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Facile Synthesis and Cytotoxic Activity of the First Ferrocene-Resveratrol Conjugate

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Abstract: The bioorganometallic **III** containing trimethylene chain between ferrocene and resveratrol (3,5,4'-trihydroxystilbene, **RSV**) moieties connected *via* ester bond has been synthesized. The novel bioconjugate was characterized using IR and NMR (^{1}H, ^{13}C, COSY, NOESY, HMBC) spectroscopy, ESI-MS and HRMS. The **RSV** and ferrocene-**RSV** conjugate **III** were screened *in vitro* for their inhibitory effects against proliferation of hepatoblastoma (Hep G2) cells by MTT assay. Also, possible cytotoxicity towards normal ovary cells (CHO-K1) was evaluated. The obtained data revealed profound effects in biological/cytotoxic activity of **III** *vs*. **RSV** in Hep G2 cell line. Lower cytotoxicity of **III** was observed in normal ovary cells as compared to hepatoblastoma cells.

Keywords: ferrocene, ferrocene-resveratrol conjugate, hepatoblastoma, normal ovary cells, resveratrol.

INTRODUCTION

OWING to its therapeutic and protective role, resveratrol [3,5,4'-trihydroxystilbene (**RSV**) (Figure 1a)] came into focus of research in recent years. Among numerous health benefits, **RSV** improves cardioprotection, cancer prevention and therapy, immune regulation and metabolic and neuroprotective functions.[1–3] In order to enhance its activity, **RSV** was derivatized by: (*i*) modification of the number and position of the phenolic groups, (*ii*) insertion of a long alkyl chains or functionalized chains[4] and (*iii*) the addition of acyl chains to free hydroxyl groups.[5] The improved biological activity of the so-obtained **RSV** derivatives in comparison to those of the parent molecule is likely to be due to their lipophilicity and facilitated transport through cell membrane. Recently, **RSV**-derivatives **I** and **II** containing lipophilic ferrocene moiety instead of one benzene ring were synthesized (Figure 1b).[4] The heteroannularly disubstituted derivative **IIb**, containing additional aromatic unit linked by an ester bond, was found to exhibit more than 10-fold higher inhibitory activity in SW480 and HepG2 cell lines compared to those of **RSV**. Prompted by these results, we have prepared and tested conjugate **III** containing trimethylene alkyl chain between ferrocene and **RSV** connected *via* ester linkage (Figure 1c).

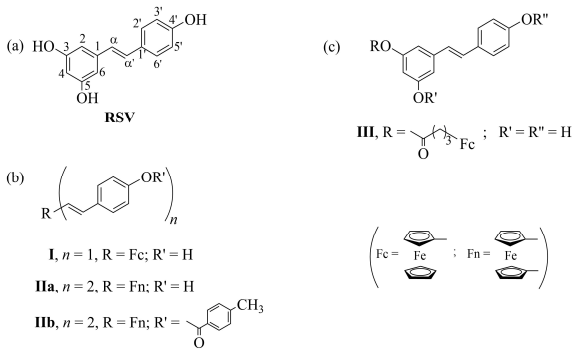
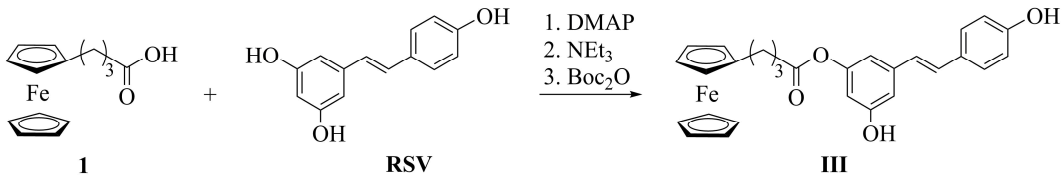


Figure 1. The chemical structures of (a) resveratrol (**RSV**), (b) ferrocene-**RSV** analogs **I** and **II** and (c) ferrocene-**RSV** conjugate **III**. The numbering of the carbon atoms is according to IUPAC nomenclature.

EXPERIMENTAL

The material and methods data are provided in Supplementary Materials.



