Preparation of novel bioactive semi-synthetic ecdysteroid derivatives

Summary of Ph.D. Thesis

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Szeged
2017

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Szeged
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INTRODUCTION

Ecdysteroids represent a large and diverse family of steroid hormones in nature, with representatives in both insects and plants. They regulate molting and reproduction in arthropods, and play defensive role against herbivores in plants. The most abundant ecdysteroid in nature is 20-hydroxyecdysone (20E, **1**), which is also recognized as the major biologically active ecdysteroid, and usually it is accompanied by a large number of minor phytoecdysteroids with lower quantity in plants. More than 480 ecdysteroids have been isolated from plant sources in the past five decades, and *ca.* one quarter of them have been discovered by the research group of Prof. Báthori at the University of Szeged.

Their structure can be characterized with a steroid skeleton, in which the A/B ring junction is normally *cis*, while the C/D ring junction is almost always *trans.* A 7-en-6-one chromophore group can be found in ring B, and a side-chain is usually attached to C-17. Several hydroxyl groups make these compounds rather polar, and occasionally form glycosidic, ester or ether bonds.

Due to their ability to disrupt hormonal processes in arthropods, ecdysteroids have been investigated to develop safe natural insecticides, however they failed to reach the market for this use. These compounds show extremely low toxicity in mammals, and do not appear to interact with the vertebrate steroid hormone system, still they have been found to exert numerous beneficial effects. Anabolic activity is such an example, and has been investigated in numerous studies, presuming Akt activation as the background of the observed increased protein synthesis. It is suggested that metabolites of 20E (such as poststerone) might be responsible for this activity. Because of the "general strengthening" activity of 20E, it has been marketed all around the world in the form of dietary supplementary products. Wound-healing, antidiabetic, hypocholesterolaemic activities have also been attributed to this compound.

20E is also a good candidate for semi-synthetic transformations, and in a number of studies it has been subjected to chemical modifications to obtain other, less accessible ecdysteroids in larger quantities. Previously, our research group has put a large number of natural ecdysteroids and some of their semi-synthetic derivatives under investigation to observe their effect on multi-drug resistance (MDR) in cancer. Based on these

observations, relevant structure-activity relationships (SARs) have been established, which served as a starting point for the hereby presented Ph.D. study.

OBJECTIVES

In search for novel ecdysteroid derivatives as potential modulators of MDR, the following goals were set up:

1. Preparation of new semi-synthetic ecdysteroid derivatives with potent biological activity. The proposed structural modifications include the formation of dioxolanes, base-catalyzed autoxidation, side-chain cleavage and fluorination.

2. Biological evaluation of the obtained ecdysteroids. Testing compounds *in vitro* for their 1) anti-proliferative activity on various sensitive and MDR cancer cell lines; 2) functional ABCB1 efflux pump inhibition; 3) chemo-sensitizing activity in combination with doxorubicin or other chemotherapeutics; and/or 4) effect on Akt-phosphorylation.

3. Establishing new structure-activity relationships (SARs). Observing how structural modifications on the tested compounds affect their biological activity related to MDR.

MATERIALS AND METHODS

Starting materials

The starting material, 20E (**1**, 90% originated from the roots of *Cyanotis arachnoidea*) was purchased from Shaanxi KingSci Biotechnology Co. Ltd. (Shanghai, China), and further purified by crystallization. Dacryhainansterone (**2**), 2-deoxy-20-hydroxyecdysone (**3**), ajugasterone C (**4**) and polypodine B (**5**) were previously isolated from *Ajuga*, *Serratula* and *Silene* species.

Preparation of ecdysteroid derivatives

Ecdysteroid dioxolanes (**6-28**) were obtained from 20E in one step by applying various aldehydes/ketones under acidic conditions – provided by phosphomolybdic acid – in methanol. The reaction mixtures were purified by means of column chromatography (CC), rotational planar chromatography (RPC), reversed-phase high performance liquid chromatography (RP-HPLC), or recrystallization from acetonitrile. Compounds **29-36** were

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obtained in a similar fashion, but in two consecutive steps, in which a 20,22-monosubstituted ecdysteroid dioxolane was subjected to substitution at the 2,3-diol. Compounds **37-42** were obtained from 20E by first protecting the more reactive 20,22-diol with phenylboronic acid, which protecting group was later removed by applying H_2O_2 after dioxolane formation at the 2,3 position. Pure products were obtained by means of RPC.

Base-catalyzed autoxidation of 20E was performed by applying NaOH in aqueous methanol, yielding oxidized ecdysteroid derivatives (**43-50**) after multiple steps of purification with centrifugal partition chromatography (CPC), CC, preparative thin layer chromatography (prep. TLC) and RP-HPLC. This reaction was also monitored by HPLC in a longitudinal study, and concentrations of compounds were determined at various times.

Compounds **51-55** were obtained by oxidative side-chain cleavage by applying [bis(trifluoroacetoxy)iodo]benzene (PIFA) on corresponding ecdysteroids (**1-5**, respectively), and means of CPC, RPC and RP-HPLC were used for purification. Poststerone (**51**) was subjected to dioxolane formation involving the 2,3-diol, yielding compounds **56-64**. These reactions were performed by applying *p*-toluenesulfonic acid as catalyst, and reaction products were purified by RPC and RP-HPLC.

Compound **20** was subjected to fluorination with (diethylamino)sulfur trifluoride (DAST) yielding compounds **65-68**, which were further purified by CC and RP-HPLC.

Procedures for structure elucidation

The obtained compounds were characterized and their structures were elucidated by means of different spectroscopic methods (MS, 1D- and 2D-NMR).

Biological evaluation of the compounds

In the experiments on bioactivity, ten human derived cell lines were used (breast cancer cells: MCF7, MCF7$_{dox}$, MDA-MB-231, MDA-MB-361, T47D; prostate cancer cells: PC3 and LNCaP; epidermal carcinoma cells: KB-3-1 and KB-C-1; and a neuroblastoma cell line: SH-SY5Y). Two mouse T-cell lymphoma cell lines were used: the L5178 cell line, and its multi-drug resistant L5178$_{MDR}$ counterpart. Akt-phosphorylation was tested on C2C12 mouse skeletal myoblasts.

