Identification of a Novel Cardenolide (2″-Oxovoruscharin) from *Calotropis procera* and the Hemisynthesis of Novel Derivatives Displaying Potent in Vitro Antitumor Activities and High in Vivo Tolerance: Structure−Activity Relationship Analyses

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Analysis of the methanolic extract of *Calotropis procera* root barks enabled the identification of a novel cardenolide (2″-oxovoruscharin) to be made. Of the 27 compounds that we hemisynthesized, one (**23**) exhibited a very interesting profile with respect to its hemisynthetic chemical yield, its in vitro antitumor activity, its in vitro inhibitory influence on the Na^+/K^+-ATPase activity, and its in vivo tolerance. Compound **23** displayed in vitro antitumor activity on a panel of 57 human cancer cell lines similar to taxol, and higher than SN-38 (the active metabolite of irinotecan), two of the most potent drugs used in hospitals to combat cancer.

Introduction

Calotropis procera (Rubberbush, Sodom Apple) is a coarse shrub belonging to the *Asclepiadaceae* family; it is present in tropical Africa, Near to Far East (including India and China), Australia and South America. *Calotropis procera* frequently appears on plains and in the uplands, but rarely in hilly areas; it tolerates very well environmental extremes such as a semi-arid or an arid climate. For decades now, chemical extracts from *Calotropis procera* have been shown to have ascaricidal,[1] schizonticidal,[2] anti-bacterial,[3,4] anthelmintic,[5] insecticidal,[6] antiinflammatory,[7,8] anti-diarrhoeal,[9] larvicidal,[10] cytotoxic[3,11] and even analgesic[12] effects. These pleiotrophic effects are due to the fact that *Calotropis procera* contains numerous different chemical entities among which norditerpenic esters,[13] pentacyclic triterpenoids,[13] organic carbonates,[14] a cysteine protease,[15] histamine,[16] alkaloids,[17] flavonoids,[17] sterols,[17] and numerous types of cardenolides.[17−19] Apart from their very widely known cardiotonic steroid-related effects mediated through their binding to the Na^+/K^+-ATPase (the sodium pump),[20,21] cardenolides also act against a large range of cancer types.[22−25]

On the basis of preliminary data obtained by our group, we decided to perform a bioguided fractionation of the methanolic extract of the *Calotropis procera* root bark in the hope of discovering novel cardenolides with antitumor properties. We thus identified several cardenolides including calotropin[26] **1**, calactin[27] **2**, uscharin[28] **3**, and voruscharin[28] **4**, all of which have been described by others (Figure 1), and also 2″-oxovoruscharin **5**, which is a cardenolide first described by ourselves[29] (Figure 1). We hemisynthetically derived a series of nine compounds from 2″-oxovoruscharin whose structure, in vitro antitumor activity, inhibitory activity on the Na^+/K^+-ATPase, and in vivo tolerance in mice are described in the present work (Table 3). We carried out the same process with uscharin **3**, from which we hemisynthetically derived 17 novel compounds as well as the well-known voruscharin **4** (Table 2). Of the nine hemisynthetic derivatives obtained from 2″-oxovoruscharin **5**, compound **23** exhibits the most interesting profile with respect to its hemisynthetic chemical yield, its in vitro antitumor activity, its in vitro effects on the Na^+/K^+-ATPase activity, and its in vivo tolerance (Table 3, Table 4).

Chemistry

Chemical Extraction of Cardenolides from the Roots of *Calotropis procera*. A methanolic extract of the root bark of *Calotropis procera* from Burkina Faso was sequentially fractionated by LC (Si-gel) into hexane, hexane/acetone 8:2, hexane/acetone 5:5, acetone, acetone/methanol 8:2, and methanol fractions. The most potent cytotoxic (see below) fraction was further purified by two successive flash chromatographies (Si-gel; eluent: CH_2Cl_2/MeOH: 98:2 with increasing amounts of MeOH) to give two pure compounds corresponding to the previously identified uscharin **3**[28] and a slightly more polar compound identified as 2″-oxovoruscharin **5** on the basis

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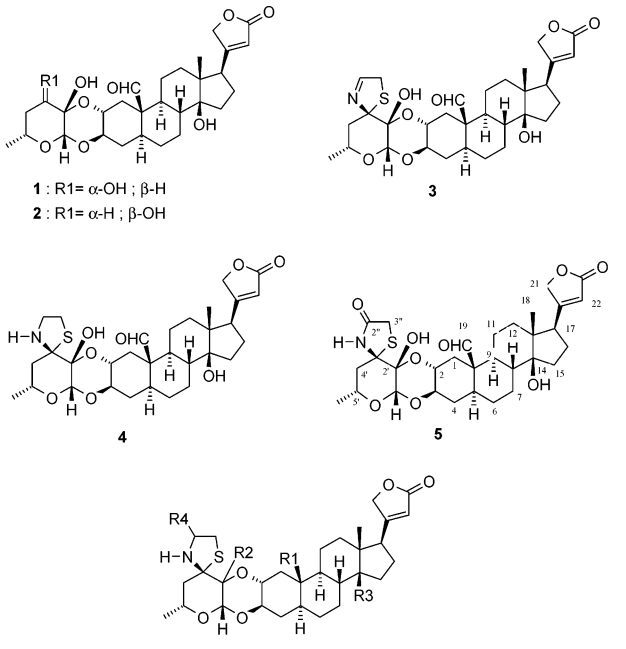


Figure 1. Structures of the isolated cardenolides.

