Bioorganic Chemistry 106 (2021) 104485

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

In vitro adjuvant antitumor activity of various classes of semi-synthetic poststerone derivatives

Rimma G. Savchenko [a,1], Márta Nové [b,1], Gabriella Spengler [b], Attila Hunyadi [c,d,*], Lyudmila V. Parfenova [a,*]

[a] Institute of Petrochemistry and Catalysis of Russian Academy of Sciences, 141, Prospekt Oktyabrya, 450075 Ufa, Russia
[b] Department of Medical Microbiology and Immunobiology, University of Szeged, Dóm sq. 9, 6720 Szeged, Hungary
[c] Institute of Pharmacognosy, Interdisciplinary Excellence Centre, University of Szeged, Eötvös str. 6, 6720 Szeged, Hungary
[d] Interdisciplinary Centre for Natural Products, University of Szeged, Eötvös str. 6, 6720 Szeged, Hungary

ARTICLE INFO

Keywords:
Ecdysteroids
Dioxolanes
Adjuvant
Chemo-sensitization
Multidrug resistance

ABSTRACT

Various classes of semi-synthetic analogs of poststerone, the product of oxidative cleavage of the C20-C22 bond in the side chain of the phytoecdysteroid 20-hydroxyecdysone, were synthesized. The analogs were obtained by reductive transformations using L-Selectride and H_2-Pd/C, by molecular *abeo*-rearrangements using the DAST reagent or ultrasonic treatment in the NaI-Zn-DMF system, and by acid-catalyzed reactions of poststerone derivatives with various aldehydes (*o*-FC_6H_4CHO, *m*-$CF_3C_6H_4CHO$, $CO_2Me(CH_2)_8CHO$). The products were tested on a mouse lymphoma cell line pair, L5178 and its ABCB1-transfected multi-drug resistant counterpart, $L5178_{MDR}$, for their *in vitro* activity alone and in combination with doxorubicin, and for the ability to inhibit the ABCB1 transporter. Among the tested compounds, new 2,3-dioxolane derivatives of the pregnane ecdysteroid were found to have a pronounced chemosensitizing activity towards doxorubicin and could be considered as promising candidates for further structure optimization for the development of effective chemosensitizing agents.

1. Introduction

Ecdysteroids represent an abundant class of mainly the cholestane type natural compounds [1]. It is known that ecdysteroids are non-toxic in mammals ($LD_{50} > 6$ g/kg) and have a broad spectrum of non-hormonal, beneficial effects: they may act as adaptogenic, anabolic, anti-hyperglycemic, hepato- and immunoprotective agents [2,3]. The most abundant and best studied representative of this class is the phytoecdysteroid 20-hydroxyecdysone (20E) (1), a molting and metamorphosis hormone in arthropods [4], which is available from plant sources [3,5,6].

Chemical modifications of ecdysteroids are directed towards the synthesis of both rare ecdysteroids that are poorly available from natural sources and their analogs promising for medicinal chemistry. For example, alkylated ecdysteroids were considered as new types of actuators for switch-activated gene therapy [7]. Dioxolane derivatives of ecdysteroids sensitize cancer cells of various origin towards the action of anticancer agents, and it is especially important that activity may be particularly strong on multidrug-resistant (MDR) cancer cells [8]. High antiproliferative activity was found for some ecdysterone–peptide conjugates [9]. Some nitrogen-containing 20E 2,3;20,22-diacetonide derivatives (oximes, oxime ethers, and lactams) show highly potent chemosensitizing activity towards doxorubicin accompanied by moderate cytotoxicity on human and mouse cancer cell lines [10]. The modern technology of using nanoparticles for the design of ecdysteroid conjugates with doxorubicin may become the next step in overcoming the multiple resistance of cancer cells to anticancer agents [11,12].

The product of oxidative C20-C22 cleavage of 20E side chain, that is, the 17-acetylecdysteroid poststerone, was first isolated from the plant *Cyathula capitata* [13]. It was found that poststerone is an *in vivo* metabolite of 20E [14] that acts as an anabolic agent in rats [15] and increases the activation of protein kinase B in C2C12 murine skeletal muscle myotubes [16]. It was also shown that poststerone dioxolanes act as potent chemosensitizers with a high MDR selectivity in ABCB1-transfected cancer cells but without expressing a functional inhibitory activity on the efflux transporter [17].

* Corresponding authors at: Institute of Pharmacognosy, Interdisciplinary Excellence Centre, University of Szeged, Eötvös str. 6, 6720 Szeged, Hungary (A. Hunyadi).
E-mail addresses: hunyadi.a@pharmacognosy.hu (A. Hunyadi), luda_parfenova@ipc-ras.ru (L.V. Parfenova).
[1] Equal contribution by the first two authors.

https://doi.org/10.1016/j.bioorg.2020.104485
Received 16 July 2020; Received in revised form 20 August 2020; Accepted 16 November 2020
Available online 19 November 2020

Previously, we demonstrated that poststerone derivatives undergo skeletal transformations on treatment with the organohydride reagent L-Selectride [18] or via ultrasonically assisted 2,3-dideoxygenation reaction (NaI-Zn-DMF) to give 13(14 → 8)-*abeo*-ecdysteroids resulting from intramolecular rearrangement [19]. This type of transformations accompanied by migration of the C13-C14 bond with simultaneous contraction/expansion of the steroid C/D-rings are driven by the presence of the reactive allylic 14-OH group and could be classified as semipinacol rearrangement of allylic tertiary alcohols initiated by Lewis or Brønsted acids [20]. Another skeletal modification of pregnane 20*R*-hydroxyecdysteroids is induced by DAST (diethylaminosulfur trifluoride) resulted in the diastereoselective formation of (20*R*)-13(17 → 20)-D-homo- or 13,14-*seco*-androstane structures [18].

In continuation of our studies on chemical transformations of the pregnane ecdysteroids and to elucidate structure–activity relationships concerning the antitumor potential of such compounds, in particular those with a transformed steroid core, we expanded the series of poststerone derivatives and studied their *in vitro* antiproliferative and cytotoxic effects on a pair of mouse lymphoma cancer cell lines, L5178 and its multidrug-resistant counterpart $L5178_{MDR}$, inhibition of the ABCB1 transporter, and their interaction with doxorubicin on MDR cells.

2. Results and discussion

The starting substrate poststerone (**2**) was prepared from 20E (1) [21], isolated from the plant *Serratula coronata* [5]. The molecule contains a conjugated Δ7-6-keto group in ring B, an important chromophore present in virtually all ecdysteroid molecules. The reductive transformations of this group studied for cholestane type ecdysteroids [22] offer the prospects for the synthesis of brassinosteroid type hybrid molecules, some of which have a natural antitumor potential [23]. Hydrogenation of cholestane and pregnane type ecdysteroids with 10% Pd-C as a catalyst in the presence of sodium nitrite in ethanol was earlier reported [24]. We developed an efficient method of alkaline hydrogenation of the Δ7-bond of the conjugated keto group (MeONa, H_2, 10% Pd-C), which provides the target 7,8α-dihydro ecdysteroid derivatives in high yields [25]. In this work using the alkaline hydrogenation, we prepared for the first time 7,8α-dihydro poststerone acetonide **4**, the structure of which was proved by 1D and 2D NMR spectroscopy.

The hydride reduction of poststerone **2** with an equimolar amount of L-Selectride resulted in the regio- and stereospecific formation of 20*R*-alcohol **5**. A similar reduction of the 20-oxo moiety of pregnane ecdysteroids on treatment with a 1.5-molar amount of organohydride reagent was carried out previously for compound **6** [18], whereas the reduction with a two molar amount of L-Selectride led to the transformation of 6,20-diketo groups accompanied by epimerization at 5-H and gave 6α,20*R* alcohol **7** of 5α-pregnane series [18].

The introduction of fluorine atoms into a natural molecule is known to increase the lipophilicity and is often used in drug delivery for increasing the drug metabolic stability [26]. In the case of ecdysteroids, fluorination of 2,3;20,22-diacetonide of 20E increased the inhibitory activity of derivatives against overexpression of ABCB1, but in most cases, it affected the chemosensitizing activity of compounds [27]. Fluorination of pregnane 20*R*-alcohols with the DAST reagent yielded products with a transformed carbon skeleton, instead of the expected fluoro derivatives [18]. The 13(14 → 17a)-*abeo*-rearrangement of 20*R*-alcohol **6** resulted in a skeletal transformation and gave 13,14-*seco*-androstane structure **8**. A similar molecular rearrangement with a shift of the C13–C14 bond with simultaneous C/D-ring contraction contraction/expansion also occurred for pregnane derivatives; the rearrangement induced by Brønsted or Lewis acids involved the allylic 14-hydroxy group [18,19]. It was established that ultrasonically assisted deoxygenation of poststerone dimesylate **10** on treatment with NaI-Zn-DMF afforded non-polar derivatives **11** and/or **12** [19], the analogs of cytotoxic marine steroids [28,29]. However, the deoxygenation of monomesylate **9** under the same conditions did not produce the desired products. The reduction of diketosteroid **12** with an excess of L-Selectride resulted in 6α,20*R*-diol **13**. The absolute configurations of the new stereogenic centers at C5, C6, and C20 of diol **13** were assigned according to NOESY data (Scheme 1).