Compounds were tested for their anti-proliferative activity by a serial-dilution method in 96-well flat-bottom microtiter plates, using the MTT assay. Inhibition of ABCB1 function was investigated on L5178$_{MDR}$ cells through the intracellular retention of rhodamine 123, a fluorescent dye, evaluated by flow cytometry. Combination assays were performed using the checkerboard microplate method, where cells were incubated with an anticancer agent (doxorubicin, paclitaxel, cisplatin or vincristine) and the tested compound. Where it was possible, combination indices (CI) were calculated by means of the median-effect equation, where CI < 1, CI = 1 and CI > 1 represent synergism, additive effect and antagonism, respectively. Oxidized ecdysteroid derivatives were tested for their effect on Akt-phosphorylation on C2C12 mouse skeletal myoblasts.

RESULTS AND DISCUSSION

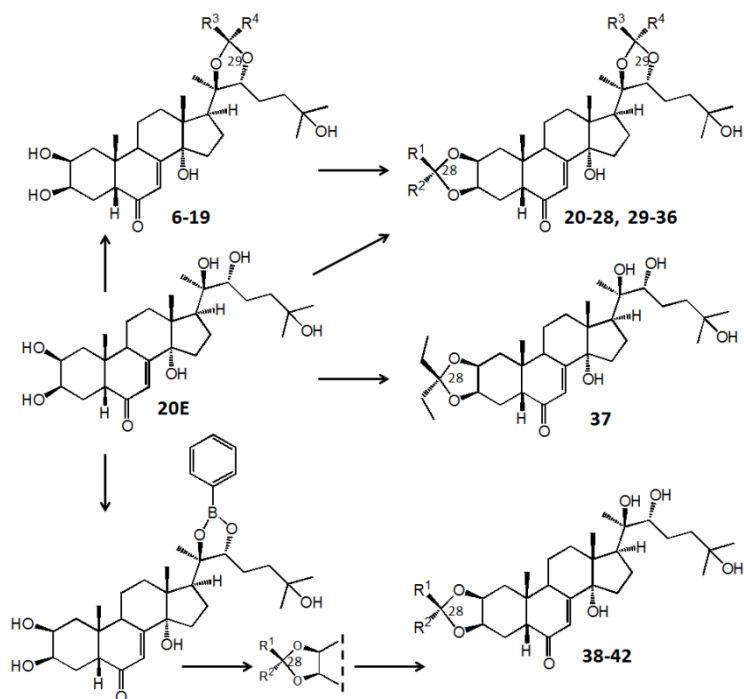Preparation of semi-synthetic ecdysteroid derivatives

The diols of 20E (namely at C-2,3 and C-20,22) were targeted by acid catalyzed reaction with aldehydes or ketones, in which a dioxolane ring was formed at C-20,22 (compounds **6-19**), at C-2,3 (compounds **37-42**) or at both positions (compounds **20-36**). From the two diols, the one at C-20,22 appeared to be more reactive. At the newly created stereogenic centers, the larger substituent attached to C-29 preferentially took the R[4]-position, while at C-28, R[1]-position was preferred. Structures can be seen on **Fig. 1.**

The autoxidation of 20E by molecular oxygen was catalyzed by applying a base (NaOH) in relatively low concentration, and yielded compounds **43-50** (see **Fig. 2.**). The reaction was carried out and monitored, and the formation of each isolated product over various reaction times was studied. Compounds **47** and **48** represent a very rare case of desmotropism, a type of tautomerism where both forms may be isolated in a stable form without detectable coexistence of the other tautomer when dissolved in the same solvent.

Side-chain cleavage between the hydroxylated C-20 and C-22 carbon atoms of various ecdysteroids (**1-5**) was performed by applying PIFA, yielding compounds **51-55** (see **Fig. 3.**).

Dioxolane formation performed on the 2,3-diol of poststerone (**51**) was carried out in a similar way to the synthesis of dioxolane derivatives of 20E, however, longer reaction times and larger amounts of the reagent were applied to yield compounds **56-64** (see **Fig. 4.**)

Reaction of compound **20** with DAST resulted in fluorination or elimination of the 14-OH and fluorination of the 25-OH group, yielding compounds **65-68** (see **Fig. 5.**).

	R^1	R^2	R^3	R^4		R^1	R^2	R^3	R^4
6	-	-	-Me	-Me	24	-Ph	-H	-H	-Ph
7	-	-	-H	-n-Pr	25	-H	-Ph	-H	-Ph
8	-	-	-H	-n-Bu	26	-n-Pr	-H	-H	-n-Pr
9	-	-	-Et	-Et	27	-n-Bu	-H	-H	-n-Bu
10	-	-	-Me	-i-Bu	28	-Et	-Et	-Et	-Et
11	-	-	-H	-3-MeO-4-OH-Ph	29	-Me	-H	-Me	-Me
12	-	-	-3-MeO-4-OH-Ph	-H	30	-Et	-Me	-Me	-Me
13	-	-	-H	-E-ethenylbenzyl	31	-Me	-Me	-H	-n-Pr
14	-	-	-H	-Z-ethenylbenzyl	32	-Me	-Me	-Et	-Et
15	-	-	-H	-4-benzyloxyphenyl	33	-Me	-Me	-Me	-i-Bu
16	-	-	-4-benzyloxyphenyl	-H	34	-Me	-Me	-H	-Ph
17	-	-	-Me	-Ph	35	-Me	-Me	-Me	-Ph
18	-	-	-Me	-Et	36	-n-Pr	-H	-Me	-Me
19	-	-	-H	-Ph	37	-Et	-Et	-	-
20	-Me	-Me	-Me	-Me	38	-Me	-Me	-	-
21	-Me	-Et	-Me	-Et	39	-Et	-H	-	-
22	-Et	-Me	-H	-Me	40	n-Bu	-H	-	-
23	-Me	-H	-Me	-Et	41	-Me	-i-Bu	-	-
					42	-i-Bu	-Me	-	-