Table 1. Comparison of the NMR Data from the Glycoside Moiety of **3**[28] and **5**

	δ_C		δ_H	
position	**3**	**5**	**3**	**5**
1′	95.1	94.2	5.07 (s)	4.76 (s)
2′	98.8	91.5	–	–
3′	91.5	72.5	–	–
4′	42.7	45.7	2.23 /1.72 (m)	1.96/2.10 (m)
5′	68.2	67.5	4.27 (m)	3.90 (m)
6″	20.7	21.0	1.23 (d)	1.24 (d)
1″	47.1	32.1	3.88 (bs)	3.51 (AB system)
2″	160.6	176.1	7.53 (bs)	–
NH	–	–	–	8.85 (bs)

of its spectral properties (MS, IR, 1D and 2D ^{1}H and ^{13}C NMR). Compound **5** is a new natural compound.

Structure Determination of 5. Compound **5** has the molecular formula $C_{31}H_{41}NO_9S$ (corresponding to 12 degrees of insaturation) deduced from the HREIMS and supported by the presence in the ^{13}C NMR spectrum of 31 signals corresponding to 2 methyl, 11 methylene, and 10 methine groups and to 8 quaternary carbon atoms. The ^{13}C NMR spectrum of **5** was very similar to that of **3**. In addition, the ^{1}H NMR of both compounds displayed the characteristic signals of a butenolactone ring (AB system at δ 4.94 and 4.78, s at 5.86), an aldehydic proton (s at δ 9.97, HOC-19), a tertiary methyl group (s at δ 0.82, H_3C-18), and a secondary methyl group (d at δ 1.24, H_3C-6′). All these data suggested that, structurally, **3** and **5** are closely related. In particular, all the signals assignable to the steroid moiety of **3** were present in the spectra of **5**, the sole significant differences being observed in the signals attributable to the H and C atoms of the glycosidic part of the molecule (Table 1). These differences are in agreement with the replacement in **5** of the 2,5-dihydrothiazole ring of uscharin **3** by a 4-thiazolidinone ring. The molecular formula of **5** and the presence in its IR spectrum of an intense carbonyl band at 1683 cm^{-1} confirmed this hypothesis. The complete assignments of the ^{13}C NMR signals and of the most characteristic proton signals were established by the 2D NMR studies (^{1}H−^{1}H COSY, HMQC, HMBC) and reported in the experimental part of the present manuscript. On the basis of the biogenetic arguments, the stereochemistry at C′3 in **5** is suggested as being identical to that of compound **3**.[28]

Hemisyntheses from Uscharin (3) and 2″-Oxovoruscharin (5). On the discovery of marked in vitro antitumor activity on the part of uscharin **3** and 2”-oxovoruscharin **5** we decided to perform a structure−activity-relationship study in the hope of discovering novel compounds capable of displaying in vitro antitumor activity and inhibitory effects on Na^+/K^+-ATPase activity while remaining well tolerated in vivo. The chemical modulations realized on both compounds **3** and **5** are summarized in Tables 2 and 3 and are described in the Supporting Information.

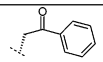
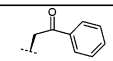
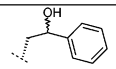
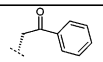
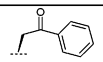
Pharmacology

In Vitro Determination of the Drug-Induced Inhibition of Human Cancer Cell Line Growth, the Na^+/K^+-ATPase Activity, and in Vivo Tolerance in Healthy Mice. For each compound under study (uscharin **3** and its 17 novel derivatives and 2″-oxovoruscharin **5** and its nine novel derivatives), nine concentrations were tested for 72 h on five different human cancer cell lines including one non-small-cell lung cancer (A549), two glioblastoma (Hs683 and U373), and two colon cancer (HCT-15 and LoVo) models (see Tables 2 and 3). We made use of the colorimetric MTT assay, which indirectly assesses the effect of potentially anticancer compounds on the overall growth of adherent cell lines as we detailed when we characterized the cytotoxic and/or cytostatic activity of a large set of compounds including neuropeptides,[30] quinolone[31] and ellipticine[32] derivatives, pyrrolo-benzodiazepines,[33] and alkaloids from marine invertebrates.[34−36] The MTT colorimetric assay does not enable any distinction to be made between drug-induced cytostatic and cytotoxic effects.[30−36] If such a distinction is required, what must be employed are flow cytometry[32] and DNA binding[32,37] analyses. Thus, in the present case we characterized the effects of various cardenolides with respect to the overall growth of human cancer cell lines. The individual IC_{50} values, i.e., the concentration which reduced the overall growth value of each of the five individual cell lines by 50% after 72 h of culture in the presence of the various drugs, and the mean IC_{50} overall growth values calculated for these five cell lines are illustrated in Table 2 for uscharin **3** and its derivatives and in Table 3 for 2″-oxovoruscharin **5** and its derivatives.

Cardenolide-mediated antitumor activities in vitro were then analyzed in parallel with those data relating to cardenolide-mediated effects with respect to the Na^+/K^+-ATPase activity. We used the Na^+/K^+-ATPase from porcine cerebral cortex because of its great similarity with human's Na^+/K^+-ATPase (amino acid sequence alignments indicated 99% of similarity between these two proteins).

