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Novel alkylaminoethyl derivatives of androstane 3-oximes as anticancer candidates: synthesis and evaluation of cytotoxic effects†

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Steroid anticancer drugs are the focus of numerous scientific research efforts. Due to their high cytotoxic effects against tumor cells, some natural or synthetic steroid compounds seem to be promising for the treatment of different classes of cancer. In the present study, fourteen novel *O*-alkylated oxyimino androst-4-ene derivatives were synthesized from isomerically pure 3*E*-oximes, using different alkylaminoethyl chlorides. Their *in vitro* cytotoxic activity was evaluated against eight human cancer cell lines, as well as against normal fetal lung (MRC-5) and human foreskin (BJ) fibroblasts, to test the efficiency and selectivity of the compounds. Most derivatives displayed strong activity against malignant melanoma (G-361), lung adenocarcinoma (A549) and colon adenocarcinoma (HT-29) cell lines. Angiogenesis was assessed *in vitro* using migration scratch and tube formation assays on HUVEC cells, where partial inhibition of endothelial cell migration was observed for the 17α-(pyridin-2-yl)methyl 2-(morpholin-4-yl)ethyl derivative. Among the compounds that most impaired the growth of lung cancer A549 cells, the (17*E*)-(pyridin-2-yl)methylidene derivative bearing a 2-(pyrrolidin-1-yl)ethyl substituent induced significant apoptosis in these cells. In combination with low cytotoxicity toward normal MRC-5 cells, this molecule stands out as a good candidate for further anticancer studies. In addition, *in vitro* investigations against cytochrome P450 enzymes revealed that certain compounds can bind selectively in the active sites of human steroid hydroxylases CYP7, CYP17A1, CYP19A1 or CYP21A2, which could be important for the development of novel activity modulators of these enzymes and identification of possible side effects.

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Introduction

New global cancer data (from December 2020) suggests that cancer diagnoses have increased to 19.3 million, while cancer mortality has risen to 10 million. The International Agency for Research on Cancer (IARC) estimated that one in five people worldwide will suffer from cancer during their lifetime, where lung and prostate cancers are the most commonly diagnosed in men, while breast cancer is the most common form among women.[1]

The main reasons for the failure of available chemotherapy for cancer treatment are the lack of selectivity of conventional drugs, metastatic spreading of initial tumors, multidrug resistance and the heterogeneity of the disease. These disadvantages have inspired medicinal chemists to design and develop safer, target-specific, and effective steroid anticancer agents.[2–4] Structurally diverse cytotoxic and cytostatic steroids are very relevant as lead compounds and molecular probes for anticancer drug discovery programs and the elucidation of the molecular mechanisms of anticancer compounds.[4]

Steroids are currently considered to be relevant scaffolds for the development of new anticancer drugs, thanks to their selectivity, suitable physicochemical properties, and reduced side effects when applied as drugs. Synthetic analogues of natural steroids are widely used in the treatment of cancers of the reproductive tissues.[5,6] Besides, several steroids have been reported to exert pronounced anticancer effects in hormone-independent tumors.[7] 2-Methoxyestradiol, an endogenous

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metabolite of estradiol without hormonal activity, exhibits potent antiproliferative activity against various tumor cell lines *in vitro* and inhibits tumor growth *in vivo*.[8] It was also demonstrated to induce programmed cell death in endothelial cells and suppresses cancer-related angiogenesis.[9,10]

Since many chemotherapeutics used in oncological praxis express nonselective cytotoxicity, today one of the most commonly used targets for the study of the side effects of new drug candidates are cytochrome P450 enzymes (CYP). Human steroid hydroxylases, cholesterol 7α-hydroxylase (CYP7A1), 25-hydroxycholesterol 7α-hydroxylase (CYP7B1), steroid 17α-hydroxylase/17,20-lyase (CYP17A1), aromatase (CYP19A1) and 21-hydroxylase (CYP21A2) (Fig. 1) are key CYP enzymes involved in the biosynthesis of cholesterol, bile acids, neurosteroids, progestins, androgens, estrogens and corticosteroids. Consequently, changes in their endogenous levels and activity are connected with different diseases, including prostate and breast cancer.[11–13] Their key importance for normal human physiology is therefore related to their use as targets in the development of highly effective and selective drugs (especially CYP17A1, CYP19A1). Based on this, there are two important aspects of testing the effects of novel compounds against CYPs: their antihormonal effects, and the establishment of new types of biological activity for synthetic steroid derivatives, particularly for prospective anticancer agents, which can help to diminish possible problems during the development of novel drugs.

Oxyimino ethers have attracted much interest as important biologically active compounds and precursors for the preparation of a wide variety of drugs and natural products.[14–16] Some steroid oximes and oxime-ethers have been shown to have antioxidant,[17] antimicrobial,[17–19] antineoplastic[20–22] or neuromuscular blocking[23] activities. *O*-Alkylated oximes derivatized with an alkylaminoethyl side chain have also been reported as effective candidates for cytotoxic drugs. In view of these findings and in continuation of our previous work[7,24–27] on the synthesis and biological activity of androstane derivatives, we have synthesized novel oxime–ether derivatives in 17α-(pyridin-2-yl) methyl series (**4–10**) and in (17*E*)-(pyridin-2-yl)methylidene series (**14–20**), by the reaction of androst-4-ene 3*E*-oxime **2** and **12**, using various alkylaminoethyl chlorides. The biological effects of these new compounds were then studied *in vitro* on several types of human steroid-converting CYPs and on human cancer cell lines.

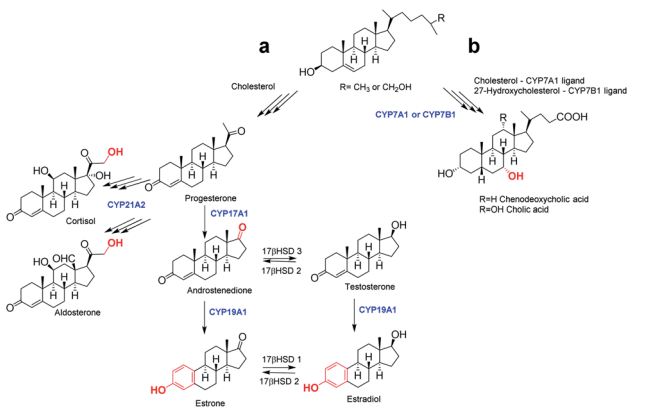


Fig. 1 Simplified scheme of biosynthesis of steroid hormones (a) and bile acids (b).

Results and discussion

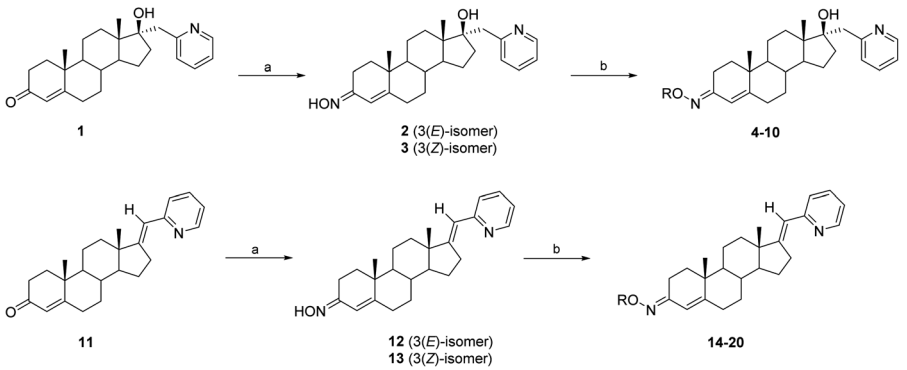
Chemistry

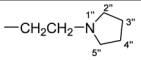
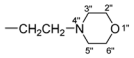
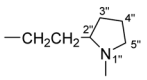
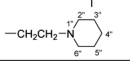
Interest in the development of steroid derivatives remains the focus of many synthetic chemists,[28–30] due to their significant antitumor potential. Heterocyclic systems with a nitrogen atom in the steroid nucleus have been consistently confirmed as structural features that have led to improved biological activity for steroid compounds.[31–38] Based on relevant literature data, as well as our previous research, the main objective of the present study was to introduce a new structural element with a nitrogen atom in the A ring of 17-substituted androst-4-ene compounds, in order to obtain new derivatives with selective anticancer properties.

The 17α-(pyridin-2-yl)methyl 3-oximes **2** and **3**, or (17*E*)-(pyridin-2-yl)methylidene 3-oximes **12** and **13**, were synthesized from the corresponding androst-4-en-3-ones **1** and **11**,[39] the oximation of which was carried out with hydroxylamine-hydrochloride and sodium-acetate in refluxing ethanol, according to the sequence shown in Scheme 1.[26] The *E*/*Z* configuration in these isomeric oximes has been assigned on the basis of the NMR data reported in the literature, for compounds with a similar A-ring, which are in good correlation with our data.[40] In the ^{1}H NMR spectra, protons from the oxyimino groups in *E*-isomers were found at 10.48 and 10.47 ppm (for compounds **2** and **12**), while for the *Z*-isomers these were registered at lower chemical shifts, at 10.22 and 10.21 ppm (for compounds **3** and **13**). This can be explained by the reduced electron density in *E* isomer, as evident by the observed "deshielding" effect.

To synthesize new *O*-alkylated derivatives, only 3*E*-isomeric oximes **2** and **12** were subjected to further transformation following previously published procedures.[23–25] Compounds **2** and **12** were condensed with different alkylaminoethyl chlorides to obtain desired oxime ethers **4–10** and **14–20**, respectively (Scheme 1). A total of fourteen alkylaminoethoxyimino androstane derivatives were prepared at different reaction times and yields (Table 1).

