

Substituted Steroidal Compounds Containing Amino and Amido Groups Reverse Multidrug Resistance of Mouse T-Lymphoma and Two Human Prostate Cancer Cell Lines *In Vitro*

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Abstract. *Background: Resistance to chemotherapy is a main problem in cancer. The search for new effective compounds that can increase sensitivity of resistant cells to existing chemotherapeutics is an urgent need. In previous studies, it has been demonstrated that steroid derivatives showed promising results concerning their capacity to modulate resistance of multidrug-resistant cell lines. Materials and Methods: Steroid derivatives were studied for their growth-inhibitory effect, cytotoxicity, reversal of multidrug resistance, apoptosis induction, and interaction with doxorubicin on multidrug resistant human ATP-binding cassette, sub-family B, member 1 (ABCB1) gene-transfected mouse T-lymphoma cell line, and human PC-3 and LNCaP prostate cancer cell lines in vitro. The steroidal interaction with P-glycoprotein (ABCB1) was investigated by molecular docking. Results: Both the activity of steroid derivatives on inhibition of the ABCB1 pump and their interaction with doxorubicin are dependent on the substituent groups of the investigated steroidal structures. Even though the investigated steroid derivatives were found to have limited antiproliferative effect on the three different cancer cell lines, in combination with doxorubicin, most of them acted as good potentiators. The binding energies from*

molecular docking ranged from –6.43 to –9.88 kcal/mol. The predicted inhibition constants ranged from 0.1 to 10.1 μ M. A significant negative correlation was found between binding energy and fluorescence activity ratio ($R=-0.5$, $p=0.015$). Conclusion: The effective compounds can be candidates of model molecules for possible application in the treatment of multidrug resistant cancer in rational drug design.

Prostate cancer has become the most common malignant disease among men, and the second leading cause of male cancer deaths in the USA. In the EU, prostate cancer is the most common cancer in men and over 417,000 new cases are diagnosed every year (1). Mortality from prostate cancer is increasing disproportionately with the aging of the male population within an overall population growth, and the explanation for this alarming trend is still elusive (2). In Hungary, for example, approximately 1250 men die from prostate cancer each year (3).

It is known that testosterone and 5 α -dihydrotestosterone (5 α -DHT) are androgens required for the development of both normal prostate and prostate cancer. Prostate-specific antigen (PSA), also known as gamma-seminoprotein or kallikrein-3 (KLK3), is a glycoprotein enzyme encoded in humans by the *KLK3* gene. PSA is a member of the kallikrein-related peptidase family and is secreted by the epithelial cells of the prostate gland (4). PSA is present in small quantities in the serum of men with healthy prostates, but is often elevated in the presence of prostate cancer or other prostate disorders (4). Since the PSA test is used for prostate cancer diagnosis, prostate cancer is detected at more localized/early stages. Nevertheless, depending on the tumor

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Key Words: steroids, multidrug resistance, ABCB1 transporter, mouse T-lymphoma, PC-3 prostate cancer cell line.