Scheme 1. The synthesis of ferrocene-**RSV** conjugate **III.**

Chemistry

The *trans*-resveratrol (**RSV**) (95 mg, 0.42 mmol) was dissolved in dry THF (7 ml) and added to a solution of ferrocene butyric acid **1**[6] (374 mg, 1.37 mmol) in the same solvent (10 ml), followed with addition of DMAP (7.6 mg, 0.06 mmol) and NEt_3 (0.35 ml, 2.49 mmol)[7] (Scheme 1). The reaction mixture was cooled to −15 °C and solution of Boc_2O (354 mg, 1.62 mmol) in dry THF (8 ml) was added. After 5 minutes, the reaction mixture was allowed to warm to room temperature and then stirred at ambient temperature during 5 days. After the total consumption of the starting material, as monitored by TLC, the mixture was diluted with EtOAc (10 ml) and washed with 2M HCl (30 ml), 5 % $NaHCO_3$ (50 ml) and saturated NaCl. The organic layer was dried over Na_2SO_4 and evaporated *in vacuo* to leave the crude product. Since the numerous yellow and UV visible spots were seen on TLC plate, the TLC purification of the crude material was repeated for several times using different solvent systems (petroleum ether/ diethyl ether = 2/1; petroleum ether / diethyl ether = 5/1; CH_2Cl_2 / hexane = 3/1) until satisfactory purity of the compound **III** was obtained and confirmed by ESI-LC method. Yellow-orange oil; 43 mg (21 %); R_f = 0.68 in petroleum ether/ diethyl ether = 2/1; IR (CH_2Cl_2) $\tilde{\nu}_{max}$/cm^{-1}: 3676 w (OH), 1759 s (C=O), 1137 s (CO); ^{1}H NMR ($CDCl_3$) δ/ppm: 1.54 (brs, 2H, OH), 1.96 (m, 2H, $CH_{2\gamma}$), 2.48 (t, J = 2.5 Hz, 2H, $CH_{2\delta}$), 2.58 (t, J = 2.6 Hz, 2H, $CH_{2\beta}$), 4.08 (s, 2H, H_{Fn}), 4.11 (s, 2H, H_{Fn}), 4.12 (s, 5H, H_{Fn}), 6.90 (pt, 1H, H4), 6.97 (d, J = 16.3 Hz, 1H, Hα), 7.06 (d, J = 16.3 Hz, 1H, Hα'), 7.11 (brs, 1H, H2), 7.17 (d, J = 8.5 Hz, 2H, H3', H5'), 7.19 (brs, 1H, H2), 7.47 (d, J = 8.5 Hz, 2H, H2', H6'); ^{13}C NMR APT ($CDCl_3$) δ/ppm: 171.70 (C=O), 151.89 (C3), 151.88 (C5), 151.47 (C4'), 139.63 (C1), 134.53 (C1'), 129.78 (Cα'), 172.75 (C2', C6'), 129.38 (Cα), 121.74 (C3', C5'), 116.90 (C6), 116.68 (C2), 116.59 (C4), 84.03 (C_{qFn}), 68.70, 68.32, 67.48 (C_{Fn}), 34.05 ($CH_{2\beta}$), 29.11 ($CH_{2\delta}$), 26.28 ($CH_{2\gamma}$); ESI-MS m/z = 583.2 [(M+2MeOH+2H_2O+H)$^+$]; MALDI-HRMS m/z = 482.118 (calculated for $C_{28}H_{26}O_4Fe$ = 482.118).

Cytotoxic Activity

Treatment: Stock solutions of **RSV** and ferrocene-**RSV** conjugate **III** were prepared as 20 mM solutions in ethanol (EtOH) and stored at 4° C. Prior to use in cytotoxicity assay, the stock solutions were further diluted with culture medium to obtain final concentrations (10−100 µM). Cytotoxic effects in Hep G2 and CHO-K1 cells were evaluated after 48 h of exposition. Samples with ethanol without test compounds were used as controls.