Easy-to-obtain semi-synthetic dioxolane derivatives of cholestane ecdysteroids demonstrated their potential as MDR-reverting agents based on their strong synergistic activity with doxorubicin for developing. A study of 2,3;20,22-dioxolane derivatives of cholestane ecdysteroids demonstrated that the decreasing of polarity (lipophilicity) of these compounds is the major factor of their significant action on the resistance to doxorubicin in mammalian cancer cells [8]. We continued this study by preparing new 2,3-dioxolane derivatives of the pregnane ecdysteroid poststerone (**14**–**17**). These compounds were synthesized in 70–80% yields via acid-catalyzed reactions of poststerone with appropriate aldehydes (*o*-FC_6H_4CHO, *m*-$CF_3C_6H_4CHO$, $CO_2Me(CH_2)_8CHO$). The products were formed as mixtures of epimers because of the appearance of a new stereogenic center in the cyclic 2,3-acetal moiety. The hydride reduction of compound **16** with $LiAlH_4$ gave alcohol **17**. According to 1D and 2D NMR spectroscopy data for **17**, the reduction of the ester group was accompanied by 1,4-reduction of the conjugated (6-oxo-7-ene) ketone of ring B and 20-keto group, giving rise to 7,8α-dihydro-20*R*-alcohol **17**. The structure and configuration of the newly formed stereogenic centers in compound **17** were established by NOESY and by comparison with the NMR data (δ_H and δ_C) of analogs **6** and **7**. As follows from the NMR study, the structure of compound **17** corresponds to a 20*R*-hydroxy-substituted 5*β*- steroid. The molecular composition for adducts **14**–**17** was confirmed by positive-ion MALDI TOF/TOF spectrometry, which spectra exhibited the $[M + Na]^+$ and/or $[M + K]^+$ ions.

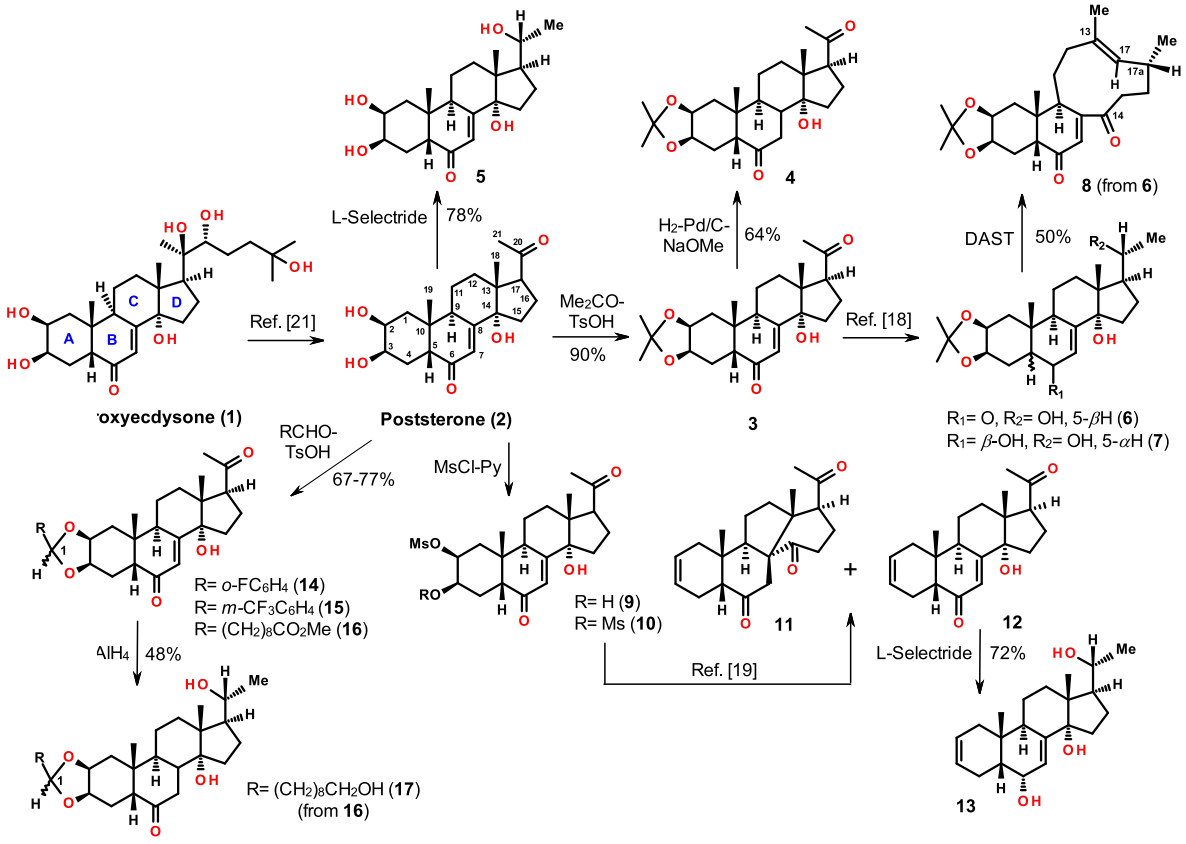
Ecdysteroids **2**–**17** were tested for their activity on a pair of mouse lymphoma cancer cell lines, namely, L5178 and its multidrug-resistant counterpart $L5178_{MDR}$. The latter cell line had been transfected to express the human ABCB1 transporter, commonly referred to as P-glycoprotein (Pgp); hence it is a good model of a frequently occurring mechanism for multidrug resistance in cancer [30]. Antiproliferative and cytotoxic activities of the compounds are summarized in Table 1.

Samples **2**–**17** had mild to weak antiproliferative activities, and several of them can be considered as inactive ($IC_{50} > 100$ μM) in terms of cytotoxicity. A comparison of the activities exerted on the two different cell lines shows that ABCB1 overexpression confers neither cross-resistance nor collateral sensitivity towards most of these compounds. It was also observed that 2,3-dioxolane derivatives of the pregnane ecdysteroid (**3**, **14**, **15**, **17**) were more potent antiproliferative agents on the parental cell line than the other compounds. However, a clear tendency for cross-resistance was observed for all these (i.e. they were less active on the MDR cell line), and this may be considered relevant (i.e. at least 2-fold resistance) for the 20*R*-diols **13** and **17**, and for poststerone acetonide **3**. This suggests that these compounds are likely ABCB1 substrates, but this was not specifically tested (Table 1).

In order to assess the activities of test compounds as functional inhibitors of the efflux transporter, they were tested for their activity on the intracellular accumulation of rhodamine 123, a fluorescent dye that is an ABCB1 substrate. The results of this test are summarized in Table 2.

The compounds were virtually inactive at the tested concentrations, except for samples **8** and **14**–**17**. These 2,3-dioxolane derivatives, especially **15** and **17**, were able to inhibit ABCB1 function even though the activity was still moderate, ca. 50–60% inhibition at 20 μM. However, it is worth noting that compound **17** showed a three times greater activity than 20-hydroxyecdysone 2,3;20,22-diacetonide (20.91% inhibition at 20 μM [10]). The inhibitory effect of compounds **8** and **16** corresponds to the level of 20-hydroxyecdysone 2,3;20,22-diacetonide. However, all compounds turned out to be less active than previously reported oxime ether derivatives of 20-hydroxyecdysone 2,3;20,22-diacetonide, some of which exerted 50–60% inhibition at a 10-times lower, 2 μM concentration and a complete inhibition at 20 μM [10].

The synthesized compounds were tested for their activity on the MDR cell line in combination with doxorubicin by using the



Scheme 1. 20-Hydroxyecdysone in the synthesis of new pregnane derivatives.

Table 1
Antiproliferative and cytotoxic activities of compounds **2**–**17**. Results are expressed as mean ± S.D., doxorubicin was used as positive control.

