University of Szeged

Faculty of Pharmacy

Department of Pharmacognosy

Expanding the chemical space of enone-containing natural products: studies on ecdysteroid and protoflavone oxime derivatives

Ph.D. Thesis Summary

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Introduction

Oximes and their derivatives have remarkable significance both in chemistry and biology. In organic synthesis, they are attractive substrates in the preparation of various nitrogen-containing species, and accordingly, they can be promising research subjects in the development of novel drug candidates. Our scientific interest has recently turned towards the semi-synthetic preparation and investigation of such compounds from the derivatives of two, naturally occurring, bioactive compound groups, ecdysteroids and protoflavonoids.

Ecdysteroids are widespread in plants and invertebrates, exerting various, mild, non-hormonal bioactivities in mammals. Our group has previously discovered the ability of less polar ecdysteroid derivatives to sensitize both multi-drug resistant (MDR) and drug-susceptible cancer cells to various chemotherapeutics (i.e., "chemo-sensitizing" activity). In this regard, we found that semi-synthetic modifications on the ecdysteroid skeleton, such as the introduction of heteroatoms (e.g., fluor-substitution), can modify this activity; however, to the best of our knowledge, ecdysteroid oximes and their derivatives have not been tested for their effects before. Nevertheless, the presence of a 2,3-acetonide moiety on the ecdysteroid skeleton appears to be necessary for their antitumor activity; however, unfortunately, chemical sensitivity of this function, particularly in an acidic environment, might severely limit the potential therapeutic applicability of a derivative.

A simple, yet efficient approach to overcome this obstacle, and accordingly, to improve the *in vivo* efficacy and safety of an antitumor ecdysteroid, can be a compound's nano-formulation through the preparation of its self-assembling drug conjugates. One particularly interesting variation of this technique utilizes squalene to form self-assembling conjugates. Accordingly, squalene is used in the role of self-assembly inducer and is synthetically attached to a bioactive agent, through biodegradable covalent bonds (e. g., esters). This way, nanoparticles obtained from such conjugates act typically as pro-drugs that, once they are administered, get dissolved in lipoproteins (particularly that of LDL in humans) that will transport them in the bloodstream, and this allows targeting cancer cells displaying high expression and activity of LDL receptors.

Protoflavonoids represent a unique, bioactive group of flavonoids, possessing a non-aromatic B-ring and a hydroxyl moiety at C-1′. Many protoflavonoids are known for their potent antitumor effects and certain derivatives, such as protoapigenone, can interfere with crucial DNA-damage response mechanisms, which confers these compounds chemo-sensitizing property towards DNA-damaging chemotherapeutics (e.g., cisplatin). Besides,

there is evidence suggesting that the possible use of protoflavones might exceed their antitumor potential; however, cytotoxicity of these compounds represents a limitation in their in-depth pharmacological investigation. Therefore, the regioselective preparation of protoflavone oximes on the carbonyl group of their B-ring, mostly responsible for their cytotoxicity, may be an attractive strategy to obtain new, potentially bioactive compounds; prior to this Ph.D. study, no such derivatives have been reported.

Objectives

In search for novel, bioactive ecdysteroid and protoflavonoid derivatives and to investigate the relevance of the oxime moiety in the chemical and pharmacological space of these compound groups, the following goals were set up:

1. Preparation of oxime derivatives from antitumor ecdysteroids. The proposed transformations included the synthesis of oximes, oxime ethers and the subsequent preparation of lactams through Beckmann-rearrangement.

2. The preparation of self-assembled nanoparticles from an ecdysteroid oxime. We planned to select one of our synthesized ecdysteroid derivatives possessing advantageous chemical and/or biological properties to prepare squalene-derived pro-drug nanoparticles.

3. Biological evaluation of the semi-synthetic ecdysteroids. The synthesized products were planned to be investigated for their *in vitro* anticancer properties in research collaborations.

4. Preparation of B-ring modified protoflavone derivatives including oximes. We planned to extend the chemical–pharmacological space of protoflavones towards non-cytotoxic derivatives. We aimed to perform the flow chemical hydrogenation or deuteration of the protoapigenone B-ring, and/or the preparation of 4′-oxime derivatives.

5. Biological evaluation of the synthetically obtained protoflavones. We planned to study the compounds' cytotoxic, ATR inhibitory and antiviral activities in research collaborations.

Materials and methods

Starting materials: 20-hydroxyecdysone (20E, **1**) was purchased from Shaanxi KingSci Biotechnology Co., Ltd (Shanghai, China) in a purity of 90%, and was further purified by recrystallization. Apigenin (**27**) was purchased from Changsa Inner Natural Inc. (Hunan, China) in a purity of 98% and was used for semi-synthesis without any further purification.

Procedures for chromatographic purification: Numerous, structurally diverse ecdysteroid and protoflavonoid derivatives were prepared through semi-synthesis. The compounds were

purified by means of normal- or reversed phase flash chromatography, or reversed-phase high performance liquid chromatography (RP-HPLC).

Procedures for structure elucidation: The chemical structure of the obtained compounds was elucidated by means of different spectroscopic methods (MS, HR-MS, 1D- and 2D-NMR).

Colloid chemical characterization of self-assembled nanoparticles: Ecdysteroid nanoparticles (NPs) and hetero-nanoparticles (H-NPs) were obtained and characterized in research collaborations. The average diameter and polydispersity index (PdI) of each nano assembly was determined by dynamic light scattering (DLS) technique. Morphology of NPs and H-NPs was studied by transmission electron microscopy (TEM).

Biological evaluation of ecdysteroid derivatives: Selected ecdysteroid derivatives were tested for their anti-proliferative activity on four human adherent cancer cell lines; MDA-MB-231, MCF-7 (breast cancers), HeLa (cervical adenocarcinoma) and SiHa (cervical carcinoma) by a serial-dilution method in 96-well flat-bottom microliter plates, using the MTT assay.

Each obtained compound was tested for its ability to inhibit the expression of the ABCB1-efflux transporter by measuring the intracellular ($L5178_{MDR}$ cells) accumulation of rhodamine 123, a fluorescent dye that is an ABCB1-substrate. As a positive control, tariquidar was used.

In vitro cytotoxic activity of ecdysteroid analogs was tested alone, or in combination with doxorubicin on two mouse lymphoma cell lines, a drug-susceptible mouse T-cell lymphoma (L5178) and its multi-drug-resistant counterpart ($L5178_{MDR}$) that had been transfected with pHa MDR1/A retrovirus to express the human ABCB1 transporter. Combination assays were performed using the checkerboard microplate method. Where it was possible, combination indexes (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.

The *in vitro* antiproliferative activity of NPs and doxorubicin-containing H-NPs on $A2780_{ADR}$ cancer cells (doxorubicin-resistant human ovarian carcinoma) was investigated by the Trypan blue assay. Results were expressed as GI_{50} (GI: grow inhibition) values, i.e., the concentration of the test agent inducing 50% reduction in cell number compared with control cultures.