All new compounds were fully characterized by IR, ^{1}H NMR and ^{13}C NMR spectra (available in ESI†), as well as by mass spectrometry analysis. In the ^{1}H NMR spectra of all *O*-alkylated compounds, the appearance of triplets for –CH_2N< group at about 2.80 ppm, and for –OCH_2– group at about 4.20 ppm was observed. The presence of the pyrrolidine ring in compounds **4** and **14** was confirmed by the ^{13}C NMR spectrum, where C-3″ and C-4″ carbons are equivalent and give one signal located at 23.52 or 23.47 ppm, respectively. An intense singlet originating from two *N*-methyl groups was observed in the ^{1}H NMR spectrum of compounds **5** and **15** at 2.24 ppm. Singlets at 2.36 and 2.35 ppm, corresponding to protons from the *N*-methyl groups of the pyrrolidine ring, were observed in the ^{1}H NMR spectra of



Compound	R	Compound	R
4, 14		**8, 18**	
5, 15	$-CH_2CH_2CH_2N(CH_3)_2$	**9, 19**	$-CH_2CH_2N(CH_2CH_3)_2$
6, 16		**10, 20**	$-CH_2CH_2N(CH_3)_2$
7, 17			

Scheme 1 Synthetic routes to compounds **2–10** and **12–20**. Reagents and conditions: (a) $NH_2OH \cdot HCl$, AcONa, 95% EtOH, reflux, 1.5 h; isomer separation by flash chromatography; (b) R–Cl·HCl, anh. KOH, anh. K_2CO_3, anh. butanone, reflux.

Table 1 Reaction times and yields

Compound	t (h)	Yield (%)	Compound	t (h)	Yield (%)
4	48	52.4	**14**	72	35.6
5	48	71.8	**15**	48	43.0
6	72	26.0	**16**	72	9.1
7	48	47.2	**17**	96	53.2
8	96	54.9	**18**	96	56.2
9	48	47.9	**19**	115	14.6
10	49	76.3	**20**	48	30.5

compounds **6** and **16**. The presence of the piperidine ring in compounds **7** and **17** is indicated by signals in the ^{13}C NMR spectrum located at 25.80 and 54.85 ppm (for **7**), and at 25.59 and 54.76 ppm (for **17**), which correspond to the equivalent carbons C-3″ and C-5″, as well as C-2″ and C-6″, respectively. Further, two multiplets at 2.52 and 3.73 ppm were observed in the ^1H NMR spectra of compounds **8** and **18**, indicating the presence of equivalent H-3″ and H-5″ protons, and H-2″ and H-6″ protons, respectively, from the morpholine ring. In the ^1H NMR spectra of compounds **9** and **19**, triplets at 1.05 and 1.07 ppm, and quartets at 2.63 and 2.65 ppm, respectively, corresponding to protons from two *N*-ethyl groups were detected. The presence of two nitrogen-bonded methyl groups in compounds **10** and **20** is indicated by intense signals in the ^1H NMR spectrum at 2.33 and 2.24 ppm, as well as in the ^{13}C NMR spectrum at 45.85 or at 45.43 ppm, respectively. Finally, in the ^1H NMR spectra of all new derivatives **4–10** and **14–20**, the absence of a signal at approximately 10 ppm (from the oxyimino group) was observed, which further confirms the presence of substitution on C_3-oxime by the corresponding alkyl group.

Cytotoxic activity

The first steps in investigating the potential of newly synthesized *O*-alkylated androstane compounds for the development of effective anticancer compounds were cytotoxicity assays. The cytotoxic activity of selected compounds was evaluated against several common human tumor cell lines, as well as on normal fetal lung cells (MRC-5) and human foreskin fibroblasts (BJ), in order to determine the antitumor activity and selectivity of the compounds. Cytotoxic activity was assessed *in vitro* after 72 h treatment using the MTT[41] or alamarBlue (resazurin)[42] assay. The results were compared with the effect of the widely used nonselective chemotherapeutic drug cisplatin (Cis-Pt) and the steroid aromatase inhibitor formestane (For), which was used as a control for general steroid toxicity.

Previously, we reported the cytotoxic activities of parent compounds in the 17α-(pyridin-2-yl)methyl (**1**) and (17*E*)-(pyridin-2-yl)methylidene series (**11**).[43] Interestingly, the majority of the newly synthesized compounds in both series appear to be most active against lung adenocarcinoma cells (A549) (Table 2), which is in contrast to results published earlier.[26] Of these, both isomeric oximes **12** and **13** of the (17*E*)-(pyridin-2-yl)methylidene series, and pyrrolidine derivative **14** showed the strongest cytotoxicity (IC_{50} 1.5, 1.8 and 2.0 μM, respectively), and all of these substances were more cytotoxic than cisplatin. Compounds **7** and **18**, containing piperidine or morpholine moiety, also displayed significant cytotoxic activity (IC_{50} 5.4 and 5.6 μM), while derivatives **4**, **6**, **9** and **10** of the 17α-(pyridin-2-yl)methyl series as well as (17*E*)-(pyridin-2-yl) methylidene derivative **15** with 3-(*N*,*N*-dimethylamino)propyl group exhibited moderate cytotoxicity for A549 (IC_{50} 11.8, 14.1, 11.8, 13.0 and 18.9 μM, respectively).

Table 2 The IC_{50} values (50% inhibitory concentration) of the tested steroid compounds **2–10** and **12–20**, and reference compounds cisplatin (Cis-Pt) and formestane (For) after 72 h of treatment. Standard deviations of the mean results are within ±10%[a]

	IC_{50} [μM], after 72 h incubation									
Assay	MTT							alamarBlue		
Cell line/compound	MCF-7	MDA-MB-231	PC-3	HeLa	HT-29	A549	MRC-5	CEM	G-361	BJ
2	>100	>100	>100	13.8	>100	30.3	>100	>50	48.0	>50
3	>100	38.9	>100	>100	>100	52.4	N/A	>50	>50	>50
4	87.5	>100	>100	22.2	>100	11.8	>100	19.5	3.6	14.2
5	>100	>100	>100	18.4	>100	>100	>100	16.6	1.9	7.6
6	10.5	>100	49.5	68.0	18.4	14.1	>100	13.0	2.6	6.1
7	>100	47.4	14.0	>100	>100	5.4	>100	16.5	2.5	7.2
8	>100	30.7	>100	>100	>100	>100	N/A	21.1	7.1	>50
9	>100	23.9	20.2	N/A	11.6	11.8	>100	14.9	2.5	7.3
10	>100	>100	14.5	>100	>100	13.0	>100	34.6	3.4	16.7
12	41.0	47.3	>100	>100	4.4	1.5	>100	>50	45.3	>50
13	44.9	5.2	57.7	>100	10.6	1.8	>100	>50	46.6	>50
14	>100	4.7	77.1	22.6	3.3	2.0	>100	30.4	8.9	25.3
15	15.1	44.6	>100	>100	>100	18.9	>100	13.9	2.8	7.5
16	7.0	>100	>100	>100	>100	>100	>100	21.9	13.6	27.6
17	>100	19.1	>100	32.2	23.9	21.3	>100	18.0	4.7	20.0
18	>100	26.8	>100	44.1	7.8	5.6	>100	21.9	11.4	>50
19	>100	>100	>100	>100	>100	26.6	86.4	23.8	3.8	14.7
20	>100	>100	69.5	>100	13.0	82.8	>100	22.7	10.6	34.2
Cis-Pt	1.6	2.6	4.5	2.1	4.1	3.2	0.2	0.8	4.5	9.6
For	>100	19.6	26.4	3.4	>100	38.6	>100	–	–	–

[a] N/A – IC_{50} value was not available due to nonlinear dose dependence or hormetic effect.

Colon cancer cells HT-29 were more sensitive to (17*E*)-(pyridin-2-yl)methylidene series, where 3*E*-oxime **12**, pyrrolidine **14** and morpholine derivative **18** exhibited low micromolar IC_{50} at 4.4, 3.3 and 7.8 μM, respectively, while compounds **6**, **9**, **13** and **20** showed moderate cytotoxicity to HT-29 (IC_{50} 18.4, 11.6, 10.6 and 13.0 μM, respectively). Compounds **13** (with 3*Z*-oxyimino function) and **14** (with pyrrolidine ring) showed strong cytotoxic activity (IC_{50} 5.2 and 4.7 μM, respectively) against estrogen receptor negative (ER−) breast cancer cell line (MDA-MB-231). It is not the first time that we have encountered derivatives that can not only stop the proliferation of triple-negative breast cancer cells but also do it selectively.[7] In addition, compound **16** with *N*-methylpyrrolidin ring expressed significant cytotoxicity (IC_{50} 7.0 μM) against the ER+ breast cancer cell line MCF-7, while formestane, aromatase inhibitor used in clinical praxis in the treatment of estrogen-dependent breast carcinoma, showed no toxicity toward these tumor cells. The cytotoxicity results also revealed that 17α-(pyridin-2-yl)methyl derivative **7** with a piperidine ring and derivative **10** with a (*N*,*N*-dimethylamino)ethyl group showed mild cytotoxicity against androgen receptor negative (AR-) prostate cancer PC-3 cells (IC_{50} 14.0 and 14.5 μM, respectively), similar to 3*E*-oxime **2** and 3-(*N*,*N*-dimethylamino)propyl derivative **5**, which were moderately but also selectively toxic when tested on HeLa cervix carcinoma cells (IC_{50} 13.8 and 18.4 μM, respectively). Finally, it should be noted that all newly synthesized compounds were confirmed to be non-toxic to the normal MRC-5 cells, while cisplatin was very toxic to these cells.