Figure 1 – Structures of the semi-synthesized ecdysteroid dioxolane derivatives (**6-42**)
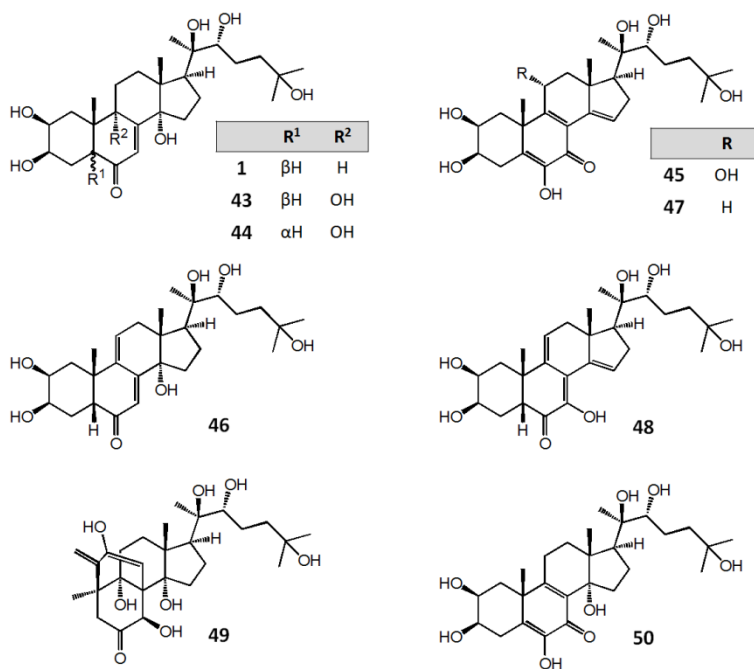
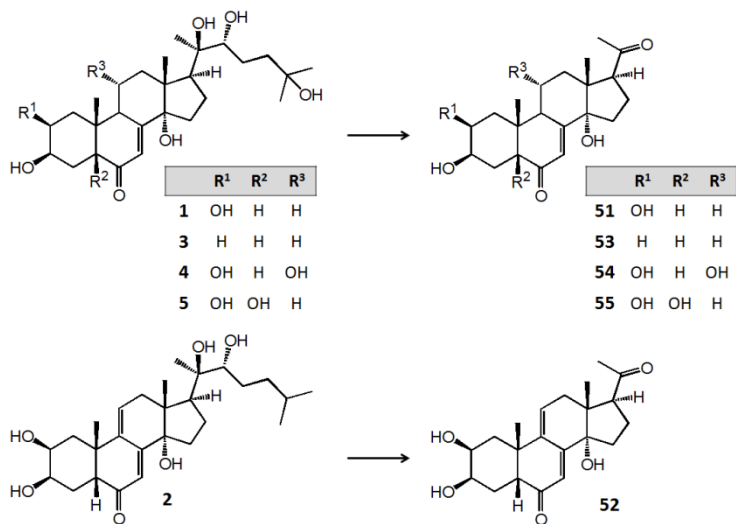**Figure 2** – Oxidized ecdysteroid derivatives (**43-50**) obtained from the autoxidation of 20E

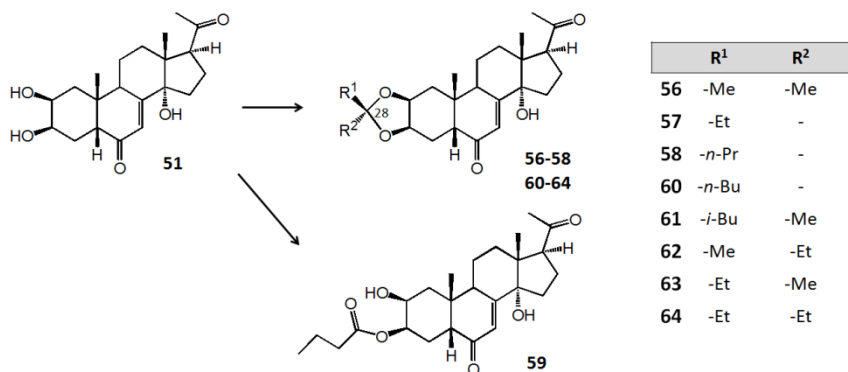**Figure 3** – Ecdysteroid derivatives (**51-55**) obtained by side-chain cleavage

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Figure 4 – Ecdysteroid derivatives (**56-64**) synthesized from poststerone

	R^1	R^2
56	-Me	-Me
57	-Et	-
58	-*n*-Pr	-
60	-*n*-Bu	-
61	-*i*-Bu	-Me
62	-Me	-Et
63	-Et	-Me
64	-Et	-Et

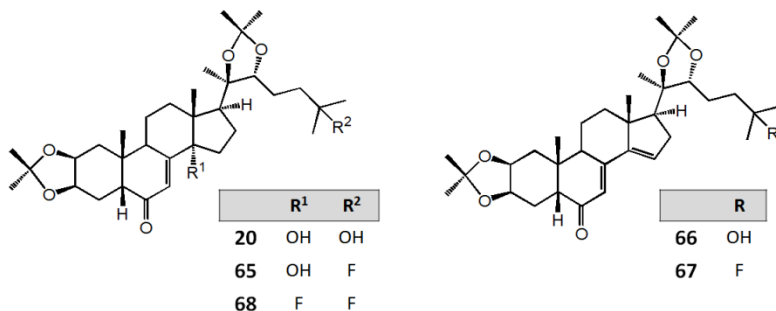|	R^1	R^2
20	OH	OH
65	OH	F
68	F	F

	R
66	OH
67	F

Figure 5 – Ecdysteroid derivatives (**65-68**) obtained from the DAST-mediated fluorination of **20**