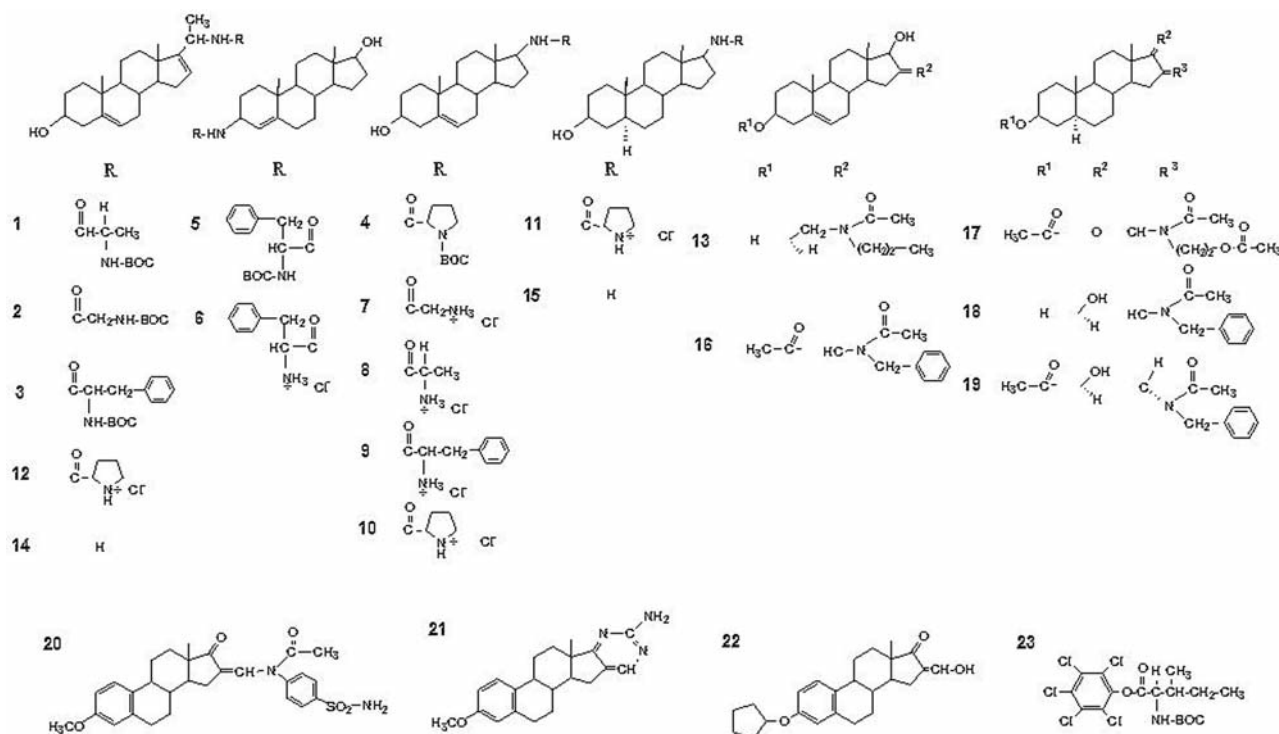


Figure 1. Chemical structure of the steroid derivatives: **1**: 20-*N*-(*N*'-BOC-*L*-alanyl)-aminopregna-5,16-dien-3 β -ol (MW=486.7); **2**: 20-*N*-(*N*'-BOC-glycidyl)-aminopregna-5,16-dien-3 β -ol (MW=472.67); **3**: 20-*N*-(*N*'-BOC-*L*-phenylalanyl)-aminopregna-5,16-dien-3 β -ol (MW=562.8); **4**: 17 β -*N*-(*N*'-BOC-*L*-prolyl)-aminoandrost-5-en-3 β -ol (MW=486.7); **5**: 3 β -*N*-(*N*'-BOC-*L*-phenylalanyl)-aminoandrost-4-en-17 β -ol (MW=536.76); **6**: 3 β -*N*-(*L*-phenyl-alanyl)-aminoandrost-4-en-17 β -ol hydrochloride (MW=473.11); **7**: 17 β -*N*(glycidyl)-aminoandrost-5-en-3 β -ol hydrochloride (MW=382.98); **8**: 17 β -*N*(*L*-alanyl)-aminoandrost-5-en-3 β -ol hydrochloride (MW=397.01); **9**: 17 β -*N*(*L*-phenyl-alanyl)-aminoandrost-5-en-3 β -ol hydrochloride (MW=473.11); **10**: 17 β -*N*(*L*-prolyl)-aminoandrost-5-en-3 β -ol hydrochloride (MW=423.04); **11**: 17 β -*N*(*L*-prolyl)-amino-5 α -androst-3 β -ol hydrochloride (425.06); **12**: 20-*N*(*L*-prolyl)-aminopregna-5,16-dien-3 β -ol hydrochloride (MW=449.09); **13**: 16-*N*(*n*-propyl)-*N*-acetyl-aminomethyl-androst-5-en-3 β ,17 β -diol (MW=403.5); **14**: 20-amino-pregna-5,16-dien-3 β -ol (MW=315.5); **15**: 17-amino-5 α -androst-3 β -ol (MW=291.48); **16**: 3 β -acethoxy,16*N*-acetyl,*N*-benzylaminomethylen-androst-5-en-17-one (MW=489.66); **17**: 3 β -acethoxy,16-(*N*-acethoxyethyl,*N*-ethyl)-aminomethylen-5 α -androst-17-one (MW=487.64); **18**: 16-(*N*-acetyl,*N*-benzyl)-aminomethylen-5 α -androst-3 β ,17 β -diol (MW=451.65); **19**: 3 β -acethoxy,16*E*-(*N*-acetyl,*N*-benzyl)-aminomethylen-5 α -androst-17 β -ol (MW=493.69); **20**: 16-(*N*-ethyl,*N*-*p*-sulfonylamidophenyl)-aminomethylene,3-methoxy-estra-1,3,5(10)-trien-17-one (MW=504.7); **21**: 16,17[*d*]-2'-aminopyrimidino,3-methoxy-estra-1,3,5(10)-triene (MW=335.45); **22**: 3-cyclopentyloxy,16-hydroxymethylen-estra-1,3,5(10)-trien-17-one (MW=366.5); **23**: *N*-BOC-*L*-isoleucine-*Opcp* (MW=477.68); where BOC is *tert*-butyl-oxy-carbonyland *Pcp* is pentachloro-phenylester.

differentiation stage, there is recurrence of the disease in 10-40% of cases, which require further treatment. Once hormonal therapy fails and disease progress to a castration-resistant stage, the question of chemotherapy arises (5).

Multidrug resistance plays a crucial role in the failure of treatment of all kinds of cancer, including prostate cancer. For the past 40 years, researchers have been investigating the various mechanisms by which cancer cells grown in tissue culture become resistant to anticancer drugs (6). These mechanisms include loss of a cell surface receptor acting as a transporter for a drug to the inside of the cell, increased metabolism of a drug and its detoxification, alteration by mutation of its specific target(s) (7), and the elevated expression of ATP-dependent drug-efflux pumps, therefore

reducing the accumulation of anticancer agents inside the cell, among others (8).

