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Direct antiproliferative effect of nonsteroidal 17 β -hydroxysteroid dehydrogenase type 1 inhibitors *in vitro*

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Abstract

Inhibition of the local formation of estrogens seems to be an attractive strategy for pharmacological intervention in hormone-dependent disorders. The direct antiproliferative properties of ten nonsteroidal 17 β -hydroxysteroid dehydrogenase type 1 (17 β -HSD1) inhibitors were investigated on human cancer cell lines of gynecological origin. The mechanism of the antiproliferative action was approximated by cell cycle analysis, fluorescent microscopy, BrdU assay, determination of caspase-3 activity and quantification of the expression of cell cycle regulators at mRNA level. Treatment of HeLa cells with some of the compounds resulted in a concentration-dependent inhibition of the G1 – S transition and an increase in the apoptotic population. The most effective agents increased the expression of tumor suppressors p21 and p53, while CDK2 and Rb were down-regulated. The reported anticancer actions of the tested compounds are independent of the 17 β -HSD1-inhibiting capacity. These results indicate that it is possible to combine direct antiproliferative activity and 17 β -HSD1 inhibition resulting in novel agents with dual mode of action.

Introduction

The most important estrogen, 17 β -estradiol, is involved in many hormone-dependent proliferative disorders in humans, including cancers of gynecological origin and endometriosis. The suppression of estrogen exposure at the targeted tissue is therefore a part of the rational therapy and is nowadays successfully applied in patients using SERMs or aromatase inhibitors [1]. An alternative approach, aiming at decreasing the level in active 17 β -estradiol, is inhibition of 17 β -hydroxysteroid dehydrogenase type 1 (17 β -HSD1) [2]. 17 β -HSD1 is responsible for the conversion of the much less potent estrogen estrone into 17 β -estradiol [3].

Since most of the targeted hormone-dependent disorders share a proliferative character, it is conceivable that a direct antiproliferative action combined to the enzyme inhibition effect could be beneficial for the treatment of these diseases.

The present aim was to characterize the direct antiproliferative action of a set of previously synthesized nonsteroidal compounds with excellent 17 β -HSD1 inhibitory capacity (IC₅₀ values: 8 – 143 nM) [4-10]. All of the tested agents possessed a hydroxyphenyl structural moiety and were therefore chemically related to a wide range of natural polyphenol compounds (e.g. flavonoids) exhibiting well-characterized anticancer efficacy [11].

17 β -HSD1 inhibitors **1-10** (Fig. 1) were tested on human adherent cell lines of gynecological origin (HeLa, MCF-7 and A2780) by means of the MTT assay. Compounds that proved to exhibit appreciable IC₅₀ values were subjected to further experiments targeting the mechanism of the antiproliferative action, including cell cycle analysis, fluorescent microscopy, and the determination of caspase-3 activity and the expression of crucial cell cycle-regulating factors.

Materials and methods

Synthesis of the tested substances

The nonsteroidal compounds were synthesized as published previously [7, 9, 12]. All of the tested agents possessed an aromatic core with two phenolic substituents (Fig. 1). Five of the molecules contained thiophene (**1-4**) or thiazole (**5**) as central part, compound **6** was a substituted *para*-terphenyl, while agents **7-10** were diphenylnaphthols. 10 mM stock solutions of the tested compounds were prepared with dimethyl sulfoxide (DMSO). The highest DMSO concentration of the medium (0.3%) did not have any significant effect on the determined cellular functions. All the chemicals, if otherwise not specified, were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary).

Cell cultures

Human cancer cell lines (HeLa and MCF-7 isolated from cervical and breast carcinomas, respectively) and noncancerous MRC-5 human lung fibroblasts were maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS) and 1% non-essential amino acids and an antibiotic-antimycotic mixture (AAM). A2780 cells (isolated from ovarian cancer) were maintained in RPMI medium supplemented with 10% FBS, 1% AAM and 1% L-glutamine. All cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK).