In vivo tolerance was determined by means of intraperitoneal (ip) injection-related toxicity (defined by the maximum tolerated dose (MTD) in vivo index) (Tables 2 and 3). It must be emphasized that the use of mice to generate an actual MTD value (the purpose of which is to predict the dose which could be assayed in a Phase I

Table 2. Description of the 17 Novel Compounds and Voruscharin 4 Hemisynthesized from 3 (uscharin)

	Reactional site [a]				IC_{50} (nM) [b]						**MTD** [c] **(mg/kg)**	**NaK Activity IC_{50} (nM)** [d]
	R1	**R2**	**R3**	**R4**	**Hs683**	**U373**	**HCT-15**	**LoVo**	**A549**	**mean ± SEM**		
3	-CHO	-OH	-OH	DDB[e] with N	4	40	28	10	25	21 ± 6	5	68
4	-CHO	-OH	-OH	-H	4	32	27	17	5	17 ± 6	1.25	nd
6	$-CH_2OH$	-OH	-OH	DDB with N	4	34	27	5	9	16 ±6	10	nd
7	$-CH_2OAc$	-OH	-OH	DDB with N	1282	4152	4126	3542	3705	3361±533	>40	12105
8	$-CH_2OCO\Phi$	-OH	-OH	DDB with N	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	>40	> 50000
9	$-CH_2OCO(CH_2)_2\Phi$	-OH	-OH	DDB with N	> 10000	> 10000	> 10000	4836	4731	★	>40	nd
10	$-CH_2OSO_2\Phi CH_3$	-OH	-OH	DDB with N	1434	9955	5797	1059	2720	4193 ±1664	10	nd
11	$-CH_2OH$	-OH	-OH	-H	3	31	21	13	4	14 ± 5	5	nd
12	$-CH_2OH$	-OH	-OH		4	34	34	18	3	19 ± 7	80	nd
13	$-CH_2OH$	-OH	-OH		4	38	44	18	4	21 ± 8	80	nd
14	$-CH_2OH$	-OH	-OH		3	28	31	19	2	17 ± 6	nd	nd
15	$-CH_2OCOOMe$	-OH	-OH	DDB with N	49	320	442	245	42	220 ± 78	> 40	nd
16	-CHO	-OH	-OH		7	41	49	33	4	27 ± 9	10	nd
17	-CHO	-OH	-OH		22	84	92	100	18	63 ± 18	10	nd
18	$-CH_2OSiMe_3$	$-OSiMe_3$	$-OSiMe_3$	DDB with N	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	>160	> 50000
19	$-CH_2OSiMe_3$	$-OSiMe_3$	-OH	DDB with N	1110	4618	3658	3853	2580	3164 ± 608	>160	4076
20	$-CH_2OAc$	$-OSiMe_3$	$-OSiMe_3$	DDB with N	7919	> 10000	> 10000	9570	> 10000	★	>160	> 50000
21	-CHO	$-OSiMe_3$	$-OSiMe_3$	DDB with N	8595	> 10000	> 10000	> 10000	7544	★	>160	50000
22	$-CH_2OH$	$-OSiMe_3$	$-OSiMe_3$	DDB with N	346	925	454	340	126	438 ± 133	>160	1888

[a] Chemical modification brought about in each of the reaction sites of **3**. [b] The in vitro antitumor activities of the 19 compounds are reported as IC_{50} values (in nM), i.e., the concentration (determined by means of the MTT colorimetric assay) needed to decrease the overall growth of the cell line by 50% 3 days after culturing the tumor cells in the presence of the drug. This was carried out on five different human cancer cell lines including the Hs683 and U373 glioblastoma, the HCT-15 and LoVo colon, and the A549 non-small-cell-lung cancer models. ★ The mean IC_{50} value could not be determined since one or more of the corresponding pieces of data was higher than the threshold value. [c] In vivo tolerance was determined by means of the maximum tolerated dose (MTD in mg/kg), which constitutes the highest single dose of the compound that can be administered i.p. to groups of three healthy mice over a minimum period of 28 days without causing their death (nd = not determined). [d] The in vitro inhibitory activity on the Na^+/K^+-ATPase is reported as IC_{50} values (in nM), i.e., the cardenolide concentration (determined by spectrophotometric assays) needed to decrease the Na^+/K^+-ATPase activity by 50%. The test was carried out on purified porcine Na^+/K^+-ATPase. [e] DDB means "displays double bond with".