The efficient synthesis of substituted androstane by A-ring modification of picolinylideneandrostane derivatives has been reported (9). It has been suggested that this compound specifically interacts with cytochrome P450 17 α -hydroxylase/17,20-lyase (CYP17) with similar affinity to the abiraterone type of androstane drug clinically used in the treatment of prostate cancer. It has been found that different (17*E*)-picolinylidene-androstanes have selective antiproliferative activity against PC-3 prostate cancer cells, suggesting that substituted steroidal compounds containing amino and amido groups can be exploited as lead compounds in the development of new anticancer compounds for the treatment

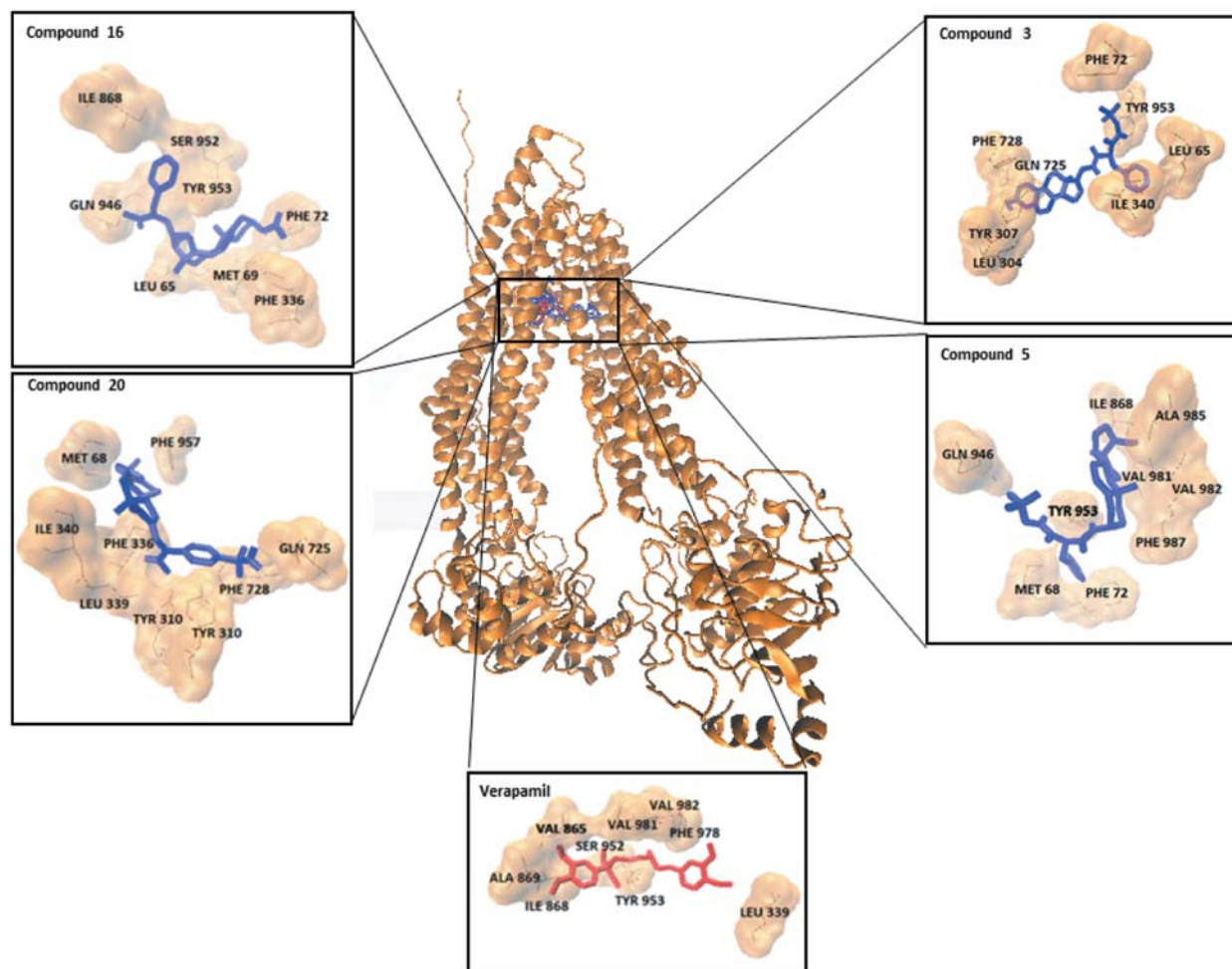


Figure 2. Interaction of four steroidal compounds (3, 5, 16 and 20) and verapamil at the transmembrane drug-binding pocket of ABCB1 (P-glycoprotein). The amino acids involved in the interactions and their positions are shown.

of prostate cancer (9, 10). Testosterone, progesterone and other important natural steroids contain ketone groups in position 3. Reduction of these groups into 3 β -hydroxy causes the loss of hormonal activity. In the case of estrogens, alkylation of the 3-phenolic hydroxyl group results in similar disappearance of their activity, therefore we also prepared derivatives of 3 β -hydroxy-pregnenes, -androstenes and 3-alkoxy-estratrienes. Amide groups were chosen as substituents. We considered that amide groups were able to attach to the peptide groups of proteins in cell membranes by hydrogen bridges, initiating an effect on the cell membrane components, including the ATP-binding cassette, sub-family B, member 1 (ABCB1) transporter. The starting steroids were transformed into 3-, 16-, and 21- amino derivatives and were acylated first with *N*-protected natural L amino acids. More simple compounds were also prepared in which the amide group is not a peptide-like moiety, but a simple acyl

amide, produced by acetylation of an alkyl-amino group (compounds 13, 16-19, and 20, Figure 1).

In the present study, substituted steroids were synthesized by using amido substituents and tested for their *in vitro* activity, including antiproliferative and cytotoxic effect, their capacity to inhibit the function of the ABCB1 membrane transporter, apoptosis induction effect and activity in combination with known chemotherapeutic agents, such as doxorubicin.