Antiproliferative activity measured by MTT assay

The antiproliferative effect was determined *in vitro* on three human cancer cell lines: HeLa, MCF-7 and A2780. The cells were grown in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were seeded onto 96-well plates at a density of 5000 cells/well and allowed to stand overnight, after which the medium containing the tested compound was added. After a 72 h incubation period, viability was determined by the addition of 20 µL MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) solution (5 mg/mL). The precipitated formazan crystals were solubilized in DMSO and the absorbance was read at 545 nm with an ELISA reader. In some of the MTT assays, steroid-free medium was used which contained charcoal-stripped FBS and no phenol red. Two independent experiments were performed with five parallel wells, and cisplatin, an agent clinically used in some gynecological malignancies, was used as positive control. Sigmoidal dose-response curves were fitted to the measured points, and the IC₅₀ values were calculated by means of GraphPad Prism 4.0 (GraphPad Software; San Diego, CA, USA) [13].

Analysis of cell cycle by flow cytometry

Flow cytometry analysis was performed in order to characterize the cellular DNA content of treated HeLa cells. After treatment for 24 h cells (200,000 per condition) were trypsinized (Gibco BRL, Paisley, U.K.), washed with phosphate-buffered saline (PBS) and fixed in 1.0 mL of cold 70% ethanol for 30 min on ice. After two washing steps in cold PBS, DNA was stained with propidium iodide (PI) (10 µg/mL) in the presence of RNA-ase (50 µg/mL). The samples were then analyzed with a FACStar (Becton-Dickinson; Mountain View, CA, USA). In each analysis, 20,000 events were recorded, and the percentages of the cells in the different cell-cycle phases (subG1, G1, S and G2/M) were calculated by using winMDI2.9. The subG1 fractions were regarded as the apoptotic cell population [14].

Hoechst 33258 – PI double staining

Near-confluent HeLa cells were seeded into a 96-well plate (5000 cells/well). After incubation for 24 h with the test compound, Hoechst 33258 and PI were added to the culture medium to give final concentrations of 5 µg/mL and 2 µg/mL, respectively. The cells were incubated for 1 h at 37 °C with the staining mixture and were then photographed by means of a Nikon Eclipse microscope equipped with an epifluorescence attachment containing the appropriate optical blocks and a QCapture CCD camera. The staining allowed the identification of live, early-apoptotic, late-apoptotic and necrotic cells. Hoechst 33258 permeates all the cells and makes the nuclei appear blue. Apoptosis was revealed by nuclear changes such as chromatin condensation and nuclear fragmentation. The necrotic and the late-apoptotic cells were identified as cells with PI uptake, which indicates the loss of membrane integrity, the cell nuclei being stained red [15].

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay

BrdU incorporation into the cellular DNA was determined with the BrdU Labeling and Detection Kit III (Roche Diagnostic, Mannheim, Germany) on HeLa cells treated with the test compound for 24 h. The incorporation of BrdU in place of thymidine was monitored as a parameter for DNA synthesis. In accordance with the manufacturer's instructions, cells were labeled with BrdU for 2 h, followed by fixation. The cellular DNA was partially digested by nuclease treatment and peroxidase labeled antibody was added, which bound to BrdU. Finally, the peroxidase activity was determined colorimetrically by the addition of the

substrate. The absorbance was measured with a microplate reader at 405 nm with a reference wavelength at 492 nm. Two independent experiments were performed with 4 parallel wells.

Caspase-3 assay

The activity of caspase-3 from treated cells was determined in triplicate by means of a commercially available colorimetric kit in accordance with the instructions of the provider (Sigma-Aldrich, Budapest, Hungary). Briefly, HeLa cells (16 million per condition) were exposed to the test item for 48 h and then scraped, counted and resuspended in lysis buffer (10 μ L for 1 million cells). The caspase-3 activity was measured by the addition of substrate (Ac-DEVD-pNA), and the amount of product (pNA) was measured at 405 nm after incubation for 17 h. Results on treated cells are given as fold-increase by direct comparison with the untreated control results.

Reverse transcription-polymerase chain reaction (RT-PCR) studies

The effects of the tested compounds on the mRNA expression pattern of retinoblastoma protein (Rb), cyclin-dependent kinase 2 (CDK2), and regulator factors p21 and p53, which play a crucial role in the transition from the G1 to the S phase, were determined by RT-PCR in HeLa cells. After a 24 h incubation period, the media containing the different test compounds were discarded and the total RNA was isolated from the cells (5×10^5) using TRIzol Reagent in accordance with the instructions of the manufacturer (Csertex Ltd; Budapest, Hungary) [16]. The pellet was resuspended in 100 μ L DNase- and RNase-free distilled water. The RNA concentrations of the samples were determined from their absorbances at 260 nm. The RNA (0.5 μ g) was mixed with DNase- and RNase-free distilled