MTT Cytotoxicity Assay: For experimental purposes cells were seeded in multiwell plates (5×10^4 cells/mL), then treated with **RSV** and **III** at a range of concentrations (10−100 µM) and after 48 h cell viability was determined by MTT assay.[8] The experiments were performed two times with at least three parallels for each concentration and data were expressed as the means ± SEM. Cell viability was expressed as percentage of treated cells *vs.* control cells. The IC_{50} values, defined as the concentrations of the tested compound that result in 50 % cell growth inhibition, were derived from the equations of related trend lines.

Statistical Analysis: A two-tailed Student's *t*-test was applied to evaluate the significant differences between control and treated cells. The results are reported as means ± SEM, $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The conjugate **III** was prepared by esterification[7] of ferrocene butyric acid (**1**)[6] with C3-OH group of *trans*-**RSV** in the presence of DMAP, NEt_3 and Boc_2O (Scheme 1). Although the reaction conditions were adjusted to provide the simultaneous formation of mono- (**III**), di- and three esterified conjugates, we were able to isolate and confirm the presence of only one pure compound, *i.e.* monoester **III** in yield of 21 %. However, TLC-plate indicated the presence of a several yellow-coloured ferrocene-containing compounds that had proved to be difficult to purify to the required level.

The NMR assignments in the present study are in a good agreement with previously published data for **RSV**[9,10] and its 3-substituted **RSV**-glycoside[11] (Table 1).

The ^1J(H,H) coupling between two alkene hydrogens α and α' of 16.5 Hz is consistent with the *trans* orientation of the phenolic rings. MS and NMR data unambiguously confirmed the presence of one single ferrocene moiety, introduced by esterification of C3-OH group of **RSV**. As it was shown for 3-substituted **RSV**-glycoside[11], the clearly separated NMR signals of C2 and C6, C3 and C5 and H2 and H6 in comparison to magnetically equivalent assignments for H2'/6', H3'/5', C2'/6' and C3'/5' indicate the alteration of their chemical environment due to the esterification of

Table 1. NMR (δ in ppm) spectroscopic data of **RSV**,[9,10] **RSV**-glycoside[11] and ferrocene-**RSV** conjugate **III** ($c = 1\times10^{-3}$ mol dm^{-3})

C and attached ^{1}H	Carbon, δ / ppm			Proton, δ / ppm			
	RSV*	**RSV**-glycoside*	**III****	**RSV***	**RSV****	**RSV**-glycoside*	**III****
1	139.19	139.3	139.59	-	-	-	-
2,6	104.23	*102.7 (C2)* *107.0 (C6)*	*116.87 (C2)* *116.54 (C6)*	6.34	6.51	*6.73 (H2)* *6.55 (H6)*	*7.19 (H2)* *7.11 (H6)*
4	101.67	104.7	114.20	6.07	6.21	6.33	6.90
α	125.60	125.1	127.35	6.76	6.80	6.86	6.97
α'	128.02	128.5	129.75	6.87	6.97	7.01	7.06
1'	128.07	129.9	134.49	-	-	-	-
2',6'	127.75	127.9	127.73	7.35	7.35	7.39	7.47
3',5'	115.43	115.5	121.68	6.71	6.81	6.76	7.17
4'	157.16	157.2	151.43	-	-	-	-
3,5	158.28	*158.2 (C3)* *158.8 (C5)*	*151.86 (C3)* *151.85 (C5)*	-	-	-	-

*NMR spectra were recorded in DMSO-d_6.
**NMR spectra were recorded in $CDCl_3$.

C3-OH group. Furthermore, the NOE contacts between free hydroxyl protons on C4' and C5 with H3'/5' and H4, respectively, and $CH_{2\beta}$ and $CH_{2\gamma}$ with H2 and H4 support the proposed structure (Figure 2).

The six protons from alkyl chain were observed as two triplets at δ 2.85 and 2.48 ppm and one multiplet at 1.96 ppm. Multiple resonances detected for the same proton during the course of NMR analysis are attributed to the presence of 5:95 *cis-trans* mixture of **RSV**.

The **RSV** and ferrocene-**RSV** conjugate **III** were evaluated *in vitro* as inhibitors of hepatoblastoma (Hep G2) cell proliferation. Also, possible cytotoxicity towards normal ovary cells (CHO-K1) was evaluated. The concentration-dependent analysis of the cytotoxicity was evaluated by the widely used MTT method. The summarized results of cytotoxicity evaluation in Hep G2 and CHO-K1 cell lines with MTT bioassay are presented in Figure 3. IC_{50} values shown in Table 2 were derived from the equations of related polynomial trend lines for **RSV** and **III**, in both cell lines.

