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Significant Activity of Ecdysteroids on the Resistance to Doxorubicin in Mammalian Cancer Cells Expressing the Human ABCB1 Transporter

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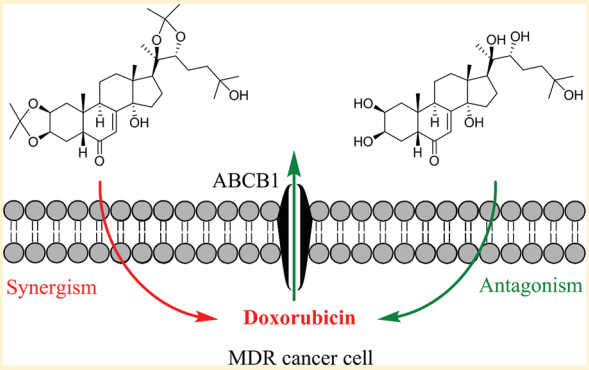
Supporting Information

ABSTRACT: Multidrug resistance (MDR) is a major cause of failure of cancer chemotherapy. Fifty-eight ecdysteroids, herbal analogues of the insect molting hormone and their semisynthetic derivatives, were tested for their activity against L5178 mouse T-cell lymphoma cells (non-MDR) and their subcell line transfected with pHa *MDR*1/A retrovirus overexpressing the human ABCB1 efflux pump (MDR cell line). The compounds showed very low antiproliferative activities but modulated the efflux of rhodamine 123 mediated by the ABCB1 transporter. Roughly depending on the polarity, mild to strong synergism or antagonism was observed by combining ecdysteroids with doxorubicin, and specific structure–activity relationships were also found. Our results show the effect of ecdysteroids on MDR cancer cells for the first time. Less polar derivatives may serve as valuable leads toward a potent and safe resistance modulator. Biological significance of the resistance-increasing activity of the most abundant phytoecdysteroids including 20-hydroxyecdysone is yet to be clarified.



INTRODUCTION

In 2008, 7.6 million people died from cancer according to the World Health Organization (WHO).[1] The majority (approximately 70%) of world cancer deaths occurs in low- and middle-income countries, and this number is increasing.[1] Resistance is one of the major factors that promote failure in cancer chemotherapy.[2] Every cancer expresses a different array of drug-resistance genes and exhibits an enormous amount of heterogeneity with respect to drug resistance.[3] Even if tumors are not intrinsically resistant to chemotherapy, selection by potent anticancer drugs can result in rapid acquisition of drug resistance.[3] This situation has commonly been described as the result of excretion of the drug from the cell as a consequence of up-regulation of efflux pumps (EPs)[4,5] that for the majority of cases results in the acquisition of multidrug resistance (MDR). Much research has been performed to discover strong EP inhibitors (EPIs),[6] and a large number of active natural compounds have been identified.[7] Despite the high expectations, no compound has become available for therapy, because of either intrinsic toxicity or changes in the pharmacokinetic properties of the chemotherapeutics resulting in strong toxic side effects.[8] Therefore, the mechanisms of modulation and reversal of resistance, other than direct inhibition of EPs, seem more promising therapeutical targets. Nevertheless, new approaches for the treatment of cancer are urgently needed.

Ecdysteroids are hydroxysteroids with a characteristic 7-en-6-one moiety in their B-ring. These compounds are analogues of the molting hormones of arthropods and are also frequently found in plants including spinach and quinoa,[9−11] where they apparently play a defensive role.[12] The most common phytoecdysteroid is 20-hydroxyecdysone (20E, compound **1**). The ecdysteroid composition of plants is generally dominated by this compound, sometimes accompanied by a few other major ecdysteroids, and typically by a large number of minor derivatives that are present in much lower amounts.[13] The

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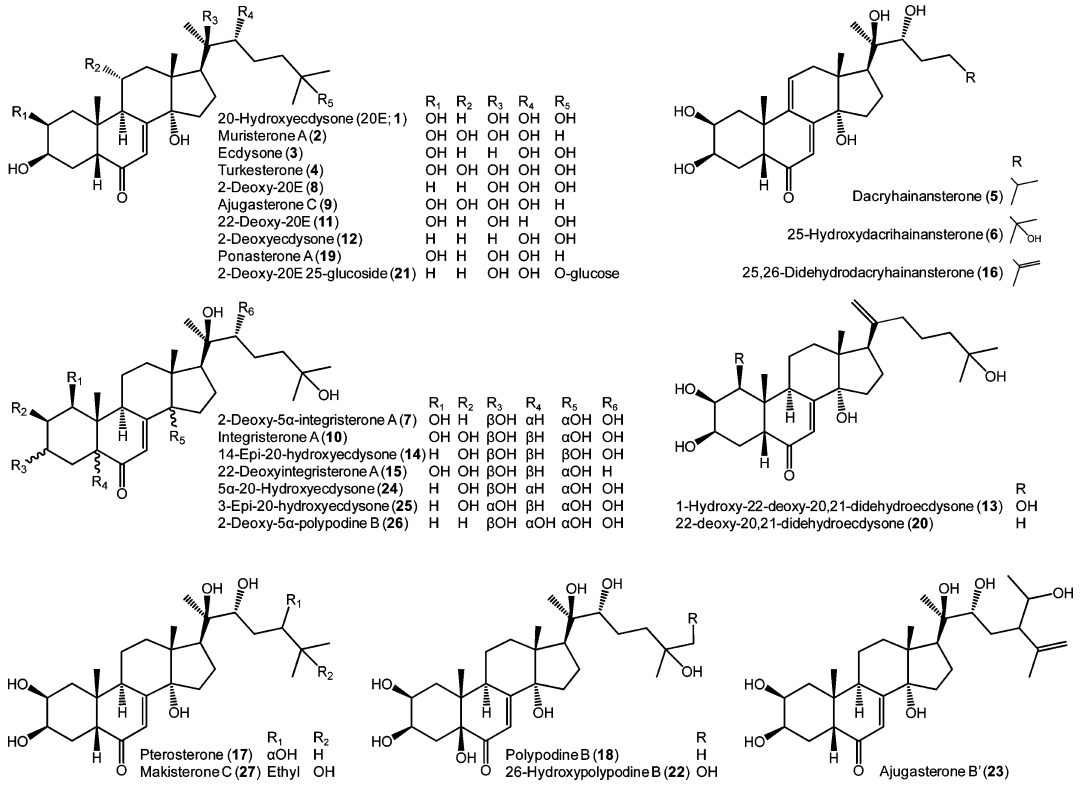

Figure 1. Structures and trivial names of compounds **1**−**27**.

positive effects of ecdysteroids on mammals have been studied over the years, but the significance of the observed benefits remains unclear.[12] It was previously shown that ecdysteroids are nontoxic in mammals. An oral LD_{50} higher than 6 g/kg in mice and a wide range of beneficial pharmacological effects (including adaptogenic, anabolic, antihyperglycemic, hepatoprotective, immunoprotective, wound-healing) were described for compound **1**.[9] Moreover, Oehme and coauthors demonstrated that muristerone A protects the human colon carcinoma cell line RKO from apoptosis, acts in a wide variety of apoptosis related pathways, and regulates gene expression in these cells.[14] Structural differences between ecdysteroids and vertebrate steroid hormones result in no in vitro or in vivo interactions with the vertebrate steroid hormone system.[9,10,15]

Food supplements containing ecdysteroids as supposedly safe "green anabolics" represent a large, worldwide, and mostly uncontrolled market, as can be seen from a simple Internet search on "ecdysterone". Such products are typically accompanied with advertisements of their safety, and because of their adaptogenic and immune-modulatory activity, there are cases when anticancer properties are also advertised.