Materials and Methods

Compounds. Aminosteroids 14 and 15 were prepared from oximes of the corresponding ketosteroids by sodium tetrahydroborate reduction in the presence of NiCl₂, as described previously (11). The obtained aminosteroids were acylated with *N*-tert-butyl-oxy-carbonyl (BOC)-protected amino acids by mixed anhydride method (1-5) and the protecting group was eliminated with dry hydrogen chloride in dioxane solution, affording amine hydrochlorides 6-12. Alkyl-

Table I. Antiproliferative effect of steroid derivatives on L5178 mouse T-lymphoma parental cell line (PAR) and its L5178Y human ABCB1 gene- transfected subline (MDR), LNCaP and PC-3 prostate cancer cells. Each experiment was repeated at least three times (data are presented as the mean of three experiments).

Compound	PAR		MDR		LNCaP		PC3	
	IC ₅₀ (µg/ml)	SEM	IC ₅₀ (µg/ml)	SEM	IC ₅₀ (µg/ml)	SEM	IC ₅₀ (µg/ml)	SEM
1	10.87	0.63	15.82	0.78	13.41	0.99	12.04	1.24
2	9.80	0.24	19.13	0.1	13.09	1.41	12.49	1.09
3	>12.5	-	20.56	1.02	3.18	0.08	14.40	0.33
4	11.38	0.70	15.12	2.74	9.53	1.65	13.26	1.55
5	>12.5	-	26.20	0.91	15.82	0.51	>25	-
6	9.97	0.04	12.33	0.57	>25	-	>25	-
7	9.36	0.40	11.75	0.30	>25	-	>25	-
8	2.89	0.04	4.81	0.83	13.74	0.31	13.31	1.78
9	5.78	0.14	10.22	0.75	15.59	0.56	16.13	1.16
10	1.34	0.05	1.34	0.13	7.77	1.21	13.93	0.50
11	3.91	0.04	5.08	0.83	11.72	2.16	>12.5	-
12	0.99	0.01	1.12	0.16	4.68	0.41	5.21	0.66
13	1.47	0.23	8.83	2.84	>25	-	>25	-
14	4.19	0.04	5.57	0.47	16.61	1.93	13.22	0.55
15	>12.5	-	16.32	1.52	>25	-	>25	-
16	11.63	0.27	14.67	1.38	9.57	2.22	16.86	2.68
17	10.66	0.48	11.05	11.05	8.93	4.85	18.52	0.47
18	>12.5	-	>25	-	3.62	0.01	15.29	2.01
19	>12.5	-	>25	-	7.48	0.03	13.22	7.06
20	>12.5	-	>25	-	16.27	6.69	>25	-
21	>12.5	-	>25	-	>25	-	>25	-
22	11.71	0.16	6.80	1.86	10.15	2.57	14.54	0.61
23	>12.5	-	>25	-	9.23	1.60	15.00	0.70

IC₅₀: Half maximal inhibitory concentration; SEM: standard error of mean.

aminomethylene steroids were prepared from 16-hydroxymethylene-17-ketosteroids and primary amines and acetylated with acetic anhydride (compounds **16-20**) (12, 13). The D-condensed heterocyclic steroid **21** was prepared from 16-hydroxymethylene-3-methoxy-estra-1,3,5(10)trien-17-one with guanidine (14, 15). The 16-aminomethyl-androstene derivative **13** was synthesized from the corresponding 16-methylene-17-ketone by addition of *n*-propylamine and selective *O,O*-desacetylation of the full acetylated product (15). The chemical structures of the steroid derivatives evaluated in this study are shown in Figure 1. Samples of the above mentioned compounds were dissolved in dimethyl sulfoxide (DMSO) as 2.0 mg/ml stock solutions. Verapamil as positive control, DMSO and *N*-BOC-isoleucine-*O*-pentachloro-phenylester (compound **23**) were obtained from Sigma-Aldrich, St. Louis, MO, USA.

Cell cultures. The parental L5178 mouse T-cell lymphoma cells (L5178 PAR) (ECACC cat. no. 87111908, obtained from the Food and Drug Administration, Silver Spring, MD, USA) were transfected with pHa ABCB1/A retrovirus, as previously described by Cornwell *et al.* (16). The multidrug- resistant (MDR) ABCB1-expressing cell line L5178Y was selected by culturing the transfected cells in medium containing 60 ng/ml colchicine. L5178 PAR mouse T-cell

Table II. The comparison of the effect of aminosteroid derivatives on the activity of the ATP-binding cassette, sub-family B, member 1 (ABCB1) transporter in mouse T-lymphoma cells by flow cytometry. Compounds were tested at 2 µg/ml. Verapamil was used as positive control at 10 µg/ml.

Compound	FAR	Compound	FAR
Verapamil	17.21	13	58.79
1	54.28	14	2.20
2	37.64	15	1.76
3	59.28	16	75.48
4	37.92	17	16.69
5	29.07	18	77.41
6	9.21	19	52.90
7	10.21	20	45.82
8	1.47	21	1.07
9	39.14	22	10.43
10	1.19	23	12.26
11	2.77	DMSO	0.78
12	7.41		

FAR: Fluorescence activity ratio, calculated using the equation given in the Material and Methods; DMSO: dimethyl sulfoxide.

lymphoma cells and the MDR L5178Y human ABCB1-transfected subline were cultured in McCoy's 5A medium supplemented with 10% heat inactivated horse serum, 200 mM L-glutamine, and penicillin-streptomycin mixture at 100 U/l and 10 mg/l, respectively.