water and 20 μ M oligo(dT) (Invitrogen, Carlsbad, USA), in a final reaction volume of 10 μ L and was incubated at 70 °C for 5 min. After the mixture had been cooled to 4 °C, 20 U RNase inhibitor (Promega, Madison, USA), 20 U MMLV reverse transcriptase (Promega, Madison, USA), 200 μ M dNTP (Sigma-Aldrich; Budapest, Hungary) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 5 mM MgCl₂ in a final reaction volume of 10 μ L were added. The mixture was incubated at 37 °C for 60 min. The PCR was carried out with 5 μ L cDNA, 25 μ L ReadyMix Taq PCR reaction mix, 2 μ L 20 pM sense and the antisense primer of Rb, CDK2, p21 or p53, and 16 μ L DNase- and RNase-free distilled water. The primer sequences used to amplify Rb, CDK2 and p21 were described by Gao et al. and that for p53 was proved by Matsushashi et al. [17, 18]. Human glyceraldehyde 3-phosphate dehydrogenase (hGAPDH) primers were used as internal control in all samples (Supplementary table). The PCR was performed with an ESCO SWIFT MAXI thermal cycler (Esco Technologies Inc, Philadelphia, PA, USA) and the products were separated on 2% agarose gels, stained with ethidium bromide and photographed under a UV transilluminator. Semiquantitative analysis was performed by densitometric scanning of the gel with Kodak IMAGE STATION 2000R (Csertex Ltd; Budapest, Hungary).

Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA), followed by the Dunnet post-test. RT-PCR data were analyzed by ANOVA, followed by the Neuman-Keuls post-test. All statistical analyses of the data were performed with GraphPad Prism 4.0 (GraphPad Software; San Diego, CA, USA).

Results

Antiproliferative assay

The antiproliferative effects of the investigated compounds are presented in Table 1. Compounds **1-7** exhibited antiproliferative activities comparable to that of the reference agent cisplatin on the HeLa cells (IC_{50} for cisplatin: 5.66 μ M), while the MCF-7 and A2780 cells were generally less sensitive. When the assays of these agents were repeated in steroid-free milieu, the calculated IC_{50} values were not substantially different from those observed in the standard cell culture medium. Human fetal fibroblast MRC-5 cells were utilized for an additional set of experiment in order to characterize the selectivity of compounds **1-7**. None of these molecules exerted appreciable antiproliferative action against the noncancerous MRC-5 cells up to 30 μ M. Compound **6** proved to be the most potent antiproliferative agent, exhibiting a lower IC_{50} than that of the reference compound cisplatin. Test substances with a hydroxyphenylnaphthalene structure (compounds **8-10**) exhibited substantially weaker action against the cell lines used.

Morphological studies and cell cycle distribution

HeLa cells were incubated with 3 and 10 μ M of compounds **1-3** and **5-7** for 24 h. The development of apoptosis or necrosis was determined according to the cell morphology and membrane integrity. Separate pictures were taken, illustrating Hoechst 33258 and PI fluorescence as morphological markers (Supplementary fig. 1). Concentration-dependent increases in nuclear condensation and in cell membrane permeability were generally detected. As nuclear condensation did not coincide with increased membrane permeability in the corresponding picture, an early apoptotic event is indicated. The morphological evidence demonstrated that compound **3** was the most potent apoptosis inducer: it elicited a pronounced

condensation in the nuclei of the treated HeLa cells even at 3 μ M without affecting the membrane function to a substantial extent at 10 μ M. Treatment with compound **5** resulted in the most pronounced deterioration in membrane integrity, as evidenced by intensive staining with PI.

Treatment with the tested compounds resulted in the distribution of the cells in the cell cycle phases (subG1, G1, S, and G2/M phases) as illustrated in Fig. 2. HeLa cells were treated with the investigated compounds at 3 μ M and 10 μ M concentrations for 24 and 48 h. After 24 hours, there was no essential change in the number of apoptotic cells. All the tested compounds decreased the population of the synthetic phase, resulting in an increase either of the G1 phase (compounds **1-3** and **5**) or the G2/M population (compounds **3, 5** and **7**). After treatment for 48 h, an unequivocal cumulation of hypodiploid (subG1) populations was detected for all items, accompanied by a decrease of the G1 population. The actions on later cell cycle phases were less conclusive, except for clear increases in the S and G2/M populations on the action of compounds **2** and **5-6**, respectively.