Structure elucidation of the prepared ecdysteroids

Mass spectra of the compounds were recorded by APCI and ESI measurements in positive mode. Structure determination was performed by comprehensive 1D and 2D NMR techniques. In case of the dioxolane derivatives, a comparison of the ^{1}H and ^{13}C chemical shifts of 20E in positions 2, 3, 20 and 22 with the corresponding values of the dioxolane compounds showed pronounced deshielding, thereby proving that dioxolane formation took place. The configuration of the newly obtained stereogenic centers at C-28 and C-29 was elucidated by 2D ROESY and sel-ROE experiments. Structural modifications in the steroid skeleton during the autoxidation of 20E manifested in the appearance of additional double bonds and hydroxyl groups, whose presence was reflected in the downfield shift of

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corresponding signals in the ^{1}H and ^{13}C spectra. The unique α-configuration of H-5 in compound **44** was unequivocally established by sel-ROESY measurements. Side-chain cleavage led to the formation of a carbonyl group at C-20, with a characteristic signal ($δ_C$ = 212.5 ppm) appearing in the ^{13}C NMR spectra. In case of poststerone dioxolanes, the presence of a dioxolane ring at C-2,3 was reflected by the pronounced deshielding observed in the chemical shifts of H-2,3 and C-2,3 in the corresponding spectra. Similarly to the case of other ecdysteroid dioxolanes, the larger substituent connected to the newly created stereogenic center preferred to take the R^1-position, which was confirmed by 1D ROESY experiments. In case of fluorinated ecdysteroid derivatives, fluorine substitution manifested in characteristic changes in the NMR spectra. In the α-position, namely directly on the substituted carbon, a *ca.* 20-25 ppm paramagnetic shift and in the β-position a *ca.* 2-3 ppm diamagnetic shift was observed, which effect decreased below 1 ppm in the γ-position. In addition to these, both the ^{1}H and the ^{13}C spectra showed signal splitting caused by characteristic direct, geminal and vicinal couplings. Evidence was found that no configurational change took place at C-14 during fluorine substitution, thus supporting the expected S_N1 reaction mechanism.

Biological activity and related SAR of the obtained compounds

The ecdysteroid dioxolanes (**6-42** and **56-64**) were tested for their anti-proliferative activity on various cell lines. The majority of the tested compounds – in contrast with the parental 20E – showed anti-proliferative activity in the bioassays. However, in most cases, this activity proved to be weak on the L5178 cell line, and often somewhat even less pronounced on its resistant counterpart, L5178$_{MDR}$. It was observed, that from the two possible substitutions of 20E leading to dioxolane formation, the one taking place at C-20,22 has greater effect on anti-proliferative activity, while the impact of 2,3-substitution is negligible. For example, the 2,3-monosubstituted derivatives of 20E (compounds **37-42**) – with an intact side chain – showed no anti-proliferative activity on either cell lines, just like 20E. However, dioxolane formation at C-20,22 might lead to marked increase in cell growth inhibition, which becomes more pronounced when an apolar aromatic group is part of the substituent (as in case of compounds **13-16**, **24, 25, 34** and **35**). Removal of the side-chain

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does not appear to contribute significantly to the anti-proliferative activity of the compounds. In case of compounds **20** and **65-68**, fluorine substitution at C-25 or at C-14 and C-25 had little effect on the very mild anti-proliferative activity of **20**, however, presence of a $\Delta^{14,15}$ double bond markedly increased the activity. Results are shown on **Table 1** and **2**).

Table 1 – IC_{50} values of ecdysteroid derivatives (**6-36** and **56-68**) tested on the parental L5178 and multi-drug resistant L5178$_{MDR}$ cell line, presented as the average of 3 independent experiments ± standard error of the mean (SEM).

compound	IC_{50} (μM)		compound	IC_{50} (μM)	
	L5178	L5178$_{MDR}$		L5178	L5178$_{MDR}$
1 (20E)	> 150	> 150	27	19.9 ± 0.03	25.5 ± 3.4
6	> 150	> 150	28	52.6 ± 12.9	49.7 ± 3.9
7	40.6 ± 3.6	> 150	29	> 150	> 150
8	21.4 ± 1.4	57.8 ± 5.7	30	> 150	> 150
9	77.0 ± 1.5	92.7 ± 2.0	31	75.2 ± 12.1	64.4 ± 13.7
10	18.5 ± 1.8	35.7 ± 0.5	32	77.5 ± 20.7	75.9 ± 3.1
11	92.3 ± 23.1	> 150	33	72.2 ± 9.8	66.8 ± 4.7
12	98.9	> 150	34	42.0 ± 18.9	42.7 ± 2.6
13	36.7 ± 0.8	89.4 ± 14.4	35	41.6 ± 6.7	46.5 ± 7.0
14	45.0 ± 9.6	> 150	36	62.6 ± 16.8	64.7 ± 7.3
15	31.6 ± 5.3	43.2 ± 2.7	56	95.7 ± 0.6	> 150
16	41.0 ± 6.8	59.5 ± 5.8	57	91.1 ± 4.2	> 150
17	34.1 ± 3.3	38.8 ± 0.4	58	74.3 ± 2.3	> 150
18	68.9 ± 2.1	> 150	59	97.8 ± 2.1	> 150
19	50.0 ± 3.4	> 150	60	72.2 ± 0.6	> 150
20	82.9 ± 1.0	106.1 ±3.3	61	59.6 ± 0.8	55.4 ± 4.9
21	51.2 ± 1.5	56.0 ± 6.4	62	92.5 ± 1.0	85.7 ± 1.1
22	99.6 ± 5.2	> 150	63	95.2 ± 1.4	87.7 ± 1.1
23	52.6 ± 1.5	87.8 ± 10.9	64	97.0 ± 5.8	91.6 ± 7.0
24	20.3 ± 0.8	22.4 ± 0.8	65	48.3 ± 0.2	50.1 ± 2.1
25	30.2 ± 1.2	38.3 ± 1.1	66	14.6 ± 0.3	19.3 ± 0.5
26	19.5 ± 2.6	30.6 ± 1.4	67	14.1 ± 0.8	17.4 ± 1.2
			68	88.6 ± 1.1	82.3 ± 1.4