The human prostate cancer cell lines PC-3 (ATCC® CRL-1435) and LNCaP (ATCC® Cat. No. CRL-1740) were purchased from LGC Standards GmbH, Wesel, Germany. The cells were grown in RPMI-1640 medium with 2 mM L-glutamine and 10% fetal bovine serum supplemented with antibiotics. Adherent human cancer cells were detached with 0.25% trypsin and 0.02% EDTA for 5 min. The cell lines were incubated in a humidified atmosphere (5% CO₂, 95% air) at 37°C. The LNCaP cell line possesses androgen and oestrogen receptors, while the PC-3 cell line does not contain either of these receptors and is a suitable model for the hormone-resistant state.

Assay for antiproliferative effect of the compounds. The effects of increasing concentrations of compounds on cell growth were determined in 96-well flat-bottomed microtiter plates. The compounds were serially diluted in 100 µl of McCoy's 5A or RPMI-1640 medium, respectively. A total of 6×10³ mouse T-cell lymphoma cells (PAR or MDR) or 1×10⁴ PC-3 prostate cancer cells in 100 µl of medium were then added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37°C under 5% CO₂ for 72 h. At the end of the incubation period, 20 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) solution (from a 5 mg/ml stock) were added to each well and after a further 4 h, 100 µl of 10% sodium dodecyl sulfate (Sigma) in 0.01 N HCl were measured into each well. The culture plates were further incubated at 37°C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystem, Cheshire, WA, USA). In the assay, the solvent did not have any effect on the cell growth at the concentrations used for half maximal inhibitory concentration (IC₅₀) calculations. IC₅₀ values and the standard error of the mean (SEM)

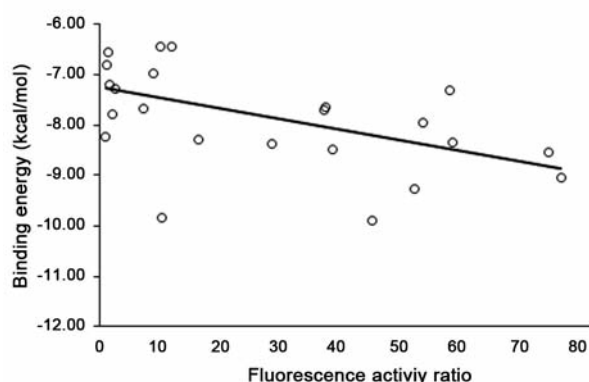


Figure 3. Correlation between lowest binding energies calculated by molecular docking and drug uptake data shown in Table II. (Pearson correlation test; $R=-0.5$; $p=0.015$).

of triplicate experiments were calculated using GraphPad Prism software version 5.00 for Windows with nonlinear regression curve fit (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

Assay for reversal of MDR in mouse T-lymphoma cells. The cell density of L5178 MDR and L5178Y PAR cell lines was adjusted to 2×10^6 cells/ml, and they were re-suspended in serum-free McCoy's 5A medium and distributed in 0.5 ml aliquots into Eppendorf centrifuge tubes. The tested compounds were added at a final concentration of 2 $\mu\text{g/ml}$ and the samples were incubated for 10 minutes at room temperature. Verapamil (Sigma) was applied as positive control (16) at 10 $\mu\text{g/ml}$. DMSO was added to the negative control tubes at the same volume used for the tested compounds. No activity of DMSO was observed. Next, 10 μl (5.2 μM final concentration) of the fluorochrome and ABCB1 substrate rhodamine 123 (Sigma) was added to the samples and the cells were incubated for a further 20 minutes at 37°C, then washed twice and re-suspended in 0.5 ml phosphate buffer saline (PBS) for analysis. The fluorescence of the cell population was measured with a Partec CyFlow® flow cytometer (Partec, Münster, Germany). The percentage mean fluorescence intensity was calculated for the treated MDR cells as compared with the untreated cells. A fluorescence activity ratio (FAR) was calculated based on the following equation (16) on the basis of the measured fluorescence values:

$$FAR = \frac{MDR_{treated} / MDR_{control}}{Parental_{treated} / Parental_{control}}$$

A checkerboard microplate method was applied to study the effect of drug interactions between the steroidal derivatives and chemotherapeutic drug doxorubicin on PC-3 cancer cells. The dilutions of doxorubicin were made in a horizontal direction in 100 μl , and the dilutions of the compounds vertically in the microtiter plate in 50 μl . The cells were re-suspended in culture medium and distributed into each well in 50 μl containing 6×10^3 cells. The plates were incubated for 72 h at 37°C in a CO₂ incubator. The cell growth rate was determined after MTT staining, as described above. Combination index (CI) values at 50% growth inhibition (ED₅₀) were determined using CompuSyn software (<http://www.combosyn.com>; ComboSyn,

Table III. Type of interaction between 16 amidosteroid derivatives and doxorubicin against the PC-3 prostate cancer cell line.

Compound	Ratio ^a	CI ^b	Interaction
1	5:1	0.44	Synergism
2	5:1	0.47	Synergism
3	10:1	0.91	No interaction
4	5:1	0.28	Strong synergism
8	10:1	0.40	Synergism
9	10:1	0.17	Strong synergism
10	10:1	0.26	Strong synergism
12	5:1	0.55	Synergism
14	10:1	0.29	Strong synergism
16	10:1	0.53	Synergism
17	10:1	0.38	Synergism
18	10:1	0.96	No interaction
19	20:1	0.61	Synergism
22	20:1	0.27	Strong synergism
23	20:1	0.30	Synergism

^aData are shown as the best combination ratio between doxorubicin and the tested compounds, respectively. ^bCombination index (CI) values at 50% of growth inhibition (ED₅₀) were determined by using CompuSyn software to plot four to five data points for each ratio. CI values were calculated by means of the median-effect equation, where $CI < 1$, $CI = 1$, and $CI > 1$ represent synergism, additive effect (*i.e.* no interaction), and antagonism, respectively.