Biological evaluation of protoflavonoid derivatives: Selected protoflavonoids were tested for their *in vitro* cytotoxicity on MCF-7, HeLa, and SiHa human adherent cancer cell lines by a serial-dilution method in 96-well flat-bottom microliter plates, using the MTT assay. The same analogs were tested for their potential to interfere with the ATR/ATM signaling pathways. The investigation was performed by pre-treating MCF-7 cells with or without 5, 10 or 20 μM of compounds for 30 min, and DNA damage was induced by exposing the cells to 1

μm of doxorubicin for 6 h. The detection of DNA damage response was achieved by Western blot assay.

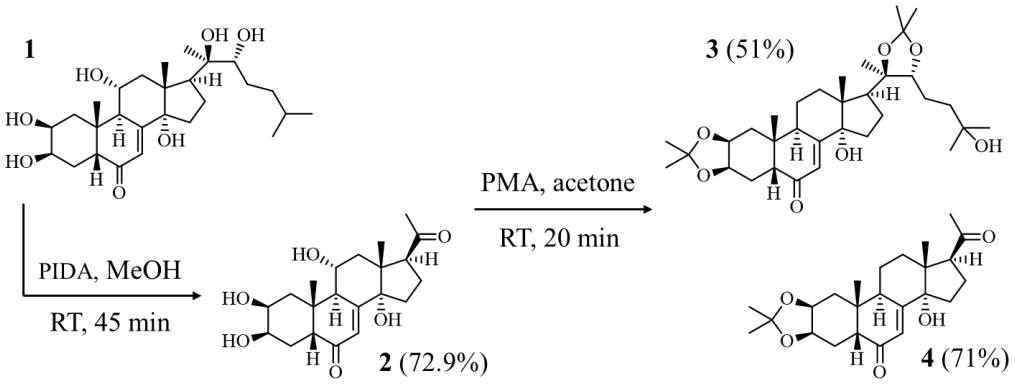
Selected protoflavonoids were tested against HIV-1 using a pseudotype virus assay on U373-CD4-CCR5 cells. Drug cytotoxicity was evaluated by using the MTT assay. Furthermore, some of the obtained compounds were tested for their effects on the immunoblot assays of Epstein–Barr Virus (EBV) Rta Protein. Additionally, we examined the cytotoxicity of the active analogs on P3HR1 cells. For the analysis, 96-well plates were used, and the compounds' cytotoxicity was determined by Cell Proliferation Kit I.

Results and discussion

ECDYSTEROIDS

Semi-synthetic preparation of ecdysteroid derivatives

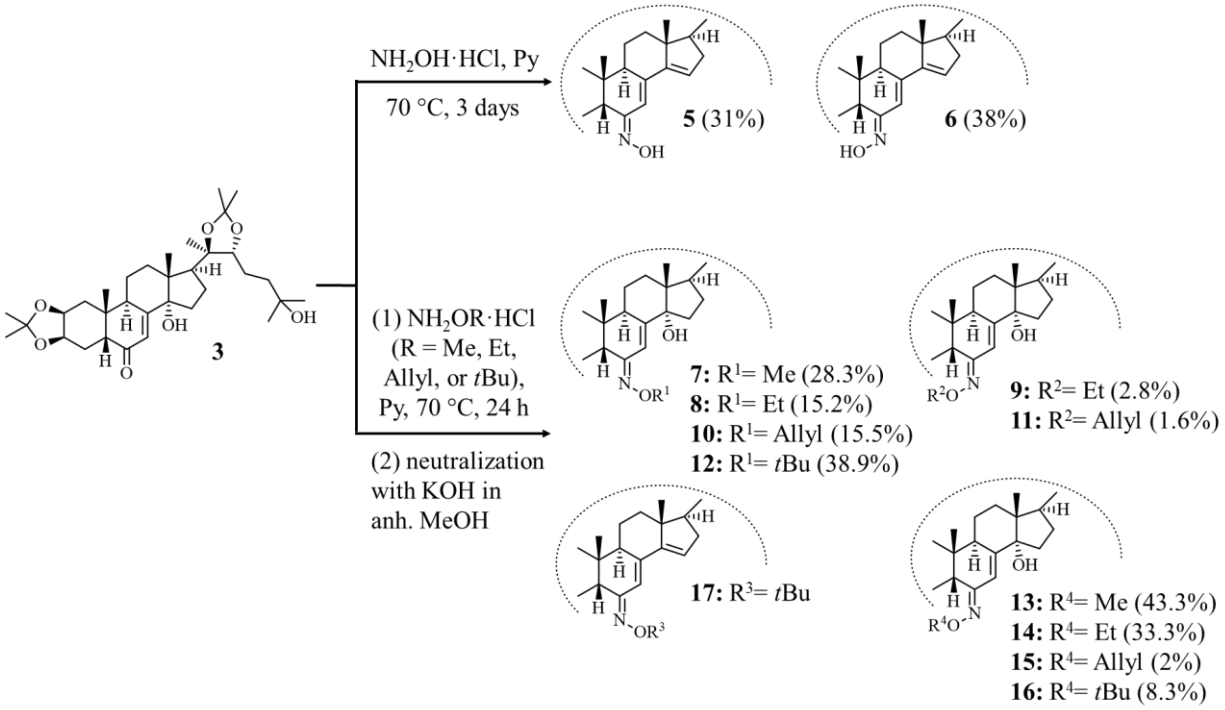
The presence of the 2,3-acetonide moiety is necessary for the antitumor activity of ecdysteroids and through the oxidative cleavage of the sterol-side chain, we can completely abolish any, otherwise mild, interfering inhibitory effect of a derivative on the efflux function of P-gp. Considering this, we planned to perform semi-synthetic modifications relevant to our scientific aims on two different antitumor ecdysteroids, one that possesses a sterol-side chain and another one that represented the former's side-chain cleaved analog. Accordingly, oxidative cleavage of the sterol side-chain of 20E (**1**) was achieved using the hypervalent iodine reagent PIDA in methanol that afforded poststerone (**2**). Acetonide-formation on the vicinal diols of compounds **1** and **2** was performed by using phosphomolybdic acid as catalyst in acetone that afforded products **3** and **4** as potential intermediates for further transformations. The reactions are shown in **Scheme 1**.



Scheme 1. Preparation of ecdysteroid acetonide analogs.

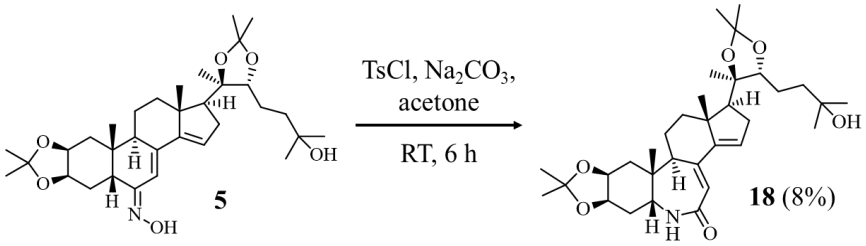
The 6-oxime or oxime ether analogs of 20E 2,3;20,22-diacetonide (**3**) were obtained by reacting the 6-carbonyl moiety of the substrate with the appropriate hydroxylamine or

alkoxyamine reagent in pyridine. During the preparation of ecdysteroid 6-oxime ethers, we neutralized the reaction mixtures with methanolic solutions of KOH that allowed the semi-synthesis of various, structurally diverse products, including intact, and 14,15-anhydro derivatives. The transformations are shown in **Scheme 2**.