Furthermore, two additional human cancer cell lines (CEM, acute leukemia cell line; G-361, malignant melanoma cell line) and normal human skin fibroblasts (BJ) were also used to evaluate the cytotoxicity of eighteen derivatives after 72 h treatment (Table 2). Seven compounds (**4**, **5**, **6**, **7**, **9**, **15** and **17**) were active against CEM cells in the lower micromolar range; seven exhibited moderate cytotoxicity (**8**, **10**, **14**, **16**, **18**, **19** and **20**); while four compounds (**2**, **3**, **12** and **13**) showed zero activity. The similar series of compounds (**4–10**, **14–15**, **17** and **19**) was also very toxic to melanoma G-361 cells after 72 h (<10 μM), and their cytotoxicity was comparable to that of Cis-Pt. Of all *O*-

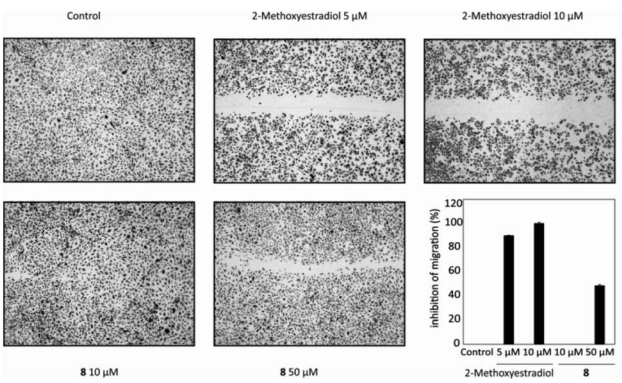


Fig. 2 Migration of HUVECs after 20 h of treatment with **8** (10 and 50 μM) compared with untreated control and positive control (2-methoxyestradiol). Experiments were repeated three times in triplicate.

alkylated compounds, only morpholine derivatives **8** and **18** showed no cytotoxicity against normal human BJ fibroblasts. Moreover, 17α-(pyridin-2-yl)methylandrostene **8** expressed stronger cytotoxic activity against melanoma G-361 cells (IC_{50} 7.1 μM), and therefore it was selected for further experiments to test anti-angiogenic and anti-inflammatory activity *in vitro*.

To examine the influence of new androstane-derived compounds on angiogenesis *in vitro*, migration scratch and tube formation assays on HUVEC cells treated with 10 and 50 μM of morpholine derivative **8** for 20 hours were performed. Compound **8**, which was selected for this experiment thanks to its strong antiproliferative effect against melanoma G-361 cells, inhibited the migration of endothelial cells after application of 50 μM of this compound by 48% compared to 10 μM 2-methoxyestradiol as a positive control (Fig. 2). However, both tested concentrations did not affect the creation of tube-like structures by HUVECs (data not shown). Derivative **8** had also no influence on the expression of the inflammatory adhesion molecule E-selectin on the cell surface after 4 h of HUVECs' treatment (data not shown).

Apoptosis induction

Some of the tested compounds have been shown to have an antiproliferative effect on human tumor cell lines that respond poorly to hormone therapy. Here we tried to detect the presence of apoptosis and compare in, as much as possible, the same conditions for samples, rather than to precisely determine the percentage of apoptosis. Since compounds **12**, **13** and **14** showed potent micromolar cytotoxicity against lung adenocarcinoma A549 cells, we investigated whether the selected test compounds induced programmed cell death in this cell line as a model system. Apoptosis in cells was monitored by a fluorescent double-staining method using acridine orange and ethidium bromide dyes after cells treatment for 72 h with equitoxic concentrations of steroid compounds, equal to their IC_{50}s. Results are shown in Fig. 3 as the ratio of fluorescent signals (red to green) for a control sample of untreated cells (**Ctrl**) and samples treated with selected compounds **12**, **13** and **14**, and were obtained using the program *ImageJ*.

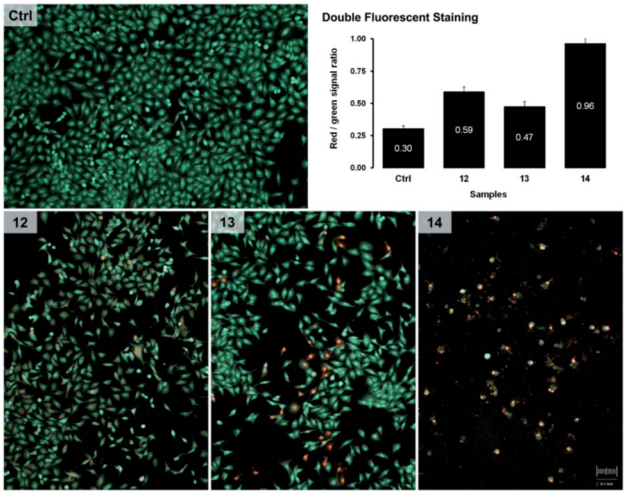


Fig. 3 Results of apoptosis obtained by the double-fluorescent staining method. The chart shows the ratio of red and green channel density measured in *ImageJ* computer program for samples of untreated A549 cells (**Ctrl**) and samples treated 72 h with IC_{50} concentrations of the selected compounds **12**, **13** and **14**. Images of stained cells are photographed from 6-well plates turned bottom up, using a fluorescence microscope (Olympus BX51) at 400× magnification.

As shown in Fig. 3, fluorescent signals indicating apoptotic changes were detected in all samples of A549 cells treated with test compounds, where derivative 14 was the most effective in inducing apoptosis. The intensity of apoptotic changes in cells treated with compounds **12–14** can also be observed on microphotographs of specimens stained with fluorescent dyes.

The oxime derivatives **12** and **13**, as well as *O*-alkylated pyrrolidine derivative **14**, showed strong cytotoxic activity and the potential to induce apoptosis in A549 lung adenocarcinoma cells, while at the same time being non-toxic to normal lung fibroblasts MRC-5. These findings classify them in the group of rare selective antitumor compounds whose steroid core is likely to have low general cytotoxicity in humans. Although other compounds have shown lower cytotoxicity, they have also been selective, and such properties are desirable in candidates for the development of new antitumor drugs.

In vitro screening toward human steroid hydroxylases

Screening of novel steroid oximes against a palette of human steroid hydroxylases including CYP7A1, CYP7B1, CYP17A1, CYP19A1 and CYP21A2 (Fig. 1) was performed to identify novel ligands of these enzymes. The results obtained in such binding tests may direct further research towards the design of novel compounds or further biomedical studies, depending on whether the compounds bind to the desired CYP enzyme. The key importance of these enzymes for normal human physiology is therefore related to their use as targets in the development of highly effective and selective drugs, or in the identification of possible side effects during administration. Binding affinities of CYPs for new androstane-derived compounds are presented in Table 3.

It was found that novel compounds are able to bind either as substrate-like ligands (leading to the displacement of the water molecule from the Fe coordination sphere) or as inhibitor-like molecules (leading to the replacement of the water molecule from the Fe coordination sphere). In all cases, the amplitude of the spectral response is quite small (compared to known ligands of these enzymes). This means that only a minor fraction of corresponding P450 enzymes (in terms of different protein conformations) can bind substrate molecules.

In the case of CYP7A1 and CYP7B1 enzymes, that are involved in the synthesis of bile acids, compounds **5**, **10** and **18** were detected as inhibitors based on spectral data, meaning that the nitrogen atom of these compounds shares unpaired electrons with the Fe^{2+} of the cofactor. Moreover, in the case of CYP7B1, a dual-type binding mode was detected, which means that there are two possible orientations of the ligands in the active site of the enzyme.

Table 3 *In vitro* binding affinity of novel steroid compounds **2–10** and **12–20** toward steroid-converting CYPs

Compound	CYP7A1	CYP7B1	CYP17A1	CYP19A1	CYP21A2
5	Type II binding (inhibitor-like molecule) $\Delta A = 0.004$ C = 0–98 μM $K_d \sim 0.2$ μM[a] ($R^2 = 0.6$)	—	—	—	—
10	—	Type I + type II binding[b]	—	—	—
17	—	—	—	—	Type I binding (substrate-like molecule) $\Delta A = 0.004$ C = 0–49 μM $K_d = 3.6 \pm 1.0$ μM ($R^2 = 0.93$)
18	—	Type I + type II binding[b]	—	—	—
19	—	—	Type I binding (substrate-like molecule) $\Delta A = 0.012$ C = 0–49 μM $K_d = 8.6 \pm 1.5$ μM ($R^2 = 0.97$)	—	—
20	—	—	—	Type I binding (substrate-like molecule) $\Delta A = 0.004$ C = 0–15 μM $K_d \sim 0.2$ μM[a] ($R^2 = 0.77$)	—
2–4, 6–9, 12–16	No binding				

[a] Binding was detected, but spectral response is too low. [b] K_d and ligand type cannot be estimated because of two maxima, corresponding to binding of substrate-like molecule (λ = 393 nm) and inhibitor-like molecule (λ = 433 nm).