Compound	Antiproliferative IC_{50} ± SD (μM)		Cytotoxic IC_{50} ± SD (μM)	
	L5178	$L5178_{MDR}$	L5178	$L5178_{MDR}$
2	74.73 ± 1.41	55.3 ± 2.85	>100	>100
3	28.02 ± 2.5	58.74 ± 2.33	>100	>100
4	53.64 ± 3.51	50.43 ± 2.14	>100	>100
5	86.82 ± 3.47	73.89 ± 2.08	>100	>100
6	60.33 ± 2.89	51.51 ± 0.42	>100	>100
7	54.76 ± 2.38	>100	>100	>100
8	42.12 ± 0.27	45.77 ± 1.19	65.80 ± 4.32	82.06 ± 0.01
9	54.01 ± 2.68	67.59 ± 4.58	60.42 ± 4.52	>100
10	62.15 ± 2.08	58.56 ± 5.01	>100	>100
11	56.44 ± 2.87	48.56 ± 2.08	>100	81.64
12	37.99 ± 2.05	36.24 ± 1.74	39.23 ± 2.75	70.75 ± 3.36
13	35.8 ± 2.05	98.15 ± 0.5	38.52 ± 0.25	86.39 ± 3.65
14	26.01 ± 1.93	34.12 ± 0.85	40.72 ± 3.95	54.66 ± 3.73
15	13.1 ± 0.73	22.04 ± 0.56	31.13 ± 2.29	34.25 ± 1.82
16	71.96 ± 2.39	>100	>100	>100
17	15.58 ± 1.02	36.85 ± 3.73	12.19 ± 1.83	24.88 ± 2.25
Doxorubicin	0.28 ± 0.06	1.75 ± 0.38	0.7 ± 0.56	2.14 ± 0.76

Table 2
Functional inhibition of the ABCB1 transporter via the effect on the intracellular accumulation of rhodamine 123. Positive control: 20 nM tariquidar, exerting 99.1% inhibition. Results were obtained from single-point measurements.

Compound	Inhibition at 2 μM (%)	Inhibition at 20 μM (%)
2	0.03	0.23
3	0.09	0.51
4	0.33	6.86
5	0.05	0.01
6	0.21	0.09
7	−0.07	0.03
8	0.31	22.75
9	0.03	0.04
10	0.04	0.08
11	0.11	0.27
12	0.06	1.21
13	−0.01	2.35
14	0.33	14.43
15	0.67	47.44
16	0.59	27.38
17	5.91	66.47

checkerboard microplate method. Most significant results observed on each plate are shown in Table 3, and the dataset concerning other ecdysteroid vs. doxorubicin ratios is available as Supporting Information, Table S1.

Two new compounds, **14** and **15** were identified to act in strong synergism with doxorubicin (CI_{avg} < 0.3 [31]), and they were more potent in this regard than the previously reported poststerone acetonide (**3**). This suggests the benefit of a fluorine-substituted aromatic ring present at the newly formed dioxolane function. According to our previous results, a much weaker chemosensitizing activity was observed for bis-2,3;20,22-methylidene derivatives of 20E containing a phenyl substituent at both new stereogenic centers C-28 and C29, regardless of the configuration at C-28 [32]. The improved activity observed in the current study is therefore a result of the fluorine (**14**) or trifluoromethyl (**15**) substitution of the phenyl group, and/or the lacking sidechain. Interestingly, if the 2,3-methylidene is substituted with a C9 side chain ending with a carboxymethyl group (**16**) the compound becomes inactive as chemosensitizer and rather acts as a weak antagonist when combined with doxorubicin. Reduction of the carboxymethyl group (as in **17**), however, restores a rather potent chemosensitizing activity (CI_{avg} = 0.32), indicating that it is not the length of the newly attached sidechain but the carboxymethyl group that makes compound **16** interacting differently with doxorubicin.

Table 3
Interaction of compounds **2**–**17** with doxorubicin on the $L5178_{MDR}$ cell line. Dox: doxorubicin; CI: combination index at 50, 75 and 90% growth inhibition; CI_{avg}: weighted average CI value; 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 the antilog of the x-intercept, slope, and linear correlation coefficient of the median-effect plot, respectively.

Compound	Compound vs. Dox ratio[a]	CI_{50}	CI_{75}	CI_{90}	CI_{avg}	Dm	m	r
2	278.4:1[b]	1.17	1.18	1.20	1.19	63.098	2.419	0.993
3[c]	46.4:1	0.50	0.29	0.17	0.26	9.365	2.891	0.978
4	34.8:1	0.78	0.45	0.30	0.43	9.912	2.688	0.983
5	139.2:1	1.23	1.29	1.37	1.32	55.665	2.250	0.996
6	34.8:1	0.53	0.43	0.35	0.41	10.140	2.628	0.971
7	46.4:1	1.07	0.67	0.42	0.61	23.010	1.653	0.997
8	17.4:1	0.71	0.49	0.35	0.46	5.671	2.277	0.974
9	34.8:1	0.91	1.09	1.33	1.18	18.303	1.662	0.980
10	139.2:1	0.95	0.84	0.77	0.82	39.147	3.185	0.951
11	139.2:1	0.97	0.79	0.65	0.75	31.880	2.419	0.974
12	17.4:1	0.81	0.69	0.59	0.66	6.374	2.502	0.949
13	185.6:1	1.88	1.49	1.20	1.41	44.406	2.356	0.986
14	46.4:1	0.31	0.24	0.20	0.23	6.976	1.877	0.999
15	23.2:1	0.40	0.29	0.21	0.27	5.040	3.205	0.958
16	92.8:1	1.61	1.28	1.01	1.20	44.364	1.659	0.988
17	23.2:1	0.52	0.35	0.24	0.32	6.986	2.775	0.988

[a] Drug ratios are given in molarity; serial dilutions of doxorubicin were initiated from a commercially available injection of 2 mg mL^{-1} (doxorubicin hydrochloride, Teva).

[b] When interpreting the results, we followed our previously used "best ratio" principle, i.e. the ratio where the CI_{avg} indicated the highest difference from 1 is presented here and considered for SAR. A complete dataset is available as Supporting Information, Table S1.

[c] Compound **3**, poststerone 2,3-acetonide, was previously published by us as a chemo-sensitizer interacting with doxorubicin on the same cell line [17], and it was our positive control in this work.

In addition to the above, an overview of the CI values reveals several further new structure–activity relationships. Even though the 2- and 3-OH group elimination shifts the activity towards chemosensitizing (as expected from the decreased polarity at the A-ring) (**12** vs. **2**), this effect is still weaker than that in the presence of a 2,3-acetonide moiety (**3** vs. **12**). C-D ring rearrangement markedly decreased the chemosensitizing activity (**11** vs. **12**), but joining these two rings into a macrocycle (**8**) led to a marked increase of activity as compared to compound **12**. Saturation of the 7,8-olefin also decreased the strength of synergism with doxorubicin (**4** vs. **3**). However, it is of interest that if this is accompanied by reduction of the keto group at C-20 to *R*-OH group, a high increase in the chemosensitizing activity can be observed (**6** vs. **4**). This suggests that a selective reduction of the 20-oxo group would be even more beneficial in terms of this bioactivity.

When comparing the compounds' activity on the resistance to doxorubicin, i.e. the strength of synergism, with that on the functional efflux inhibition, i.e. the activity on the efflux of rhodamine 123, it seems to be clear that there is no correlation. For example, compound **16** inhibited ABCB1 function similarly as compound **8**, still, compound **16** rather protects the cells from the effect of the ABCB1 substrate doxorubicin, while **8** significantly enhances its killing effect. This further strengthens our previous findings that the adjuvant antitumor effect of less polar ecdysteroid derivatives is not due to efflux pump inhibition, but through another mechanism that may still be connected to the presence of over-expressed efflux transporters [8,10,17]. This may be considered as a promising feature of these compounds, since the now several decades-long development of ABCB1 inhibitors has been failing to deliver a clinically applicable adjuvant anticancer drug [33,34]. Because of this, it is an emerging concept that, instead of a targeted inhibition of drug efflux, new strategies are needed to combat MDR cancer [34–36]. Further, due to the many possible drug-drug interaction-related problems that may arise from treatment with a potent ABCB1 inhibitor [37], at this point we consider efflux inhibition by ecdysteroids as a rather unwanted side-effect. Therefore, we believe our most promising leads are those compounds that exert the strongest sensitizing activity and the weakest functional inhibition of ABCB1, i.e. compound **14** in the herein reported study.

3. Conclusions

In this study, we synthesized a series of semi-synthetic analogs of poststerone and examined their antitumor activities. The bioactivity testing confirmed the ability of poststerone 2,3-dioxolane derivatives to overcome the multidrug resistance of tumor cells. Among the analogs with a transformed steroid core, the potential of 13,14-*seco*-androstane structures as P-gp inhibitors was shown. A comparison of closely related compounds activities revealed several structural elements that influence the bioactivity, and showed that the introduction of fluorophenyl substituents in the 2,3-dioxolane groups enhances the chemosensitizing ability of ecdysteroids.

4. Materials and methods

One-dimensional (^{1}H and ^{13}C) and two-dimensional (COSY, NOESY, HSQC, and HMBC) NMR spectra of compounds were recorded on *Bruker Avance 400* spectrometer (400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C) and *Bruker Avance II 500 HD Ascend* spectrometer (500.17 MHz for ^{1}H and 125.77 MHz for ^{13}C) using standard Bruker pulse sequences. Chemical shifts are given in ppm using TMS as the internal standard. MALDI TOF/TOF mass-spectra were obtained on *Bruker Autoflex III* spectrometer with the registration of positive ions; 3,5-dimethoxy-4-hydroxycinnamic (sinapic) acid and α-cyano-4-hydroxycinnamic acids were used as matrix.