Table 3. Description of the **9** Novel Compounds Hemisynthesized from **5** (2″-Oxovoruscharin)[a]

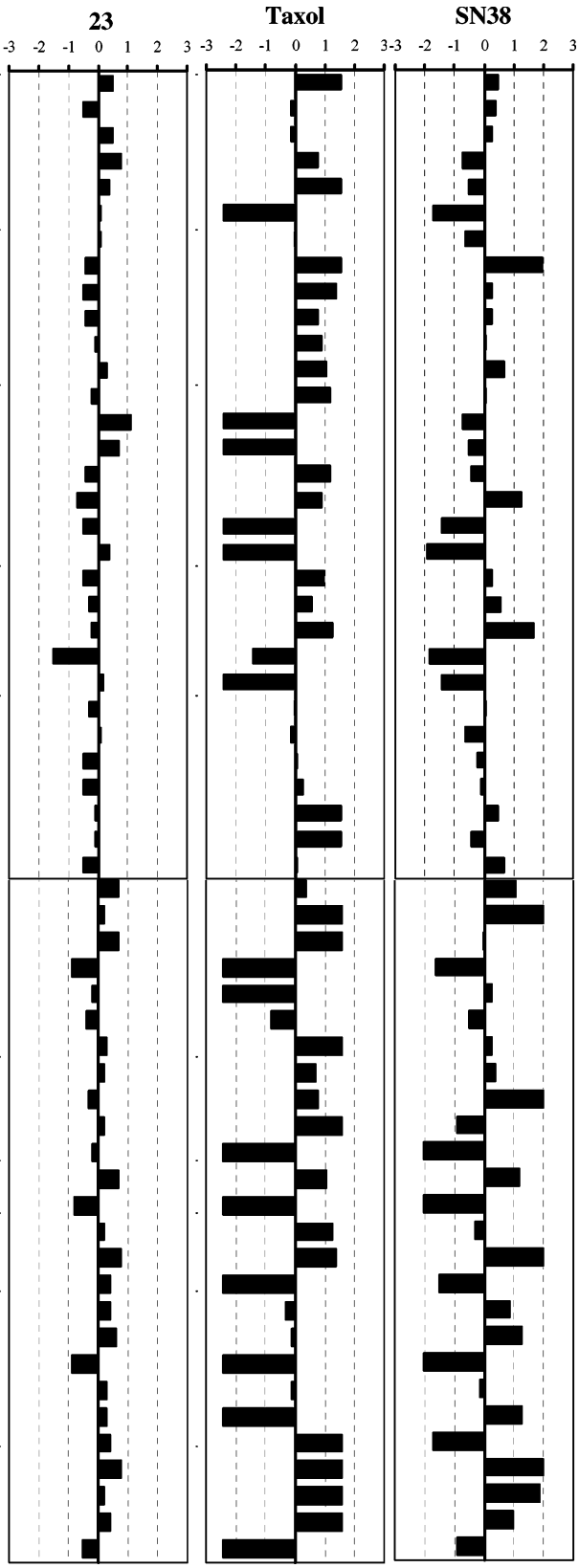
					IC_{50} (nM)						MTD	Na/K activity
	R1	R2	R3	R4	Hs683	U373	HCT-15	LoVo	A549	mean ± SEM	(mg/kg)	IC_{50} (nM)
5	see Figure 1	3	32	24	8	15	16 ± 5	10	74			
23	CH_2OH	OH	OH	=O	3	9	24	10	8	11 ± 4	80	75
24	CH_2Oac	OH	OH	=O	2778	4599	4432	2772	1861	3288 ± 529	>40	nd
25	$CH_2OCO\Phi$	OH	OH	=O	778	4170	3621	2367	625	2312 ± 720	>40	nd
26	$CH_2OCO(CH_2)_2\Phi$	OH	OH	=O	>10 000	4189	>10000	>10000	>10000	*a*	>40	nd
27	$CH_2OSO_2\ \Phi CH_3$	OH	OH	=O	45	326	382	89	48	178 ± 73	nd	nd
28	$CH_2OSO_2CH_3$	OH	OH	=O	3417	7310	>10 000	8392	3652	*a*	nd	nd
29	$CH_2OCO(CH_2)_2COCH_3$	OH	OH	=O	758	3234	3238	2997	463	2138 ± 627	20	nd
30	$CH_2OCO(CH_2)_2CHOHCH_3$	OH	OH	=O	370	1190	1499	1557	414	1006 ± 258	>40	nd
31	CH_2OSiMe_3	$OSiMe_3$	$OSiMe_3$	=O	525	3724	2686	931	657	1705 ± 638	nd	nd

[a] The mean IC_{50} value could not be determined since one or more of the corresponding pieces of data was higher than the threshold value.

clinical trial) was not appropriate since both mice and rats are known to be less sensitive to cardenolides than dogs or humans. Thus, the determination of a cardenolide-related MTD value for later therapeutical application in humans ought to be carried out on dogs, rather than on rodents. However, we kept to mice in the present study (for an obvious reason of compound availability) in order to investigate (i) whether the various cardenolides that we produced had any significantly different in vivo tolerance in mice, and (ii)

Table 4. Global Cytotoxicity on 57 Human Cancer Cell Lines[a]