Presented data reveal profound effects in biological activity of ferrocene-**RSV** conjugate **III** *vs.* **RSV** in hepatoblastoma cell line. **III** in concentrations of 35, 75 and 100 μM significantly (p<0.001 – p<0.5) decreased Hep G2 cell proliferation (Figure 3a) and in all tested concentrations inhibitory effect is more pronounced for **III** compared to the same doses of **RSV**. This finding is also supported by comparison of IC_{50} values in Hep G2 cells: IC_{50} value for **III** is 20 % lower than IC_{50} value for **RSV**[4] (Table 2). The results in normal CHO-K1 cells (Figure 3b), as opposite to those obtained in Hep G2 carcinoma cells, indicate much lower cytotoxicity of **III** to normal cells – in fact, there was no statistically significant cytotoxicity observed in normal cells, except for the highest tested concentration (100 μM) and IC_{50} value was out of the applied concentration range.

CONCLUSION

The previously described ferrocene-**RSV** derivative **IIb**, containing two benzene, one ferrocene and one ester group, displayed higher inhibitory activity in SW480 and HepG2 cell lines as compared to **RSV**, owing to its enhanced lipophilicity. With that in mind, we have synthesized conjugate **III** comprised of the same elements but with different structural and spatial patterns and equipped with additional trimethylene alkyl chain in order to improve its lipophilicity as a crucial requirement for biological activity. Although the novel conjugate **III** is found to be less active than **IIb**, it is more potent in comparison to **RSV**. Therefore, the herein presented preliminary results hold potential for further studies of ferrocene-**RSV** conjugates based on multi-method approach in order to obtain more detailed information.

Table 2. IC_{50} values (μM) for **RSV** and ferrocene-**RSV** conjugate **III** *vs.* hepatoblastoma (Hep G2) cells and normal ovary cells (CHO-K1) revealed by MTT cytotoxicity assay

Compound	IC_{50} (μM)	
	Hep G2	CHO-K1
RSV	121.5	99.0
III	98.3	n.d.

n.d. – non determinated IC value for the corresponding incubation period, cannot be calculated from the equations of related polynomial trend lines.