Column chromatography and TLC were performed using silica gel (<0.06 mm) and pre-coated silica gel (*Silufol* plates), respectively; spots were processed by treatment with a solution of 4-hydroxy-3-methoxybenzaldehyde in ethanol, acidified with sulfuric acid. Melting points were determined on *Boetius* hot-stage microscope. Specific rotations were measured on *Perkin-Elmer-341* polarimeter.

4.1. Synthesis

20-Hydroxyecdysone (1) was isolated from the juice of *Serratula coronata* L. [5] (mp 239–240 °C, $[\alpha]_D^{20}$ + 54.3° (c 1.45, MeOH); literature: mp 246 °C (EtOAc-MeOH, 9:1), $[\alpha]_D^{20}$ + 65.3° (c 1.0, MeOH). Poststerone (**2**) was synthesized from **1** as described in Ref. [21] [(mp: 233–235 °C, $[\alpha]_D^{18}$ + 137.2° (c 1.13, MeOH), literature: mp: 242–246 °C. Poststerone acetonide **3** was synthesized according to Ref. [18]. 2*β*,3*β*-

Dimesyloxy-14α-hydroxy-5*β*-pregn-7-en-6,20-dione (**10**), (8*R*)-8,13-*cyclo*-13,14-seco-5*β*-pregn-2-ene-6,14,20-trione (**11**) and 14*α*-hydroxy-5*β*-pregn-2(3),7-dien-6,20-dione or 2,3-didesoxy-$\Delta^{2(3)}$-poststerone (**12**) were synthesized accordingly to Ref. [19]. (20*R*)-14*α*,20-Dihydroxy-2*β*,3*β*-isopropylidenedioxy-5*β*-pregn-7-en-6-one (**6**), (20*R*)-2*β*,3*β*-isopropylidenedioxy-6*β*,14*α*,20-trihydroxy- 5*α*- pregn-7-ene (**7**), 2*β*,3*β*-isopropylidenedioxy-(17a)*α*-methyl-16,17(a)-homo-13,14-seco-5*β*-androsta-7,12(13E)-dien-6,14-dione (**8**) were synthesized accordingly to Ref. [18]. Aldehydes *o*-FC_6H_4CHO and *m*-$CF_3C_6H_4CHO$ are commercially available from Merck. $CO_2Me(CH_2)_8CHO$ is available from Abcr.

4.1.1. Synthesis of 14α-hydroxy-2β,3β-isopropilydendioxy-5β,8α-pregna-6-one (4)

Metallic sodium (0.028 g, 0.5 mmol) was added under stirring to a solution of poststerone acetonide **3** (0.1 g, 0.25 mmol) in MeOH (10 mL). The mixture was stirred for 30 min at room temperature. Then 10% Pd-C (0.046 mmol) was added and hydrogen gas from a balloon was bubbled through the reaction mixture for 3 h. The reaction mixture was filtered through a short acidic alumina column: the residue was treated with MeOH and the solvent was evaporated. The crude product was purified by column chromatography on SiO_2 (eluent $CHCl_3$) to afford **4**.

Compound **4**. Yield 64% (0.064 g); white solid; R_f 0.7 ($CHCl_3$-MeOH, 10:1); $[\alpha]_D^{20}$= +62.4, (*c* 1.38, CH_2Cl_2); mp 106–108 °C. ^{1}H NMR (500 MHz, $CDCl_3$): *δ* 0.86 (s, 3H, 18-CH_3), 1.26 and 1.92 (m, 2H, 4-CH_2), 1.28 and 1.49 (s, Me_2C), 1.39 (s, 3H, 19-CH_3), 1.52 and 2.22 (m, 2H, 15-CH_2), 1.66 and 1.92 (m, 2H, 16-CH_2), 1.70 (m, 2H, 12-CH_2), 1.78 and 2.26 (m, 2H, 11-CH_2), 1.82 and 2.12 (m, 2H, 1-CH_2), 2.05 (m, 1H, 8-CH), 2.09 (s, 3H, 21-CH_3), 2.29 and 2.61 (m, 2H, 7-CH_2), 2.44 (m, 1H, 9-CH), 2.55 (m, 1H, 5-CH), 3.24 (m, 1H, 17-CH), 4.19 (m, 1H, 2-CH), 4.47 (m, 1H, 3-CH). ^{13}C NMR (125 MHz, $CDCl_3$): *δ* 18.31 (16-CH_2), 19.03 (18-CH_3), 20.87 (11-CH_2), 25.41 (15-CH_2), 25.93 (CH_3), 26.78 (19-CH_3), 28.62 (CH_3), 31.32 (21-CH_3), 31.69 (4-CH_2), 32.14 (1- CH_2), 34.38 (12- CH_2), 39.59 (10-C), 41.16 (7-CH_2), 41.68 (8-CH), 44.29 (9-CH), 47.28 (13-C), 50.62 (5-CH), 60.33 (17-CH), 70.83 (3-CH), 73.52 (2-CH), 84.80 (14-C), 107.73 (C*), 210.33 (20-C), 211.59 (6-C). MALDI-TOF: *m/z* 443.207 $[M + K]^+$ calcd for $C_{24}H_{38}O_5K$ 443.219; *m/z* 427.259 $[M + Na]^+$ calcd for $C_{24}H_{38}O_5Na$ 427.246.

4.1.2. Synthesis of (20R)- 2β,3β,14α,20-tetrahydroxy-5β-pregn-7-en-6-one (5)

To a solution of poststerone **2** (0.1 g, 0.28 mmol) in 5 mL of anhydrous THF, 0.3 mmol of L-Selectride (1 M solution in THF) was added at −70 °C. The reaction mixture was stirred under argon at the same temperature for 0.5 h and at ambient temperature for another 2 h. The reaction was quenched by adding of H_2O (1 mL), 6 M sodium hydroxide (1 mL) and 30% H_2O_2 (1 mL), successively. The reaction mixture was extracted with ethyl acetate. The organic layer was collected, dried over anhydrous Na_2SO_4 and evaporated. Crude product was purified by column chromatography on SiO_2 (eluent $CHCl_3$-MeOH: 95–5) to afford **5**.

Compound **5**. Yield 78% (0.076 g); white solid; R_f 0.4 ($CHCl_3$-MeOH, 5:1); $[\alpha]_D^{20}$=+37, (*c* 1.44, CH_2Cl_2); mp 165–167 °C. ^{1}H NMR (500 MHz, CD_3OD): *δ* 0.94 (s, 3H, 18-CH_3), 1.04 (s, 3H, 19-CH_3), 1.31 (d, *J* = 5.5 Hz, 3H, 21-CH_3), 1.64 and 1.82 (m, 2H, 11-CH_2), 1.84 and 2.10 (m, 2H, 12-CH_2), 1.86 and 2.05 (m, 2H, 1-CH_2), 1.89 and 2.04 (m, 2H, 4-CH_2), 1.86 and 2.10 (m, 2H, 15-CH_2), 2.02 (m, 2H, 16-CH_2), 2.67 (m, 1H, 17-CH), 3.00 (m, 1H, 5-CH), 3.57 (t, 1H, 9-CH), 3.96 (m, 1H, 20-CH), 4.12 (m, 1H, 2-CH), 4.25 (m, 1H, 3-CH), 6.24 (d, *J* = 14.2 Hz, 1H, 7-CH). ^{13}C NMR (125 MHz, CD_3OD): *δ* 16.16 (18-CH_3), 20.90 (11-CH_2), 24.19 (19-CH_3), 24.22 (21-CH_3), 24.64 (16-CH_2), 31.04 (12-CH_2), 31.89 (15-CH_2), 32.26 (4-CH_2), 34.44 (9-CH), 37.59 (1-CH_2), 38.49 (10-C), 47.19 (13-C), 51.21 (5-CH), 53.26 (17-CH), 67.83 (3-CH), 67.87 (2-CH), 69.16 (20-CH), 83.71 (14-C), 121.20 (7-CH), 165.99 (8-C), 203.66 (6-C). MALDI-TOF: *m/z* 403.189 $[M + K]^+$ calcd for $C_{21}H_{32}O_5K$ 403.171; *m/z* 387.215 $[M + Na]^+$ calcd for $C_{21}H_{32}O_5Na$ 387.216.