BrdU incorporation

The amount of thymidine analog BrdU incorporated was determined as a marker of DNA synthesis. HeLa cells were treated with two concentrations of investigated compounds (3 μ M or 10 μ M) for 24 h. All of the selected compounds substantially and statistically significantly inhibited the incorporation of BrdU into the DNA in a concentration-dependent manner, a property comparable to that of cisplatin. Compound **6** was the most potent in this regard, exerting more than 50% inhibition even at 3 μ M (Supplementary fig. 2).

Theoretically, the marked inhibition of BrdU incorporation might be attributed to a substantial decrease of the viability of the treated cells independently of the mechanism of the

intervention. In order to exclude this possibility, the antiproliferative assay was repeated under identical conditions. Although most of the selected compounds resulted in a statistically significant inhibition of cell proliferation, apart from agents **2** and **5**, the highest inhibition was ~ 30% exerted by 10 μ M of compound **6**. A modest (0-20%) decrease in cell viability was generally detected after incubation for 24 h at 10 μ M.

Caspase-3 activity

On the basis of the cell cycle results, two compounds (**5** and **6**) were selected for additional experiments in order to determine their effects on the activity of caspase-3. Both of these agents induced a statistically significant and substantial increase in the activity of this apoptosis-executing enzyme (Supplementary fig. 3). While **5** resulted in a clear concentration-effect relationship, **6** led to slightly lower activity at 10 μ M than at 3 μ M.

RT-PCR studies

The expressions of four cell cycle-regulating factors (CDK2, p21, p53 and Rb) that play key roles in the orchestration of the G1 – S transition were additionally determined by means of a semiquantitative RT-PCR technique. In view of the results of the cell cycle analyses and the BrdU incorporation assays, three compounds (**1**, **2** and **6**) were included and two concentrations (3 μ M and 10 μ M) were used during exposure for 24 h (Fig. 3). Tumor suppressor gene p53 was substantially and statistically significantly increased at a mRNA level under all the tested conditions. Although the concentration-response relationships were not always highly consequent, the ~2-fold induction by **1** and **6** proved to be the most pronounced effects. The other tumor suppressor p21 was also induced by **2** and **6** at a

concentration of 3 μ M, and by **1** at 10 μ M. CDK2 is regarded as the main regulating factor promoting the transition from the G1 to the S phase. Treatment with the selected agents resulted in the concentration-dependent repression of CDK2 at the level of mRNA expression. Compound **1** seemed to be more potent than **2** and **6** in this respect. Rb was significantly repressed by **1** and **6**, but **2** did not exert any appreciable action at the concentrations used.

Discussion

Cancers of the female reproductive organs, including the breast, the cervix and the ovaries, are frequently hormone-dependent malignancies. Estrogens play a crucial role in the development and progression of these disorders. In postmenopausal women, adrenal steroids are converted to estrogens by a set of enzymes such as aromatase and the reductive isoforms of 17 β -HSD [19]. 17 β -HSD1 is the best-characterized isoform of reductive 17 β -HSDs responsible for the local generation of the most potent natural estrogen 17 β -estradiol, while the oxidative isoforms (17 β -HSD2, 4 and 14) govern the opposite reaction, leading to the substantially less potent estrone. It is generally considered that the tissue-specific expressions of the reductive and oxidative isoforms play pivotal roles in the *in situ* estrogen exposure and consequently determine the progression of hormone-dependent disorders. It has been reported that tumor growth in a murine xenograft model was stimulated by estrone when 17 β -HSD1-expressing MCF-7 cells were used, while proliferation of parental MCF-7 cells can be stimulated by estradiol only. Consequently, this growth stimulation could be prevented by a specific 17 β -HSD1 inhibitor without exerting substantial effect on the uterine weight [20]. These data clearly demonstrate that 17 β -HSD1 blockade is an attractive and selective point for intervention in estrogen-dependent cancers.

Since the targeted disorders are estrogen-dependent proliferative states (e.g. gynecological cancers and endometriosis), a direct antiproliferative property of these pharmacons can be considered advantageous. It has not yet been investigated if the combination of 17 β -HSD1 inhibition and antiproliferative property in one molecule could lead to superior effects compared to pure 17 β -HSD1 inhibitors. It might have a positive impact delaying drug resistance as observed nowadays in case of SERMs or aromatase inhibitor treatment [21].

