

In early studies on structure–activity relationships in the guinea-pig enzyme [28], digitoxin was found to have lost most of its inhibitory potency after removal of the lactone ring. This finding was interpreted to mean that the lactone side chain is the "effective grouping", and the steroid nucleus operates as the "fixing group". The present more systematic study confirms that the lactone side chains of cardenolides and bufadienolides greatly contribute to the integral interaction energy (cf. Table 3), but moreover proves that their steroid nucleus serves as lead structure also in the guinea-pig as in the human enzyme [25], although the two enzyme variants differ in the chemotopology of the steroid binding subsite as shown in the present paper.

In conclusion, estimates of the inhibitory potency of steroidal compounds obtained on the guinea-pig cardiac Na/K-ATPase can neither quantitatively nor qualitatively be easily extrapolated to the human cardiac enzyme so that the latter enzyme variant derserves preference.

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