It was also found that some of the tested compounds can bind microsomal steroid hydroxylases CYP17A1, CYP19A1 and CYP21A2, necessary for the biosynthesis of steroid hormones. The most prominent results were obtained for CYP17A1 and 2-(*N*,*N*-diethylamino)ethyl derivative **19**. The data show that this molecule interacts with CYP17A1 with an affinity of $K_d = 8.6 \pm 1.5$ μM, which is comparable with the affinity of natural ligands of the protein (progesterone and 17α-hydroxyprogesterone).[13]

In silico analysis of compound **19** binding in the CYP17A1 active site showed that the pose of the steroid molecule is similar to that of abiraterone and galeterone. The pyridine fragment of the modified steroid occupies a hydrophobic pocket, formed by residues V366, A367, I371 and V483, like a benzimidazole moiety of galeterone.[13] Derivative **19** is localized in such a way that the steroid core of the molecule interacts with amino acids from α-helix I (A301, G302), while the large substituting group at C3 interacts with amino acid residues A105, I205, R239 and D298 from α-helices B′, F, G and I (Fig. 4). Unfavorable interactions of the highly hydrophobic part of the substitution group with polar residues could be the reason for the relatively low affinity (compared to known ligands of the enzyme) of the modified steroid. It was also found that the oxygen atom of the oxyimino group forms bonds with conserved N202 from α-helix F. It is well-known that such interactions are crucial for the stabilization of CYP17A1 ligands.[13]

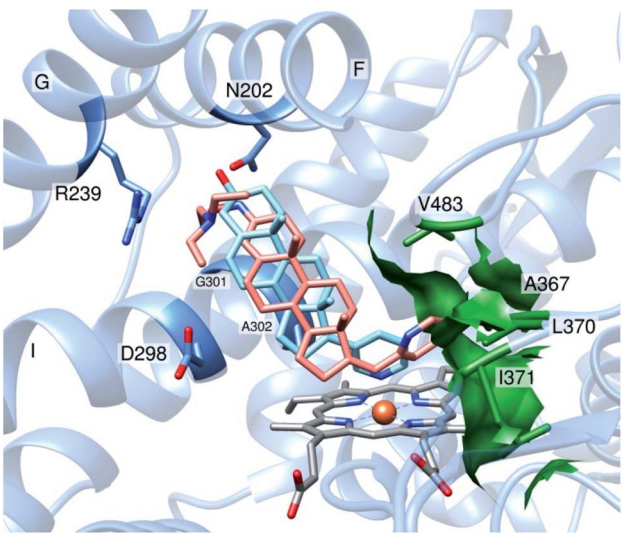


Fig. 4 Fragment of active site of CYP17A1 in complex with compound **19** and abiraterone (PDB ID: 3RUK). In the stick and sphere representations, non-carbon atoms are indicated in blue (N) and red (O), Fe atom is indicated as an orange sphere. Molecules are colored by dark grey (heme), salmon (compound **19**) and light blue (abiraterone). Surface and key residues, forming hydrophobic pocket are colored by forest green. Key residues, forming bonds with derivative **19**, are colored cornflower blue.

Based on these findings, compound **19** could be considered as a promising candidate for the development of highly efficient inhibitors of the enzyme, and in the development of drug candidates for the treatment of androgen-dependent diseases, especially prostate cancer. On the other hand, compounds expressing strong cytotoxicity based on apoptosis induction and showed no binding to steroid-converting CYP, thus bypassing possible side-effects, impose as molecules whose structural features enable their potential in drug development.

Conclusions

An efficient synthesis of four isomerically pure oximes and fourteen new oxime ether steroid derivatives in 17α-(pyridin-2-yl)methyl and (17*E*)-(pyridin-2-yl)methylideneandrost-4-ene series was performed. *In vitro* cytotoxic activity of all compounds was first evaluated against eight human cancer cell lines and two normal human fibroblasts. Most of the compounds tested showed significant cytotoxicity against tumor cell lines, especially A549, HT-29 and G-361 cells. *In vitro* screening also revealed that compounds **12**, **13** and **14** proved to be the most promising candidates for further examination, due to their markedly strong cytotoxic effects on A549, HT-29 and MDA-MB-231 cells and their demonstrated pro-apoptotic potential against A549 cells. Compound **8**, which was very cytotoxic against melanoma G-361 cells, but non-toxic toward normal human fibroblasts (BJ), was evaluated *in vitro* for anti-angiogenic and anti-inflammatory activity, where partial inhibition of endothelial cell migration was observed after treatment with 50 μM. Overall, the lung adenocarcinoma cell line (A549) appears to be the most sensitive to the majority of the compounds tested, which is a very encouraging result, due to the fact that lung cancer has recently become the leading cause of death for both women and men. In addition, compounds in the (17*E*)-(pyridin-2-yl)methylidene series showed stronger cytotoxic effects against the tested tumor cells lines, compared to the 17α-(pyridin-2-yl)methyl derivatives. *In vitro* screening toward cytochrome P450 enzymes, as possible molecular targets, suggests that compounds **5**, **10** and **17–20** can selectively bind to the active site of human steroid hydroxylases CYP7, CYP17A1, CYP19A1 or CYP21A2. This information could be important for the development of highly effective anticancer drug candidates with low side effects based on these newly synthesized compounds.

The very high complexity of biological model systems such as cell lines, especially from metastatic cancers, limits us to boldly make a hypothesis. So, the exact mechanism of action of the compounds remains unknown. Indications are that the structure of the compounds plays a significant role since small changes in the structure lead to a different biological effect. Although further studies are necessary, collected data highlight the importance of oxyimino and *O*-alkylated oxyimino functionality for the development of new candidates for tumor treatment. Combining the alkylaminoethyl side chain and pyridine heterocycle with steroid nucleus seems to be the right step for the development of potent and selective antineoplastic agents.

Experimental

General information

Infrared spectra (wavenumbers in cm^{-1}) were recorded on a PerkinElmer Spectrum Two spectrometer. NMR spectra were recorded on a Bruker AVANCE III HD 400 spectrometer operating at 400 MHz (^1H) and 100 MHz (^{13}C). Chemical shifts are given in ppm (δ-scale). For chemical shift calibration, internal TMS or residual solvent signals were used. High-resolution mass spectrometry (HRMS) measurements were recorded on a Thermo LTQ Orbitrap XL instrument using (ESI+) ionisation. Chromatographic separations were performed on silica gel columns (Kieselgel 60, 0.04–0.063 mm and 0.063–0.20 mm, Merck). All the reagents used were of analytical grade.

General synthetic procedure for 17α-(pyridin-2-yl)methyl and (17*E*)-(pyridin-2-yl)methylidene 3-hydroxyimino derivatives 2, 3 and 12, 13

Compound **1** (0.190 g, 0.50 mmol) or **11** (0.262 g, 0.72 mmol) was dissolved in 95% ethanol (30 mL), and sodium acetate (5 equivalents) and hydroxylamine hydrochloride (5 equivalents) were added. The reaction mixture was stirred under reflux for 1.5 h, then poured into water and adjusted to pH 8 with saturated $NaHCO_3$. The white precipitate that formed was filtered off and washed with water. Obtained mixtures of the isomeric *E* and *Z* oximes **2** and **3** (molar ratio **2 : 3** = 2.8 : 1 by NMR), or **12** and **13** (molar ratio **12 : 13** = 2.6 : 1 by NMR) were then separated by flash chromatography (petroleum ether/ethyl acetate 8 : 5 for **2** and **3**, or petroleum ether/ethyl acetate 5 : 2 for **12** and **13**), yielding pure 3*E* isomer **2** and pure 3*Z* isomer **3**, as well as pure 3*E* isomer **12** and pure 3*Z* isomer **13**, as white solid.

17β-Hydroxy-17α-(pyridin-2-yl)methylandrost-4-en-(3*E*)-one oxime (2). Yield 55%. IR (film, ν, cm^{-1}): 3273, 2939, 1634, 1598, 1570, 1474, 1436, 1377, 1052, 1022, 969, 755; ^1H NMR (400 MHz, DMSO-d_6) δ 0.85 (s, 3H, H-18), 1.02 (s, 3H, H-19), 2.78 (d, 1H, J = 13.7 Hz, CH_2Py), 2.88 (d, 1H, J = 13.7 Hz, CH_2Py), 5.29 (s, 1H, 17β-OH), 5.71 (s, 1H, H-4), 7.23 (m, 1H, H-3′, Py), 7.38 (m, 1H, H-5′, Py), 7.69 (m, 1H, H-4′, Py), 8.46 (d, 1H, J = 4.9 Hz, H-6′, Py), 10.48 (s, 1H, =NOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 14.83 (C-18), 18.00 (C-19), 18.99, 21.20, 23.97, 31.73, 32.20 (2× CH_2), 34.00, 34.90, 36.71, 37.98, 43.82, 46.61, 49.89, 53.75, 82.96 (C-17), 118.35 (C-4), 121.77 (C-5′, Py), 125.90 (C-3′, Py), 136.77 (C-4′, Py), 148.40 (C-6′, Py), 53.14 (C-3), 154.61 (C-5), 160.80 (C-2′, Py); HRMS (*m*/*z*): for $C_{25}H_{35}N_2O_2$ $[M + H]^+$ calcd 395.26985, found 395.26941.