Since no direct antiproliferative effect was considered during the development of the tested 17 β -HSD1 inhibitors (it was only checked that the compounds did not induce any proliferative effect after estrogen receptors activation), identification of compounds combining these two properties was the goal of this study.

The currently investigated ten compounds are highly active 17 β -HSD1 inhibitors (IC₅₀ 8 – 143 nM, Table 1) [7, 9, 12]. Although representing different chemical classes, the chosen 17 β -HSD1 inhibitors, designed as steroidomimetics, all share a hydrophobic central core, mimicking the B and C steroidal ring, and two phenolic substituents, mimicking the two polar oxygens of estrone. In the optimization process to increase their potency, it appeared that these moieties are necessary for high 17 β -HSD1 inhibition [4, 6, 8]. These ten compounds differ in the nature of the hydrophobic central core, which influence not only the electronic density on the whole compound but also the overall geometry of the compounds: thiophene **1-4**, thiazole **5** and phenyl rings **6** lead to a linear shape while the derivatives **7-10** with a naphthalene group are more globular.

Many of the chemically related natural product classes (e.g. flavonoids, lignans and chalcones) exhibit pronounced antiproliferative capacities which are reflected in diet-related morbidity of some cancers [22]. In addition, a reduced risk of breast, ovarian and colorectal cancer was found for high intake of flavonoids [23].

The antiproliferative effects of the 17 β -HSD1 inhibitors (compounds **1-10**) were tested in 4 different cell-lines: HeLa, A2780, MCF-7 and MRC-5 under standard condition. Based upon their antiproliferative activity in HeLa cells where higher responses are observed, the compounds can be classified in three groups: highly active (**6** and **3**), middle active (**1**, **2**, **5**, **7**) and low or inactive (**4**, **8**, **9**, **10**). Interestingly, the structure activity relationship (SAR) study on the central core shows that both phenyl **6** and 2,5-substituted thiophene **3** as central core are good, while the naphthalene (**8**, **9**, **10**) is not. In addition, the 2,4-thiophene **4** is 3 times less active than the thiazole **5**. As the different central cores will induce different electronic repartitions in the molecule, these results highlight the influence of the electronic density on the activity. Comparison of **3** (highly active) and **2** (middle active) also indicates the importance of the substitution pattern on the hydroxyphenyl rings: only a fluorine atom leads to good activity and only one, and this molecular feature seems to be valid for **6** as well.

Additionally, it was determined on HeLa and MCF-7 cells in steroid-free medium. Although the calculated IC₅₀ tended to be higher in that latter case, the differences were not sufficiently large to indicate a pivotal role of the estrogen exposure of the utilized cells. The estrogen receptor expressions and steroid metabolic capacities of these cell lines are in good agreement with our findings. HeLa and A2780 cells express no estrogen receptors while MCF-7 cell line is reported as estrogen receptor positive [24, 25]. This crucial difference is not reflected in the IC₅₀ values. Furthermore, most of our experiments were performed on HeLa cells, because this cell line proved to be the most sensitive toward the tested agents. HeLa cells have recently been characterized with very low activities of aromatase and reductive 17 β -HSD (types 1, 5, 7 and 12) and a moderate steroid sulfatase capacity [26]. In MCF-7 breast cancer cells, reductive 17 β -HSD12 (able to transform estrone into estradiol) and steroid sulfatase are highly expressed, but mRNA levels of 17 β -HSD1 and 17 β -HSD2 were low or not detected, respectively [27]. It was concluded that in these cells estradiol can

be formed from estrone and estrone sulfate. Human ovarian cell line A2780 expresses functional reductive 17 β -HSD, which can be substantially stimulated by interleukin-6 and basic fibroblastic growth factor, but the increased rate of the estrone – estradiol conversion has no impact on the viability of the treated cells [28]. All these published data indicated that no relationship can be revealed between 17 β -HSD1 activity and the proliferative character of the cancer cell lines used. Moreover, all of these compounds inhibited 17 β -HSD1 and 17 β -HSD2 in the nM and nM- μ M ranges, respectively, *in vitro* without exhibiting substantial binding affinity to the α and β types of human estrogen receptors [7, 12]. As antiproliferative action of the compounds was found for cancer cell lines not or hardly expressing 17 β -HSD1 this effect seems to be an additional, beneficial feature independent of the interference with the metabolism of endogenous estrogens.