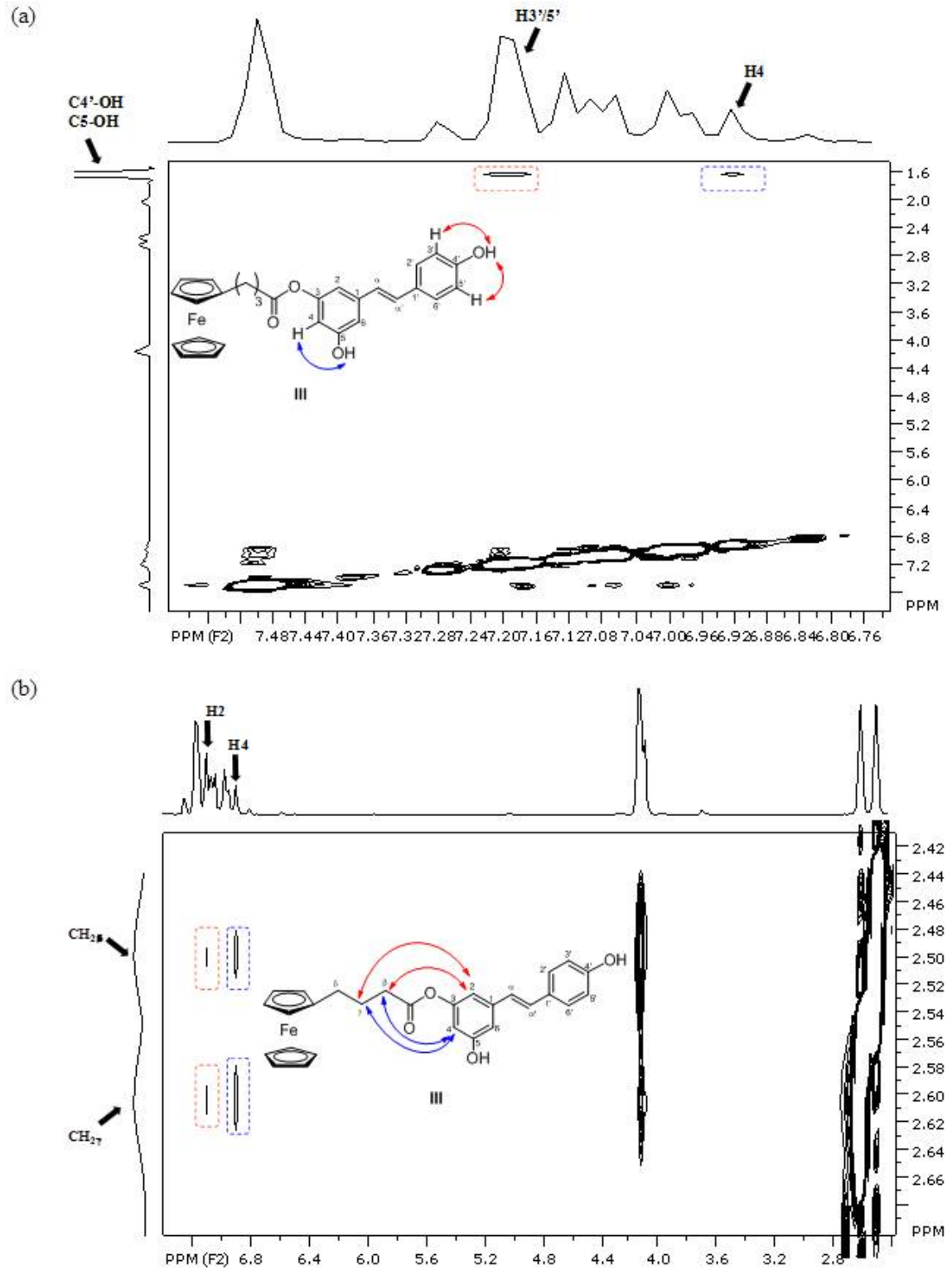


Figure 2. The NOE contacts between (a) free hydroxyl protons on C4' and C5 with H3'/5' and H4 and (b) $CH_{2\beta}$ and $CH_{2\gamma}$ with H2 and H4.

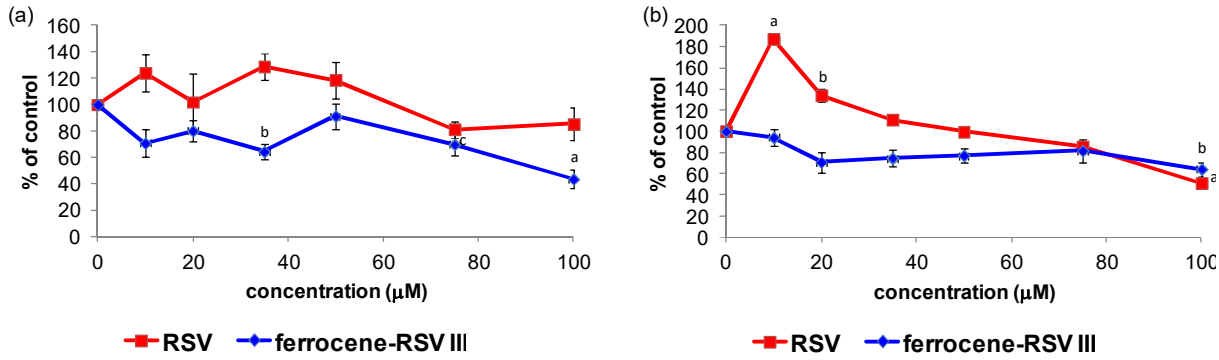


Figure 3. *In vitro* anti-proliferative effect of **RSV** and ferrocene-**RSV** conjugate **III** against (a) hepatoblastoma (Hep G2) cells and (b) normal ovary cells (CHO-K1), obtained with MTT assay after 48 h exposure. Data are presented as percentage of control: mean ± SEM of 2 experiments with at least three measurements within each experiment for each concentration. Statistical significance *vs*. control: $^{a}p < 0.001$; $^{b}p < 0.025$; $^{c}p < 0.05$.