17β-Hydroxy-17α-(pyridin-2-yl)methylandrost-4-en-(3*Z*)-one oxime (3). Yield 31.8%. IR (film, ν, cm^{-1}): 3267, 2940, 1631, 1597, 1570, 1474, 1437, 1024, 999, 971, 944, 910, 754, 665; ^1H NMR (400 MHz, DMSO-d6) δ 0.85 (s, 3H, H-18), 1.08 (s, 3H, H-19), 2.77 (d, 1H, J = 13.6 Hz, CH_2Py), 2.87 (d, 1H, J = 13.6 Hz, CH_2Py), 5.29 (s, 1H, 17β-OH), 6.34 (s, 1H, H-4), 7.23 (m, 1H, H-3′, Py), 7.38 (m, 1H, H-5′, Py), 7.69 (m, 1H, H-4′, Py), 8.46 (d, 1H, J = 4.8 Hz, H-6′, Py), 10.22 (s, 1H, =NOH); ^{13}C NMR (100 MHz, DMSO-d6) δ 14.83 (C-18), 18.29 (C-19), 21.04, 23.95, 24.79, 31.68, 32.60, 32.82, 33.99, 36.58, 36.62, 38.94, 43.82, 46.65, 49.83, 54.01, 82.93 (C-17), 111.78 (C-4), 121.78 (C-5′, Py), 125.90 (C-3′,

Py), 136.77 (C-4′, Py), 148.40 (C-6′, Py), 151.47 (C-3), 156.98 (C-5), 160.77 (C-2′, Py); HRMS (*m*/*z*): for $C_{25}H_{35}N_2O_2$ $[M + H]^+$ calcd 395.26985, found 395.26968.

(17*E*)-(Pyridin-2-yl)methylideneandrost-4-en-(3*E*)-one oxime (12). Yield 47.5%. IR (film, ν, cm^{-1}): 3186, 3052, 2940, 2878, 2854, 1632, 1591, 1469, 1436, 1373, 1240, 1216, 971, 941, 909, 874, 754, 666. ^1H NMR (400 MHz, DMSO-d6) δ 0.88 (s, 3H, H-18), 1.06 (s, 3H, H-19), 2.74–2.90 (m, 2H, H-16a and H-16b), 5.72 (s, 1H, H-4), 6.15 (s, 1H, H-20), 7.12 (m, 1H, H-5′, Py), 7.31 (m, 1H, H-3′, Py), 7.70 (td, 1H, $J_1 = 7.8$ Hz, $J_2 = 1.9$ Hz, H-4′, Py), 8.52 (m, 1H, H-6′, Py), 10.47 (s, 1H, =NOH); ^{13}C NMR (100 MHz, DMSO-d6) δ 18.00 (C-18), 18.97, 19.16 (C-19), 21.49, 25.00, 30.35, 32.13, 32.28, 34.86, 35.55, 35.90, 38.00, 45.73, 53.43, 53.93, 117.63 (C-20), 118.44 (C-4), 120.84 (C-5′, Py), 123.48 (C-3′, Py), 136.61 (C-4′, Py), 149.44 (C-6′, Py), 152.98 (C-3), 154.58 (C-5), 157.38 (C-17), 160.37 (C-2′, Py); HRMS (*m*/*z*): for $C_{25}H_{33}N_2O$ $[M + H]^+$ calcd 377.25929, found 377.25960.

(17*E*)-(Pyridin-2-yl)methylideneandrost-4-en-(3*Z*)-one oxime (13). Yield 33.2%. IR (film, ν, cm^{-1}): 3180, 3009, 2962, 2939, 2877, 2853, 1653, 1590, 1468, 1435, 1373, 969, 909, 872, 863, 848, 754. ^1H NMR (400 MHz, DMSO-d6) δ 0.88 (s, 3H, H-18), 1.10 (s, 3H, H-19), 2.68–2.89 (m, 2H, H-16a and H-16b), 6.14 (s, 1H, H-20), 6.35 (s, 1H, H-4), 7.12 (m, 1H, H-5′), 7.30 (m, 1H, H-3′), 7.70 (td, 1H, $J_1 = 7.6$ Hz, $J_2 = 1.9$ Hz, H-4′), 8.52 (m, 1H, H-6′), 10.21 (s, 1H, =NOH); ^{13}C NMR (100 MHz, DMSO-d6) δ 18.27 (C-18), 19.17 (C-19), 21.33, 24.77, 24.97, 30.35, 32.64, 32.75, 35.47, 35.84, 36.55, 38.95, 45.77, 53.35, 54.22, 111.27 (C-4), 117.66 (C-20), 120.84 (C-5′, Py), 123.47 (C-3′, Py), 136.60 (C-4′, Py), 149.44 (C-6′, Py), 151.45 (C-3), 156.80 (C-5), 157.38 (C-17), 160.30 (C-2′, Py); HRMS (*m*/*z*): for $C_{25}H_{33}N_2O$ $[M + H]^+$ calcd 377.25929, found 377.25942.

General synthetic procedure for the synthesis of *O*-alkylated compounds 4–10 and 14–20

To a solution of 3*E*-oxyimino derivative **2** or **12** (0.28 mmol) in dry butanone (25 mL), anhydrous K_2CO_3 was added (15 equivalents) and the reaction mixture was refluxed for the next 2 h. Respective hydrochlorides of alkylaminoethyl chlorides (10 equivalents) and KOH (7 equivalents) were added and the reaction mixture was further refluxed with stirring. After reaction completion (Table 1), the mixture was cooled, filtered, and the solvent was removed under reduced pressure. Iced water was added to an oily residue and it was stirred for 1 h, to remove inorganic compounds. The entire rinsing process was repeated twice more. The obtained resinous product was filtered, dissolved in dichloromethane and dried. The solvent was removed under vacuum and the crude product was purified by column chromatography, to afford the corresponding alkylaminoethyloxyimino derivatives **4–10** and **14–20** as light yellow oil, in different yields (Table 1).

17β-Hydroxy-17α-(pyridin-2-yl)methylandrost-4-en-(3*E*)-one *O*-[2-(pyrrolidin-1-yl)ethyl] oxime (4). Column chromatography (5 g silica gel, 95% ethanol). IR (film, ν, cm^{-1}): 3347, 2938, 1596, 1473, 1437, 1376, 1249, 1052, 1032, 964, 851, 835, 754, 663; ^1H NMR (400 MHz, $CDCl_3$) δ 0.98 (s, 3H, H-18), 1.07 (s, 3H, H-19), 2.78 (1H, C$\underline{H}_2$Py), 2.80 (2H, NC$\underline{H}_2$), 3.05 (d, 1H, $J = 14.7$ Hz, CH_2Py), 4.20 (t, 2H, $J = 6.0$ Hz, OCH_2), 5.78 (s, 1H, H-4), 7.15 (m, 2H, H-3′ and H-5′, Py), 7.63 (m, 1H, H-4′, Py), 8.45 (d, 1H, $J =$ 3.6 Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.16 (C-18), 17.83 (C-19), 19.62, 21.08, 23.52 (C-3″ and C-4″), 23.91, 32.11, 32.21, 32.54, 34.87, 35.96, 36.59, 38.03, 43.14, 46.39, 50.32, 53.88 (NCH$_2$), 54.78 (C-2″ and C-5″), 54.95, 73.02 (OCH$_2$), 83.39 (C-17), 117.31 (C-4), 121.37 (C-5′, Py), 124.76 (C-3′, Py), 136.79 (C-4′, Py), 148.03 (C-6′, Py), 155.04 (C-3), 156.34 (C-5), 160.82 (C-2′, Py); HRMS (*m*/*z*): for $C_{31}H_{46}N_3O_2$ $[M + H]^+$ calcd 492.35900, found 492.35737.

17β-Hydroxy-17α-(pyridin-2-yl)methylandrost-4-en-(3*E*)-one *O*-[3-(*N*,*N*-dimethylamino)propyl] oxime (5). Column chromatography (5 g silica gel, 95% ethanol). IR (film, ν, cm^{-1}): 3352, 2857, 2939, 1967, 1596, 1569, 1437, 1376, 1154, 1052, 916, 853, 753, 657; ^1H NMR (400 MHz, $CDCl_3$) δ 0.98 (s, 3H, H-18), 1.08 (s, 3H, H-19), 2.24 (s, 6H, N(C$\underline{H}_3$)$_2$), 2.35 (2H, NC$\underline{H}_2$), 2.97 (d, 1H, J = 14.0 Hz, CH_2Py), 3.06 (d, 1H, $J = 14.0$ Hz, CH_2Py), 4.08 (t, 2H, J = 6.4 Hz, OCH_2), 5.77 (s, 1H, H-4), 7.15 (m, 2H, H-3′ and H-5′, Py), 7.61 (m, 1H, H-4′, Py), 8.45 (d, 1H, $J = 3.5$ Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.15 (C-18), 17.83 (C-19), 19.46, 21.09, 23.92, 27.47, 32.11, 32.21, 32.53, 34.87, 35.95, 36.59, 38.03, 43.14, 45.47 (N(C$\underline{H}_3$)$_2$), 46.39, 50.32, 53.88 (NC$\underline{H}_2$), 56.54, 71.96 (OC$\underline{H}_2$), 83.40 (C-17), 117.38 (C-4), 121.37 (C-5′, Py), 124.78 (C-3′, Py), 136.79 (C-4′, Py), 148.03 (C-6′, Py), 155.26 (C-3), 156.24 (C-5), 160.82 (C-2′, Py); HRMS (*m*/*z*): for $C_{30}H_{46}N_3O_2$ $[M + H]^+$ calcd 480.35900, found 480.35855.