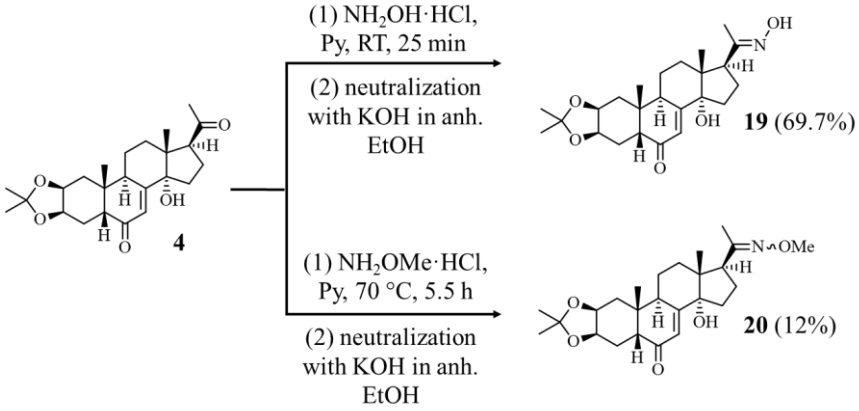
Scheme 2. Semi-synthesis of compounds **5-17** through the transformation of substrate **3**.

Beckmann-rearrangement is known as a stereospecific reaction that involves the migration of the chemical group located in *anti-position* with respect to the oxime's hydroxyl. Since in the case of ecdysteroid (6*Z*)-oxime **6**, the corresponding group is C_7H with an *sp2* -hybridized carbon atom, migration of the moiety is chemically disabled. Accordingly, we could perform the Beckmann-rearrangement solely of ecdysteroid (6*E*)-oxime **5**. This way, we found that the use of *p*-toluenesulfonyl chloride (TsCl) in acetone can effectively furnish the desired 7-membered lactam ring within 6 hours. Since the transformation gives rise to HCl, the use of Na_2CO_3 alkali was particularly important in our case, to prevent the acid-promoted cleavage of the acetonide pharmacophore. **Scheme 3** shows the rearrangement.



Scheme 3. Beckmann-rearrangement of ecdysteroid (6*E*)-oxime **5** to ecdysteroid lactam **18**.

The preparation of 20-oxime and oxime ether analogs of poststerone 2,3-acetonide (**4**) was achieved by slightly modifying the reaction conditions used for the semi-synthesis of ecdysteroid 6-oximes and oxime ethers **5-17**. Our preliminary small-scale test reactions indicated that under the applied synthetic conditions, oxime formation proceeds significantly faster at the 20-carbonyl than that of at C-6 that allowed the regioselective preparation of the desired products as a function of reaction time. Ecdysteroid derivative **20** was afforded as a pair of (*E*/*Z*)-isomeric 20-*O*-methyl oxime ethers at a 95:5 ratio (*E* and *Z*, respectively). The transformations are presented in **Scheme 4**.

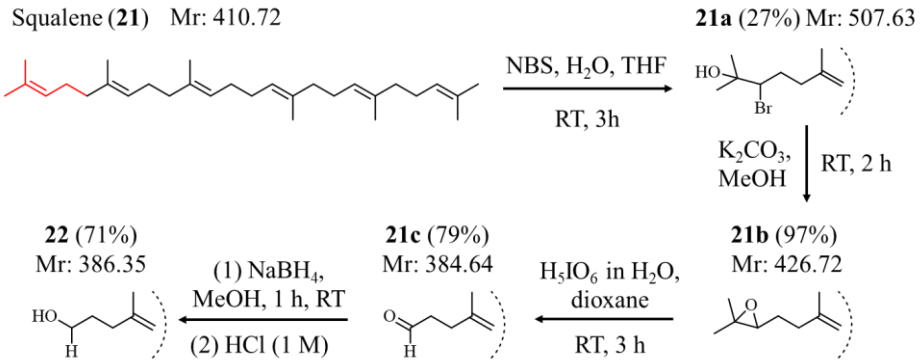


Scheme 4. Preparation of compounds **19**-**20** from poststerone 2,3-acetonide (**4**).

To the best of our knowledge, self-assembling drug conjugates of ecdysteroids have not been prepared and studied before. Since ecdysteroids represent relatively hydroxylated molecules, we found it reasonable to obtain their bioconjugates through esterification. To assure regioselectivity, this required the presence of possibly one single sterically accessible, reactive hydroxyl group on the antitumor ecdysteroid. Taking this into account, poststerone 2,3-acetonide 20-oxime (**19**) was a particularly attractive substrate for functionalization from multiple-aspects: 1) its adjuvant antitumor effect was not accompanied by efflux pump inhibitory activity (presented and discussed later); and 2) the hydroxyl on its 20-oxime group appeared as free and easily accessible. Considering this, nano-formulation of compound **19** was performed in three major steps.

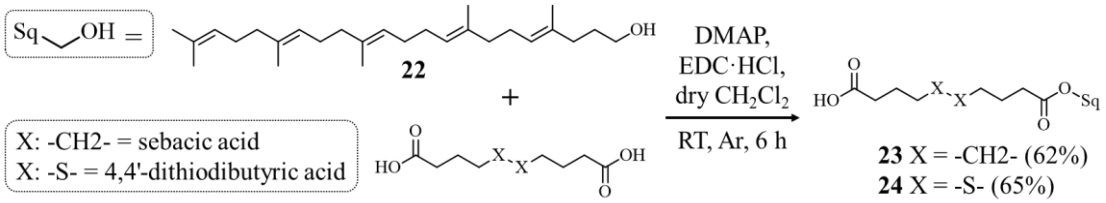
Since squalene (**21**) lacks a proper anchor point for the chemical linkage of the drug, it requires preliminary functionalization at its terminal double bond to serve the task. Therefore, at first, we transformed it to 1,1′,2-tris-norsqualene alcohol (**22**) in four synthetic steps. Accordingly, we oxidized squalene (**21**) to 2-hydroxy-3-bromosqualene (**21a**) employing NBS in an aqueous-organic medium, and subsequently converted the molecule's terminal bromohydrin moiety to epoxide using K_2CO_3 as a base in the substrate's methanolic solution

to afford 2,3-oxidosqualene (**21b**). Then, oxidative opening of the epoxide ring of 2,3-epoxysqualene was performed by aq. periodic acid in dioxane medium to afford 1,1′,2-trisnorsqualene aldehyde (**21c**). Eventually, we achieved reduction of the aldehyde moiety of intermediate **21c** to alcohol using $NaBH_4$ in methanol that resulted in the formation of 1,1′,2-trisnorsqualene alcohol (**22**). The reactions are presented in **Scheme 5**.