Table 2 – IC_{50} values of the ecdysteroid derivatives **65-68** compared to that of the parental compound **20**, tested on breast cancer (MCF-7, MDA-MB-231, MDA-MB-361 and T47D) and neuroblastoma (SH-SY5Y) cell lines. Values are presented as the average of 3 independent experiments ± SEM.

compound	IC_{50} (μM)				
	MCF-7	T47D	MDA-MB-231	MDA-MB-361	SH-SY5Y
20	75.1 ± 3.4	84.7 ± 3.9	106.1 ± 7.2	69.2 ± 6.0	126.8 ± 9.8
65	63.1 ± 2.3	70.3 ± 1.3	48.9 ± 2.0	30.9 ± 2.6	70.7 ± 4.1
66	30.1 ± 0.8	10.9 ± 0.3	38.5 ± 3.8	13.8 ± 0.4	20.8 ± 0.3
67	43.8 ± 1.7	17.4 ± 1.0	50.2 ± 2.6	11.8 ± 1.2	18.8 ± 1.2
68	127.5 ± 4.3	49.2 ± 5.0	98.4 ± 17.2	53.8 ± 5.7	125.6 ± 13.2

The dioxolane derivatives (**6-36** and **56-68**) were also tested for their inhibiting activity on the ABCB1 efflux transporter. Usually, applying higher concentration (20 µM) of the compound led to more potent inhibition, and in some cases resulted in cytotoxicity. It seems that side-chain cleavage does not lead to the appearance of pump inhibition even if it is accompanied by dioxolane formation at C-2,3, as none of the poststerone derivatives (**56-64**, with the exception of **61**) showed any activity in this bioassay, similarly to 20E. In case of compounds **65-68**, elimination of 14-OH, as well as fluorination at C-14 or C-25, manifested in a significant increase in the ABCB1 inhibitory activity (see **Table 3**).

Table 3 – Fluorescence activity ratio (FAR) values in presence of 2 and 20 µM of compounds (**6-36** and **56-68**). Asterisk (*) represent that the compound showed cytotoxicity at this concentration and it was irrelevant to calculate the FAR value. FAR values of the positive control verapamil (20.4 µM) and the negative control DMSO (0.2%) were 5.73 and 0.72, respectively.

compound	FAR		compound	FAR	
	2 µM	20 µM		2 µM	20 µM
1 (20E)	1.70	1.76	**27**	114.64	*
6	-	1.53	**28**	14.71	*
7	1.02	4.04	**29**	3.88	14.07
8	2.40	40.76	**30**	1.41	17.67
9	1.42	1.40	**31**	1.68	60.67
10	1.53	98.74	**32**	2.21	68.46
11	0.87	7.85	**33**	51.67	75.17
12	0.87	0.87	**34**	10.98	67.78
13	0.77	1.01	**35**	61.67	*
14	0.76	0.91	**36**	3.47	63.22
15	1.11	*	**56**	1.06	1.54
16	1.00	109.40	**57**	1.20	1.25
17	1.21	94.56	**58**	1.02	4.24
18	0.95	1.04	**59**	0.84	1.02
19	1.04	1.69	**60**	0.95	2.95
20	3.33	11.28	**61**	2.63	30.85
21	29.96	45.35	**62**	0.84	1.16
22	6.47	53.49	**63**	0.73	0.87
23	10.00	55.81	**64**	0.75	1.10
24	51.97	*	**65**	1.59	38.62
25	1.08	82.68	**66**	1.28	57.36
26	43.81	*	**67**	31.15	109.37
			68	11.02	89.78

20E and its dioxolane derivatives (with the exception of compounds **37-42**) were tested in a combination assay with doxorubicin on L5178$_{MDR}$ cells, and all the derivatives showed synergistic activity, increasing the sensitivity of multi-drug resistant mouse lymphoma cells towards doxorubicin. This sensitizing effect was more pronounced in case of 2,3;20,22-disubstituted dioxolanes compared to that of 20,22-monosubstituted ones. Based on the comparison of the C-28 and C-29 epimer pairs, it was found that at C-28, the larger substituent needs to take the R^2-position while at C-29 the R^3-position for a stronger activity. Increasing the length of the substituent coupled to C-29 led to a significant increase in activity till the length of three carbon atoms, however, a longer alkyl substituent appeared to be less preferable. Introducing larger aromatic groups did not lead to stronger activities, although further substituents on the aromatic ring were able to increase activity. Addition of a β-methyl group to C-29 could significantly increase the activity as compared to that of the 29α-phenyl substituted derivatives (see **Table 4**).

Table 4 – Combination index (CI) values at 50%, 75% and 90% of inhibition at the most active constant ratio of compounds **1**, **6-36** and **65-68**, combined with doxorubicin on the ABCB1 transfected L5178$_{MDR}$ cell line. 0 < CI <1, CI = 1, CI > 1 represent synergism, additivity and antagonism, respectively. CI$_{avg}$ = (CI$_{50}$ + 2 x CI$_{75}$ + 3 x CI$_{90}$)/6, r represents the linear correlation coefficient of the median-effect plot.