In this work, it is shown for the first time that some ecdysteroids can effectively be used, in vitro, to reverse efflux mediated resistance developed by cancer cells while others, typically the most abundant ones including **1**, are able to increase multidrug resistance.

RESULTS

The 58 natural and semisynthetic ecdysteroids presented in this work showed very weak antiproliferative activity or cytotoxicity to the cells. The majority of these compounds had IC_{50} values higher than 90 μM. Compounds **9**, **14**, **22**, and **25** had IC_{50} values between 30 and 90 μM. Similar results were observed for the ecdysteroid benzoate **40**, the ecdysteroids acetonides **53**−**56**, and the ecdysteroids acetates **35** and **41**−**43**.

Flow cytometry measurements revealed that several compounds were able to increase the intracellular accumulation of rhodamine 123 by MDR mouse lymphoma cells compared to the parental non-MDR cells and the MDR untreated controls. Fluorescence activity ratio (FAR) values were used for the accumulation measure and were calculated according to the following formula:

$$\mathrm{FAR} = (\mathrm{Fl}_{\mathrm{MDRtreated}}/\mathrm{Fl}_{\mathrm{MDRcontrol}})/(\mathrm{Fl}_{\mathrm{PARtreated}}/\mathrm{Fl}_{\mathrm{PARcontrol}})$$

where Fl represents the fluorescence intensities observed for the MDR and non-MDR (PAR) cell lines in presence (treated) and absence (control) of the analyte. The highest FAR value represents the highest intracellular accumulation of rhodamine 123. Verapamil was used as positive control, and a FAR of 8.60 was obtained when used at 22 μM. Among the tested ecdysteroids, the highest activities in this model were found for the less polar derivatives (**53**−**58**) with the following FAR values when tested at 20 μM: 46.49 (**55**), 41.15 (**56**), 26.53 (**58**), 24.39 (**54**), 13.32 (**53**), and 11.28 (**57**).

With the exception of muristerone A (**2**), which was only available in a limited amount, all compounds were investigated for their capacity to modulate the activity of doxorubicin on the MDR cell line. This cell line is resistant to doxorubicin because of the overexpression of the human ABCB1 efflux pump, commonly known as P-gp1. To investigate MDR modulation on this cell line, cytotoxicity of each ecdysteroid was tested in combination with doxorubicin by using the checkerboard microplate method. Each compound was tested in separate 96-well microplates containing duplicate dilutions of doxorubin and the ecdysteroid as well as the corresponding combinations; thus, the results for constant ecdysteroid−doxorubicin ratios

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Table 1. Fluorescence Activity Ratios (FAR) and Selected Combination Index (CI) Values for Compounds 1−27[a]

			CI at						
compd	FAR	drug ratio	ED_{50}	ED_{75}	ED_{90}	Dm	*m*	*r*	CI_{avg}
1	1.76	20.4:1	2.00	2.02	2.04	35.52	2.855	0.997	2.03
2	0.74		nd	nd	nd	nd	nd	nd	nd
3	1.38	81.5:1	2.31	4.89	12.16	65.42	0.825	0.961	8.10
4	0.93	81.5:1	5.19	4.53	4.20	188.26	1.883	0.994	4.48
5	1.07	40.8:1	1.76	2.92	5.46	26.48	0.854	0.967	3.99
6	1.24	81.5:1	2.66	3.35	4.61	57.75	1.186	0.976	3.86
7	0.68	20.4:1	2.48	1.70	1.97	41.16	1.658	0.962	1.97
8	0.55	40.8:1	1.05	1.48	2.26	28.14	0.894	0.934	1.80
9	1.13	20.4:1	1.79	1.66	1.88	32.04	1.641	0.978	1.79
10	0.94	81.5:1	0.80	1.31	2.42	31.73	0.877	0.918	1.78
11	0.67	81.5:1	2.04	1.75	1.59	92.55	2.556	0.949	1.72
12	0.94	81.5:1	2.05	1.27	0.81	75.06	2.291	0.961	1.17
13	1.58	20.4:1	0.79	0.86	0.95	13.45	1.605	0.954	0.89
14	1.03	81.5:1	1.18	0.91	0.69	34.33	2.962	0.969	0.85
15	0.66	40.8:1	1.37	0.90	0.60	41.98	1.663	0.975	0.83
16	1.43	40.8:1	1.75	0.85	0.43	40.44	2.793	0.996	0.79
17	0.56	40.8:1	1.32	0.84	0.54	44.09	2.576	0.995	0.77
18	0.77	20.4:1	1.11	0.80	0.58	17.04	1.547	0.908	0.75
19	1.26	81.5:1	1.41	0.78	0.44	38.66	1.449	0.961	0.72
20	0.88	40.8:1	1.05	0.71	0.53	18.24	1.515	0.975	0.68
21	0.68	81.5:1	1.05	0.72	0.51	38.93	1.825	0.974	0.67
22	1.32	81.5:1	2.24	0.47	0.12	47.54	2.391	0.997	0.59
23	0.70	40.8:1	1.21	0.59	0.30	35.19	5.137	0.953	0.55
24	0.81	81.5:1	0.99	0.57	0.37	53.65	2.996	0.998	0.54
25	0.89	81.5:1	1.16	0.45	0.22	52.11	3.015	0.983	0.46
26	1.21	81.5:1	0.78	0.45	0.27	68.38	2.938	0.999	0.41
27	0.66	40.8:1	0.98	0.42	0.20	28.72	2.157	0.958	0.40

[a]FAR values were calculated at 20 μM ecdysteroids, and 22 μM verapamil was used as positive control (FAR = 8.60). CI values for each compound are presented at 50%, 75%, and 90% of inhibition (ED_{50}, ED_{75}, and ED_{90}, respectively) at the most active (as either synergism or antagonism) constant ratio of compound vs doxorubicin (denoted as drug ratio in the table) by means of the weighted average CI (CI_{avg}). $CI_{avg} = (CI_{50} + 2CI_{75} + 3CI_{90})/6$. CI < 1, CI = 1, and CI > 1 represent synergism, additivity, and antagonism, respectively. Dm, *m*, and *r* represent antilog of the *x*-intercept, slope, and linear correlation coefficient of the median-effect plot, respectively. These parameters show the activity (IC_{50}), shape of the dose−effect curve, and conformity of the data, respectively, according to Chou.[16]