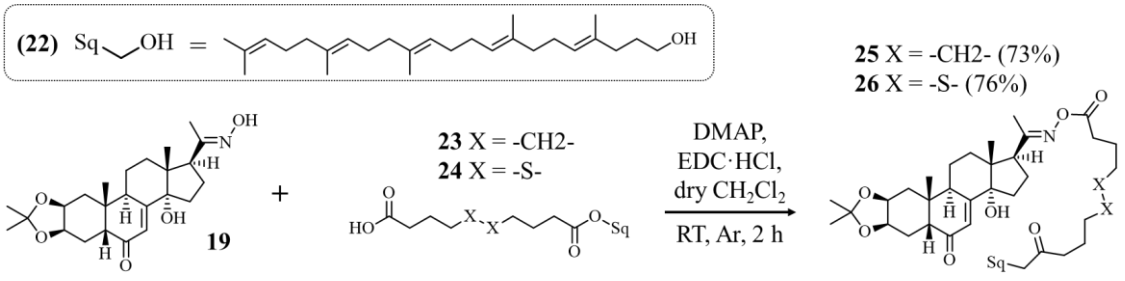
Scheme 5. Preparation of 1,1',2-tris-norsqualene alcohol (**22**) from squalene (**21**).

In some cases, the linkage of the molecular units through a so-called linker function may further improve the *in vivo* release of the drug. Taking this into account, we condensated 1,1',2-tris-norsqualene alcohol (**22**) with two different types of linker entities: sebacic acid or the disulfide-bridge containing 4,4'-dithiodibutyric acid. **Scheme 6** shows the reactions.



Scheme 6. Preparation of compounds **23** and **24** from 1,1′,2-tris-norsqualene alcohol (**22**).

Subsequently, condensation of compound **19** with the obtained side-chain entities **23** and **24** was performed using similar reaction conditions as presented above, which resulted in the preparation of ecdysteroid drug conjugates **25** and **26**, respectively (**Scheme 7**).



Scheme 7. Synthesis of drug conjugates **25** and **26** from their substrates.

Preparation and characterization of ecdysteroid nanoparticles (NPs)

We further studied the potential of ecdysteroid conjugates **25** and **26** to self-assemble into NPs, and the antitumor effects of the NPs within the frameworks of scientific research collaborations. The NPs were obtained by nanoprecipitation. Accordingly, a 250 μg aliquot of ecdysteroid conjugate **25** or **26** was dissolved in 125 μl of THF (2 mg/ml), and under mild stirring (400 rpm), this solution was added dropwise to a double volume of ultrapure water. Self-assembly of the conjugates occurred spontaneously as a consequence of local interactions between the hydrophobic molecules. Subsequently, the organic solvent was evaporated that afforded nanosuspension $\mathbf{25}_{\mathbf{NP}}$ or $\mathbf{26}_{\mathbf{NP}}$.

Besides, aliquots of compounds **25** and **26** were (250 μg in 125 μl of THF; 2 mg/ml) mixed with squalene-functionalized doxorubicin solution (2 mg/ml) in THF in a 1:50 molar ratio of ecdysteroid/doxorubicin to obtain multi-component solutions in 2 mg/ml concentrations. The samples' self-assembly was performed similarly to the above described and afforded the aqueous nanosuspensions of H-NPs $\mathbf{25}_{\mathbf{NP\text{-}DOX}}$ or $\mathbf{26}_{\mathbf{NP\text{-}DOX}}$.

The four different nanosuspensions were characterized by DLS and TEM techniques that confirmed the formation of nano assemblies in an aqueous medium. In general, the samples were found monodisperse (theoretical optimum: PdI < 0.2) and showed acceptable colloid chemical stability. The hydrodynamic diameters were as follows: $\mathbf{25}_{\mathbf{NP}}$ (366.3 ± 20.17 nm), $\mathbf{26}_{\mathbf{NP}}$ (221.8 ± 4.879 nm), $\mathbf{25}_{\mathbf{NP\text{-}DOX}}$ (187.7 ± 14.48 nm), $\mathbf{26}_{\mathbf{NP\text{-}DOX}}$ (298.7 ± 11.43 nm).

Biological evaluation of ecdysteroid derivatives

Antiproliferative activities against human gynecological cancer cell lines

Compounds **5-18** were tested in collaboration for their antiproliferative effects on HeLa, SiHa, MDA-MB-231 and MCF7 human gynecological cancer cell lines. Briefly, the analogs exhibited weak to moderate activities against the tested cell lines with IC_{50} values in the range of ca. 8-30 μM or above, while activity of the positive control cisplatin was exceeded by only one compound (**12**) on the HeLa and MDA-MB-231 models (IC_{50}=8.4 and 12.4 μM, respectively).

Cytotoxic activity, ABCB1-inhibition, and interaction with doxorubicin

Compounds **5-19** were investigated for their cytotoxic activity and ability to interfere with the efflux function of the ABCB1 transporter on a murine lymphoma cell line pair, i.e. a drug susceptible cell line (L5178) and its multi-drug-resistant counterpart ($L5178_{MDR}$)

transfected to express the human ABCB1 transporter. **Table 1** shows the results of these bioassays.

Table 1. Cytotoxicity of compounds **3-19** on L5178 and $L5178_{MDR}$ cells, and functional inhibition of the ABCB1 transporter. Dox = doxorubicin; for the ABCB1 inhibition, *(+)* control: 100 nM of tariquidar (112.4% inhibition), *(-)* control: 2% DMSO (-0.07% inhibition).

Compound	Change in the B-ring of **3** [a]	14-OH or $\Delta^{14,15}$	IC_{50} (μM) [95% confidence intervals] [b]		ABCB1 inhibition (%)	
			L5178	$L5178_{MDR}$	2 μM	20 μM
3	-	14-OH	110.3 [77.50-157.1]	97.69 [71.07-134.3]	2.54	20.91
4	-	14-OH	>75	>75	0.08	0.64
5	*(E)*-oxime	$\Delta^{14,15}$	20.91 [17.68-24.74]	24.63 [19.82-30.63]	10.57	82.95
6	*(Z)*-oxime	$\Delta^{14,15}$	34.22 [28.21-41.51]	28.35 [21.97-36.58]	7.15	81.09
7	*(E)*; R=Me	14-OH	40.92 [35.66-46.97]	55.05 [41.53-72.98]	2.25	25.05
8	*(E)*; R=Et	14-OH	35.02 [25.35-48.38]	47.00 [31.14-70.93]	17.54	78.79
9	*(Z)*; R=Et	14-OH	37.26 [25.65-54.11]	42.16 [41.24-43.10]	18.96	75.03
10	*(E)*; R=Allyl	14-OH	31.48 [23.71-41.80]	51.91 [42.69-63.13]	20.98	89.39
11	*(Z)*; R=Allyl	14-OH	36.66 [28.32-47.44]	49.29 [43.07-56.40]	24.17	81.80
12	*(E)*; R=*t*-But	14-OH	28.06 [21.30-36.98]	29.12 [25.12-33.76]	38.75	112.4
13	*(Z)*; R=Me	$\Delta^{14,15}$	45.95 [36.97-57.11]	53.14 [43.54-64.86]	33.36	106.2
14	*(Z)*; R=Et	$\Delta^{14,15}$	53.20 [38.64-73.26]	58.94 [45.86-75.74]	56.41	107.7
15	*(Z)*; R=Allyl	$\Delta^{14,15}$	55.28 [46.21-66.13]	52.72 [39.97-65.53]	61.13	102.7
16	*(Z)*; R=*t*-But	$\Delta^{14,15}$	63.23 [58.57-68.26]	51.22 [39.13-67.04]	58.99	78.76
17	*(E)*; R=*t*-But	$\Delta^{14,15}$	63.84 [45.70-89.19]	65.44 [55.66-76.94]	67.46	93.95
18	δ-lactam	$\Delta^{14,15}$	63.42 [47.51-84.65]	72.35 [64.39-81.29]	1.16	4.27
19	-	14-OH	162.3 [82.41-319.7]	142.1 [77.47-260.5]	0.68	1.53
Dox	-	-	0.080 [0.053-0.12]	4.49 [3.43-5.89]	-	-