	drug ratio	CI values at ED$_{50}$	ED$_{75}$	ED$_{90}$	CI$_{avg}$	r		drug ratio	CI values at ED$_{50}$	ED$_{75}$	ED$_{90}$	CI$_{avg}$	r
1	20.4 : 1	2.00	2.02	2.04	*2.03*	0.997	23	20.4 : 1	0.31	0.22	0.17	*0.21*	0.978
6	20.4 : 1	0.84	0.54	0.35	*0.49*	0.955	24	20.4 : 1	0.70	0.69	0.74	*0.71*	0.998
7	81.6 : 1	0.91	0.31	0.11	*0.31*	0.989	25	20.4 : 1	0.54	0.40	0.31	*0.38*	0.947
8	40.8 : 1	0.56	0.58	0.78	*0.68*	0.870	26	20.4 : 1	0.44	0.32	0.27	*0.31*	1.000
9	40.8 : 1	0.50	0.26	0.14	*0.24*	0.959	27	20.4 : 1	0.39	0.38	0.39	*0.39*	0.979
10	81.6 : 1	0.70	0.44	0.28	*0.40*	0.936	28	40.8 : 1	0.26	0.30	0.35	*0.32*	0.992
11	20.4 : 1	0.95	0.53	0.34	*0.51*	0.945	29	20.4 : 1	0.31	0.18	0.15	*0.19*	0.962
12	40.8 : 1	0.86	0.48	0.27	*0.44*	0.958	30	20.4 : 1	0.34	0.18	0.15	*0.19*	0.956
13	81.6 : 1	0.82	0.68	0.74	*0.73*	0.950	31	20.4 : 1	0.21	0.12	0.07	*0.11*	0.956
14	40.8 : 1	0.92	0.81	0.94	*0.89*	0.991	32	20.4 : 1	0.20	0.13	0.10	*0.13*	0.958
15	20.4 : 1	0.66	0.61	0.70	*0.66*	0.954	33	20.4 : 1	0.23	0.08	0.06	*0.09*	0.984
16	20.4 : 1	0.20	0.25	0.33	*0.28*	0.946	34	20.4 : 1	0.12	0.10	0.12	*0.11*	0.963
17	20.4 : 1	0.63	0.47	0.61	*0.57*	0.820	35	20.4 : 1	0.18	0.12	0.12	*0.13*	0.999
18	20.4 : 1	0.92	0.59	0.41	*0.55*	0.968	36	20.4 : 1	0.15	0.13	0.14	*0.14*	0.999
19	40.8 : 1	0.84	0.81	0.91	*0.87*	0.967	65	20.4 : 1	0.19	0.19	0.19	*0.19*	0.908
20	40.8 : 1	0.26	0.14	0.08	*0.13*	0.996	66	20.4 : 1	0.54	0.44	0.37	*0.42*	0.989
21	20.4 : 1	0.31	0.25	0.24	*0.25*	0.999	67	20.4 : 1	0.48	0.36	0.28	*0.34*	0.983
22	20.4 : 1	0.23	0.16	0.15	*0.16*	0.956	68	20.4 : 1	0.21	0.12	0.07	*0.11*	0.957

Compounds **37-42** and **56-64** did not show considerable anti-proliferative activity on their own, therefore combination indices (CIs) were not calculated for them. However, they were still tested in combination with doxorubicin. These compounds exerted significant effect in the bioassay, which was even more pronounced in case of the side-chain cleaved poststerone derivatives. Generally, with larger substituents connected to C-2,3, higher activity was observed, which appeared to be most significant in case of an *iso*-butyl group (see **Table 5**, results for compounds **37-42** are incomplete and are not listed).

Table 5 – Chemo-sensitizing activity of poststerone (**51**) and its derivatives (**56-64**) on L5178 and L5178$_{MDR}$ cell lines. Values are presented as the average of 3 independent experiments ± standard error of the mean (SEM). With the exception of 10 µM of compound **51** (either cell line), and **56, 57** and **63** (L5178), all IC$_{50}$ values differ from that found for doxorubicin alone at $p < 0.01$ by means of one-way ANOVA followed by Dunnett's post-hoc test.

compound	IC$_{50}$ (µM) of doxorubicin			
	L5178		L5178$_{MDR}$	
	10 µM	25 µM	10 µM	25 µM
control	0.41 ± 0.02	0.41 ± 0.02	11.83 ± 0.64	11.83 ± 0.64
51	0.37 ± 0.01	0.29 ± 0.01	13.22 ± 0.44	7.55 ± 0.56
56	0.34 ± 0.02	0.23 ± 0.01	3.80 ± 0.10	1.77 ± 0.08
57	0.33 ± 0.01	0.15 ± 0.004	2.74 ± 0.66	1.39 ± 0.06
58	0.26 ± 0.02	0.17 ± 0.007	1.74 ± 0.11	0.87 ± 0.08
59	0.28 ± 0.02	0.20 ± 0.01	6.81 ± 0.33	3.00 ± 0.09
60	0.21 ± 0.002	0.15 ± 0.01	1.56 ± 0.08	0.79 ± 0.05
61	0.18 ± 0.003	0.12 ± 0.002	0.43 ± 0.02	0.17 ± 0.005
62	0.19 ± 0.01	0.11 ± 0.003	2.60 ± 0.11	1.40 ± 0.08
63	0.34 ± 0.02	0.26 ± 0.04	2.23 ± 0.14	1.47 ± 0.08
64	0.24 ± 0.02	0.23 ± 0.02	1.59 ± 0.02	0.97 ± 0.03

Considering that some 20,22-monosubstituted dioxolane derivatives of 20E (namely compounds **7**, **9**, **12** and **18**), and particularly most derivatives of poststerone (compounds **57-60** and **62-64**) sensitized the multi-drug resistant mouse lymphoma cells towards doxorubicin, but had very low FAR values, these compounds should exert their effect in a way other than a functional inhibition of the ABCB1 efflux pump. This makes these compounds particularly valuable, considering the several decades-long unsuccessful efforts for developing a clinically applicable inhibitor, there is a great need for new approaches against MDR. The observed structure-activity relationships are shown on **Figure 6**.
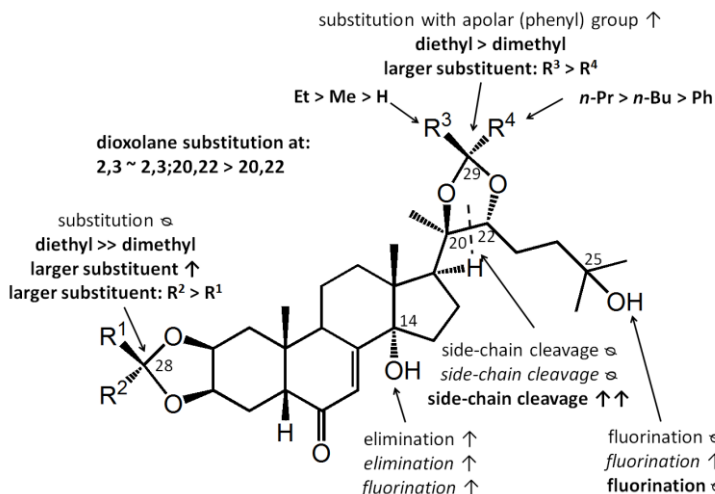
Figure 6 – SAR summary for ecdysteroid dioxolanes (**6-42** and **56-68**). Font types represent: normal – effect on anti-proliferative activity, *italic – effect on ABCB1-inhibiting activity*, **bold – effect on chemo-sensitizing activity to doxorubicin.** "Greater than" symbols denote stronger synergistic activities, ↑ refers to increase in activity, and ⌀ means no effect.