The cancer selectivity of the tested agents deserves special consideration. Although the MTT assay on intact human fibroblasts can not substitute a comprehensive preclinical toxicological evaluation, it is undoubtedly advantageous that none of the molecules substantially inhibited the growth of fetal fibroblast MRC-5 cells.

Since many of active agents inhibited cell growth in relevant concentration range (i.e. the μ M range), the mechanism of their action was also approximated. Most of the currently available anticancer drugs exert their effect by promoting programmed cell death by either potentiating apoptotic or inhibiting antiapoptotic signaling [29]. The apoptosis-inducing property of the tested drugs was therefore regarded as a crucial point of our experiments. Fluorescent staining with Hoechst 33258 and with PI is suitable for the qualitative detection of nuclear condensation and perturbation of the membrane functions, respectively. An apparent contradiction between the results of these morphological studies and the flow cytometry analyses after the 24 h treatment was revealed: in spite of the clear nuclear condensation and increase in membrane permeability, no substantial subG1 cell populations

were evidenced. It is concluded that 24 h is not enough for complete activation of the enzymatic machinery of the apoptotic self-decomposition. Instead, a significant depression of the synthetic phase of the cell cycle was displayed. After a longer incubation period (48 h), a concentration-dependent increase in the hypodiploid fraction was detected, which is a generally accepted hallmark of apoptosis [14]. Induction of programmed cell death was confirmed by demonstration of significantly increased caspase-3 activities in the cases of **5** and **6**. Although caspase-independent cell death can manifest apoptotic morphology, and crucial caspases, including caspase-3, may exert non-lethal functions as intracellular signaling molecules, the assessment of executioner caspase activity remains an important part of apoptosis detection [30].

The inhibition of nucleic acid synthesis was confirmed by means of BrdU incorporation assay. Compounds **1-3** and **5-7** decreased the DNA turnover in a comparable manner to the reference agent cisplatin. This behavior indicates a point of intervention in the upstream events of the G1 – S transition. In the next set of experiments, therefore, the expressions of four relevant cell cycle-regulating factors were followed through a RT-PCR technique with three selected compounds (**1**, **2** and **6**) at the mRNA level.

The G1 – S transition is governed by an orchestrated interaction of a set of regulating factors, including the four determined crucial proteins. Entry into the S phase, and therefore cell proliferation, is inhibited as long as Rb remains unphosphorylated by a complex containing cyclin E and CDK2. Phosphorylated Rb dissociates from a heterodimeric complex allowing the transcription of S-phase-specific genes [31]. The principal regulator of the cyclin E – CDK2 complex is tumor suppressor p21, which is transcriptionally activated by p53 encoded by the TP53 gene. The importance of this pathway is illustrated by the finding that nearly all cancers have a mutation in the TP53 gene or in some components of its downstream

events [32]. Although it is proved that compounds **1**, **2** and **6** exert their action via this pathway, the exact site of action can not be deduced from our results.

Genistein, one of the most extensively investigated isoflavones, has been reported to induce apoptosis in breast cancer cell lines with the up-regulation of p21 and p53 tumor suppressors. Inhibition of protooncogene HER-2 protein tyrosine phosphorylation has been reported recently and the tyrosine-kinase blockade has been suggested as the basis of the anticancer effect of genistein [33]. Many of the polyphenol-type phytochemicals isolated from green tea, including catechins and resveratrol, exert their cytostatic action by a direct inhibition of specific receptor tyrosine kinases [34]. On the basis of these data, kinase inhibition can be speculated as a basic antiproliferative mechanism of the currently investigated agents.

In addition, as the mode of action of the compounds could not be completely elucidated, it is not clear if the antiproliferative effects observed are either off-target effects or specific effects. From the experiments described in this study, it is believed that the compounds studied can interfere with the cell cycle regulating machinery presumably at multiple levels in cancer cells without affecting the viability of intact cells.

17 β -HSD1 inhibition is a very promising approach for the treatment of estrogen-dependent diseases as it will decrease the level of the active estrogen estradiol in the target cell. However, estradiol is so potent that only traces are able to activate cell proliferation. As it might difficult to abolish completely the estradiol presence by 17 β -HSD1 inhibition, it will be beneficial to use an agent which shows also antiproliferative properties and suppresses the cell growth via another mechanism. The antiproliferative potency of these compounds is limited but it might be sufficient to complete the 17 β -HSD1 inhibitory effect. Moreover, moderately potent cytostatic 16,17-secoandrostane derivatives with remarkable aromatase

inhibitory action have been suggested as anticancer drug candidates indicating the favorable combination of direct antiproliferative and endocrine disruptor properties [35].