4.1.3. Synthesis of 2β-mesyloxy-3β,14α-dihydroxy −5β-pregn-7-en-6,20-dione (9)

To a solution of poststerone **2** (0.5 g, 1.38 mmol) in 5 mL of a dry pyridine 0.64 mL (8.28 mmol) MsCl was added at 0–5 °C. The reaction mixture was stirred at 0–5 ^{0}C for 1 h and at ambient temperature for another 1 h. Water was added and the mixture extracted with $CHCl_3$ (3x25 mL). The extract was evaporated and the residue was purified by column chromatography (SiO_2; $CHCl_3$-MeOH: 95–5) to provide 0.06 g of 2.3-dimesylate **10** (43%) and 0.02 g of **9** (30%).

Compound **9.** Yield 30% (0.02 g); white solid; R_f 0.34 ($CHCl_3$-MeOH, 10:1); $[\alpha]_D^{20}$= +45.3, (*c* 1.004, $CHCl_3$); mp 160–162 °C; ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD): *δ* 0.55 (s, 3H, 18-CH_3), 0.95 (s, 3H, 19-CH_3), 1.56 and 1.80 (m, 2H, 11-CH_2), 1.66 and 1.95 (m, 2H, 1-CH_2), 1.60 and 2.10 (m, 4H, 4, 12-CH_2), 2.10 (s, 3H, 21-CH_3), 2.44 (m, 1H, 5-CH), 3.05 (m, 1H, 9-CH), 3.06 (s, 3H, OMs-CH_3), 3.25 (t, *J* = 8.5 Hz, 1H, 17-CH), 4.84 (m, 1H, 2-CH), 4.18 (br s, $\omega_{1/2}$ = 11 Hz, 1H, 3-CH), 5.78 (d, *J* = 3.5 Hz, *J* = 14 Hz, 1H, 7-CH); ^{13}C NMR (100 MHz, $CDCl_3$ + CD_3OD): *δ* 16.95 (18-CH_3), 20.32 (11-CH_2), 21.16 (16-CH_2), 23.52 (19-CH_3), 24.79 (15-CH_2), 29.69 (12-CH_2), 31.17 (4-CH_2), 31.28 (21-CH_3), 33.40 (9-CH), 33.95 (1-CH_2), 38.77 (OMs-CH_3, 10-C), 47.45 (13-C), 49.41 (5-CH), 58.67 (17-CH), 65.41 (3-CH), 78.79 (2-CH), 83.88 (14-C), 121.71 (7-CH), 164.34 (8-C), 203.44 (6-C), 210.40 (20-C). MALDI-TOF: *m/z* 442.136 $[M + H]^+$ calcd. for $C_{22}H_{32}O_7S$ 442.203.

4.1.4. Synthesis of 6α,14α,20R-trihydroxy-5α-pregna-2(3),7-diene (13)

To a solution of the steroid substrate **12** [19] (0.1 g, 0.3 mmol) in 5 mL of anhydrous THF, 0.4 mmol of L-Selectride (1 M solution in THF) was added at −70 °C. The reaction mixture was stirred under argon at the same temperature for 0.5 h and at ambient temperature for another 2 h and the reaction was quenched by adding of H_2O (1 mL), 6 M sodium hydroxide (1 mL) and 30% H_2O_2 (1 mL), successively. The reaction mixture was extracted with ethyl acetate. The organic layer was collected and dried over anhydrous Na_2SO_4. Then the solvent was evaporated and the residue was purified by column chromatography (SiO_2; $CHCl_3$) to provide alcohol **13**.

Compound **13**. Yield 0.072 g (72%); white solid; R_f 0.26 ($CHCl_3$-MeOH, 10:1); $[\alpha]_D^{20}$ = +57.8^0 (c 1.54; $CHCl_3$); mp 106–107 °C. ^{1}H NMR (500 MHz, CD_3OD): 0.77 (s, 3H, 18-CH_3), 1.00 (s, 3H, 19-CH_3), 1.16 (d, 3H, *J* = 6.3 Hz, 21-CH_3), 1.49 (m, 2H, 16-CH_2), 1.58 and 1.62 (m, 2H, 15-CH_2), 1.75 and 1.79 (m, 2H, 4-CH_2), 1.75 and 2.17 (m, 2H, 1-CH_2), 1.90 (m, 2H, 12-CH_2), 1.92 (m, 1H, 5-CH), 2.00 (m, 2H, 11-CH_2), 2.08 (m, 1H, 17-CH), 2.74 (m, 1H, 9-CH); 3.69 (m, 1H, 20-CH), 4.62 (m, 1H, 6-CH), 5.32 (m, 1H, 7-CH), 5.54 (m, 1H, 2-CH), 5.69 (m, 1H, 3-CH); ^{13}C NMR (125 MHz, $CDCl_3$): 15.66 (18-CH_3), 20.03 (16-CH_2), 22.06 (11-CH_2), 22.43 (21-CH_3), 23.39 (19-CH_3), 24.17 (4- CH_2), 30.91 (12-CH_2), 31.18 (15-CH_2), 32.81 (9-CH), 34.31 (10-C), 35.95 (1-CH_2), 45.08 (5-CH), 46.41 (13-C), 52.53 (17-CH), 66.69 (6-CH), 69.47 (20-CH), 84.67 (14-C), 120.68 (7-CH), 123.67 (2-CH), 126.39 (3-CH), 143.02 (8-C); MALDI TOF *m/z* 355.246 $[M + Na]^+$ calcd for $C_{21}H_{32}O_3Na$ 355.224; *m/z* 371.215 $[M + K]^+$ calcd for $C_{21}H_{32}O_3K$ 371.198.

4.1.5. General procedure of the synthesis of poststerone 2,3-dioxolane derivatives 14–16

The aldehyde 0.4 mmol (*o*-FC_6H_4CHO, *m*- $CF_3C_6H_4CHO$ or $CO_2Me(CH_2)_8CHO$) was added to a solution of poststerone **2** (0.1 g, 0.28 mmol) in 5 mL of anhydrous DME (dimethoxyethane), and then TsOH (50 mg) was added. The reaction mixture was stirred for 1 h (TLC control) and neutralized by a saturated solution of $NaHCO_3$, and then extracted with ethyl acetate. The combined organic extracts were dried (Na_2SO_4) and evaporated. Crude product was purified by column chromatography on SiO_2 to afford **14**–**16**.

Compound **14** (epimer ratio 1:1.1). Yield 77% (0.101 g); white solid; R_f 0.77 ($CHCl_3$-MeOH, 20:1); $[\alpha]_D^{20}$= +69.0, (*c* 1.43, CH_2Cl_2); mp 118–120 °C. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 0.61 and 0.63 (s, 3H, 18-CH_3), 0.96 and 1.03 (s, 3H, 19-CH_3), 1.20 and 2.02 (m, 2H, 1-CH_2), 1.62–1.80 (m, 2H, 11-CH_2), 1.64–2.09 (m, 2H, 12-CH_2), 1.88 and 2.30

(m, 2H, 4-CH_2), 1.90 and 2.30 (m, 2H, 16-CH_2), 2.15 (s, 3H, 21-CH_3), 2.28–2.30 (m, 2H, 15-CH_2), 2.38 (dd, $J = 5.1$ Hz, $J = 15.0$ Hz, 1H, 5-CH), 2.92 (t, $J = 7.6$ Hz, 1H, 9-CH), 3.30 (t, $J = 8.8$ Hz, 1H, 17-CH), 4.28–4.37 (m, 1H, 3-CH), 4.40–4.54 (m, 1H, 2-CH), 5.82 (s, 1H, 7-CH), 6.14 (s, 0.53H, 1′-CH), 6.38 (s, 0.47H, 1′-CH), 7.02–7.10 (m, 1H, 4′-CH), 7.13 (dd, $^3J_{HH} = 7.4$ Hz, $^3J_{HH} = 8.4$ Hz, 1H, 6′-CH) and 7.18 (dd, $^3J_{HH} = 7.6$ Hz, $^3J_{HH} = 8.5$ Hz, 1H, 6′-CH), 7.29–7.39 (m, 1H, 5′-CH), 7.45 (dd, $^3J_{HH} = 8.4$ Hz, $^4J_{HF} = 7.2$ Hz, 0.47H, 6′-CH) and 7.60 (dd, $^3J_{HH} = 8.5$ Hz, $^4J_{HF} = 7.1$ Hz, 0.53H, 7′-CH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.13 (18-CH_3), 20.66 (11-CH_2), 21.18 (16-CH_2), 23.44 and 23.56 (19-CH_3), 26.50 (4-CH_2), 29.90 (15-CH_2), 31.39 (21-CH_3), 31.92 (12-CH_2), 34.76 (10-C), 34.42 and 34.77 (9-CH), 37.72 and 37.79 (1-CH_2), 47.89 (13-C), 50.55 and 50.92 (5-CH), 58.73 (17-CH), 71.91 and 72.04 (3-CH), 73.23 and 74.11 (2-CH), 84.53 (14-C), 97.82 (d, $^3J_{CF} = 2.7$ Hz, 1′-CH) and 98.35 (d, $^3J_{CF} = 3.7$ Hz, 1′-CH), 115.59 (d, $^2J_{CF} = 21.0$ Hz, 4′-CH) and 115.75 (d, $^2J_{CF} = 21.1$ Hz, 4′-CH), 121.66 and 121.77 (7-CH), 123.90 (d, $^4J_{CF} = 3.5$ Hz, 6′-CH) and 124.17 (d, $^4J_{CF} = 3.3$ Hz, 6′-CH), 125.95 (d, $^2J_{CF} = 146.0$ Hz, 2′-CH) and 126.05 (d, $^2J_{CF} = 146.0$ Hz, 2′-CH), 127.29 (d, $^3J_{CF} = 35.8$ Hz, 7′-CH) and 127.31 (d, $^3J_{CF} = 36.2$ Hz, 7′-CH), 130.65 (d, $^3J_{CF} = 8.3$ Hz, 5′-CH) and 130.80 (d, $^3J_{CF} = 8.2$ Hz, 5′-CH), 160.88 (d, $^1J_{CF} = 248.9$ Hz, 3′-CF) and 161.06 (d, $^1J_{CF} = 248.9$ Hz, 3′-CF), 162.36 and 162.59 (8-C), 202.47 and 202.53 (6-C), 209.64 (20-C). MALDI-TOF: *m/z* 507.206 $[M + K]^+$ calcd for $C_{28}H_{33}FO_5K$ 507.195; *m/z* 491.223 $[M + Na]^+$ calcd for $C_{28}H_{33}FO_5Na$ 491.221.