Compounds **1**, **6** and **20** were tested on other, human derived cell lines (two breast cancer and two prostate cancer cell lines) beside the mouse lymphoma cells, in combination with other chemotherapeutics (paclitaxel, cisplatin) than doxorubicin. Compound **20** could effectively sensitize non-MDR human cell lines with no detectable (MCF7) or very low (LNCaP) expression of the ABCB1 transporter, which supports our assumption that less polar ecdysteroids do not exclusively act as ABCB1 inhibitors. Compound **6** showed less potent activity than **20**, and in most cases, the activity of compound **1** was considered to be irrelevant. Interestingly, however, all three compounds decreased the sensitivity of the cells towards cisplatin (see **Table 6**).

Some dioxolane derivatives (**20**, **31**, **33**, **34**, **36**) – identified as potentially being able to penetrate the blood-brain barrier – were also tested on the SH-SY5Y neuroblastoma cell line towards vincristine. All the tested compounds showed dose-dependent sensitizing activity, and compound **31** proved to be most potent: when applying at 10 µM, it decreased the IC_{50} value of vincristine by three orders of magnitude, to as low as 0.056 ± 0.03 nM. This dramatic increase in the observed cytotoxic activity suggests the high sensitivity of this neuroblastoma cell line towards such combinational treatment.

The oxidized ecdysteroid derivatives (**43-50**, with the exception of **45** and **49**) were tested for their capacity to influence the Akt-phosphorylation in C2C12 myotubes. Compounds **44** and **47** significantly increased Akt-phosphorylation, while compound **48** – the desmotropic pair of **47** – showed the opposite effect at the applied concentration. This puzzling difference between the bioactivities of these isomers is of particular interest for further studies (see **Figure 7**).
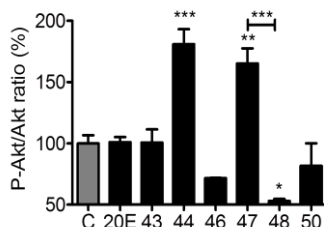

Figure 7 – Activity of compounds **43-44**, **46-48** and **50** on the Akt-phosphorylation in murine skeletal muscle cells. Quantification of Western blots was performed by ImageJ; error bars represent SEM; **: $p < 0.01$; ***: $p < 0.001$ by one-way ANOVA followed by Bonferroni's post hoc test; *: $p < 0.05$ by one-way ANOVA performed in a planned comparison of **47** and **48** to the control (C) by using Dunnett's post hoc test; n = 2-5.

SUMMARY

- 63 ecdysteroid derivatives have been synthesized from 20E or other natural ecdysteroids, including 46 new compounds. These compounds were obtained by dioxolane formation, base-catalyzed autoxidation, side-chain cleavage or fluorination.

- Most of the dioxolane compounds, including the 2,3-monosubstituted derivatives of 20E (**37-42**) and those of poststerone (**56-60** and **62**-**64**) showed very weak or no anti-proliferative activity on cancer cell lines.

- Compounds **6-36** and **56-68** were tested for their ability to inhibit the ABCB1 transporter: while some compounds showed marked inhibiton, most of them were weak in this regard; others (**56-60** and **62-64**) proved to be completely inactive.

- Ecdysteroid dioxolanes (**6-42** and **56-64**) were found to act synergistically with doxorubicin on mouse T-cell lymphoma cells and particularly on the MDR cell line, with more pronounced activity in case of disubstituted dioxolanes and poststerone derivatives. Selected compounds showed synergistic activity on other cell lines as well, in combination with paclitaxel or vincristine, but exerted antagonistic effect with

cisplatin. SH-SY5Y neuroblastoma cells proved to be most sensitive towards the chemo-sensitizing activity of ecdysteroid dioxolanes when combined with vincristine.

- Our SAR studies on ecdysteroid dioxolanes revealed that they can be engineered to become inactive as functional ABCB1 inhibitors, while retaining a strong chemo-sensitizing activity with a strong selectivity towards the ABCB1-transfected cancer cells.
- Oxidized ecdysteroid derivatives (**43-50**) were tested for their activity on the Akt-phosphorylation in C2C12 myotubes, and the difference observed between the bioactivities of a desmotropic pair (**47** and **48**) is of particular interest. To our best knowledge, our work represents the first report of such a phenomenon.
- Further SARs were discovered concerning the anti-proliferative, ABCB1-inhibiting and chemo-sensitizing activity of ecdysteroid dioxolanes (**6-42** and **56-68**). In general, dioxolane formation and side-chain cleavage increases chemo-sensitizing activity.
- Based on these findings, poststerone dioxolanes (**56-64**) can be highlighted as the most prospective leads for further research.

I am grateful to my co-authors for their collegial support. Without them, it would not have been possible to conduct this research.

My thanks are likewise due to all my colleagues in the Department of Pharmacognosy for their support and for providing such a wonderful atmosphere to work in. I thank my fellow labmates for the stimulating discussions and all the help they gave, and for all the fun we have had in the past few years.

Last, but not the least, I would like to thank my family and friends for supporting me spiritually throughout my Ph.D. study and my life in general.