[a] *R groups refer to the alkyl substituents of ecdysteroid 6-oxime ethers **7-17***

[b] *IC_{50} values were calculated by the CompuSyn software as the median cytotoxic activities (Dm) from the control lanes on the checkerboard plates of the combination*

Although the tested compounds demonstrated weak to moderate cytotoxic effects on the mouse lymphoma cell line pair, all derivatives obtained through the transformation of 20E 2,3;20,22-diacetonide (**4**), ecdysteroids **5-18**, respectively, were stronger than the parental compound. In contrast, we observed feeble cytotoxic effect for poststerone 2,3-acetonide 20-oxime (**19**); in this analogue's case, the formation of the oxime moiety at C-20 could almost two-fold-decrease the cytotoxicity compared to its parental compound **4**.

Cytotoxic properties of the compounds were also tested in combination with doxorubicin, to which the MDR cells show efflux-mediated resistance, to study possible drug-drug interactions. Results of the combination assays are summarized in **Table 2**.

As it is displayed in the table, all tested compounds acted in synergism with doxorubicin, in other words, they exerted a chemo-sensitizing activity towards the cytotoxic effect of doxorubicin. The most promising compound we identified was the ecdysteroid lactam **18**, which was slightly stronger than our previous lead (compound **3**) on both the MDR and the non-MDR cell lines. It is of further interest that this derivative, along with poststerone

2,3-acetonide 20-oxime (**19**), was found practically inactive as a functional inhibitor of the efflux transporter unlike compound **3** that was a weak inhibitor.

Table 2. Chemo-sensitizing activity of compounds **3** and **5-19** on the L5178 and L5178$_{MDR}$ cell lines towards doxorubicin at 50, 75 and 90% of growth inhibition (ED_{50}, ED_{75} and ED_{90}, respectively). CI: combination index; 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 antilog of the x-intercept, slope, and linear correlation coefficient of the median-effect plot, respectively.

Compound	Cell line	Drug ratio	CI at			Dm	m	r	CI_{avg}
			ED_{50}	ED_{75}	ED_{90}				
3 [91]	L5178$_{MDR}$	20.4 : 1	0.27	0.14	0.07	11.678	3.246	0.964	0.13
	L5178	163 : 1	0.67	0.55	0.46	11.236	2.103	0.942	0.53
5	L5178$_{MDR}$	15 : 1	0.26	0.16	0.12	4.454	6.638	1.000	0.16
	L5178	150 : 1	0.80	0.79	0.78	10.748	2.572	0.997	0.78
6	L5178$_{MDR}$	30 : 1	0.32	0.25	0.20	7.595	3.981	0.994	0.24
	L5178	150 : 1	0.98	0.76	0.61	16.049	3.239	0.986	0.72
7	L5178$_{MDR}$	15 : 1	0.17	0.16	0.16	6.605	3.721	0.978	0.16
	L5178	150 : 1	1.06	0.79	0.62	14.306	2.947	0.971	0.75
8	L5178$_{MDR}$	7.5 : 1	0.18	0.14	0.12	5.001	5.858	1.000	0.14
	L5178	37.5 : 1	0.55	0.58	0.60	8.598	2.495	0.972	0.59
9	L5178$_{MDR}$	3.75 : 1	0.27	0.16	0.13	3.030	3.329	0.993	0.16
	L5178	37.5 : 1	0.63	0.52	0.45	8.078	3.858	0.952	0.50
10	L5178$_{MDR}$	15 : 1	0.17	0.13	0.13	4.939	3.193	0.955	0.14
	L5178	150 :1	1.03	0.81	0.69	8.970	2.178	0.991	0.79
11	L5178$_{MDR}$	15 : 1	0.17	0.16	0.17	7.338	3.771	0.947	0.17
	L5178	75 : 1	0.70	0.83	1.03	8.202	1.722	0.956	0.91
12	L5178$_{MDR}$	7.5 : 1	0.30	0.20	0.17	3.928	4.610	1.000	0.20
	L5178	37.5 : 1	0.58	0.63	0.70	7.606	2.502	0.966	0.66
13	L5178$_{MDR}$	7.5 : 1	0.17	0.16	0.15	5.224	3.722	0.971	0.16
	L5178	37.5 : 1	0.77	0.47	0.31	8.165	3.044	0.982	0.44
14	L5178$_{MDR}$	7.5 : 1	0.21	0.14	0.11	6.133	4.890	0.992	0.14
	L5178	75 : 1	0.49	0.50	0.52	7.864	2.094	0.961	0.51
15	L5178$_{MDR}$	3.75 : 1	0.25	0.15	0.11	5.614	5.805	1.000	0.15
	L5178	37.5 : 1	0.46	0.47	0.47	8.295	2.882	0.981	0.47
16	L5178$_{MDR}$	7.5 : 1	0.34	0.26	0.23	8.365	3.378	0.939	0.26
	L5178	37.5 : 1	0.53	0.59	0.66	9.652	2.400	0.961	0.62
17	L5178$_{MDR}$	7.5 : 1	0.27	0.24	0.23	8.739	3.813	0.960	0.24
	L5178	37.5 : 1	1.16	0.85	0.64	7.199	3.273	0.977	0.80
18	L5178$_{MDR}$	15 : 1	0.20	0.12	0.09	6.419	4.953	0.970	0.12
	L5178	150 : 1	0.40	0.42	0.46	10.477	2.033	0.966	0.44
19	L5178$_{MDR}$	30 : 1	0.34	0.20	0.13	30.423	2.306	0.968	0.22
	L5178	300 : 1	0.56	0.45	0.40	84.393	1.665	0.995	0.47

Antiproliferative effect of self-assembled ecdysteroid NPs on A2780$_{ADR}$ cells

Ecdysteroid NPs and H-NPs **25$_{NP}$**, **26$_{NP}$**, **25$_{NP\text{-}DOX}$** and **26$_{NP\text{-}DOX}$** were tested for their potential to inhibit the cell growth of A2780$_{ADR}$ cells, a human ovarian carcinoma doxorubicin-resistant cell line. The results are shown in **Table 3**.

The results indicated remarkable differences between the cytotoxicity of the NPs and H-NPs of conjugates **25** and **26**. Accordingly, ecdysteroid NPs **25$_{NP}$** and **26$_{NP}$** exerted a measurable, yet low antiproliferative effect, while **25$_{NP\text{-}DOX}$** and **26$_{NP\text{-}DOX}$** displayed cytotoxicity in the sub-micromolar range, and presence of the ecdysteroid in these H-NPs

resulted in a nearly four-times increase of activity as compared to that of squalene-coupled doxorubicin NPs. The obtained results may provide a promising basis for further investigations on the use of ecdysteroid acetonides in similar or other types of antitumor nanostructures.