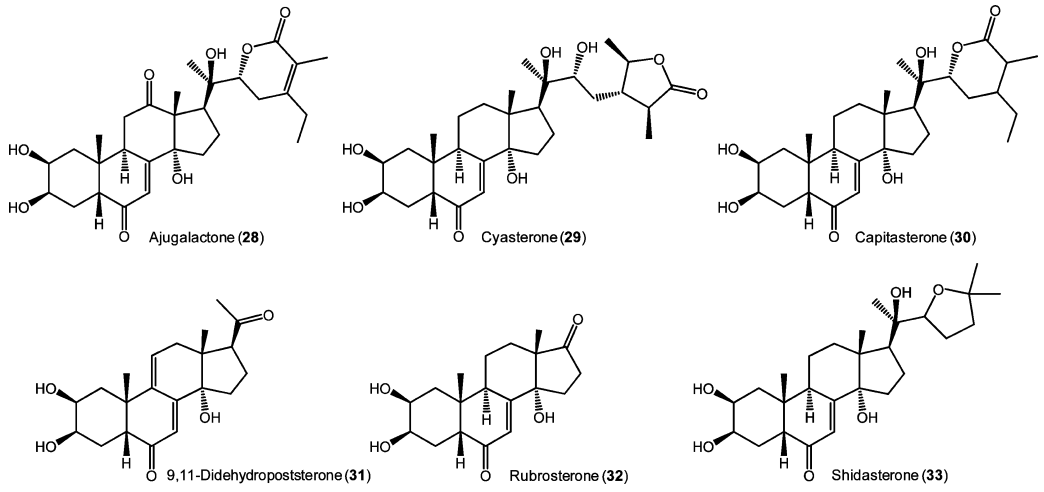

Figure 2. Structures and trivial names of ecdysteroid derivatives formed by ring closure in their side chains (**28**−**30** and **33**) or by side chain cleavage (**31** and **32**).

needed[16] for computing combination index (CI) values at all effect levels were available in the diagonals and parallel to them. This means that for a selected ratio, four to five data points could be utilized in the calculation. The combination index plots (or Fa−CI plots, where Fa is the fraction affected) were generated for each compound by the CompuSyn software from these data points on the basis of a serial deletion analysis, which deletes one data point corresponding to a dose and performs repeated recalculations in order to determine variability of data along with computing CI values to each activity levels other

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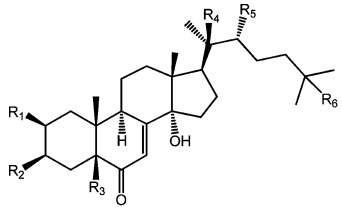
Table 2. Selected Combination Index (CI) Values for Compounds 28−33, Side Chain Shortened Ecdysteroid Derivatives, and Those Formed by Ring-Closure in the Side Chain[a]

			CI at						
compd	FAR	drug ratio	ED_{50}	ED_{75}	ED_{90}	Dm	*m*	*r*	CI_{avg}
28	0.87	81.5:1	1.43	1.96	2.89	58.20	1.352	0.966	2.34
29	0.75	40.8:1	1.54	0.85	0.47	54.31	2.666	0.994	0.78
30	0.63	40.8:1	1.35	0.74	0.49	35.16	1.437	0.952	0.71
31	5.40	81.5:1	1.21	0.67	0.37	59.65	2.452	0.996	0.61
32	0.89	40.8:1	1.12	0.65	0.41	35.13	2.77	0.956	0.61
33	0.88	81.5:1	1.44	0.32	0.096	52.97	2.515	0.995	0.40

[a]CI values for each compound are presented at 50%, 75%, and 90% of inhibition (ED_{50}, ED_{75}, and ED_{90}, respectively) at the most active constant ratio of compound versus doxorubicin by means of the weighted average CI (CI_{avg}). $CI_{avg} = (CI_{50} + 2CI_{75} + 3CI_{90})/6$. CI < 1, CI = 1, and CI > 1 represent synergism, additivity, and antagonism, respectively. Dm, *m*, and *r* represent antilog of the *x*-intercept, slope, and linear correlation coefficient of the median-effect plot, respectively. These parameters show the activity (IC_{50}), shape of the dose−effect curve, and conformity of the data, respectively, according to Chou.[16]



	R_1	R_2	R_3	R_4	R_5	R_6
20E 22-acetate (**34**)	OH	OH	H	OH	OAc	OH
20E 2,3,22-triacetate (**35**)	OAc	OAc	H	OH	OAc	OH
Polypodine B 2,22-diacetate (**36**)	OAc	OH	OH	OH	OAc	OH
2-Deoxyecdysone 3,22-diacetate (**37**)	H	OAc	H	H	OAc	OH
2-Deoxy-20E 22-acetate (**38**)	H	OH	H	OH	OAc	OH
Polypodine B 2,3,22,25-tetraacetate (**39**)	OAc	OAc	OH	OH	OAc	OAc
20E 22-benzoate (**40**)	OH	OH	H	OH	Benzoate	OH
Polypodine B 2,3,22-triacetate (**41**)	OAc	OAc	OH	OH	OAc	OH
20E 2,3,22,25-tetraacetate (**42**)	OAc	OAc	H	OH	OAc	Oac
2-Deoxyecdysone 3,22,25-triacetate (**43**)	H	OAc	H	H	OAc	OAc

Figure 3. Structures and trivial names of ecdysteroid esters: acetates (**34−39** and **41−43**) and a benzoate (**40**).

Table 3. Selected Combination Index (CI) Values for Compounds 34−43, Ecdysteroid Esters[a]

			CI at						
	FAR	drug ratio	ED_{50}	ED_{75}	ED_{90}	Dm	*m*	*r*	CI_{avg}
34	1.54	81.5:1	1.60	0.97	0.64	37.85	3.117	0.983	0.91
35	1.25	40.8:1	1.26	0.91	0.70	36.67	2.963	0.986	0.86
36	0.84	40.8:1	1.22	0.86	0.60	24.33	1.709	0.963	0.79
37	0.97	20.4:1	1.50	0.86	0.50	17.44	1.389	0.933	0.79
38	1.21	81.5:1	1.03	0.66	0.44	47.63	3.121	0.957	0.61
39	1.48	81.5:1	0.65	0.46	0.38	24.82	2.411	0.980	0.45
40	2.18	40.8:1	0.58	0.43	0.32	32.54	1.690	0.914	0.40
41	47.95	40.8:1	0.35	0.30	0.26	7.39	2.179	0.946	0.29
42	8.55	20.4:1	0.73	0.29	0.13	12.84	3.679	0.973	0.28
43	25.07	20.4:1	0.41	0.23	0.14	7.02	3.109	0.981	0.22

[a]CI values for each compound are presented at 50%, 75%, and 90% of inhibition (ED_{50}, ED_{75}, and ED_{90}, respectively) at the most active constant ratio of compound versus doxorubicin by means of the weighted average CI (CI_{avg}). $CI_{avg} = (CI_{50} + 2CI_{75} + 3CI_{90})/6$. CI < 1, CI = 1, and CI > 1 represent synergism, additivity, and antagonism, respectively. Dm, *m*, and *r* represent antilog of the *x*-intercept, slope, and linear correlation coefficient of the median-effect plot, respectively. These parameters show the activity (IC_{50}), shape of the dose−effect curve, and conformity of the data, respectively, according to Chou.[16]

than the experimental data points.[16] The CI calculations were performed by means of the median-effect equation proposed by Chou,[16] a widely used and accepted method for quantitative description of synergism/antagonism, where CI < 1, CI = 1, and CI > 1 represent synergism, additive effect (or no interaction), and antagonism, respectively.