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Supplementary Information. Supporting information to the paper is enclosed to the electronic version of the article at: http://dx.doi.org/10.5562/cca2992.

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Supplementary Materials

Materials and methods

Chemistry: Reaction was carried out under argon atmosphere. The THF used for synthesis was dried and distilled over CaH_2 and stored over molecular sieves (4Å). The synthesis of ferrocene butyric acid was performed according to literature data[6]. *Trans*-resveratrol (**RSV**) (Sigma-Aldrich), di-*tert*-butyldicarbonate (Acros Organics), NEt_3 (Fischer Chemical) and DMAP (Sigma-Aldrich) were used as received. Product was purified by preparative thin layer chromatography on silica gel (Merck, Kieselgel 60 HF_{254}). Infrared spectrum was recorded as CH_2Cl_2 solution between NaCl windows by using a Bomem MB 100 mid FTIR spectrometer. (s) = strong, (w) = weak. The ^1H NMR spectra measured at 600.133 MHz and ^{13}C NMR spectra measured at 150.917 MHz using Bruker Avance spectrometer, were referenced to the residual solvent peak ($CDCl_3$, ^1H: 7.26 ppm, ^{13}C: 77.16 ppm). Double resonance experiments (COSY, NOESY, HMBC) were performed in order to assist in signal assignment. (s) = singlet, (brs) = broad singlet, (d) = doublet, (t) = triplet, (m) = muliplet, (pt) = pseudotriplet (unresolved doublet of doublets). Mass spectra were measured on a LC-MS system coupled with triple-quadrupole mass spectrometer, operating in a positive ESI mode (H_2O/MeOH = 80/20). High-resolution mass spectrum was acquired using 4800 MALDI TOF/TOF-MS Analyzer.

Cell culture: Hep G2 (human hepatoblastoma cell line; ATCC® HB-8065™) and CHO-K1 (chinese hamster ovary; ATCC® CCL61™) cell lines were purchased from American Type Culture Collection (ATCC, USA). Hep G2 and CHO-K1 cells were maintained in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 with 15 mM HEPES buffer and L-glutamine (DMEM/F-12 (1:1); Gibco, Paisley, UK). Heat inactivated Fetal Bovine Serum (Gibco, Paisley, UK) was added to make the complete growth medium for both cell cultures in final concentration of 10 %. Cells were routinely cultured in 80-cm^2 cell flasks (Nunc, Roskilde, Denmark) at 37° C and humidified in atmosphere of 5 % CO_2 in air. After reaching 70-90 % confluence cells were disaggregated using a trypsin/EDTA (0.25 % trypsin, 1mM EDTA·4Na), counted and placed at the necessary density prior to sub-culture or seeding in wells for experimental needs.

Treatment: Hep G2 and CHO-K1 cells in log phase of growth were seeded in 96-well plates (100 µL of cell solution per well) at the initial concentration of 5×10^4 cells/mL and allowed 24 h to attach before treatment with **RSV** and ferrocene-**RSV** conjugate. Stock solutions of **RSV** and ferrocene-**RSV** conjugate **III** were prepared as 20 mM solutions in ethanol (EtOH) and stored at 4° C. Prior to use in cytotoxicity assay, the stock solutions were further diluted with culture medium to obtain final concentrations (10-100 µM). After 24 h, the media was replaced with fresh one containing different concentrations of **RSV** or ferrocene-**RSV** conjugate **III**. Cytotoxic effects were evaluated after 48h of exposition. Samples with ethanol without test compounds were used as controls. Final

concentration of ethanol did not exceed 0.5 % and had no interference with the biological activities tested. Cytotoxic effects were evaluated after 48h of exposition.

MTT Cytotoxicity Assay: The cytotoxicity of **RSV** and ferrocene-**RSV** conjugate **III** was determined by MTT assay. Cells were incubated with tetrazolium salt MTT {3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide} for 4h. The absorbance was measured at 570 nm on the microplate reader (microplate reader, model LKB 5060-006, LKB Vertriebs GmbH, Vienna, Austria).