Table 3. Inhibitory activity of NPs and H-NPs of compounds **25** and **26** on the grow of $A2780_{ADR}$ cells. Dox-Sq: squalene-functionalized doxorubicin, GI: grow inhibition, GI_{50}: concentration of the test agent inducing 50% reduction in cell number compared with control cultures. Values are the mean ± SD of at least four independent experiments.

	$A2780_{ADR}$ cells (GI_{50}, μM)	
Compound (X)	**X_{NP}**	**$X_{NP\text{-}DOX}$**
25	19 ± 3	0.34 ± 0.08
26	39 ± 4	0.26 ± 0.03
Dox-Sq	1.17 ± 0.06	**----**

PROTOFLAVONOIDS

Semi-synthetic preparation of protoflavonoid derivatives

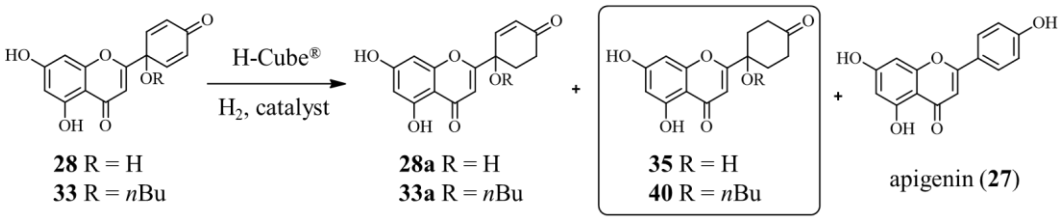
Protoflavonoids are mostly recognized for their antitumor properties; however, evidence suggests that they might likely have a more sophisticated, non-tumor related pharmacology existing as well. In this Ph.D. work, we aimed to extend their chemical and pharmacological space towards less cytotoxic derivatives to initiate exploring their pharmacological potential in new directions. We selected protoapigenone (**28**), and its 1′-*O*-alkyl analogs (**29-34**) as substrates of our studies; some of these derivatives have been previously reported to exert interesting, non-tumor-related bioactivities.

The function mainly responsible for the cytotoxicity of protoflavones is the symmetric dienone moiety on the B-ring, which confers pro-oxidant and Michael-acceptor properties to these compounds. Therefore, we chose this moiety as the target of our semi-synthetic transformations to prepare less cytotoxic, yet potentially bioactive derivatives. In this context, we employed two different synthetic strategies: 1) the selective saturation of the B-ring to obtain protoflavone analogs possessing the rare, naturally occurring dihydro- and/or tetrahydroprotoflavone structural elements, and 2) the substitution of the protoflavone 4'-oxo moiety with an oxime function.

The first step of our preparative strategy was the oxidative dearomatization apigenin (**27**), which was carried out using hypervalent iodine reagent PIFA in acetonitrile medium in the presence of water or an appropriate alcohol according to the desired alkyl substituent to be coupled at C-1′. Under reflux, the reaction resulted in protoapigenone (**28**) or its 1′-*O*-alkyl ether analogs (**29-34**) in an hour. Compounds **28-34** served as intermediates for further

transformations, while, from this set of derivatives, compound **32** was already a compound of interest for our bioactivity studies due to its very weak cytotoxicity when compared to the others. The reactions are presented in **Scheme 8**.

We carried out the selective hydrogenation of the protoflavone B-ring of starting materials **28-33** employing a modified version of H-cube® continuous flow hydrogenation reactor. For the proposed semi-synthetic task, at first, we developed a general procedure by performing a series of small-scale test reactions with compounds **28** and **33** to identify optimal synthetic conditions. **Figure 1** summarizes the results of this study.



Entry	Starting material[a]	Catalyst	Solvent	*p* (bar)	*T* (°C)	Flow (ml/min)	Conv.[b] (%)	Selectivity[b] (%)		
								28a or **33a**	**35** or **40**	**27**
1	**33**	Lindlar catalyst	EtOAc	20	25	1	19	not detected		
2	**33**	Lindlar catalyst	EtOAc	40	25	1	43	0	88	12
3	**33**	Lindlar catalyst	EtOAc	80	25	1	99	0	92	8
4	**33**	Lindlar catalyst	EtOAc	80	25	1.5	38	0	not detected	
5	**33**	Lindlar catalyst	EtOAc	40	50	1	78	0	66	34
6	**33**	Lindlar catalyst	MeOH	80	25	1	97	0	81	19
7	**33**	5% Pd/C	EtOAc	20	25	1	71	0	82	18
8	**33**	5% Pd/C	EtOAc	40	25	1	100	0	84	16
9	**28**	5% Pd/C	EtOAc	40	25	1	99	traces	77	23
10	**28**	5% Pd/C	MeOH	40	25	1	99	traces	80	20

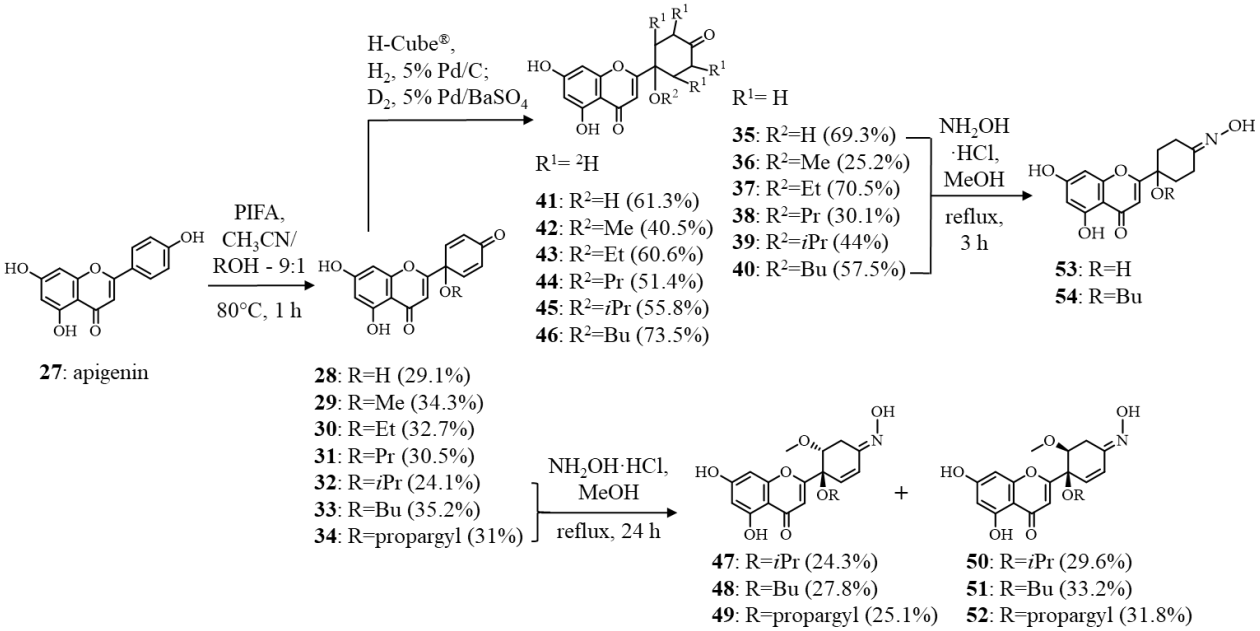
Figure 1. Results of the optimization of conditions for the continuous-flow hydrogenation of compounds **28** and **33**. [a]: $c_{\text{starting material}}$=1 mg/ml; [b]: Determined by ^{1}H NMR spectroscopic analysis of the crude product mixture.