The structures and results of all compounds studied are presented in four groups of closely related derivatives: (i) "classical" ecdysteroids (Figure 1 and Table 1); (ii) derivatives with a heterocyclic ring in their side chain or those formed after side chain cleavage (Figure 2 and Table 2); (iii) ecdysteroid esters (Figure 3 and Table 3); (iv) ecdysteroid acetonides (Figure 4 and Table 4). These tables show results at the ecdysteroid−doxorubicin ratios where the strongest activities (either synergism or antagonism) were found. This was based upon the weighted average CI values suggested as a good measure of potential for synergism/antagonism in the case of cancer,[16] and the order of compounds and their numbering in each table descend from this value. A summary of further results found for different ratios is shown in Supporting Information Table 1.

Compounds **53−58** showed a strong synergism with doxorubicin with weighted average CI values lower than 0.2, while compounds **41−43** and **49−52** have weighted average CI values between 0.2 and 0.3.

A further 10 ecdysteroids presented synergism with weighted average CI values between 0.3 and 0.5. Twenty-two compounds showed moderate or weak synergistic activity, and 11 compounds were found to be antagonists (see Table 1). Compounds **3−6** exerted the strongest antagonistic activities with weighted average CI values higher than 3.5.

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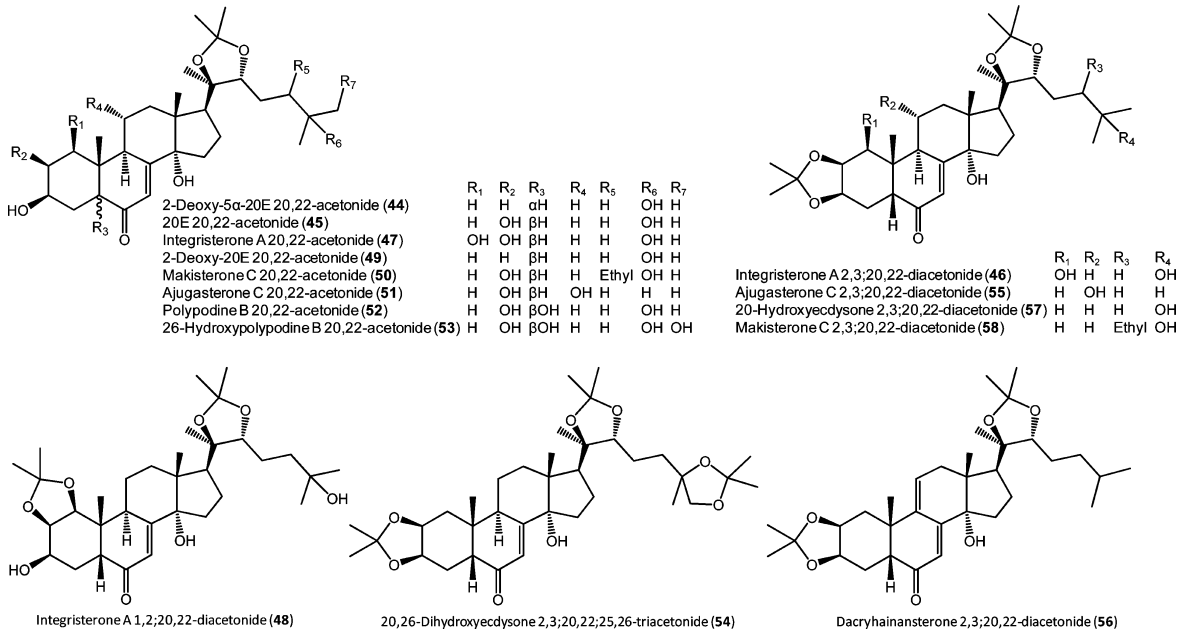

Figure 4. Structures and trivial names of ecdysteroid acetonides (**44**–**58**).

Table 4. Selected Combination Index (CI) Values for Compounds 44–58, ecdysteroid Acetonides[a]

	FAR	drug ratio	CI at ED_{50}	CI at ED_{75}	CI at ED_{90}	Dm	*m*	*r*	CI_{avg}
44	8.19	81.5:1	1.28	0.69	0.37	39.19	3.453	1.000	0.63
45	1.53	20.4:1	0.84	0.54	0.35	20.51	1.933	0.955	0.49
46	2.53	20.4:1	1.23	0.49	0.24	11.17	2.102	0.953	0.49
47	1.14	40.8:1	1.16	0.51	0.23	49.31	2.410	0.991	0.48
48	2.43	20.4:1	0.66	0.42	0.28	13.16	1.964	0.970	0.39
49	7.01	20.4:1	0.88	0.28	0.091	20.85	2.985	0.952	0.29
50	1.21	81.5:1	0.81	0.26	0.091	50.63	4.004	0.977	0.27
51	1.93	20.4:1	0.52	0.25	0.13	10.30	3.058	0.970	0.23
52	1.80	81.5:1	0.63	0.21	0.11	37.86	1.707	0.935	0.23
53	13.32	40.8:1	0.42	0.16	0.062	22.11	3.085	0.979	0.16
54	24.39	20.4:1	0.23	0.16	0.12	5.85	4.061	0.980	0.15
55	46.49	20.4:1	0.18	0.14	0.12	4.70	4.178	0.998	0.14
56	41.15	20.4:1	0.19	0.14	0.10	6.02	3.782	1.000	0.13
57	19.13	20.4:1	0.28	0.14	0.073	11.68	3.246	0.964	0.13
58	26.53	20.4:1	0.19	0.11	0.068	5.79	4.484	0.983	0.10

[a]CI values for each compound are presented at 50%, 75%, and 90% of inhibition (ED_{50}, ED_{75}, and ED_{90}, respectively) at the most active constant ratio of compound versus doxorubicin by means of the weighted average CI. $CI_{avg} = (CI_{50} + 2CI_{75} + 3CI_{90})/6$. CI < 1, CI = 1, and CI > 1 represent synergism, additivity, and antagonism, respectively. Dm, *m*, and *r* represent antilog of the *x*-intercept, slope, and linear correlation coefficient of the median-effect plot, respectively. These parameters show the activity (IC_{50}), shape of the dose–effect curve, and conformity of the data, respectively, according to Chou.[16]

A typical example for combination index plots is shown in Figure 5 for 20E (**1**) and two of its characteristic, less polar derivatives, the monoacetonide **45** and the diacetonide **57** exerting MDR reversal activity. These results show that the "classical", polar ecdysteroids including compound **1** have a tendency for increasing multidrug resistance within this system, and less polar derivatives such as acetonides or some of the acetates are able to reverse resistance of the MDR cell line.