Inc., Paramus, NJ, USA) to plot four to five data points for each ratio. CI values were calculated by means of the median-effect equation (17), where $CI < 1$, $CI = 1$, and $CI > 1$ represent synergism, additive effect (or no interaction), and antagonism, respectively.

Apoptosis assay. The capacity of the compounds to induce apoptosis was investigated using human PC-3 prostate cancer cell line. The cell density was adjusted to 5×10^5 cells/ml and cells were distributed in 0.5 ml aliquots into 24-well plates. The apoptosis inducer 12H-benzo[α]phenothiazine (M627) was kindly provided Professor Noboru Motohashi (Meiji Pharmaceutical University, Tokyo, Japan) used as positive control at 25 $\mu\text{g/ml}$. Compounds were tested at 4 $\mu\text{g/ml}$. Wells containing no M627 or steroidal compounds served as negative controls. The cells were incubated at 37°C for 3 h and at the end of the incubation period, the culture medium was removed, and the cells were washed with PBS and 0.5 ml of fresh culture medium was added to the cells. Samples were then transferred to 24-well culture plates and further incubated overnight at 37°C under 5% CO₂. Apoptotic activity of the compounds was evaluated using Annexin-V FITC Apoptosis Detection Kit (Cat. No. PF 032; Calbiochem, Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The fluorescence of cell population was analyzed immediately using a Partec CyFlow® flow cytometer (Partec).

Molecular docking. The 2D structures of the chemical entities were drawn and later energy-minimized into 3D structures using Corina Online Demo (18). All 3D structures were saved in PDB format ready to be docked. Molecular docking was conducted following a protocol previously reported by us (19). In brief, the X-ray crystallography-based structure of mouse P-glycoprotein and the homo-

Table IV. Results of molecular docking of steroidal compounds to the transmembrane proteins. Lowest binding energy, predicted inhibition constant (Pki) and amino acids (AA) involved in hydrogen bonding for each compound are shown. Each docking experiment was repeated three times (data are presented as the mean and SEM).

Compound	Binding Energy (kcal/mol)		Pki (μM)		Hydrogen bond-forming AA
	Mean	SEM	Mean	SEM	
Verapamil	-5.64	0.41	0.9	0.2	Val 862
1	-7.94	0.08	1.5	0.2	Gln 946
2	-7.71	0.19	2.4	0.7	-
3	-8.34	0.26	0.8	0.3	-
4	-7.66	0.02	2.4	0.1	Gln 347
5	-8.39	0.06	0.7	0.1	Tyr 953
6	-6.97	0.08	7.8	1.0	-
7	-6.44	0.01	18.9	0.2	Asp 188; Gln 195
8	-6.55	0.20	9.7	0.1	-
9	-8.49	0.06	0.6	0.1	Gln 347
10	-6.81	0.02	10.1	0.2	Asp 188
11	-7.30	0.06	4.5	0.4	Thr 945
12	-7.67	0.06	2.4	0.2	Asp 188; Gln 195
13	-7.31	0.09	4.4	0.7	-
14	-7.80	0.01	1.9	0.0	-
15	-7.20	0.00	5.5	0.3	Asp 188
16	-8.54	0.10	0.6	0.1	-
17	-8.30	0.18	0.9	0.2	-
18	-9.04	0.01	0.2	0.0	Asp 886; Lys 934
19	-9.27	0.03	0.2	0.0	-
20	-9.88	0.07	0.1	0.0	-
21	-8.23	0.12	1.1	0.0	-
22	-9.84	0.02	0.1	0.0	-
23	-6.43	0.10	19.5	3.5	-

logy-modelled structure of human P-glycoprotein were set as the rigid receptor molecule. The X-ray crystallography protein-based structures were first processed with AutodockTools-1.5.6rc3 (20) by the addition of essential hydrogen atoms to overcome the problem of incomplete structures due to missing atoms or water molecules. The output file after preparation was in PDBQT format, where information about atomic partial charges, torsion degrees of freedom and different atom types were added, e.g. aliphatic and aromatic carbon atoms or polar atoms forming hydrogen bonds. A grid box was then constructed to define docking spaces. The dimensions of the grid box were set around the whole P-glycoprotein molecule such that the ligand was able to freely move and rotate in the docking space. The grid box consisted of 126 grid points in all three dimensions (X, Y and Z) each separated by a distance of 1 Å. Energies at each grid point were then evaluated for each type of atom present in the ligand, and the values were then used to predict the energy of a particular ligand configuration. Docking parameters were set to 250 runs and 2,500,000 energy evaluations for each cycle. Docking was performed by Autodock4 (20) using the Lamarckian algorithm. The corresponding binding energies and the

number of conformations in each cluster were attained from the docking log files. The binding energies of the 23 compounds were correlated with their FARs using Pearson correlation coefficient.

Results

Twenty-three derivatives were synthesized and their antiproliferative (Table I) and cytotoxicity activities were studied on PAR, MDR, LNCaP, and PC-3 cell lines. Evaluation of the antiproliferative activity of the compounds revealed that compounds **8** and **10-14** were the most active against the parental mouse T-lymphoma cell line, with IC₅₀ values of less than 5 μg/ml. With the exception of compounds **9** and **13**, all the above-mentioned compounds and compound **22** were also active against the MDR cell line. With respect to the two different prostate cancer cell lines, the most effective compounds were **3**, **12**, **18**, **19**, and **23** against LNCaP cells, and compound **12** against PC-3 cells. Therefore, as per the results above, compound **12** was the only one with strong activity against all four cell lines studied.