THE THESIS IS BASED ON THE FOLLOWING PUBLICATIONS:

I. Martins, A.; **Csábi, J.**; Balázs, A.; Kitka, D.; Amaral, L.; Molnár, J.; Simon, A.; Tóth, G.; Hunyadi, A.; Synthesis and Structure-Activity Relationships of Novel Ecdysteroid Dioxolanes as MDR Modulators in Cancer. *Molecules*, **2013**, *18*(12), 15255-15275. *(Shared first co-authorship)* **IF: 2.095**

II. Balázs, A.; Hunyadi, A.; **Csábi, J.**; Jedlinszki, N.; Martins, A.; Simon, A.; Tóth, G.; ^{1}H and ^{13}C NMR investigation of 20-hydroxyecdysone dioxolane derivatives, a novel group of MDR modulator agents. *Magnetic Resonance in Chemistry*, **2013**, *51*(12), 830-836. **IF: 1.559**

III. Martins, A.; Sipos, P.; Dér, K.; **Csábi, J.**; Miklós, W.; Berger, W.; Zalatnai, A.; Amaral, L.; Molnár, J.; Szabó-Révész, P.; Hunyadi, A.; Ecdysteroids Sensitize MDR and Non-MDR Cancer Cell Lines to Doxorubicin, Paclitaxel, and Vincristine but Tend to Protect Them from Cisplatin. *BioMed Research International*, **2015**, Article ID: 895360. **IF: 2.134**

IV. **Csábi, J.**; Hsieh, T-J.; Hasanpour, F.; Martins, A.; Kele, Z.; Gáti, T.; Simon, A.; Tóth, G.; Hunyadi, A.; Oxidized Metabolites of 20-Hydroxyecdysone and Their Activity on Skeletal Muscle Cells: Preparation of a Pair of Desmotropes with Opposite Bioactivities. *Journal of Natural Products*, **2015**, *78*(10), 2339-2345. **IF: 3.662**

V. Gáti, T.; Simon, A.; Hunyadi, A.; **Csábi, J.**; Kele, Z.; Tóth, G.; New ring-rearranged metabolite of 20-hydroxyecdysone obtained by base-catalyzed auto-oxidation. *Magnetic Resonance in Chemistry*, **2016**, *54*, 391-395. **IF: 1.226***

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IF: 3.773*

VIII. Hunyadi, A.; **Csábi, J.**; Martins, A.; Molnár, J.; Balázs, A.; Tóth, G.; Backstabbing P-gp: side-chain cleaved ecdysteroid 2,3-dioxolanes hyper-sensitize MDR cancer cells to doxorubicin without efflux inhibition. *Molecules*, **2017**, *22*, 199.   **IF: 2.465***

*The impact factor for the year 2015 is given.

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I. Lai, W-C.; Dankó, B.; **Csábi, J.**; Kele, Z.; Chang, F-R.; Pascu, M-L.; Gáti, T.; Simon, A.; Amaral, L.; Tóth, G.; Hunyadi, A.; Rapid, laser-induced conversion of 20-hydroxyecdysone – A follow-up study on the products obtained. *Steroids*, **2014**, *89*, 56-62.   **IF: 2.639**

PRESENTATIONS RELATED TO THE THESIS:

1. **Csábi, J.**; Martins, A.; Simon, A.; Tóth, G.; Hunyadi, A.; Further insights into the SAR of ecdysteroids – synthesis and MDR-modulator activity of novel dioxolane derivatives. *A Meeting of COST Action CM0804: "Chemical Biology with Natrual Products"*. Izmir, Turkey, 6-8. May, 2013.

2. **Csábi, J.**; Martins, A.; Simon, A.; Tóth, G.; Hunyadi, A.; Ecdysterid dioxolane derivatives as novel MDR-modulators. *Trends in natural products research: a young scientists meeting of PSE and ÖPhG*. Obergurgl, Austria, 21-25. July, 2013.

3. **Csábi, J.**; Martins, A.; Balázs, A.; Amaral, L.; Molnár, J.; Simon, A.; Tóth, G.; Hunyadi, A.; Félszintetikus ekdiszteroid-származékok előállítása és multidrog-rezisztenciára kifejtett hatásuk vizsgálata. *Fiatal Gyógynövénykutatók Fóruma: A Magyar Gyógyszerésztudományi Társaság Gyógynövény Szakosztályának tudományos konferenciája*. Budakalász, Hungary, 14. February, 2014.

4. **Csábi, J.**; Martins, A.; Balázs, A.; Amaral, L.; Molnár, J.; Simon, A.; Tóth, G.; Hunyadi, A.; Semi-synthesis of ecdysteroid-derivatives and their effect on multidrug-resistance. *COST Action – Chemical Approaches to Targeting Drug Resistance in Cancer Stem Cells: 2nd Working Group Meeting*. Budapest, Hungary, 27-28. March, 2014.

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7. Martins, A.; **Csábi, J.**; Amaral, L.; Molnár, J.; Hunyadi, A.; Combination effect of ecdysteroid 2,3-dioxolanes and doxorubicin against MDR resistant cancer. *62nd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research – GA 2014*. Guimaraes, Portugal, 31. August – 4. September, 2014.

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10. **Csábi, J.**; Hsieh, T-J.; Hasanpour, F.; Martins, A.; Kele, Z.; Gáti, T.; Tóth, G.; Hunyadi, A.; Új, bioaktív ekdiszteroidok előállítása a 20-hidroxiekdizon bázis-katalizált autooxidációjával. *MTA Szteroid és Terpenoidkémiai Munkabizottságának ülése*. Szeged, Hungary, 31. October, 2014.

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12. **Csábi, J.**; Martins, A.; Balázs, A.; Amaral, L.; Molnár, J.; Tóth, G.; Hunyadi, A.; Synthesis and SAR of ecdysteroid derivatives as adjuvant anticancer agents. *63rd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research.* Budapest, Hungary, 23-27. August, 2015.

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