The strong synergistic activity of **57** combined with its facile production makes it a very interesting lead for further research. However, known acid sensitivity of acetonide groups may cause this compound to metabolize to **1** upon oral administration and thus to exert the opposite effect. Figure 6 shows the stability of **57** tested by using hydrochloric acid solution at a typical gastric pH.

Diacetonide **57** undergoes a rapid decomposition at pH 1.48 with a calculated half-life of 7.30 min. The primary product is monoacetonide **45**, which is among the MDR reverting derivatives, but has a weaker effect than **57**. The decomposition of **45** to **1** is very slow with only 5.64% of **1** present after 1 h.

Acute toxicity of 20-hydroxyecdysone 2,3;20,22-diacetonide (**57**) was also tested on CFLP mice, and no severe toxicity was found for 250 mg/kg or in conjuction with 1.5 mg/kg doxorubicin after a single-dose intraperitoneal administration. Our preliminary pharmacokinetical experiments also revealed that concentrations of **57** that were found active in the in vitro studies can easily be reached in the plasma of mice via

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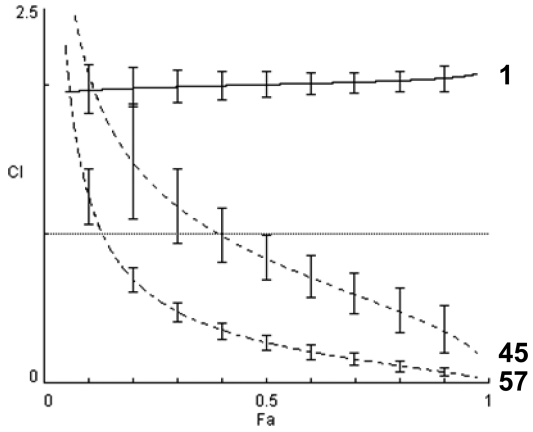

Figure 5. Fa−CI plots of compound **1** and its two acetonide derivatives: the monoacetonide **45** and the diacetonide **57**. CI < 1, CI = 1, and CI > 1 represent synergism, additive effect, and antagonism. Horizontal line represents CI = 1, and the error bars show 95% confidence intervals. The results indicate significant differences between the three derivatives.

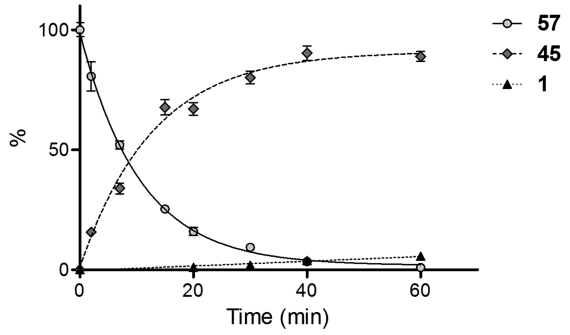

Figure 6. Stability testing of 20-hydroxyecdysone 2,3;20,22-diacetonide (**57**) at pH 1.48. Results are presented in relative % compared to the average peak area of 1.00 mg/mL of **57** (starting concentration) in three neutral pH solutions. Measurements were done in triplicate at 2, 7, 15, 20, 30, 40, and 60 min. Error bars represent standard error of mean. Kinetic curves fitted by GraphPad Prism 5.0 by using the one-phase decay exponential model are also presented.

intraperitoneal administration without any apparent toxicity. In vivo activity testing on MDR cancer xenografts is currently being performed, and results will soon be reported.

DISCUSSION

A wide variety of structurally related ecdysteroids were tested in this work, and some of them showed promising activity as modulators of resistance mediated by the overexpression of the efflux pump ABCB1 on the MDR mouse lymphoma cell line. On the basis of the results obtained for the combination indices at the IC_{50}, the reduction of resistance to doxorubicin was significantly high when doxorubicin was used in combination with ecdysteroid diacetonides such as **55**, **56**, **57**, **58**, the triacetonide **54**, and monoacetonides like **52** or **53**. Certain ecdysteroid acetates, particularly **39**−**43**, were also found to exert promising MDR decreasing activity. As it is frequently found in combination studies,[16] CI values tended to decrease at higher levels of activity, resulting in weighted average CI values showing a strong synergism in several cases. This is a promising development, as in chemotherapy the total elimination of the cancer cells is targeted. The most common ecdysteroids were in the nearly additive or practically inactive range of activity or (as for example, ecdysone (**3**), 20-hydroxyecdysone (**1**), and turkesterone (**4**)) acted as antagonists.

The antagonistic activity of the most abundant phytoecdysteroids, particularly **1**, raises the question of what biological significance these results may have in view of nutraceutical consumption. To our knowledge there is only one report available on the ecdysteroid levels reached in human plasma after per os consumption, where ecdysone (**3**) was applied in a single dose of 0.2 mg/kg, and a concentration maximum of 100 nM/L was found.[17] The lowest dose of ecdysteroids (4.688 μM/L) used in the in vitro experiments presented here was around 50 times higher. However, this plasma concentration seems to be feasible, as there are products containing as much as 500 mg of **1** per capsule and there are recommendations for taking it up to 2 g. Our results have not given enough information to judge what possible risks (if any) that excessive consumption would have to a cancer patient. Future research will certainly clarify this.

Regarding structure−activity relationships (SARs), no significant correlation was observed with any of the 333 built-in QSAR descriptors of CCG MOE (data not shown), although there was an observed tendency of activity type and strength for the less polar ecdysteroids (acetonides, acetates) as synergistic and those of higher polarity as nearly additive or antagonistic when applied together with doxorubicin. Nevertheless, the activity profile found for ecdysteroids on MDR is even more interesting in view of previous results published for other types of steroids, where less polar derivatives were found to be able to bind to the ATP binding pocket of the ABCB1, hence inhibiting it, while others of higher polarity were ligands of the pump (substrates) getting excreted from the cell.[18] More recently, it was also shown that certain human sexual steroid hormone analogues are able to induce the expression of the ABCB1 pump.[19] Such a mechanism could explain the ability of ecdysteroids to exert either MDR decreasing or increasing activity roughly depending on the polarity and would also provide a very interesting link to the mammalian steroid hormone system.

Despite the effects of polarity in this study, the structural diversity of the ecdysteroids tested also reveals specific SAR on the basis of comparing the activities of several compounds that differ from each other only in one functional group. Because of the large amount of data available (see Supporting Information Table 1), this comparison was performed by means of the strongest activity that could be found for the compounds to be compared (for the corresponding data see Tables 1−4), regardless of the ecdysteroid/doxorubicin ratio where they exerted that particular activity.