Cell Lines	Log(IC50) 23	Taxol	SN38
MCF-7[HTB-22]	-8.4	-9.0	-7.5
ZR-75-1	-7.4	-7.3	-7.4
T-47D	-8.4	-7.3	-7.3
Hs578T	-8.7	-8.2	-6.3
BT-20	-8.3	-9.0	-6.5
MDA-MB-231	-8.0	-5.0	-5.3
SK-LMS-1	-8.0	-7.4	-6.4
SK-UT-1B	-7.5	-9.0	-9.0
HT1080	-7.4	-8.8	-7.3
Hs729	-7.5	-8.2	-7.3
MES-SA	-7.8	-8.3	-7.1
A204	-8.2	-8.5	-7.7
BxPC-3	-7.7	-8.6	-7.1
Capan-2	-9.0	-5.0	-6.3
Su.86.86	-8.6	-5.0	-6.5
MiaPaca-2	-7.5	-8.6	-6.6
CFPAC-1	-7.2	-8.3	-8.3
Hs766T	-7.4	-5.0	-5.6
Panc-1	-8.3	-5.0	-5.1
SKMEL-28	-7.4	-8.4	-7.3
HT-144	-7.6	-8.0	-7.6
C32	-7.7	-8.7	-8.7
Malme-3M	-6.4	-6.0	-5.2
G361	-8.1	-5.0	-5.6
HCT-15	-7.6	-7.4	-7.1
LoVo	-8.0	-7.3	-6.4
DLD-1	-7.4	-7.5	-6.8
Hs174T	-7.4	-7.7	-6.9
HT29	-7.8	-9.0	-7.5
WiDr	-7.8	-9.0	-6.6
SW948	-7.4	-7.5	-7.7
U-87MG	-8.6	-7.8	-8.1
U-373MG	-8.1	-9.0	-9.0
Hs683	-8.6	-9.0	-7.0
SW1088	-7.0	-5.0	-5.4
SW1783	-7.7	-5.0	-7.3
H4	-7.5	-6.6	-6.5
T98G	-8.2	-9.0	-7.3
A549	-8.1	-8.1	-7.4
A427	-7.6	-8.2	-9.0
AL-12T	-8.1	-9.0	-6.1
NCI-H727	-7.7	-5.0	-5.0
T24	-8.6	-8.5	-8.2
HT-1197	-7.1	-5.0	-5.0
PC-3	-8.1	-8.7	-6.7
DU145	-8.7	-8.8	-9.0
LNCaP	-8.3	-5.0	-5.5
769-P	-8.3	-7.1	-7.9
786-O	-8.5	-7.3	-8.3
A-704	-7.0	-5.0	-5.0
A-498	-8.2	-7.3	-6.9
ACHN	-8.2	-5.0	-8.3
CAKI-2	-8.3	-9.0	-5.3
RPMI2650	-8.7	-9.0	-9.0
FaDu	-8.1	-9.0	-8.9
Detroit 562	-8.3	-9.0	-8.0
SCC-9	-7.4	-5.0	-6.1



[a] Average IC_{50} values on all (n = 57) cell lines for compounds **23**, taxol, and SN-38 are 2.7 nM, 2.5 nM, and 19.5 nM, respectively. Illustration for each cell line analyzed (Y-axis) of the differences (X-axis) between the mean value of all the log IC_{50} values obtained for a given drug on the 57 human cancer cell lines under study, and the log IC_{50} value characterizing the effect of a given drug on a given cell line (X axis). A negative value indicates that the IC_{50} value on this latter cell line was above the mean and, inversely, a positive value means that the IC_{50} value for this cell line was below the mean. More particularly, a value i displayed in this chart means that the IC_{50} value of the cell line is equal to the geometric mean of all the IC_{50} values multiplied by a factor 10^{-i}.

whether any different in vivo tolerance in mice correlated with different effects on porcine Na^+/K^+-ATPase activity. The fact of comparing in vivo tolerance in mice with in vitro porcine Na^+/K^+-ATPase activity as well

as with in vitro antitumor activity with respect to human cell lines limited the caveat engendered by the use of mice in our SAR analysis-related reasoning. It is interesting to note that even if mice are considered to be less sensitive to cardenolides than dogs or humans, the MTD values in mice that we obtained for two well-known cardenolides, i.e., ouabain and digitoxin, were 5 and 20 mg/kg, respectively, and 10 mg/kg for 2″-oxovoruscharin. These data contrast with the fact that the MTD value for compound **23** is 120 mg/kg in mice. All together, the data from the present study thus reveal that the IC_{50} values with respect to antitumor activities for **23**, ouabain and digitoxin were relatively similar (11, 41, and 43 nM, respectively) as it was also the case with respect to the IC_{50} values for Na^+/K^+-ATPase activity inhibition (74, 121, and 65 nM, respectively). In sharp contrast, **23** was markedly best tolerated in vivo by mice than ouabain and digitoxin.

Structure−Activity-Relationship Analyses. The data in Tables 2 and 3 show that the IC_{50} values range over 3 logarithmic concentrations. As indicated by the values of the mean IC_{50}, all the esters (in position R1) synthesized from the two starting products (**3** and **5**) exhibited both weak cytotoxic activity and weak Na^+/K^+-ATPase activity inhibition in vitro, and relatively high in vivo tolerance (MTD > 40 mg/kg). In contrast, other compounds presenting a formyl or a hydroxyalkyl moiety on C_{10} and a free 14-β-hydroxy on the steroid skeleton showed the greatest in vitro antitumor activity, along with marked inhibition of Na^+/K^+-ATPase activity. These results highlight the importance of the presence of a sterically nonpolar hindered group in R1 position. The R3 position plays also an important role on these pharmacological properties, as revealed when comparing compounds **18** and **19** (Table 3). The free hydroxy of **19** is necessary to induce the inhibition of tumor cell proliferation and to maintain a certain level of inhibition of Na^+/K^+-ATPase activity. These data are in good agreement with the model proposed by Höltje and Anzali[38] who postulated the existence of a hydrogen bond between this 14 β-hydroxy and the protein.