17β-Hydroxy-17α-(pyridin-2-yl)methylandrost-4-en-(3*E*)-one *O*-[2-(*N*-methylpyrrolidin-2-yl)ethyl] oxime (6). Column chromatography (5 g silica gel, 95% ethanol). IR (film, ν, cm^{-1}): 2939, 2872, 1596, 1473, 1437, 1376, 1250, 1125, 1051, 1020, 918, 854, 753; ^1H NMR (400 MHz, $CDCl_3$) δ 0.93 (s, 3H, H-18), 0.97 (s, 3H, H-19), 2.36 (s, 3H, NC$\underline{H}_3$), 2.96 (d, 1H, $J = 15.0$ Hz, CH_2Py), 3.05 (d, 1H, $J = 15.0$ Hz, CH_2Py), 4.30 (t, 2H, $J = 8.0$ Hz, OCH_2), 5.77 (s, 1H, H-4), 7.15 (m, 2H, H-3′ and H-5′, Py), 7.62 (m, 1H, H-4′, Py), 8.45 (d, 1H, $J = 4.0$ Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.15 (C-18), 17.83 (C-19), 19.63, 21.09, 22.85, 23.19, 29.40, 32.15, 32.17, 32.21, 32.52, 32.66, 34.89, 35.94, 36.59, 38.03, 43.14, 46.38 (NC$\underline{H}_3$), 50.32, 52.76, 53.87, 58.87 (C-2″), 81.31 (OCH$_2$), 83.40 (C-17), 117.56 (C-4), 121.37 (C-5′, Py), 124.76 (C-3′, Py), 136.78 (C-4′, Py), 148.02 (C-6′, Py), 155.02 (C-3), 156.10 (C-5), 160.80 (C-2′, Py); HRMS (*m*/*z*): for $C_{32}H_{48}N_3O_2$ $[M + H]^+$ calcd 506.37465, found 506.37289.

17β-Hydroxy-17α-(pyridin-2-yl)methylandrost-4-en-(3*E*)-one *O*-[2-(piperidin-1-yl)ethyl] oxime (7). Column chromatography (5 g silica gel, hexane/acetone 4 : 1). IR (film, ν, cm^{-1}): 3325, 2935, 2855, 1596, 1470, 1438, 1302, 1250, 1125, 1050, 852, 836, 754; ^1H NMR (400 MHz, $CDCl_3$) δ 0.98 (s, 3H, H-18), 1.07 (s, 3H, H-19), 2.50 (m, 4H, H-2″ and H-6″), 2.70 (t, 2H, $J = 6.0$ Hz, NCH$_2$), 2.79 (d, 1H, $J = 14.4$ Hz, CH_2Py), 3.05 (d, 1H, $J = 14.5$ Hz, CH_2Py), 4.21 (t, 2H, $J = 6.0$ Hz, OCH_2), 5.77 (s, 1H, H-4), 7.15 (m, 2H, H-3′ and H-5′, Py), 7.62 (m, 1H, H-4′, Py), 8.45 (d, 1H, $J =$ 3.6 Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.15 (C-18), 17.82 (C-19), 19.60, 21.07 (C-4″), 23.91, 24.10, 25.80 (C-3″ and C-5″), 32.10, 32.20, 32.53, 34.85, 35.94, 36.58, 38.02, 43.13, 46.38, 50.31, 53.86 (NCH$_2$), 54.85 (C-2″ and C-6″), 57.71, 71.61 (OCH$_2$), 83.39 (C-17), 117.27 (C-4), 121.37 (C-5′, Py), 124.76 (C-3′, Py), 136.79 (C-4′, Py), 148.02 (C-6′, Py), 155.47 (C-3), 156.41 (C-5),

160.80 (C-2′, Py); HRMS (*m*/*z*): for $C_{32}H_{48}N_3O_2$ $[M + H]^+$ calcd 506.37465, found 506.37295.

17β-Hydroxy-17α-(pyridin-2-yl)methylandrost-4-en-(3*E*)-one *O*-[2-(morpholin-4-yl)ethyl] oxime (8). Column chromatography (5 g silica gel, hexane/acetone 5 : 1). IR (film, ν, cm^{-1}): 3402, 2938, 1633, 1596, 1377, 1118, 1021, 953, 871, 854, 753, 708, 657; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.98 (s, 3H, H-18), 1.08 (s, 3H, H-19), 2.55 (m, 4H, H-3″ and H-5″), 2.72 (t, 2H, J = 5.6 Hz, NCH_2), 2.78 (d, 1H, J = 14.4 Hz, CH_2Py), 3.05 (d, 1H, J = 14.4 Hz, CH_2Py), 3.73 (m, 4H, H-2″ and H-6″), 4.22 (t, 2H, J = 5.6 Hz, OCH_2), 5.77 (s, 1H, H-4), 7.16 (m, 2H, H-3′ and H-5′, Py), 7.62 (m, 1H, H-4′, Py), 8.45 (d, 1H, J = 3.6 Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.15 (C-18), 17.82 (C-19), 19.64, 21.07, 23.91, 32.09, 32.20, 32.55, 34.83, 35.94, 36.57, 38.05, 43.13, 46.38, 50.31, 53.87 (NCH_2), 53.99 (C-3″ and C-5″), 57.49, 66.87 (C-2″ and C-6″), 71.49 (OCH_2), 83.38 (C-17), 117.16 (C-4), 121.37 (C-5′, Py), 124.75 (C-3′, Py), 136.79 (C-4′, Py), 148.03 (C-6′, Py), 155.73 (C-3), 156.57 (C-5), 160.79 (C-2′, Py); HRMS (*m*/*z*): for $C_{31}H_{46}N_3O_3$ $[M + H]^+$ calcd 508.35392, found 508.35436.

17β-Hydroxy-17α-(pyridin-2-yl)methylandrost-4-en-(3*E*)-one *O*-[2-(*N*,*N*-diethylamino)ethyl] oxime (9). Column chromatography (5 g silica gel, hexane/acetone 1 : 1). IR (film, ν, cm^{-1}): 3325, 2937, 1596, 1472, 1437, 1377, 1250, 1201, 1050, 1000, 963, 836, 754; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.98 (s, 3H, H-18), 1.05 (t, 6H, J = 7.2 Hz, $N(CH_3CH_2)_2$), 1.07 (s, 3H, H-19), 2.63 (q, 4H, J = 7.2 Hz, $N(CH_3CH_2)_2$), 2.78 (m, 1H, CH_2Py), 2.80 (t, 2H, J = 4.5 Hz, NCH_2), 3.05 (d, 1H, J = 14.4 Hz, CH_2Py), 4.16 (t, 2H, J = 4.5 Hz, OCH_2), 5.77 (s, 1H, J = 4.5 Hz, H-4), 7.15 (m, 2H, H-3′ and H-5′, Py), 7.61 (m, 1H, H-4′, Py), 8.45 (d, 1H, J = 3.6 Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.77 ($N(CH_3CH_2)_2$), 14.15 (C-18), 17.81 (C-19), 19.57, 21.07, 23.91, 32.09, 32.20, 32.53, 34.86, 35.95, 36.58, 38.02, 43.14, 46.38, 47.82 ($N(CH_3CH_2)_2$), 50.31, 51.26, 53.86 (NCH_2), 72.09 (OCH_2), 83.38 (C-17), 117.29 (C-4), 121.36 (C-5′, Py), 124.75 (C-3′, Py), 136.78 (C-4′, Py), 148.02 (C-6′, Py), 155.39 (C-3), 156.34 (C-5), 160.80 (C-2′, Py); HRMS (*m*/*z*): for $C_{31}H_{48}N_3O_2$ $[M + H]^+$ calcd 494.37465, found 494.37502.

17β-Hydroxy-17α-(pyridin-2-yl)methylandrost-4-en-(3*E*)-one *O*-[2-(*N*,*N*-dimethylamino)ethyl] oxime (10). Column chromatography (5 g silica gel, hexane/acetone 1 : 2). IR (film, ν, cm^{-1}): 3351, 2940, 1596, 1569, 1438, 1375, 1249, 1034, 999, 963, 916, 851, 836, 754, 657; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.98 (s, 3H, H-18), 1.07 (s, 3H, H-19), 2.33 (s, 6H, $N(CH_3)_2$), 2.66 (t, 2H, J = 5.6 Hz, NCH_2), 2.79 (d, 1H, J = 14.4 Hz, CH_2Py), 3.05 (d, 1H, J = 14.4 Hz, CH_2Py), 4.18 (t, 2H, J = 5.6 Hz, OCH_2), 5.77 (s, 1H, H-4), 7.15 (m, 2H, H-3′ and H-5′, Py), 7.62 (m, 1H, H-4′, Py), 8.45 (d, 1H, J = 3.6 Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.15 (C-18), 17.81 (C-19), 19.60, 21.07, 23.91, 32.09, 32.20, 32.54, 34.82, 35.95, 36.58, 38.08, 43.14, 45.85 ($N(CH_3)_2$), 46.38, 50.31, 53.86 (NCH_2), 58.10, 71.77 (OCH_2), 83.39 (C-17), 117.24 (C-4), 121.37 (C-5′, Py), 124.76 (C-3′, Py), 136.79 (C-4′, Py), 148.02 (C-6′, Py), 155.58 (C-3), 156.50 (C-5), 160.81 (C-2′, Py); HRMS (*m*/*z*): for $C_{29}H_{44}N_3O_2$ $[M + H]^+$ calcd 466.34335, found 466.34560.