The SARs discussed here show the potential of each compound to modulate multidrug resistance during the conditions of the assay and put less emphasis on direct comparisons of their activities at the same ratios of compound versus doxorubicin. Although this approach certainly allows only a limited SAR interpretation, based on the overview on the activity data, there seems to be an "optimal ratio" for each compound, at which the CI value shows the highest difference to 1. Moreover, as no fundamental changes in the activity profiles for the different groups of compounds is made by this interpretation, specific conclusions on qualitative SAR can be made as shown in Figure 7.

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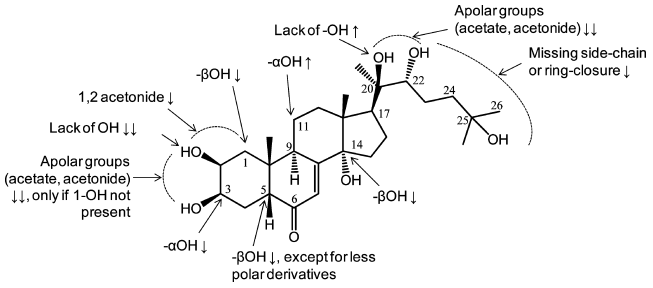

Figure 7. Structure–activity relationships for the effect of ecdysteroids in our experimental model. As a core structure, 20E (**1**) is presented along with structural modifications that result in characteristic changes in the activity. Arrows (↑ and ↓) indicate the corresponding change in the weighted average CI value.

On the basis of comparisons between the activities of selected compound pairs, the following structure–activity relationships can be observed:

(1) In comparisons of compound **10** with **1**, **15** with **11**, **13** with **20**, and **47** with **45**, the presence of a 1-OH group, even though increases polarity, can decrease the CI value and shift the activity toward synergism. On the other hand, a hydroxyl group at C-1 cancels the CI decreasing effect of the 2,3-acetonide group, if present, as **46** exerts the same activity as **47** and not as **57**. This suggests that even if a 1-OH group may decrease MDR, low polarity that is disturbed by its presence in this region of the molecule is still more important for such an activity than this group itself. If a 1-OH group is present, a 1,2-acetonide can also be formed on the A ring, in which the moiety, as seen from the activity of **48** compared to that of **57**, is less favorable for an MDR reversal activity than a 2,3-acetonide.

(2) Comparisons of compound **8** with **1**, **12** with **3**, **38** with **34**, and **49** with **45** show that a lack of the 2-OH group markedly decreases the CI value.

(3) Activity of compound **25**, when compared to that of **1**, shows that epimerization of the 3-OH group can decrease the antagonistic activity.

(4) Evaluation of the activities of compound **18** when compared with **1**, **52** with **45**, and **41** with **35** indicates that the presence of a 5β-OH group, which decreases polarity because of intramolecular hydrogen-bond formation either with the 3-OH or 6-O group, also decreases the CI value. This is, however, not the case for compounds **39** and **42** where this OH group increases polarity (log P(o/w) of 3.97 and 3.52, respectively, as calculated by CCG-MOE) most likely because of a less favorable intramolecular H-bonding.

(5) Presence of an 11α-OH group can strongly increase the CI value, as seen from the antagonistic activities of **4** and **1** or **9** and **19**. This finding is remarkable considering that the 11α-OH group was found to increase the anabolic effect of ecdysteroids;[20] antagonistic (cell survival increasing) activity in our experimental system fits well the image of compounds that are able to shift anabolic–catabolic equilibrium toward the previous one by acting on various biochemical pathways. Considering, however, that **55** (the diacetonide of **9**) is among the strongest MDR decreasing derivatives and no diacetonides of **19** or **4** were tested, such conclusion on the role of an 11α-OH group cannot be made in the case of the apolar ecdysteroids.

(6) Epimerization of the 14-OH results in the loss of antagonistic activity for **14** compared to that of **1** and decreases the CI value representing a weak synergism instead.

(7) The role of a 5α anellation, a 9–11 olefin bond, and the 20-OH and 25-OH groups in the presented activities remains unclear.

On the basis of its strong synergistic activity with doxorubicin and its semisynthetically easy-to-obtain chemical structure, compound **57**, 20-hydroxyecdysone 2,3;20,22-diacetonide seems to be the most promising lead compound for developing an MDR-reverting agent at the present state of research. Despite the moderate acid sensitivity of an acetonide group, it is not an uncommon structural element of drugs applied for human therapy, as seen from the example of triamcinolone acetonide, a widely used corticosteroid drug that has an acceptable (23%) bioavailability even after per os administration.[21] On the other hand, in view of a generally preferred per os administration for any therapeutic drug, the opposite effects of **1** and **57** lead to a crucial question: Does acidic hydrolysis of compound **57** yield compound **1**? After investigation of the stability of compound **57** under acidic conditions, it was observed that at gastric pH, the diacetonide is mainly hydrolyzed to monoacetonide (**45**) while the amount of **1** stays very low even after a long exposure. Although this does not invert the activity, because of the weaker MDR decreasing activity of **45** compared to that of **57**, such decomposition is still undesired. Parenteral application or enterosolvent formulation of the diacetonide may assist in overcoming this problem. More acid-resistant derivatives, for example, ecdysteroid acetates such as **41**–**43**, may also provide valuable alternatives to the diacetonides.

The mechanism of action of ecdysteroids on MDR is still to be clarified. As a possibly involved signaling route, it may, however, be worth mentioning the PI3K/Akt pathway, which was found to be influenced by muristerone A and ponasterone A[22] (in our experiments **2** and **19**, respectively), resulting in an antiapoptotic effect at the level of caspase-8 activation.[14] This pathway has been proposed as a possible key for understanding the various metabolic effects of ecdysteroids in mammals,[14] while it is also closely connected to multidrug resistance mediated by the ABCB1 pump: PI3K/Akt inhibition was recently described to result in MDR modulation in murine lymphoma cell lines.[23] Nevertheless, further investigations on the regulation of expression and activities of the major efflux pump systems by ecdysteroids are needed, including the role of the above-mentioned pathway.