Tables 2 and 3 also show that the in vivo tolerance largely depends on the nature of the different chemical substitutions. A high in vivo tolerance is obtained with those compounds displaying a hydroxyalkyl in R1 and an oxygen (doubly linked to form a carbonyl) in R4. This relatively high in vivo tolerance (in mice) obtained with different substitutes in R4 is confirmed by the MTD values obtained with compounds **12**, **13**, and **23** (Tables 2 and 3). On the basis of all these results, we selected our "lead" compound, i.e., compound **23**, which has been further tested in vitro on a larger panel of 57 human cancer cell lines.

In Vitro Comparison of the Antitumor Activity of Compound 23 on 57 Human Cancer Cell Lines and That of Taxol, SN-38 (the Active Metabolite of Irinotecan), Oxaliplatin, and Etoposide, Four Clinically Active Antitumor Compounds. We made use of the mean graph technique described by Paull et al.[39] in order to investigate the individual sensitivity to **23**, taxol, and SN-38 (the active metabolite of irinotecan) of each of the 57 human cancer cell lines under study (Table 4). We chose taxol and SN-38 to challenge **23**

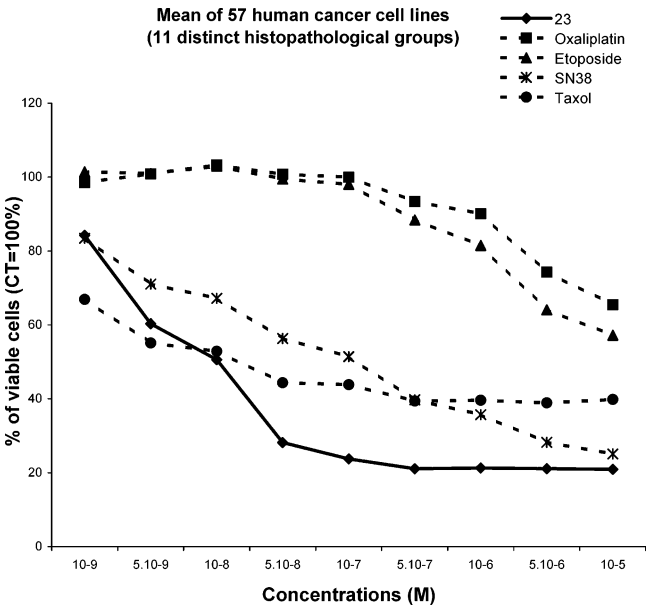


Figure 2. Illustration of the in vitro antitumor activities (evaluated at overall growth level by means of the MTT colorimetric assay) of five compounds, namely compound **23** (see Table 3), taxol, SN-38 (the active metabolite of irinotecan), etoposide, and oxaliplatin in the case of 57 human cancer cell lines (see Table 4). The drugs were assayed at nine distinct concentrations ranging from 10^{-9} to 10^{-5} M with a semilog concentration increase (see the *X*-axis). The *Y*-axis represents the percentages of surviving cells after 3 days of culture in the presence of the drug, with 100% arbitrarily indicating control.

because of the four reference compounds under study (Table 4); these two appeared to be the most potent.

Table 4 shows that the individual sensitivities—in terms of in vitro antitumor activity—of most of the 57 human cancer cell lines to **23** differed markedly from those observed with respect to taxol and SN-38. These data strongly suggest that **23** does not act either as a tubulin inhibitor (as does taxol) or as a topoisomerase I inhibitor (as does SN-38). These data were confirmed biochemically (data not shown). In fact, as detailed in the Discussion, **23** might well target the NF-κB signaling pathway.

Figure 2 shows that **23** was as potent as taxol in its ability to reduce the mean overall growth of the 57 different human cancer cells lines detailed in Table 4. SN-38, the active metabolite of irinotecan, was significantly less active than **23** and taxol, and etoposide and oxaliplatin, the remaining two compounds, had a weak antitumor effect, at least at overall growth level. Compound **23** showed similar inhibitory activity on each of the 57 human cancer cell lines under study (Table 4).

Characterization of the in Vitro Antitumor Effect of Compound 23 on a Multidrug Resistant Human Bladder Cancer Cell Line. We used a human bladder cancer cell line (the T24 model, labeled here $T24^{sens}$ for the chemosensitive parent cell line) that we made multidrug resistant (labeled $T24^{resis}$) as detailed elsewhere.[40,41] Briefly, we cultured the $T24^{sens}$ tumor cells in the presence of 10^{-13} M vincristine, and we increased the vincristine concentration in the $T24^{sens}$ tumor cell culture medium by one-half log every two months until the cells were capable of growing in the

presence of 10^{-9} M vincristine. We then took these vincristine-resistant T24 tumor cells and cultured them in the presence of 10^{-10} M adriamycin, increasing the adriamycin concentration until the vincristine-resistant T24 tumor cells were able to grow in the presence of both 10^{-9} M vincristine and 10^{-8} M adriamycin.[40,41] We labeled these T24 tumor cells as being resistant to both vincristine and adriamycin $T24^{resis}$.