(17*E*)-(Pyridin-2-yl)methylideneandrost-4-en-(3*E*)-one *O*-[2-(pyrrolidin-1-yl)ethyl] oxime (14). Column chromatography (5 g silica gel, 95% ethanol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (s, 3H, H-18), 1.09 (s, 3H, H-19), 1.81 (m, 4H, H-3″ and H-4″), 2.66 (m, 4H, H-2″ and H-5″), 2.85 (t, 2H, J = 6.0 Hz, NCH_2), 4.22 (t, 2H, J = 6.0 Hz, CH_2O), 5.78 (s, 1H, H-4), 6.21 (s, 1H, H-20), 7.02 (m, 1H, H-5′), 7.27 (m, 1H, H-3′), 7.60 (t, 1H, J = 7.5 Hz, H-4′), 8.56 (d, 1H, J = 4.5 Hz, H-6′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.81 (C-18), 18.79 (C-19), 19.61, 21.42, 23.47 (C-3″ and C-4″), 53.97 (NCH_2), 24.96, 29.79, 32.08, 32.48, 34.80, 35.65, 35.81, 38.02, 45.76, 53.28, 53.97, 54.65 (C-2″ and C-5″), 72.55 (OCH_2), 117.37 (C-20), 118.05 (C-4), 120.32, 122.79 (C-3′, Py), 135.83 (C-4′, Py), 149.17 (C-6′, Py), 155.32 (C-3), 156.39 (C-5), 157.57 (C-17), 160.18 (C-2′, Py); HRMS (*m*/*z*): for $C_{31}H_{44}N_3O$ $[M + H]^+$ calcd 474.34844, found 474.34996.

(17*E*)-(Pyridin-2-yl)methylideneandrost-4-en-(3*E*)-one *O*-[3-(*N*,*N*-dimethylamino)propyl] oxime (15). Column chromatography (5 g silica gel, 95% ethanol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (s, 3H, H-18), 1.09 (s, 3H, H-19), 2.24 (s, 6H, $N(CH_3)_2$), 2.37 (t, 2H, J = 7.4 Hz, NCH_2), 4.08 (t, 2H, J = 6.4 Hz, CH_2O), 5.78 (s, 1H, H-4), 6.21 (s, 1H, H-20), 7.02 (m, 1H, H-5′), 7.26 (m, 1H, H-3′), 7.59 (t, 1H, J = 7.8 Hz, H-4′), 8.56 (d, 1H, J = 4.7 Hz, H-6′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.81 (C-18), 18.80 (C-19), 19.44, 21.43, 24.97, 27.38, 29.80, 32.09, 32.47, 34.81, 35.66, 35.83, 38.01, 45.38 ($N(CH_3)_2$), 45.76, 53.28, 53.97 (NCH_2), 56.48, 71.93 (OCH_2), 117.51 (C-20), 118.05 (C-4), 120.22 (C-5′, Py), 122.78 (C-3′), 135.82 (C-4′, Py), 149.17 (C-6′, Py), 154.99 (C-3), 156.13 (C-5), 157.58 (C-17), 160.20 (C-2′, Py); HRMS (*m*/*z*): for $C_{30}H_{44}N_3O$ $[M + H]^+$ calcd 462.34844, found 462.35004.

(17*E*)-(Pyridin-2-yl)methylideneandrost-4-en-(3*E*)-one *O*-[2-(*N*-methylpyrrolidin-2-yl)ethyl] oxime (16). Column chromatography (5 g silica gel, 95% ethanol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (s, 3H, H-18), 1.09 (s, 3H, H-19), 2.21–2.42 (m, 3H, H-2″ and H-5″), 2.35 (s, 3H, NCH_3), 4.10 (m, 2H, OCH_2), 5.79 (s, 1H, H-4), 6.21 (s, 1H, H-20), 7.03 (m, 1H, H-5′), 7.28 (m, 1H, H-3′), 7.60 (t, 1H, J = 8.0 Hz, H-4′), 8.56 (d, 1H, J = 4.0 Hz, H-6′); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.83 (C-18), 18.80 (C-19), 19.53, 19.62, 21.44, 21.97, 24.98, 29.71, 29.80, 31.05, 32.12, 32.47, 34.88, 35.67, 35.84, 38.02, 40.50, 45.77 (NCH_3), 53.30, 53.99, 57.17 (C-5″), 59.07 (C-2″), 81.52 (OCH_2), 117.49 (C-20), 118.06 (C-4), 120.23 (C-5′, Py), 122.79 (C-3′), 135.83 (C-4′), 149.19 (C-6′), 154.71 (C-3), 155.06 (C-5), 157.59 (C-17), 160.23 (C-2′, Py); HRMS (*m*/*z*): for $C_{32}H_{46}N_3O$ $[M + H]^+$ calcd 488.36409, found 488.36457.

(17*E*)-(Pyridin-2-yl)methylideneandrost-4-en-(3*E*)-one *O*-[2-(piperidin-1-yl)ethyl] oxime (17). Column chromatography (5 g silica gel, petroleum ether/acetone 5 : 1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (s, 3H, H-18), 1.09 (s, 3H, H-19), 2.54 (m, 4H, H-2″ and H-6″), 2.75 (t, 2H, J = 2.8 Hz, NCH_2), 2.77–2.90 (m, 2H, H-16a and H-16b), 4.24 (t, 2H, J = 5.6 Hz, OCH_2), 5.78 (s, 1H, H-4), 6.21 (s, 1H, H-20), 7.03 (m, 1H, H-5′, Py), 7.26 (m, 1H, H-3′, Py), 7.60 (t, 1H, J = 6.0 Hz, H-4′, Py), 8.56 (d, 1H, J = 4.8 Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.81 (C-18), 18.79 (C-19), 19.60, 21.43, 24.97 (C-4″), 25.59, 29.79, 32.09, 32.49, 34.80, 35.66, 35.82, 38.03, 45.77, 53.28, 53.97 (NCH_2), 54.76 (C-2″ and C-6″), 57.56, 71.39 (OCH_2), 117.37 (C-20), 118.07 (C-4), 120.23 (C-5′, Py), 122.79 (C-3′, Py), 135.83 (C-4′, Py), 149.18 (C-6′, Py), 155.33 (C-3), 156.41 (C-5), 157.58 (C-17), 160.18 (C-2′, Py); HRMS (*m*/*z*): for $C_{32}H_{46}N_3O$ $[M + H]^+$ calcd 488.36409, found 488.36478.

(17*E*)-(Pyridin-2-yl)methylideneandrost-4-en-(3*E*)-one *O*-[2-(morpholin-4-yl)ethyl] oxime (18). Column chromatography (5 g silica gel, petroleum ether/acetone 1 : 1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (s, 3H, H-18), 1.09 (s, 3H, H-19), 2.55 (m, 4H, H-3″

and H-5″), 2.71 (t, 2H, $J = 6.0$ Hz, NCH_2), 2.75-2.93 (m, 2H, H-16a and H-16b), 3.73 (m, 4H, H-2″ and H-6″), 4.22 (t, 2H, $J = 6.0$ Hz, OCH_2), 5.77 (s, 1H, H-4), 6.21 (s, 1H, H-20), 7.03 (m, 1H, H-5′, Py), 7.25 (m, 1H, H-3′, Py), 7.59 (t, 1H, $J = 7.6$ Hz, H-4′, Py), 8.55 (d, 1H, $J = 4.0$ Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.82 (C-18), 18.79 (C-19), 19.61, 21.43, 24.97, 29.79, 32.08, 32.49, 34.79, 35.65, 35.82, 38.04, 45.76, 53.28 (NCH_2), 53.97, 54.01 (C-3″ and C-5″), 57.50, 66.91 (C-2″ and C-6″), 71.54 (OCH_2), 117.32 (C-20), 118.06 (C-4), 120.23 (C-5′, Py), 122.79 (C-3′, Py), 135.83 (C-4′, Py), 149.18 (C-6′, Py), 155.44 (C-3), 156.46 (C-5), 157.57 (C-17), 160.17 (C-2′, Py); HRMS (m/z): for $C_{31}H_{44}N_3O_2$ $[M + H]^+$ calcd 490.34335, found 490.34437.

(17*E*)-(Pyridin-2-yl)methylideneandrost-4-en-(3*E*)-one *O*-[2-(*N*,*N*-diethylamino)ethyl] oxime (19). Column chromatography (5 g silica gel, petroleum ether/acetone 1 : 1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.92 (s, 3H, H-18), 1.07 (t, 6H, $J = 6.4$ Hz, $N(CH_3CH_2)_2$), 1.09 (s, 3H, H-19), 2.65 (q, 4H, $J = 6.4$ Hz, $N(CH_3CH_2)_2$), 2.83 (t, 2H, $J = 6.0$ Hz, NCH_2), 4.18 (t, 2H, $J = 6.0$ Hz, OCH_2), 5.78 (s, 1H, H-4), 6.22 (s, 1H, H-20), 7.02 (m, 1H, H-5′, Py), 7.26 (m, 1H, H-3′, Py), 7.60 (m, 1H, H-4′, Py), 8.56 (d, 1H, $J = 4.8$ Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.63 ($N(CH_3CH_2)_2$), 17.81 (C-18), 18.80 (C-19), 19.57, 21.43, 24.97, 29.80, 32.09, 32.49, 34.81, 35.66, 35.82, 38.03, 45.77, 47.79 ($N(CH_3CH_2)_2$), 51.18, 53.29, 53.96 (NCH_2), 72.00 (OCH_2), 117.40 (C-20), 118.07 (C-4), 120.23 (C-5′, Py), 122.79 (C-3′, Py), 135.83 (C-4′, Py), 149.19 (C-6′, Py), 155.24 (C-3), 156.17 (C-5), 157.59 (C-17), 160.19 (C-2′, Py); HRMS (m/z): for $C_{31}H_{46}N_3O$ $[M + H]^+$ calcd 476.36409, found 476.36522.