We achieved our best results for the selective saturation of the B-ring by employing 5% Pd/C catalyst, 1 ml/min sample solution flow rate and 40 bar pressure at ambient temperature (**Figure 1**, entries 9 and 10). These conditions were then applied to prepare further B-ring reduced derivatives. The substrates were dissolved in ethyl acetate (compounds **29-33**) or methanol (compound **28**) considering their relative polarity. The transformations afforded tetrahydroprotoapigenone derivatives **35-40** in fair yields (**Scheme 8**).

In addition, we aimed to further increase the available number of interesting, potentially bioactive protoflavone derivatives by performing the selective deuteration of the B-rings of substrates **28-33**. To this, we slightly modified the procedure employed during the hydrogenations, and by using 5% Pd/$BaSO_4$ catalyst, and ethyl acetate as a sample solvent, we successfully obtained the desired tetradeuteroprotoapigenone analogs **41-46** (**Scheme 8**).

Besides, we successfully transformed the 4′-keto group of protoapigenone analogs **32-34**, and tetrahydroprotoapigenone derivatives **35** and **40** to an oxime, by using hydroxylamine

hydrochloride reagent in methanol. Each reaction was regioselective towards the 4′-keto group; however, in the cases of substrates **32-34**, the transformation was accompanied by the acid-catalyzed Michael-addition of the solvent at C-2′ that resulted in the saturation of one double bond on the *p*-quinol dienone to afford the corresponding 2′-methoxy-2′3′-dihydroprotoapigenone 4′-oxime analogs **47-52**, or tetrahydroprotoapigenone 4′-oximes **53-54** as racemates. The transformation is presented below, in **Scheme 8**.



Scheme 8. Synthesis of tetrahydro-, tetradeutero- or 4′-oxime analogs of protoapigenone and its 1′-*O*-alkyl or propargyl ether derivatives. Oximes **47-54** were obtained as racemates; for simplicity only one enantiomer is shown.

Biological evaluation of protoflavonoid derivatives

Cytotoxicity on cancer cell lines and inhibition of DNA-damage response

In vitro cytotoxic activity of compounds **35** and **40** were tested on MCF-7, HeLa, and SiHa human adherent cancer cell lines. As expected, selective saturation of the protoflavone B-ring resulted in a dramatic decrease in the compounds' cytotoxicity. Consequently, we could only determine an IC_{50} value for compound **40** on HeLa cells (55.12 ± 1.11 μM); in all other cases, the 50% inhibitory concentrations were systematically above 100 μM.

Besides, compounds **35** and **40** were also tested for their potential to inhibit the activation of DNA damage response through the ATR/ATM signaling pathways. While we found derivative **35** completely inactive inhibiting the phosphorylation of Chk1 and Chk2, its 1′-*O*-butyl ether **40** could exert measurable effect on Chk1, yet only at its highest tested dose (20 μM). To study the possible relevance of this finding, the compounds' chemo-sensitizing activity toward doxorubicin was tested by a series of combination studies, and neither

protoflavonoid derivative was found to interact with this chemotherapeutic agent.

Antiviral activities of obtained protoflavonoid products

Protoflavonoid products **28**, **32-40** and **47-54** were tested against HIV-1 by a pseudotype virus assay. Among these compounds, tetrahydroprotoapigenone (**35**) was able to inhibit viral infection by ca. 50% at a concentration of 100 μM, while no cytotoxicity was observed up to a concentration of 500 μM (data not presented).

Further, compounds **28** and **32-54** were tested for their activity against the EBV lytic cycle. To this, the expression of the EBV lytic protein Rta was studied in P3HR1 host cells. First screening was performed at a concentration of 0.25 μM, and three compounds (**32-34**) were found active. Then, dose-dependency of this activity was determined, as well as the compounds' cytotoxicity on P3HR1 cells. Results are shown in **Figure 2**.

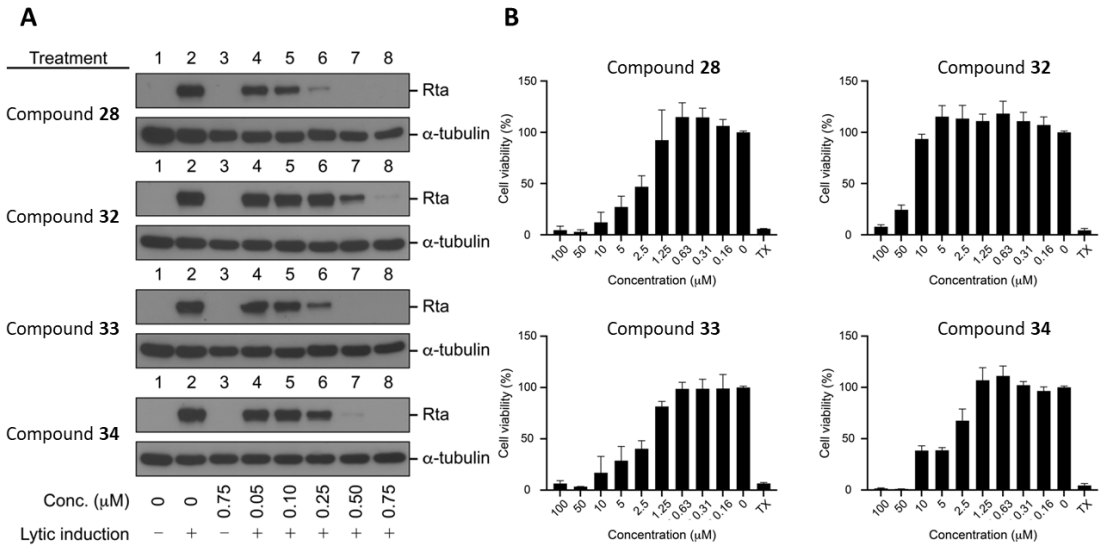


Figure 2. A) Inhibition of the expression of Epstein–Barr Virus (EBV) lytic protein Rta by protoapigenone (**28**) and its analogs **32-34**. P3HR1 cells were treated with the compounds at the time of lytic induction with sodium butyrate (SB) and tetradecanoyl phorbol acetate (TPA). Cell lysates were harvested at 24 h after lytic induction. **B**) Cytotoxicity of the compounds on P3HR1 cells. Cells were cultured for 24 h in a medium containing protoapigenone (**28**) or compounds **32-34**. Cell viability was evaluated by the MTT assay. Cells treated with 1% Triton X-100 were used as a positive control. Error bars represent SD.