The direct antiproliferative and proapoptotic effects of a chemically natural polyphenol-related set of 17 β -HSD1 inhibitors have been described. These results indicate that it is possible to combine direct antiproliferative activity with 17 β -HSD1 inhibition resulting in novel agents. Having in hand such compounds, it might be possible to further investigate if these molecules with a dual mode of action are superior to pure 17 β -HSD1 inhibitors and might lead to the discovery of superior drugs for the treatment of estrogen-dependent proliferative disorders.

Declaration of interest

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Figure legends

Figure 1. Chemical structures of the investigated 17 β -HSD1 inhibitors.

Figure 2. Effects of compounds **1-3** and **5-7** on HeLa cell cycle distribution after incubation for 24 (left panels) and 48 (right panels) h. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively, as compared with the control cells.

Figure 3. Expression of p53, p21, CDK2 and Rb mRNA after incubation for 24 h with compounds **1**, **2** and **6**. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively, as compared with the control conditions.

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Table 1. Calculated antiproliferative and 17 β -HSD1 inhibitory IC₅₀ values of the tested compounds; n.t.: not determined

Compounds	Antiproliferative IC ₅₀ values (μ M)*						17 β -HSD1 activity IC ₅₀ values (nM)**
	HeLa cells		MCF-7 cells		A2780 cells	MRC-5 cells	
	Standard medium	Steroid-free medium	Standard medium	Steroid-free medium	Standard medium	Standard medium	
Cpd 1	5.29	13.74	25.23	>30	>30	>30	38
Cpd 2	6.84	23.76	13.88	>30	>30	>30	17
Cpd 3	3.81	11.13	16.11	29.37	>30	>30	8
Cpd 4	14.53	>30	>30	>30	>30	>30	64
Cpd 5	5.66	6.05	>30	>30	>30	>30	143
Cpd 6	1.41	3.21	>30	>30	>30	>30	51
Cpd 7	5.30	12.19	25.51	>30	9.71	>30	64
Cpd 8	>30	n.t	>30	n.t	25.87	n.t	15
Cpd 9	>30	n.t	>30	n.t	>30	n.t	20
Cpd 10	>30	n.t	>30	n.t	>30	n.t	36
Cisplatin	5.66	n.t	7.99	n.t	0.86	4.13	n.t

* Mean value from two independent determinations with five parallel wells, standard deviation less than 15%.

** From references 7, 9 and 12.

Supplementary figure 1. Fluorescence microscopy images of Hoechst 33258 – propidium iodide double staining. Two separate pictures from the same field have been taken for the two markers. HeLa cells were treated with vehicle (control), or with **1-3** or **5-7** at 3 and 10 μ M. The blue fluorescence indicates Hoeschs 33258, and the red coloration is a result of cellular propidium iodide accumulation. The bar in the Hoeschs 33258 control picture indicates 100 μ m.

Supplementary figure 2. Incorporation of 5-Bromo-2'-deoxyuridine into HeLa cells (upper panel) and their viability determined by MTT assay (lower panel) after incubation for 24 h. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, as compared with the control cells.

Supplementary figure 3. Induction of caspase-3 activity after incubation for 48 h with compounds **5** and **6**. The activity of untreated cells was taken as one unit. *** indicates $p < 0.001$ as compared with the control cells.

Supplementary table. Primers and PCR conditions of cell cycle regulator genes, the Genebank access numbers and the lengths of PCR products

Name:	Primer sequence	Gene ID	Product size (bp)	Coupling temp. (°C)
CDK2	F: CATTCTCTTCCCCTCATCA R: CAGGGACTCCAAAAGCTCTG	1017	173	57
p21	F: GACACCACTGGAGGGTGACT R: CAGGTCCACATGGTCTTCCT	1026	172	59
Rb	F: GGAAGCAACCCTCCTAAACC R: TTTCTGCTTTTGCATTCGTG	5925	153	57
p53	F: GTGACACGCTTCCCTGGATT R: ATCTCCCAAACATCCCTCACAG	7157	1486	60
hGAPDH	F: ACCCAGAAGACTGTGGATGG R: TGCTGTAGCCAAATTCGTTG	2597	415	55