Compound **15** (epimer ratio 1:3.3). Yield 75% (0.105 g); white solid; R_f 0.8 ($CHCl_3$-MeOH, 20:1); $[\alpha]_D^{20} = +181$, (*c* 1.49, CH_2Cl_2); mp 84–86 °C;. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.62 and 0.64 (s, 3H, 18-CH_3), 0.95 and 1.05 (s, 3H, 19-CH_3), 1.20 and 2.05 (m, 2H, 1-CH_2), 1.64 and 2.07 (m, 2H, 12-CH_2), 1.67 and 1.83 (m, 2H, 11-CH_2), 1.72 and 2.50 (m, 2H, 16-CH_2), 1.82 and 2.32 (m, 2H, 15-CH_2), 1.92 and 2.20 (m, 2H, 4-CH_2), 2.16 (c, 3H, 21-CH_3), 2.38 (m, 1H, 5-CH), 2.92 (m, 1H, 9-CH), 3.31 (t, $J = 8.7$ Hz, 1H, 17-CH), 4.23 and 4.40 (m, 1H, 3-CH), 4.36 and 4.54 (m, 1H, 2-CH), 5.84 (s, 1H, 7-CH), 5.92 (s, 0.77H, 1′-CH), 6.18 (s, 0.23H, 1′-CH), 7.47–7.56 (m, 1H, 6′-CH), 7.58–7.66 (m, 1H, 5′-CH), 7.66–7.72 (m, 1H, 7′-CH), 7.72 and 7.75 (both s, 1H, 3′-CH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.16 (18-CH_3), 20.71 (11-CH_2), 21.16 (16-CH_2), 23.48 and 23.52 (19-CH_3), 26.46 and 26.50 (4-CH_2), 29.92 (15-CH_2), 31.37 and 31.97 (21-CH_3), 31.95 (12-CH_2), 34.82 (10-C), 34.82 and 34.95 (9-CH), 37.70 and 37.81 (1-CH_2), 47.83 and 47.91 (13-C), 50.59 and 50.81 (5-CH), 58.71 (17-CH), 71.93 and 72.30 (3-CH), 73.34 and 74.28 (2-CH), 84.64 and 84.59 (14-C), 101.14 and 102.39 (1′-CH), 121.67 and 121.76 (7-CH), 122.87 (q, $^3J_{CF} = 4.0$ Hz, 3′-CH) and 123.21 (q, $^3J_{CF} = 3.8$ Hz, 3′-CH), 123.98 (q, $^1J_{CF} = 272.6$ Hz, CF_3), 125.62 (q, $^3J_{CF} = 4.3$ Hz, 5′-CH) and 125.80 (q, $^3J_{CF} = 3.7$ Hz, 5′-CH), 128.90 and 128.96 (6′-CH), 129.52 (7′-CH), 130.76 (q, $^2J_{CF} = 32.3$ Hz, 4′-CH), 139.39 and 140.78 (2′-CH), 162.28 and 162.45 (8-C), 202.28 and 202.35 (6-C), 209.50 and 209.54 (20-C). MALDI-TOF: *m/z* 557.192 $[M + K]^+$ calcd for $C_{29}H_{33}F_3O_5K$ 557.192; *m/z* 541.223 $[M + Na]^+$ calcd for $C_{29}H_{33}F_3O_5Na$ 541.218.

Compound **16** (epimer ratio 1:1.5). Yield 67% (0.1 g); white solid; R_f 0.65 ($CHCl_3$-MeOH, 10:1); $[\alpha]_D^{20} = +42.8$, (*c* 1.47, $CHCl_3$); mp 30–32 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.58 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 1.15 and 1.95 (m, 2H, 1-CH_2), 1.20–2.05 (m, 16H, -$(CH_2)_8$-), 1.28 (m, 2H, 12-CH_2), 1.60 (m, 2H, 4-CH_2), 1.60 and 1.80 (m, 2H, 11-CH_2), 1.75 and 2.23 (m, 2H, 15-CH_2), 1.89 and 2.25 (m, 2H, 16-CH_2), 2.12 (s, 3H, 21-CH_3), 2.25 (m, 1H, 5-CH), 2.86 (m, 1H, 9-CH), 3.27 (t, $J = 8.5$ Hz, 1H, 17-CH), 3.62 (s, 3H, OCH_3), 4.08 and 4.15 (m, 1H, 3-CH), 4.05 and 4.28 (m, 1H, 2-CH), 4.90 (t, $J = 4.5$ Hz, 0.40H, 1′-CH), 5.18 (t, $J = 4.5$ Hz, 0.60H, 1′-CH), 5.78 (s, 1H, 7-CH); ^{13}C NMR (125 MHz, $CDCl_3$): δ 17.10 (18-CH_3), 20.56 and 20.60 (11-CH_2), 21.14 (16-CH_2), 23.38 and 23.52 (19-CH_3), 23.92 and 24.24 (-CH_2-), 24.68 and 24.84 (-CH_2-), 24.88 (-CH_2-), 26.52 and 26.66 (4-CH_2), 28.98 (-CH_2-), 29.04 (-CH_2-), 29.27 and 29.30 (-CH_2-), 29.38 (-CH_2-), 29.85 and 29.89 (12-CH_2), 31.40 (21-CH_3), 31.70 (15-CH_2), 33.85 (-CH_2-), 34.06 (-CH_2-), 34.19 (-CH_2-), 34.39 (-CH_2-), 34.71 (-CH_2-), 35.38 and 35.45 (9-CH), 37.68 and 37.71 (1-CH_2), 38.24 (10-C), 47.77 and 47.86 (13-C), 50.60 and 50.81 (5-CH), 51.45 (OCH_3), 58.75 (17-CH), 71.26 and 71.46 (3-CH), 72.45 and 73.38 (2-CH), 84.40 (14-C), 103,17 and 104.67 (1′-CH), 121.64 and 121.72 (7-CH), 162.48 and 162.70 (8-C), 174.43 (O-C═O), 202.83 and 202.90 (6-C), 209.75 (20-C). MALDI-TOF: *m/z* 583.317 $[M + K]^+$ calcd for $C_{32}H_{48}O_7K$ 583.304; *m/z* 567.341 $[M + Na]^+$ calcd for $C_{32}H_{48}O_7Na$ 567.330.

4.1.6. Synthesis of **17** by hydride reduction of poststerone 2,3-dioxolane derivative **16**

To a solution of compound **16** (0.065 g, 0.1 mmol) in 3 mL of anhydrous THF, $LiAlH_4$ (0.008 g, 0.2 mmol) was added at 0–5 °C. The reaction mixture was stirred under argon at same temperature for 0.5 h and at ambient temperature for another 2 h. The excess of $LiAlH_4$ was decomposed by addition of 2 mL of saturated aq. solution of NH_4Cl and the resulting solution was extracted with ethyl acetate. Then solvent was evaporated and the residue was purified by column chromatography (SiO_2; $CHCl_3$) to provide **17**.