(17*E*)-(Pyridin-2-yl)methylideneandrost-4-en-(3*E*)-one *O*-[2-(*N*,*N*-dimethylamino)ethyl] oxime (20). Column chromatography (5 g silica gel, petroleum ether/acetone 1 : 1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.94 (s, 3H, H-18), 1.10 (s, 3H, H-19), 2.24 (s, 6H, $N(CH_3)_2$), 2.78 (t, 2H, $J = 5.6$ Hz, NCH_2), 4.23 (t, 2H, $J = 5.6$ Hz, OCH_2), 5.79 (s, 1H, H-4), 6.23 (s, 1H, H-20), 7.04 (m, 1H, H-5′, Py), 7.28 (m, 1H, H-3′ Py), 7.61 (t, 1H, $J = 7.6$ Hz, H-4′, Py), 8.58 (d, 1H, $J = 4.8$ Hz, H-6′, Py); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.80 (C-18), 18.79 (C-19), 19.61, 21.42, 24.97, 29.79, 32.08, 32.50, 34.75, 35.65, 35.81, 38.04, 45.43 ($N(CH_3)_2$), 45.76, 53.27, 53.95 (NCH_2), 57.71, 71.13 (OCH_2), 117.25 (C-20), 118.05 (C-4), 120.23 (C-5′, Py), 122.80 (C-3′, Py), 135.84 (C-4′, Py), 149.17 (C-6′, Py), 155.61 (C-3), 156.66 (C-5), 157.56 (C-17), 160.17 (C-2′, Py); HRMS (m/z): for $C_{29}H_{42}N_3O$ $[M + H]^+$ calcd 448.33279, found 448.33398.

Cell culture

Six human tumor cell lines: estrogen receptor positive (ER+) breast adenocarcinoma MCF-7, estrogen receptor negative (ER−) breast adenocarcinoma MDA-MB-231, prostate cancer PC-3, cervical carcinoma HeLa, colon adenocarcinoma HT-29 and lung adenocarcinoma A549, as well as one normal fetal lung fibroblasts cell line MRC-5 were used in the present study (American Type Culture Collection – ATCC). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) with 4.5% of glucose, supplemented with 10% of fetal calf serum (FCS, Sigma) and antibiotics antimycotics solution (Sigma). Cells were cultured in flasks (Costar, 25 cm^2) at 37 °C in 100% humidity with 5% CO_2. T-Lymphoblastic leukemia (CEM) and malignant melanoma (G-361) cell lines (European Collection of Authenticated Cell Cultures ECACC, London, UK) were also used for cytotoxicity screening. Human foreskin fibroblasts (BJ) and human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection (Manassas, VA, USA). G-361 and BJ cells were cultured in DMEM medium, CEM cells in RPMI 1640 (Sigma, MO, USA) and HUVECs in endothelial cell growth medium (ECPM, Provitro, Berlin, Germany). Media used were supplemented with 10% (G-361, BJ, HUVEC) or 20% (CEM) fetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin. The cell lines were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. Cells were sub-cultured two or three times a week using the standard trypsinization procedure for adherent cells.

Cytotoxicity assay

The cytotoxicity of test and reference compounds (cisplatin and formestane) on the above-mentioned cancer and normal cells was determined after 72 h of incubation, using two cytotoxicity tests. The first, MTT test with tetrazolium salt,[41] as described in detail by Ajduković *et al.*[26] was performed on MCF-7, MDA-MB-231, PC-3, HeLa, HT-29, A549 and MRC-5 cell lines, with five different concentrations of the tested compounds ranging from 0.01 to 100 μM. The second cytotoxicity assay using resazurin[42] (manufacturer's protocol – Sigma Aldrich, St. Louis, MO, USA), was performed as described earlier for protocol with Calcein AM dye[44] on CEM, G-361 and BJ cell lines, with six concentrations of tested compounds ranging from 0.2 to 50 μM (dilution 3×). The data shown are the IC_{50} values calculated from the means of results obtained from at least two or three independent experiments performed in triplicate or quadruplicate.

Angiogenesis *in vitro*

Migration scratch assay was performed with confluent HUVECs. They were scratched and immediately treated with a full medium containing different doses of the substance for 20 h. For tube formation assay, ibidi 15-well μ-slides (Ibidi, Munich, Germany) were coated with Matrigel (Corning, Tewksbury, MA, USA). 1×10^4 HUVECs were treated with compound for 20 h. At the end of both assays, images were captured using a microscope (IX51, Olympus, Japan). Images were analyzed using in-house software. The data were obtained from at least three independent experiments performed in triplicate.

Cell-surface ELISA CD62E (E-Selectin, ELAM) and cytotoxicity

Enzyme-linked activity assay (ELISA) was used to detect the levels of cell adhesion molecule ELAM on HUVECs after 30 min of incubation with tested compounds and 4 h of stimulation with TNFα as described earlier.[44] Curcumin was used as a positive control. Experiments were repeated three times in triplicate. Calcein AM (Molecular Probes, Invitrogen, Karlsruhe, Germany) cytotoxicity assay after 4 h of treatment in the HUVECs was used to measure the cytotoxicity of compounds for

ELAM expression assay as described previously.[45] Triplicates of at least three independent experiments were used.

Apoptosis assay

The fluorescent double-staining method[46,47] with acridine orange (AO) and ethidium bromide (EB) dyes were used to detect cells in which membrane integrity is disturbed as a consequence of the apoptotic process. Cultures of A549 lung adenocarcinoma cells were seeded in 6-well flat bottom tissue culture plates (Sarstedt), with 1×10^5 cells in 5 mL of supplemented DMEM cell culture medium per well. All seeded cultures were incubated initially for 24 h at 37 °C, absolute humidity and 5% CO_2 conditions. Experimental cultures were exposed to IC_{50} concentrations of the investigated steroid derivatives over 72 h. The untreated culture was used as a control sample. After incubation, 100 μL of fluorescent mixture solution (15 mg of AO (Sigma-Aldrich) and 50 mg of EB (Serva) dissolved in the dark in 1 mL of absolute (96%) ethanol, with the addition of 49 mL of distilled H_2O, frozen in aliquots) were added to each well and the cells were incubated again with dyes for 15 min. Medium with dyes was discarded and cells were then washed once in wells with 1 mL of phosphate-buffered saline (PBS, Sigma) and immediately examined and photographed (6-well plates were turned bottom-up) using a fluorescence microscope (Olympus BX51) at 10 × 15 and 40 × 15 magnification.

Microphotographs with recorded fluorescent signals were analysed in the program *ImageJ* (NIH Image, http://imagej.nih.gov). Channels for the blue, red and green colour in each image were separated and their density was measured. The samples were compared by the numerical value of the red and green channel density ratio.

In vitro screening toward human cytochromes P450 (CYPs)

Purification of the proteins. All human proteins were obtained as described previously[11–13,48] and fully characterized using absorbance spectroscopy, MALDI mass-spectrometry and SDS-PAGE electrophoresis. The purity of the enzyme preparations was greater than 85%.

***In vitro* screening of the binding of steroid ligands to CYPs.** The novel steroid compounds were tested using a two-step approach: high throughput screening followed by spectrophotometric titration experiments. Firstly, 96-well plates were filled with a protein solution (1 μM) in 50 mM potassium–phosphate buffer (pH 7.4). Ligand in DMSO was added to each well at a final concentration of 80 μM. Compounds for which changes in difference absorbance spectrum were detected (shifting of absorbance maximum to lower or higher wavelength from 417 nm) were selected for estimation of K_d using a spectrophotometric titration technique. This experiment was performed in 50 mM potassium-phosphate buffer (pH 7.4) with a final CYP concentration of 1 μM. Ligand solution (stock solutions in DMSO with concentrations from 10^{-4} to 10^{-2} M) was added to the experimental cuvette and difference spectra were measured. Pure DMSO was used as a control. K_d was estimated from a tight-binding model using the following equation:

$$A = A_{max}\frac{[L]_t + [R]_0 + K_d - \sqrt{([L]_t + [R]_0 + K_d)^2 - 4[R]_0[L]_t}}{2[R]_0}$$

where, A – amplitude of the spectral change at $[L]_t$ ligand concentration; A_{max} – amplitude of the spectral change at saturation ligand concentration; $[L]_t$ – ligand concentration; $[R]_0$ – protein concentration.

***In silico* study.** The structure of 2-(*N*,*N*-diethylamino)ethyl derivative **19** was built using MolView web-service (http://www.molview.org) and minimized in UCSF Chimera software[49] (2000 steepest descent steps and 1000 conjugate gradient steps with step size 0.02 Å). AutoDock Vina software (v.1.1.2) was used for docking[50] with the following parameters: 20 diverse positions of the ligand in the active site of protein; exhaustiveness – 256; energy range – 4 kcal mol^{-1}. All hits were ranked according to their root-mean-square deviation (RMSD) of maximum common substructure toward similarly known ligands from the PDB and according to AutoDock Vina scoring function values. The binding of novel steroid compound **19** with CYP17A1 was compared with binding of abiraterone (PDB ID: 3RUK) and galeterone (PDB ID: 3SWZ), known inhibitors of this enzyme.[51]

Author contributions

J. Ajduković synthesized the compounds, organized the collaboration and drafted the manuscript. D. Jakimov planned, performed and evaluated *in vitro* cytotoxicity experiments on MCF-7, MDA-MB-231, PC-3, HeLa, HT-29, A549 and MRC-5 cell lines, including double-fluorescence apoptosis assays. L. Rárová planned and carried out the *in vitro* experiments on CEM, G-361, BJ and HUVEC cells. Y. Dzichenka and S. Jovanović-Šanta performed *in vitro* and *in silico* experiments toward human CYPs. M. Sakač supervised synthesis of new compounds. M. Strnad and S. Usanov supervised biological experiments. D. Škorić performed the NMR experiments and spectral analysis. All authors read, corrected and approved the manuscript.

Conflicts of interest

There are no conflicts to declare.

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