The capacity of the compounds to inhibit the ABCB1 transporter was studied on MDR mouse T-lymphoma cells that were transfected with the human *ABCB1* gene (Table II). The MDR-reversal effect was studied at 2 μg/ml. Amino-steroid compounds acylated with BOC amino acids **1-5** and **9** and even simple *N*-acetyl derivatives **13**, **16**, **18**, **19** presented remarkable activity in the reversal of MDR of the MDR mouse T-lymphoma cell line, with FAR values of between 29 and 77. Steroids containing free amino groups or amine-hydrochloride substituents showed weaker or no activity on MDR reversal as measured by accumulation of rhodamine-123. Interestingly, compound **12** presented only mild inhibition at the concentration used. Concerning the activity of compounds expressed in μM, the most active compound was compound **18** because at a concentration of 4.4 μM, the FAR of compound **18** was 77.41. Moreover, compounds **1**, **3**, **13**, **16**, and **19** were also highly active.

The apoptosis-inducing effect of the compounds was measured at the non-toxic concentration of 4 μg/ml on the PC-3 cell line (data not shown). It was observed that there was no relevant activity of these compounds on apoptosis induction: the proportion of early apoptosis was between 1-6%, late apoptosis and necrosis between 1-5%, and cell death was around 1%. There was no significant difference when comparing these data with those of the untreated control.

The activity of the steroidal compounds in combination with doxorubicin was analyzed using checkerboard assay for PC-3 cells (Table III). It was observed that the effect of these compounds on the activity of doxorubicin ranged from ineffective to strongly synergistic. Strongly synergistic effects of modified steroids with doxorubicin were produced in the case of five compounds, namely **4**, **9**, **10**, **14**, and **22**. Synergy was found with derivatives **1**, **2**, **8**, **12**, **16**, **17**, **19**, and **23**. Two

compounds, **3** and **18**, were ineffective in this study. The IC_{50} of compounds **5-7**, **11**, **13**, **15**, **20** and **21** could not be calculated and this fact did not allow the determination of the degree of interaction of these compounds with doxorubicin.

We investigated *in silico* the interaction of the panel of steroid compounds with P-glycoprotein, using verapamil as a positive control. All 23 compounds had binding energies lower than that of verapamil (Table IV). The binding energies ranged from -6.43 to -9.88 kcal/mol. The predicted inhibition constants ranged from 0.1 to 10.1 μ M. The interaction of four of the test compounds, the control compound verapamil and the amino acids involved are shown in Figure 2. The binding sites of the compounds were found to coincide with the binding site of verapamil. The binding energies of the 23 compounds were correlated with their FARs using Pearson correlation coefficient (Figure 3). A significant negative correlation was found between the binding energies of the 23 compounds and their FARs ($R=-0.5$, $p=0.015$).

Discussion

The MDR transporters are one of the main causes of cancer treatment failures (21). Modulation of efflux pump-related mechanisms, in order to increase the activity of existing chemotherapeutics to which cancer becomes resistant, is one of the possible ways to overcome resistance. In our investigation, modified steroid derivatives without hormonal activity were chosen to achieve this effect.

Based on our studies, compounds **8**, **10-12**, **14**, and **22** had effective antiproliferative activity against MDR mouse T-lymphoma cells. These compounds are aminoacylamide salts applied to three different steroid skeletons (androstane, androstene, and pregnadienes). Compound **22** is an estrone ether derivative and it has a bidentate α -hydroxymethylene-ketone arrangement on the D-ring, rendering the possibility of a 1,4-hydrogen donor-acceptor connection. On the other hand, compounds **3**, **12**, **18**, **19**, and **23** proved to be effective in the antiproliferative assays against the LNCaP prostate cell line.

In the MDR-reversal studies, the majority of the investigated compounds were effective: **1-5**, **9**, **13**, **16-20** (Table II). Compounds **6**, **8**, **10-12**, **14**, **15**, and **21** had only a weak effect. These latter molecules either contain amine hydrochloride, or have special structures; the latter three compounds (**14**, **15**, and **21**) are estrone ether derivatives. Aminosteroids with basal primary amino groups had practically no effect. The compounds of remarkable activity contain four different steroid skeletons, four different aminoacyl parts (glycyl, alanyl, phenylalanyl and prolyl) and, in the case of the simpler molecules, three different alkyl (propyl, benzyl and acetoxyethyl) moiety. The only common structural element amongst them is the amide part, deriving from an α -amino acid or acetic acid. In compounds **18** and **19**, a double bond

is present in the A and B rings, in addition to there being no H atom at the C-5 position. This H atom either induces or is responsible for conformational changes in the four condensed rings with chair-like structure. In the case of compound **13**, the hydrophobic steroid containing acyl-amide can help binding to the beta-turn of the ABCB1 protein. We suppose that the biologically active *N*-protected BOC compounds bind to particular beta-turns of the ABCB1 transporter. Possibly, there are preferred amino acids in the beta-turns that may have a key role in maintaining the functionally active conformations of the ABCB1 transporter localized in the cell membrane. Molecular docking is an effective computational technique which estimates the binding energy of a compound to the target protein. A scoring system is used to detect the ideal docking configuration. The amino acids involved in hydrophobic and hydrogen bond interactions are also predicted by the algorithm. Our results from molecular docking suggest that the compounds tested inhibit P-glycoprotein activity through binding to the drug-binding pocket, which is the same binding site as for verapamil. Most of the compounds with high activity, and verapamil, share one or more amino acids in their binding sites. It appears that there is a significant correlation between the binding energies of the compounds and their FARs. It can be suggested that molecular docking might be used as a tool predictive of the activity of steroidal compounds as inhibitors of P-glycoprotein.