In the present study the vincristine IC_{50} on the $T24^{sens}$ tumor cells was less than 1 nM, with an increase to 4,-768 nM in the case of the $T24^{resis}$ tumor cells. This means that the $T24^{resis}$ cells resisted the vincristine approximately 5000 times more effectively than did the $T24^{sens}$ tumor cells. The adriamycin IC_{50} values for the $T24^{sens}$ and $T24^{resis}$ tumor cells were 37 and 2823 nM, respectively. This means that the $T24^{resis}$ cells resist the adriamycin 70 times more effectively than did the $T24^{sens}$ tumor cells. Though the $T24^{resis}$ tumor cells were never cultured in the presence of taxol, the taxol IC_{50} values for the $T24^{sens}$ and $T24^{resis}$ tumor cells were 1 and more than 10 000 nM, respectively. This means that the $T24^{resis}$ cells resisted taxol 10 000 times more effectively than the $T24^{sens}$ tumor cells, and that they developed cross-resistance features. In sharp contrast, the IC_{50} values associated with **23** in the $T24^{sens}$ and $T24^{resis}$ bladder tumor cells were 45 and 25 nM respectively, thus indicating that **23** was as potent toward chemosensitive human bladder cancer cells as it was toward chemoresistant ones.

Discussion

One of the major classes of proteins acting as the preferred ligands for cardenolides is the Na^+/K^+-ATPase (the so-called sodium pump), which belongs to the so-called "Na^+/K^+-ATPase signalosome".[20,21] The R3 position and the butenolide part of the cardenolide structure are essential for binding to Na^+/K^+-ATPase.[42] Tables 2 and 3 clearly show that the 14β-hydroxy (R3) of the steroid is also very important for cytotoxic activity, and the modulation of the R3 position by a trimethylsilane group led to a dramatically lower level of antitumor activity on the part of compounds **19**, **22**, and **31** than in the case of **3**, **5**, or **23**, and even to a total lack of antitumor activity ($>10^{-5}$ M) in the case of **20** and **21**. Indeed, we observed that this inactivation of the R3 position prevented compounds **18**, **20**, and **21**, for example, from activating the porcine sodium pump (Table 3). Whereas modulating R4 did not seem to influence the cytotoxic activity, it did have a great deal of influence on the in vivo tolerance (Tables 2 and 3). Modulating R2 did not seem to have any fundamental effect on either the in vitro antitumor activity, the inhibitory activity toward Na^+/K^+-ATPase or the in vivo tolerance (Tables 2 and 3). While modulating R1 by reducing the formyl in a primary alcohol (in compound **23**) made it possible to maintain the in vitro antitumor activity observed with **5**, it dramatically decreased (by about one log) the in vivo toxicity (Tables 2 and 3). Compound **23** appears to be the most interesting one among the 31 compounds detailed here in terms of in vitro antitumor activity, in vitro anti-Na^+/K^+-ATPase activity, in vivo tolerance, and chemical availability from natural compound 2"-oxovoruscharin **5**.

Activation of the sodium pump by certain types of cardenolides can lead to NF-κB pathway inactivation.[43−47] NF-κB is in fact a collective designation for a family of highly regulated dimeric transcription factors.[48] Virtually all vertebral cells express at least one of the five Rel/NF-κB member: p50/p105 (NF-κB1), p52/p100 (NF-κB2), c-Rel, p65 (RelA), and RelB, which are assembled into homo- and heterodimers.[49,50] Whereas almost every one of he kinase pathways explored was found to participate in NF-κB activation—examples include PKC, AKT/PKB, JNK, MAP3Ks and MEKKs—few have ever been corroborated by genetic data.[51] With only a few exceptions (e.g. H_2O_2 and UV radiation), all the signals converge to activate IKK and so lead to IκB phosphorylation, ubiquitination, and degradation, which enable the nuclear translocation and DNA-binding of NF-κB dimers to take place.[51] Once activated, IKK phosphorylates the bulk of the cytoplasmic IκB pool and thereby promotes its degradation and NF-κB activation.[52,53] IκB is one of a family of NF-κB inhibitors of which IκBα, IκBβ, and IκBϵ are the chief regulators in mammalians.[52] Different types of cardenolides can act as more or less specific inhibitors of the NF-κB pathway.[24,43−47,52,53] We had observed that **23** inhibits the NF-κB pathway at several levels in human A549 non-small-cell lung cancer cells (manuscript submitted for publication). Indeed, **23** deactivates the NF-κB pathway in A549 tumor cells by (i) increasing the levels of expression of I-κBβ, (ii) decreasing the levels of phosphorylation of I-κBα, (iii) decreasing the levels of expression of p65, (iv) decreasing the levels of serine 276 phosphorylation on the p65 subunit which is essential for NF-κB binding to DNA, and (v) decreasing NF-κB transcriptional activity (manuscript submitted for publication[54]).