EXPERIMENTAL SECTION

Ecdysteroids. Ecdysteroids tested in our experiments were either of natural or semisynthetic origin. Natural ecdysteroids **1**–**15**, **17**–**30**, **32**, **33**, **38**, **40**, **44**, **45**, **47**, **49**, **53**, and **58** were previously isolated from *Ajuga*, *Serratula* and *Silene* species by our group.[24−29] Spectroscopic data for these compounds can be found in the continuously updated online version of The Ecdysone Handbook edited by Lafont et al.[30] For the novel semisynthetic derivatives described here, structure elucidation was performed by means of their HRMS and 1D and 2D NMR spectra. ^{1}H, ^{13}C, and 2D (COSY, NOESY, HMBC, and HMQC) NMR spectra were recorded in CD_3OD in Shigemi sample tubes at room temperature, either by using a Varian 800 MHz NMR spectrometer equipped with a ^{1}H {^{13}C/^{15}N} triple resonance ^{13}C enhanced salt tolerant cold probe operating at 800 MHz for ^{1}H and

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201 MHz for ^{13}C NMR (in the cases of compounds **35**−**37**, **39**, **41**−**43**) or by using a Bruker Avance DRX-500 spectrometer (for compounds **16**, **31**, and **56**). Chemical shifts are given on the δ-scale and referenced to the residual protosolvent (δ_C = 49.15 and δ_H = 3.31). Known semisynthesized compounds were identified by comparing their spectroscopic and chromatographic data with those published,[31] and in the cases of compounds **44**, **45**, **49**, and **57**, direct chromatographic comparisons with previously isolated compounds of natural origin were also performed. Melting points were measured by a Boetius apparatus. All compounds possessed a purity of over 95% by means of HPLC−UV.

*Synthesis of Ecdysteroid Acetonides **46**, **48**, **50**−**52**, **54**, **55**, **57**.* The corresponding ecdysteroid **1** (50 mg for **57**), **18** (21 mg for **52**), **9** (9 mg for **51** and **55**), **10** (22 mg for **46** and **48**), 20,26-dihydroxyecdysone, not listed for the present experiment (8 mg for **54**), or **27** (5 mg for **50**) was dissolved in acetone (Merck, Germany) (1 mg/mL). Double amount of acetone containing 5% phosphomolybdic acid (Merck, Germany) was added, and the mixture was kept at room temperature for 5 min. The reaction was terminated by diluting with water and alkalizing with $NaHCO_3$ (Merck, Germany). The mixture was concentrated by vacuum distillation until only water was present, and acetonides were extracted from the aqueous solution with dichloromethane (Merck, Germany). Acetonides were isolated by HPLC as published before[31] in the following yields: **46** (1.5 mg, 5.9%), **48** (1.5 mg, 5.9%), **50** (1.0 mg, 18.5%), **51** (1.5 mg, 15.6%), **52** (11.2 mg, 49.8%), **54** (2.3 mg, 22.8%), **55** (5.7 mg, 54.5%), **57** (35.1 mg, 60.2%).

Structures of the isolated ecdysteroid acetonide derivatives were verified based on their chromatographic properties and ESI-MS/MS spectra.

*Synthesis of Compounds **16**, **31**, and **56** Expressing 7,9(11)-Diene Moieties from the Corresponding 11α-Hydroxyecdysteroids.* An amount of 1 mg of starting material (0.5 mg/mL in methanol (Merck, Germany) was adsorbed onto alumina chromatographic stationary phase (Brockmann II, neutral) under vacuum at 50 °C and immediately eluted with methanol. Yields were 93.8%, 93.9%, and 96.6%, respectively, by means of HPLC−UV. Each compound showed a strong upfield shift of both ^1H and ^{13}C signals at C-11 and a characteristic increase in the UV absorbance due to the more extensive conjugation, compared to their parental ecdysteroids. Key spectroscopic data of these compounds are as follows, and detailed data are available as Supporting Information.

*25,26-Didehydrodacryhainansterone (**16**).* White solid, semisynthesized from isovitexirone (not listed for the present experiments) obtained from *Serratula wolffii*.[24] UV λ_{max} nm (log ε): 298 (3.786). ^1H NMR (CD_3OD) δ 6.29 (1H, dt; J = 6.6, 2.0 Hz, H-11). ^{13}C NMR (CD_3OD) 136.3 (C-9), 134 (C-11).

*9,11-Didehydropoststerone (**31**).* White solid, semisynthesized from 11α-hydroxypoststerone (not listed for the present experiments) obtained from *Serratula wolffii*.[32] UV λ_{max} nm (log ε): 295.7 (3.374). ^1H NMR (CD_3OD) δ 6.34 (1H, dt, J = 6.6, 2.0 Hz, H-11). ^{13}C NMR (CD_3OD) δ 136.7 (C-9), 133.2 (C-11).

*Dacryhainansterone 2,3;20,22-Diacetonide (**56**).* White solid, semisynthesized from **55**. ^1H NMR (CD_3OD) δ 6.22 (1H, dt, J = 6.5, 2.1 Hz, H-11). ^{13}C NMR (CD_3OD) δ 136.2 (C-9), 134.1 (C-11).

*Synthesis of Ecdysteroid Acetates **35**, **42** from **1**; **36**, **39**, and **41** from **18**; and **37** and **43** from **12**.* Compounds **1**, **12**, and **18** (50, 24, and 36 mg, respectively) were dissolved in acetic anhydride (0.8, 0.6, and 0.5 mL, respectively). Pyridine was added (1.5, 1, and 1 mL, respectively), and the solutions were left at room temperature for 24 h. Ice-cold H_2O (20 mL) was added to each reaction mixture, and solvent−solvent extraction was subsequently performed with dichloromethane (5 × 10 mL). Each combined organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness to obtain mixtures of acetate derivatives of the parental ecdysteroid. Derivatives of **1** were purified by using rotational planar chromatography (RPC) on silica with a gradient of dichloromethane−methanol mixtures (250:3, 250:5, 250:6, 250:8, v/v) to obtain the triacetate **35** (16 mg, 25.3%) and the tetraacetate **42** (9 mg, 13.0%), both of which gave spectroscopic data identical to those published before.[33] Derivatives of **18** were purified similarly to obtain the diacetate **36** (5 mg, 11.9%), the tetraacetate **39** (3 mg, 6.2%), and the triacetate **41** (12 mg, 26.6%). Derivatives of **12** were also subjected to RPC as described above and subsequently purified by normal phase HPLC, using CH_2Cl_2−*i*-PrOH−H_2O (250:14:1, v/v/v) to obtain the diacetate[34] **37** (4 mg, 14.0%) and the triacetate **43** (2 mg, 6.5%). ^1H NMR spectra of these derivatives justified the presence of acetate methyl singlets at 1.90−2.09 ppm. The acetylated positions were indicated by the downfield shifts of protons directly linked to the substituted positions. ^{13}C NMR data either from direct or from HSQC experiment (in case of **37**) confirmed the acetylation at the given positions showing the expected downfield and upfield shifts for ipso and for α carbons, respectively, as compared to the data available for the parental compounds. Key spectroscopic data of these compounds are as follows, and detailed data are available as Supporting Information.

*Polypodine B 2,22-Diacetate (**36**).* White solid, semisynthesized from **18**. ^1H NMR (800 MHz, CD_3OD) δ 2.09 (3H, s), 2.08 (3H, s). ^{13}C NMR (201 MHz, CD_3OD) δ 173.4 (AcO, C-22), 172.4 (AcO, C-2), 80.7 (C-22), 72.3 (C-2), 21.1, (AcO, C-2), 21.2, (AcO, C-22).