Results of the anti-EBV activity testing (**Figure 2-A**) indicated the ability of all three compounds **32-34** to cause a reduction in the Rta levels at concentrations of 0.50, 0.25 and 0.50 μM, respectively. The calculated IC_{50} values for the positive control protoapigenone (**28**) and the tested protoflavonoids **32-34** were 0.127, 0.467, 0.208 and 0.285 μM, respectively.

Regarding the results of the cytotoxicity assays (**Figure 2-B**), compounds **33** and **34** were similarly active as protoapigenone (**28**), while the isopropyl ether derivative **32** was much weaker in this regard. Based on our results, selectivity of the anti-EBV vs cytotoxic

effect of compounds **28** and **32-34**, expressed as a ratio of the corresponding IC_{50} values, were calculated as 30.1, 73.0, 9.80 and 17.3, respectively. Accordingly, we found a 73-times selectivity of antiviral over cytotoxic activity in the case of protoapigenone 1′-*O*-isopropyl ether (**32**), in contrast with the slightly stronger antiviral, yet much more cytotoxic protoapigenone (**28**).

Summary

ECDYSTEROIDS

- A total of 20 ecdysteroids, including 18 nitrogen-containing derivatives were obtained through semi-synthetic transformations. Altogether, 14 compounds were new.
- We prepared two different self-assembling ecdysteroid drug conjugates from poststerone 2,3-acetonide 20-oxime (**19**). In research collaboration, the conjugates were successfully transformed to their corresponding NPs and doxorubicin-containing H-NPs and subsequently characterized for their colloid chemical properties.
- Compounds **5-18** were tested on human gynecological cancer cell lines. In general, moderate antiproliferative activities were observed.
- Compounds **5-19** were tested for their ability to inhibit the ABCB1-mediated efflux transporter. Several SARs were revealed, and two derivatives, the ecdysteroid lactam **18** and poststerone 2,3-acetonide 20-oxime (**19**) were inactive in this regard.
- Compounds **5-19** were tested for their chemo-sensitizing activity in combination with doxorubicin. While each derivative could considerably sensitize both the MDR and the susceptible cancer cell line, the ecdysteroid lactam **18** was identified as our most interesting compound for further studies; its chemo-sensitizing activity exceeded that of our previous lead (compound **3**), while it did not inhibit the efflux function of P-gp.
- Ecdysteroid NPs (**25**$_{\mathbf{NP}}$ and **26**$_{\mathbf{NP}}$) and H-NPs (**25**$_{\mathbf{NP\text{-}DOX}}$ and **26**$_{\mathbf{NP\text{-}DOX}}$) were tested for their ability to inhibit the growth of $A2780_{ADR}$ cells. The chemo-sensitizing effect of squalenoylated ecdysteroid oxime **19** contributed to the ability of H-NPs to overcome the drug resistance of the cancer cells with cytotoxic activity in the submicromolar range.

PROTOFLAVONOIDS

- A total of 27 protoflavonoids, 19 new compounds, were semi-synthesized from apigenin. The continuous-flow method that was developed allowed the first-time semi-synthesis of compounds containing the rare, naturally occurring tetrahydroprotoflavone moiety.

- Tetrahydroprotoflavone derivatives **35** and **40** were tested for their cytotoxic effects against cancer cell lines and DNA-damage response inhibitory activities. The reduction of the double bonds in the p-quinol B-ring of the derivatives resulted in a great decrease in the bioactivity of the compounds towards the tested properties. Accordingly, the employed synthetic strategy may serve as an effective tool to knock out these bioactivities of protoflavonoids that could potentially confer them toxic side effects.
- Compounds **28**, **32-40** and **47-54** were tested against HIV-1 on a pseudotype virus assay; among these compounds, tetrahydroprotoapigenone (**35**) was found to inhibit viral infection by ca. 50% at 100 μM that was more than 5-times below its cytotoxic concentration.
- Compounds **28** and **32-54** were tested for their antiviral activity against EBV; these studies revealed a 73-times selectivity of antiviral over cytotoxic activity in the case of protoapigenone 1′-*O*-isopropyl ether (**32**).

List of publications

Publications related to the Ph.D. thesis:

I. **Vágvölgyi M**, Girst G, Kúsz N, Ötvös SB, Fülöp F, Hohmann J, Servais JY, Seguin-Devaux C, Chang FR, Chen MS, Chang LK, Hunyadi A, Less cytotoxic protoflavones as antiviral agents: protoapigenone 1′-O-isopropyl ether shows improved selectivity against the Epstein-Barr virus lytic cycle, *International Journal of Molecular Sciences* (2019) **20** 24; 6269

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II. Fumagalli G, Giorgi G, **Vágvölgyi M**, Colombo E, Christodoulou MS, Collico V, Prosperi D, Dosio F, Hunyadi A, Montopoli M, Hyeraci M, Silvani A, Lesma G, Dalla Via L, Passarella D, Heteronanoparticles by Self-Assembly of Ecdysteroid and Doxorubicin Conjugates To Overcome Cancer Resistance, *ACS Medicinal Chemistry Letters;* (2018) **9** 5; 468-471.

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III. Bogdán D, Haessner R, **Vágvölgyi M**, Passarella D, Hunyadi A, Tóth G, Stereochemistry and complete 1H and 13C NMR signal assignment of C-20-oxime derivatives of posterone 2,3-acetonide in solution state, *Magnetic Resonance in Chemistry;* (2018) **56**; 859–866.

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II. **Vágvölgyi M**, Girst G, Zomborszki ZP, Fülöp F, Ötvös SB, Hunyadi A,
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III. **Vágvölgyi M**, Girst G, Zomborszki ZP, Fülöp F, Ötvös SB, Hunyadi A,
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VI. **Vágvölgyi M**, Martins A, Tóth G, Kúsz N, Giorgi G, Passarella D, Hunyadi A,
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VIII. **Vágvölgyi M**, Ötvös SB, Kúsz N, Orbán-Gyapai O, Fülöp F, Hunyadi A,
Új protoflavon származékok előállítása
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IX. Hunyadi A, Dankó B, Csábi J, **Vágvölgyi M**, Issaadi M, Fási L, Zoofishan Z,
A brief overview of our compound library available for collaborative studies
4th Workshop of COST Action CM1106, Chemical Approaches to Targeting Drug Resistance in Cancer Stem Cells, Chioggia, Italy (2016) (Poster presentation)

X. Hunyadi A, Dankó B, Csábi J, **Vágvölgyi M**, Issaadi M, Fási L, Zoofishan Z,
What we can provide for collaboration: an overview of our available compound library
4th Workshop of COST Action CM1106, Chemical Approaches to Targeting Drug Resistance in Cancer Stem Cells, Chioggia, Italy (2016) (Poster presentation)

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I. Latif AD, Gonda T, **Vágvölgyi M**, Kúsz N, Kulmány Á, Ocsovszki I, Zomborszki ZP, Zupkó I, Hunyadi A, Synthesis and In Vitro Antitumor Activity of Naringenin Oxime and Oxime Ether Derivatives, *International Journal of Molecular Sciences,* (2019) **20** 9 2184
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