Thus, constitutive NF-κB pathway activation protects a large group of cancer cells against apoptosis and therefore against conventional chemotherapy, the only purpose of which is to induce apoptosis in cancer cells. The use of specific cardenolides, which are able to reduce the level of activation of the NF-κB pathway, can restore normal levels of apoptosis in cancer cells and also make them more sensitive to conventional chemotherapy. We also observed that **23** (at low doses, i.e., in the 10 nM range) induces paraptosis in A549 NSCLC cells (manuscript submitted for publication). Paraptosis is a non-apoptotic, nonnecrotic, and nonautophagic related cell death process.[55,56]

A number of reports have already emphasized the potential roles of cardenolides as valuable anticancer agents. With respect to breast cancer, for example, Stenkvist[25] reports that tumor cell populations in breast cancer patients on digitalis medication (for cardiac problems) are characterized by a number of cytometric features, which strongly indicate that they have a lower proliferative ability than tumor cells in patients not on digitalis treatment. Stenkvist et al.[57] also report that after a 5-year follow-up the recurrence rate among patients not on digitalis was 9.6 times higher than among patients on digitalis. In a 20-year follow-up, Stenkvist et al.[58] report that the death rate from breast carcinoma (excluding other causes of death) was 6% (two out of 32) among patients on digitalis, as compared to 34% (48 of 143) among patients not on digitalis (p = 0.002). It has also been shown that in vitro both digitoxin and digoxin have growth-inhibiting effects on both receptor-positive and receptor-negative human

breast and glioma cell lines.[25,57,58] Additional evidence of the potential use of cardenolides against various types of cancer is given in the review by Haux.[22] We are now involved in large-scale in vivo experiments in which we are investigating **23**-associated antitumor effects on both the tumor growth rates and the survival periods in the case of human-xenograft-bearing nude mice; included in this investigation are breast, prostate, melanoma, glioblastoma, and non-small-cell-lung cancer xenografts.

In conclusion, the present study shows that novel cardenolides hemisynthetically derived from 2″-oxovoruscharin display potent in vitro antitumor activity and a high level of in vivo tolerance (as compared to the original compound from which they derive). These types of compounds could therefore constitute new promising anti-cancer agents.

Experimental Section

In Vitro Characterization of the Drug-Induced Effects on Human Cancer Cell Line Growth. The 57 human tumor cell lines, which were obtained from the American Type Culture Collection (ATCC, Manassas, VA), are detailed in Table 4.

All the culture media were supplemented with a mixture of 0.6-mg/mL glutamine (Gibco), 200 IU/mL penicillin (Gibco), 200 IU/mL streptomycin (Gibco), and 0.1 mg/mL gentamycin (Gibco). The FCS was heat-inactivated for 1 h at 56 °C.

The 57 cell lines were incubated for 24 h in 96-microwell plates (at a concentration of 10 000 to 40 000 cells/mL culture medium depending on the cell type) to ensure adequate plating prior to cell growth determination. This process was carried out by means of the colorimetric MTT assay, as detailed previously.[30−36] This assessment of cell population growth is based on the capability of living cells to reduce the yellow product MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St Louis, MO) to a blue product, formazan, by a reduction reaction occurring in the mitochondria. The number of living cells after 72 h of culture in the presence (or absence: control) of the various drugs is directly proportional to the intensity of the blue, which is quantitatively measured by spectrophotometry—in our case using a DIAS microplate reader (Dynatech Laboratories) at a 570 nm wavelength (with a reference of 630 nm). Each experiment was carried out in sextuplicate. Nine concentrations ranging from 10^{-5} to 10^{-9} M (with semilog decrease in concentration) were assayed for each of the 29 compounds under study (see Tables 2 and 3).

Characterization of Na^+/K^+-ATPase Activity in the Presence of Various Types of Cardenolides. Porcine cerebral cortex sodium potassium-ATPase activity was measured using an enzyme-linked assay in which the formation of ADP by ATPase was coupled to NADH oxidation in the presence of the enzymes pyruvate kinase and lactate dehydrogenase, with the intermediate substrate phosphoenolpyruvate present in excess. The Na^+/K^+-ATPase activity was obtained using a modification of the Manunta et al. method.[59] The samples (3×10^{-3} units of porcine Na^+/K^+-ATPase, Sigma-Aldrich) were preincubated at 37 °C for 30 min in Tris-HCl 30 mM, pH 7.4, $MgCl_2$ 5 mM, with increasing concentrations of the various cardenolides under study. The reaction was then initiated by the addition of 1 mL of reaction mixture which contained (in mM or appropriate units): Tris-HCl pH 7.4, 30; NaCl, 100; KCl, 5; $MgCl_2$, 5; ATP (Sigma-Aldrich), 3; PEP (Calbiochem), 1; NADH (Calbiochem), 0.3; EGTA (Sigma-Aldrich), 0.1; lactate dehydrogenase (Sigma-Aldrich), 5; pyruvate kinase (Sigma-Aldrich), 6, with the corresponding inhibitor concentrations. The absorbance was measured after 1-h of incubation at 37 °C at 340 nm in a 1-mL cuvette (VWR international). The procedure was characterized using an inhibitor range from 1 to 50 000 nM.

In Vivo Determination of Drug-Induced Toxicity. Drug-induced toxicity can be monitored in vivo by determining the maximum tolerated dose (MTD). This MTD determination was carried out by defining the maximum dose of a drug which can be administered acutely (i.e. in one intraperitoneal single dose) to healthy animals (B6D2F1 mice, Iffa Credo), i.e., not grafted with tumors. The survival and weight of the animals were recorded over the 28 days postinjection. Six different doses of each drug (5, 10, 20, 40, 80, 120, and 160 mg/kg) were used for the MTD index determination, with each experimental group being composed of three mice for this purpose.

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Supporting Information Available: ^1H NMR, ^{13}C NMR, EIMS, and IR spectral data are available free of charge via the Internet: http://pubs.acs.org.

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