Compound **17** (epimer ratio 1:1). Yield 48% (0.03 g); white solid; R_f 0.65 ($CHCl_3$-MeOH, 10:1); $[\alpha]_D^{20} = +2.8$, (*c* 0.42, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.09 (s, 3H, 18-CH_3), 1.21 (d, $J = 6.5$ Hz, 3H, 21-CH_3), 1.36 (s, 3H, 19-CH_3), 1.40–2.00 (m, 16H, -$(CH_2)_8$-), 1.51–1.82 (m, 4H, 1-CH_2, 4-CH_2), 1.51 and 2.20 (m, 2H, 16-CH_2), 1.69–1.90 (m, 2H, 11-CH_2), 1.76–1.98 (m, 2H, 12-CH_2), 1.89 (m, 2H, 15-CH_2), 1.98 (m, 1H, 9-CH), 2.01 (m, 1H, 17-CH), 2.31 (m, 1H, 7α-CH), 2.44 (m, 1H, 8-CH), 2.61 (brs, $w_{1/2} = 10$ Hz, 1H, 5-CH), 2.72 (m, 1H, 7β -CH), 3.65 (t, $J = 6.3$ Hz, 2H, -CH_2OH), 3.80 (m, 1H, 20-CH), 4.02 and 4.10 (m, 1H, 2-CH), 4.46 and 4.64 (m, 1H, 3-CH), 4.85 (m, 0.5H, 1′-CH), 5.23 (m, 0.5H, 1′-CH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.67 (18-CH_3), 18.25 (11-CH_2), 22.38 (16-CH_2), 23.80 and 24.02 (-CH_2-), 23.89 (21-CH_3), 25.75 and 26.02 (19-CH_3), 25.71 and 25.82 (-CH_2-), 29.37, 29.42, 29.45, 29.53, 29.69 (-CH_2-), 31.73 (12-CH_2), 32.80 (15-CH_2), 32.93 (-CH_2-), 34.44 and 34.85 (1-CH_2), 35.31 (4-CH_2), 39.45 and 39.63 (10-C), 41.30 (7-CH), 41.88 (9-CH), 43.38 and 43.43 (8-CH), 46.08 (13-C), 50.33 and 50.61 (5-CH), 53.71 (17-CH), 63.06 (-CH_2OH), 69.78 (20-CH), 73.90 and 75.54 (2-CH), 69.71 and 71.50 (3-CH), 84.83 (14-C), 102.18 and 103.76 (1′-CH), 174.39 (O-C═O), 211.99 and 212.14 (6-C). MALDI-TOF: *m/z* 543.343 $[M + Na]^+$ calcd for $C_{31}H_{52}O_6Na$ 543.366.

4.2. Cell lines

The L5178Y mouse *T*-cell lymphoma cells (ECACC Cat. No. 87111908, obtained from FDA, Silver Spring, MD, USA) were transfected with pHa MDR1/A retrovirus. The *ABCB1*-expressing cell line (MDR) was selected by culturing the infected cells with colchicine. L5178Y (parental, PAR) mouse *T*-cell lymphoma cells and the $L5178Y_{MDR}$ human *ABCB1*-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat inactivated horse serum, 200 mM L-glutamine, and penicillin–streptomycin mixture in 100 U/l and 10 mg/l concentration, respectively.

4.3. Assay for cytotoxic effect

The effects of increasing concentrations of the drugs alone on cell growth were tested in 96-well flat-bottomed microtiter plates. A 10 mM concentration stock solution in DMSO was used for each compound. These were diluted in 100 μL of McCoy's 5A medium. A sum of 1×10^4 mouse *T*-cell lymphoma cells (PAR or MDR) in 100 μL of medium were then added to each well, except for the medium control wells. The culture plates were further incubated at 37 °C for 24 h; at the end of the incubation period, 20 μL of MTT solution (from a 5 mg/mL stock) was added to each well. After incubation at 37 °C for 4 h, 100 μL of SDS solution (10% in 0.01 M HCl) was added to each well and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). IC_{50} values were calculated by variable slope nonlinear regression using

the log(inhibitor) vs. normalized response of GraphPad Prism 5.01 (GraphPad Software Inc, San Diego, CA, USA) from the inhibition percentage values (Inh%) that were obtained *via* the following equation.

$$\text{Inh\%} = 100 - \left[\frac{\text{ODsample} - \text{ODmedium control}}{\text{ODcell control} - \text{ODmedium control}}\right] \quad (1)$$

4.4. Assay for antiproliferative effect

The effects of increasing concentrations of the drugs alone on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in 100 μL of McCoy's 5A medium. 6×10^3 mouse *T*-cell lymphoma cells (PAR or MDR) in 100 μL of medium were then added to each well, except for the medium control wells. The culture plates were further incubated at 37 °C for 72 h in a CO_2 incubator; at the end of the incubation period, 20 μL of MTT solution (from a 5 mg/mL stock) was added to each well. After incubation at 37 °C for 4 h, 100 μL of SDS solution (10% in 0.01 M HCI) was added to each well and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the OD at 540 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). IC_{50} values were calculated from the inhibition percentage values obtained according to Eq. (1) as described above.

4.5. Fluorescence uptake assay

The cell numbers of the L5178Y PAR and MDR cell lines were adjusted to 2×10^6 cells/mL, re-suspended in serum-free McCoy's 5A medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added at a final concentration of 2 and 20 μM and the samples were incubated for 10 min at room temperature. Tariquidar was applied as positive control at 20 nM. DMSO was added to the negative control tubes in the same volume as had been used for the tested compounds. No activity of DMSO was observed. Next, 10 μL (5.2 μM final concentration) of the fluorochrome and ABCB1 substrate rhodamine 123 was added to the samples and the cells were incubated for a further 20 min at 37 °C, washed twice and re-suspended in 1 mL PBS for analysis. The fluorescence of the cell population was measured with a PartecCyFlow® flow cytometer (Partec, Münster, Germany). The percentage of mean fluorescence intensity was calculated for the treated MDR cells as compared with the untreated cells, and inhibition percentage for the treated cells was calculated from the corresponding values of the untreated MDR (i.e. 0% inhibition) and PAR cells (i.e. 100% inhibition).

4.6. Drug combination assay

Doxorubicin (2 mg/mL, Teva Pharmaceuticals, Budapest, Hungary) was serially diluted in the horizontal direction as previously described. The ecdysteroid derivative was subsequently diluted in the vertical direction. The dilutions of doxorubicin were made in a horizontal direction in 100 mL, and the dilutions of ecdysteroids vertically in the microtiter plate in 50 mL volume. The $L5178Y_{MDR}$ mouse *T*-lymphoma cells were re-suspended in culture medium and distributed into each well in 50 mL containing 1×10^4 cells, with the exception of the medium control wells, to a final volume of 200 mL per well. The plates were incubated for 72 h at 37 °C in a CO_2 incubator and at the end of the incubation period, the cell growth was determined by MTT staining method, as earlier described. Drug interactions were evaluated using Calcusyn software. Each dose–response curve (for individual agents as well as combinations) was fit to a linear model using the median effect equation, in order to obtain the median effect value (corresponding to the IC_{50}) and slope (m). The goodness-of-fit was assessed using the linear correlation coefficient, r, and only data from analysis with $r > 0.90$ were presented. The extent of interaction between drugs was expressed using the combination index (CI), in which a CI value close to 1 indicates additivity, whilst a $CI < 1$ is defined as synergism and a $CI > 1$ as antagonism [31].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank the Russian Foundation for Basic Research (Grant No. 20-03-00649) for financial support. A part of the studies was carried out in accordance with the Federal Program No. AAAA-A19-119022290012-3. The results were obtained with the financial support of the Russian Ministry of Education and Science (project no. 2019-05-595-000-058) on unique equipment at the 'Agidel' Collective Usage Center (Ufa Federal Research Center, Russian Academy of Sciences). Financial support from the National Research, Development and Innovation Office, Hungary (NKFIH; K119770), and from the Ministry of Human Capacities, Hungary (20391-3/2018/FEKUSTRAT) is acknowledged. M.N. was supported by EFOP 3.6.3-VEKOP-16-2017-00009.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.104485.