A large number of compounds were able to enhance the activity of doxorubicin against the PC-3 cell line in combination experiments. Based on our results, compound **9** could be a lead compound for further investigations related to combined chemotherapy; in addition, compound **12** might be a promising candidate as an effective antiproliferative chemotherapeutic agent.

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References

- 1 Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, Forman D and Bray F: Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012. *Eur J Cancer* 49: 1374-1403, 2013.

- 2 Sapi Z, Bodo M, Vadasz G, Henson DE and Haas GP: The increasing incidence of prostate cancer in Hungary. *In Vivo* 8: 433-436, 1994.
- 3 Bray F, Ferlay J, Forman D, Lortet-Tieulent J and Auvinen A: Prostate cancer incidence and mortality trends in 37 European countries. *Eur J Cancer* 46: 3040-3052, 2010.
- 4 Catalona WJ, Richie JP, Ahmann FR, Hudson MA, Scardino PT, Flanigan RC, Dekernion JB, Ratliff TL, Kavoussi LR and Dalkin BL: Comparison of digital rectal examination and serum prostate-specific antigen in the early detection of prostate cancer: results of a multicenter clinical trial of 6,630 men. *J Urol* 151: 1283-1290, 1994.
- 5 Pfister D, Heidenreich A and Porres D: Biomarker docetaxel-based chemotherapy. *Urologe A* 52: 1261-1264, 2013.
- 6 Gottesman MM: Mechanisms of cancer drug resistance. *Annu Rev Med* 53: 615-627, 2002.
- 7 Longo-Sorbello GS and Bertino JR: Current understanding of methotrexate pharmacology and efficacy in acute leukemias. Use of newer antifolates in clinical trials. *Haematologica* 86: 121-127, 2001.
- 8 Szakács G, Paterson JK, Ludwig JA, Booth-Gentle C and Gottesman MM: Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* 5: 219-234, 2006.
- 9 Ajduković JJ, Djurendić EA, Petri ET, Klisurić OR, Celić AS, Sakač MN, Jakimov DS and Gaši KM: 17(E)-Picolinylideneandrostane derivatives as potential inhibitors of prostate cancer cell growth: antiproliferative activity and molecular docking studies. *Bioorg Med Chem* 21: 7257-7266, 2013.
- 10 Pintér O, Molnár J, Tóth C, Szabó Z, Lipták J, Fél P, Papp G, Hollman E, Hazay L, Sztreit B, Kisbenedek L, Fehér M, Kocsis I and Pajor L: Administration of estramustin in response to changes in prostate-specific antigen and Karnofsky Index in the treatment of prostate cancer. *In Vivo* 19: 787-792, 2005.
- 11 Szendi Z, Dombi G and Vincze I: Steroids, LIII: New routes to aminosteroids. *Monatshefte für Chemie* 127: 1189-1196, 1996.
- 12 Vincze I, Hackler L, Szendi S and Schneider G: Steroids 54. Amino acylamidosteroids. *Steroids* 61: 697-702, 1996.
- 13 Vincze I, Somlai Cs, Schneider Gy, Dombi Gy and Mák M: Steroids. XLV. Neighbouring group participation. XII. Decomposition of (Z)-16-amidomethylene-17b-hydroxy steroids mediated by neighbouring group participation. *Liebigs Annal Chemie* 3: 187-192, 1992.
- 14 Forgo P and Vincze I: Syntheses and advanced NMR structure determination of androsteno-[17,16-d]-pyrimidine derivatives. *Steroids* 67: 749-756, 2002.
- 15 Serly J, Vincze I, Somlai Cs, Hodonicki L and Molnár J: Synthesis and comparison of the antitumor activities of steroids on ABCB1-transfected mouse lymphoma and human ovary carcinoma. *Lett Drug Des Discov* 8: 138-147, 2011.
- 16 Cornwell MM, Pastan I and Gottesman MM: Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J of Biol Chem* 262: 2166-2170, 1987.
- 17 Chou TC and Martin N: CompuSyn for Drug Combinations: PC Software and User's Guide: A Computer Program for Quantitation of Synergism and Antagonism in Drug Combinations, and the Determination of IC₅₀ and ED₅₀ and LD₅₀ Values, Combo-Syn Inc. Paramus, NJ, USA, 2005.
- 18 Zeino M, Saeed MEM, Kadioglu O and Efferth T: The ability of molecular docking to unravel the controversy and challenges related to P-glycoprotein-a well-known, yet poorly understood drug transporter. *Invest New Drugs* 32: 618-625, 2014.
- 19 Zhao Q, Zeino M, Eichhorn T, Hermann J, Müller R and Efferth T: Molecular docking studies of myxobacterial disorazoles and tubulysins to tubulin. *J of Biosci Med* 3: 31-43, 2013.
- 20 Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS and Olson AJ: AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* 30: 2785-2791, 2009.
- 21 Spengler G, Molnar J, Viveiros M and Amaral L: Thioridazine induces apoptosis of multidrug-resistant mouse lymphoma cells transfected with the human ABCB1 and inhibits the expression of P-glycoprotein. *Anticancer Res* 31: 4201-4205, 2011.

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