*2-Deoxyecdysone 3,22-Diacetate (**37**).* White solid, semisynthesized from **12**. ^1H NMR (CD_3OD, 800 MHz) δ 2.06 (3H, s), 2.04 (3H, s). ^{13}C NMR (201 MHz, CD_3OD) δ 172.2 (AcO, C-22), 172.1 (AcO, C-3), 78.9 (C-22), 69.4 (C-3), 21.2 (AcO, C-22), 21.1 (AcO, C-3).

*Polypodine B 2,3,22,25-Tetraacetate (**39**).* White solid, semisynthesized from **18**. ^1H NMR (CD_3OD, 800 MHz) δ 2.03 (s, 3H), 2.02 (s, 3H), 1.94 (s, 3H), 1.90 (s, 3H). ^{13}C NMR (201 MHz, CD_3OD) 173.3 (AcO, C-22), 172.7 (AcO, C-3), 172.4 (AcO, C-25), 172.1 (AcO, C-2), 83.3 (C-22), 80.4 (C-25), 69.5 (C-2), 68.5 (C-3), 22.2 (AcO, C-25), 21.5 (AcO, C-2), 21.2 (AcO, C-22), 20.9, (AcO, C-3).

*Polypodine B 2,3,22-Triacetate (**41**).* White solid, semisynthesized from **18**. ^1H NMR (800 MHz, CD_3OD) δ 2.09 (3H, s), 2.08 (3H, s), 2.00 (3H, s). ^{13}C NMR (201 MHz, CD_3OD) δ 173.4 (AcO, C-22), 172.7 (AcO, C-2), 172.1 (AcO, C-3), 80.7 (C-22), 69.5 (C-2), 68.5 (C-3), 21.2 (AcO, C-2), 21.2 (AcO, C-22), 20.9 (AcO, C-3).

*2-Deoxyecdysone 3,22,25-Triacetate (**43**).* White solid, semisynthesized from **12**. ^1H NMR (CD_3OD, 800 MHz) δ 2.07 (3H, s), 2.04 (3H, s), 1.97 (3H, s). ^{13}C NMR (201 MHz, CD_3OD) δ 72.2 (AcO, C-22), 171.9 (AcO, C-25), 171.7 (AcO, C-3), 82.8 (C-25), 78.3 (C-22), 69.4 (C-3), 22.1 (AcO, C-25), 20.9 (AcO, C-3), 20.8 (AcO, C-22).

For bioassays, each compound was resuspended in 99.5% DMSO (Sigma, Germany). In each protocol DMSO was always tested as solvent control and no activity was found at the ratios used.

Cell Lines. Parental (PAR) and multidrug resistant (MDR) cell lines were the L5178 mouse T-cell lymphoma cells (ECACC catalog no. 87111908, U.S. FDA, Silver Spring, MD, U.S.) and the L5178 cells transfected with pHa MDR1/A retrovirus,[35] respectively. MDR cell line was selected by culturing the infected cells with 60 μg/L colchicine. Both cell lines were cultured in McCoy's 5A medium supplemented with 10% heat inactivated horse serum, L-glutamine, and antibiotics (penicillin and streptomycin) at 37 °C and 5% CO_2 atmosphere.[36] Medium, horse serum, and antibiotics were purchased from Difco, U.S.

Antiproliferative Assay. Antiproliferative activities on MDR cells were tested by the MTT assay (n = 3)[37] in serum free McCoy's 5A medium at 6 × 10^3 cells/well. After 72 h of incubation (under 5% CO_2 and at 37 °C), MTT (Sigma, Germany) was added (10% per well). After 4 h of incubation SDS 10% (Sigma, Germany) was added (5% per well). Optical density at 540 and 630 nm was read after 72 h of incubation using an ELISA reader (Multiskan EX, Lab Systems, U.S.).

Inhibition of ABCB1 Pump (P-gp) on *mdr1* Gene Transfected Mouse Lymphoma Cells. Inhibition of ABCB1 was evaluated using rhodamine 123, a fluorescent dye, and its concentration inside the cells was determined by flow cytometry.[5] Briefly, 2 × 10^6 cells/mL were treated with 2 and 20 μM each ecdysteroid and incubated for 10 min. Rhodamine 123 (Sigma, Germany) was added to a final concentration of 5.2 μM. The samples were incubated for 20 min at 37 °C in water

bath and then centrifuged (2000 rpm, 2 min). The pellet was resuspended in 0.5 mL of phosphate buffer saline (PBS) (Sigma, Germany). The washing step was repeated twice. The fluorescence of the samples was measured by flow cytometry (Becton Dickinson FACScan, BD, U.S.). Verapamil (Sanofi-Synthelabo) at 22 μM was used as positive control.

Assay for Interaction of Ecdysteroids with Doxorubicin. The combined activity of doxorubicin (Teva, Hungary) and ecdysteroids was determined using the checkerboard microplate method. Briefly, cell suspension (5×10^4 cells/well) was incubated with doxorubicin and the compound to be tested for 48 h at 37 °C under 5% CO_2. Cell growth rate was determined through MTT staining, as described above. The interaction was evaluated by using the CompuSyn software (CompuSyn, Inc., U.S.) for the constant ratios, and combination index (CI) values are presented for 50%, 75%, and 90% of growth inhibition. For all calculations, M/M ratios of compound versus doxorubicin were used. Evaluation of the results was done according to that suggested by Chou.[16]

Stability Testing of 20-Hydroxyecdysone 2,3;20,22-Diacetonide (57) in Acidic Conditions. An amount of 40 μL of a 25 mg/mL stock solution of **57** in DMSO was diluted with water (control) or 0.05 M HCl (pH 1.48, measured by an Orion 9107BNMD pH meter; Thermo Scientific, Waltham, MA, U.S.) to 1.00 mL in triplicate (final concentration of **57**, 1 mg/mL). An amount of 5 μL of the acidic solution was analyzed at 2, 7, 15, 20, 30, 40, and 60 min by HPLC by using a system of two Jasco PU-2080 pumps connected to a Jasco MD-2010 PDA detector. A gradient of aqueous methanol (40–100%) was used at 0.9 mL/min on an Agilent Poroshell 120 EC C-8 column (3 mm × 50 mm, 2.7 μm), allowing rapid analyses of 4 min. Peaks of **57** and the metabolites **45** and **1** were integrated from the maximum absorbance chromatograms taken between 239 and 300 nm, by automated peak search at a slope sensitivity of 700 μV/s. Kinetic curves were fitted by GraphPad Prism 5.0 (GraphPad Software Inc.), by using the one-phase decay exponential model.

■ ASSOCIATED CONTENT

Supporting Information

Compound characterization data for **16**, **31**, **36**, **37**, **39**, **41**, **43**, and **56** and results of the combination studies at ecdysteroid vs doxorubicin ratios of 20.4:1, 40.8:1, and 81.5:1 on the MDR cell line. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

20E, 20-hydroxyecdysone; CI, combination index; EP, efflux pump; EPI, efflux pump inhibitor; Fa, fraction affected; FAR, fluorescence activity ratio; Fl, fluorescence intensity; RPC, rotational planar chromatography

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