References

[1] R. Lafont, J. Harmatha, F. Marion-Poll, L. Dinan, I.D. Wilson, The Ecdysone Handbook (2002).
[2] R. Lafont, L. Dinan, Practical uses for ecdysteroids in mammals including humans: an update, J. Insect Sci. 3 (2003) 7.
[3] G. Laekeman, A. Vlietinck, Phytoecdysteroids: Phytochemistry and Pharmacological Activity, in: K.G. Ramawat, J.-M. Mérillon (Eds.), Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes, Springer, Berlin Heidelberg, Berlin, Heidelberg, 2013, pp. 3827–3849.
[4] L. Dinan, Phytoecdysteroids: biological aspects, Phytochemistry 57 (3) (2001) 325–339.
[5] V.N. Odinokov, I.V. Galyautdinov, D.V. Nedopekin, L.M. Khalilov, A.S. Shashkov, V.V. Kachala, L. Dinan, R. Lafont, Phytoecdysteroids from the juice of Serratula coronata L. (Asteraceae), Insect Biochem. Mol. Biol. 32 (2) (2002) 161–165.
[6] K. Vokáč, M. Buděšínský, J. Harmatha, Minor ecdysteroid components of *Leuzea carthamoides*, Collect. Czech. Chem. Commun. 67 (2002) 124–139.
[7] S. Lapenna, L. Dinan, J. Friz, A.J. Hopfinger, J. Liu, R.E. Hormann, Semi-synthetic ecdysteroids as gene-switch actuators: synthesis, structure-activity relationships, and prospective ADME properties, ChemMedChem 4 (1) (2009) 55–68.
[8] A. Martins, N. Tóth, A. Ványolós, Z. Béni, I. Zupkó, J. Molnár, M. Báthori, A. Hunyadi, Significant activity of ecdysteroids on the resistance to doxorubicin in mammalian cancer cells expressing the human ABCB1 transporter, J. Med. Chem. 55 (11) (2012) 5034–5043.
[9] G. Lesma, A. Luraghi, G. Rainoldi, E. Mattiuzzo, R. Bortolozzi, G. Viola, A. Silvani, Multicomponent approach to bioactive peptide-ecdysteroid conjugates: creating diversity at C6 by means of the Ugi reaction, Synthesis 48 (22) (2016) 3907–3916.
[10] M. Vágvölgyi, A. Martins, Á. Kulmány, I. Zupkó, T. Gáti, A. Simon, G. Tóth, A. Hunyadi, Nitrogen-containing ecdysteroid derivatives vs. multi-drug resistance in cancer: Preparation and antitumor activity of oximes, oxime ethers and a lactam, Eur. J. Med. Chem. 144 (2018) 730–739.
[11] D. Bogdán, R. Haessner, M. Vágvölgyi, D. Passarella, A. Hunyadi, T. Gáti, G. Tóth, Stereochemistry and complete 1 H and 13 C NMR signal assignment of C-20-oxime derivatives of posterone 2,3-acetonide in solution state, Magn. Reson. Chem. 56 (9) (2018) 859–866.
[12] G. Fumagalli, B. Stella, I. Pastushenko, F. Ricci, M.S. Christodoulou, G. Damia, D. Mazza, S. Arpicco, C. Giannini, L. Morosi, F. Dosio, P.A. Sotiropoulou, D. Passarella, Heteronanoparticles by self-assembly of doxorubicin and cyclopamine conjugates, ACS Med. Chem. Lett. (2017) 953–957.
[13] H. Hikino, K. Nomoto, T. Takemoto, Poststerone, a metabolite of insect metamorphosing substances from Cyathula capitata, Steroids 16 (1970) 393–400.
[14] S. Kumpun, J.P. Girault, L. Dinan, C. Blais, A. Maria, C. Dauphin-Villemant, B. Yingyongnarongkul, A. Suksamrarn, R. Lafont, The metabolism of 20-hydroxyecdysone in mice: Relevance to pharmacological effects and gene switch applications of ecdysteroids, J. Steroid Biochem. Mol. Biol. 126 (1) (2011) 1–9.
[15] J. Csábi, T. Rafai, A. Hunyadi, E. Zádor, Poststerone increases muscle fibre size partly similar to its metabolically parent compound, 20-hydroxyecdysone, Fitoterapia 134 (2019) 459–464.

[16] H.M. Issaadi, J. Csábi, T.-J. Hsieh, T. Gáti, G. Tóth, A. Hunyadi, Side-chain cleaved phytoecdysteroid metabolites as activators of protein kinase B, Bioorg. Chem. 82 (2019) 405–413.
[17] A. Hunyadi, J. Csábi, A. Martins, J. Molnár, A. Balázs, G. Tóth, Backstabbing P-gp: side-chain cleaved ecdysteroid 2,3-dioxolanes hyper-sensitize MDR cancer cells to doxorubicin without efflux inhibition, Molecules 22 (2) (2017) 199.
[18] R.G. Savchenko, S.A. Kostyleva, A.V. Apaeva, O.S. Mozgovoj, A.L. Sauchuk, V. N. Zhabinskii, E.S. Mesheryakova, L.V. Parfenova, V.N. Odinokov, Molecular rearrangements of poststerone derivative steroid core with formation of unique D-homostructures of pregnane and androstane series, Steroids 148 (2019) 28–35.
[19] R.G. Savchenko, S.A. Kostyleva, A.V. Apaeva, E.S. Mesheryakova, A. A. Tukhbatullin, V.N. Odinokov, L.V. Parfenova, Sonochemically assisted 2,3-dideoxygenation and skeletal rearrangement of ecdysteroid derivatives, Ultrason. Sonochem. 52 (2019) 505–511.
[20] Z.-L. Song, C.-A. Fan, Y.-Q. Tu, Semipinacol rearrangement in natural product synthesis, Chem. Rev. 111 (11) (2011) 7523–7556.
[21] Q.R. Petersen, R.C. Cambie, G.B. Russell, Jones oxidation of 20-hydroxyecdysone (crustecdysone), Aust. J. Chem. 46 (12) (1993) 1961–1964.
[22] R.G. Savchenko, V.N. Odinokov, Hydrogenation of ecdysteroids, Steroids 77 (14) (2012) 1523–1529.
[23] J. Malíková, J. Swaczynová, Z. Kolář, M. Strnad, Anticancer and antiproliferative activity of natural brassinosteroids, Phytochemistry 69 (2) (2008) 418–426.
[24] A. Suksamrarn, T. Tanachatchairatana, C. Sirigarn, Stereoselective catalytic hydrogenation of Δ7-6-ketosteroids in the presence of sodium nitrite, Tetrahedron 58 (30) (2002) 6033–6037.
[25] R.G. Savchenko, Y.R. Urazaeva, R.V. Shafikov, V.N. Odinokov, Effective and selective transformations of ecdysteroids into 7,8-dihydro analogs via catalytic hydrogenation under alkaline conditions, Russ. J. Org. Chem. 46 (1) (2010) 150–152.
[26] J.-P. Bégué, D. Bonnet-Delpon, Recent advances (1995–2005) in fluorinated pharmaceuticals based on natural products, J. Fluor Chem. 127 (8) (2006) 992–1012.
[27] J. Csábi, A. Martins, I. Sinka, A. Csorba, J. Molnár, I. Zupkó, G. Tóth, L.M. V. Tillekeratne, A. Hunyadi, Synthesis and in vitro evaluation of the antitumor potential and chemo-sensitizing activity of fluorinated ecdysteroid derivatives, MedChemComm 7 (12) (2016) 2282–2289.
[28] T. Amagata, M. Doi, M. Tohgo, K. Minoura, A. Numata, Dankasterone, a new class of cytotoxic steroid produced by a Gymnascella species from a marine sponge, Chem. Comm. 14 (1999) 1321–1322.
[29] Y. Miyata, T. Diyabalanage, C.D. Amsler, J.B. McClintock, F.A. Valeriote, B. J. Baker, Ecdysteroids from the Antarctic Tunicate Synoicum adareanum, J. Nat. Prod. 70 (12) (2007) 1859–1864.
[30] I. Pastan, M.M. Gottesman, K. Ueda, E. Lovelace, A.V. Rutherford, M. C. Willingham, A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells, PNAS 85 (12) (1988) 4486.
[31] T.C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies, Pharmacol. Rev. 58 (3) (2006) 621–681.
[32] A. Martins, J. Csábi, A. Balázs, D. Kitka, L. Amaral, J. Molnár, A. Simon, G. Tóth, A. Hunyadi, Synthesis and structure-activity relationships of novel ecdysteroid dioxolanes as MDR modulators in cancer, Molecules 18 (12) (2013) 15255–15275.
[33] M. Paškevičiūtė, V. Petrikaitė, Overcoming transporter-mediated multidrug resistance in cancer: failures and achievements of the last decades, Drug Delivery Translational Res. 9 (1) (2019) 379–393.
[34] A.C. Jaramillo, F.A. Saig, J. Cloos, G. Jansen, G.J. Peters, How to overcome ATP-binding cassette drug efflux transporter-mediated drug resistance? Cancer Drug Resistance 1 (1) (2018) 6–29.
[35] G. Szakács, M.D. Hall, M.M. Gottesman, A. Boumendjel, R. Kachadourian, B.J. Day, H. Baubichon-Cortay, A. Di Pietro, Targeting the achilles heel of multidrug-resistant cancer by exploiting the fitness cost of resistance, Chem. Rev. 114 (11) (2014) 5753–5774.
[36] T. Efferth, M.E.M. Saeed, O. Kadioglu, E.-J. Seo, S. Shirooie, A.T. Mbaveng, S. M. Nabavi, V. Kuete, Collateral sensitivity of natural products in drug-resistant cancer cells, Biotechnol. Adv. 38 (2020), 107342.
[37] A. Aszalos, Drug–drug interactions affected by the transporter protein, P-glycoprotein (ABCB1, MDR1): II. Clinical aspects, Drug Discovery Today 12 (19) (2007) 838–843.