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CHARACTERIZATION OF ANTINEOPLASTIC PROPERTIES OF NOVEL ANDROSTANE-BASED SYNTHETIC STEROIDS

PhD Thesis

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SCIENTIFIC PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

I. Baji Á, **Gyovai A**, Wölfling J, Minorics R, Ocsovszki I, Zupkó I, Frank É: Microwave-assisted one-pot synthesis of steroid–quinoline hybrids and an evaluation of their antiproliferative activities on gynecological cancer cell lines. *RSC Advances* 6:27501-27516. (2016)

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II. Schneider G, Kiss A, Mernyák E, Benke Z, Wölfling J, Frank É, Bózsity N, **Gyovai A**, Minorics R, Zupkó I: Stereocontrolled synthesis of the four 16-hydroxymethyl-19-nortestosterone isomers and their antiproliferative activities. *Steroids* 105:113-120. (2016)

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III. **Gyovai A**, Minorics R, Kiss A, Mernyák E, Schneider G, Szekeres A, Kerekes E, Ocsovszki I, Zupkó I: Antiproliferative Properties of Newly Synthesized 19-Nortestosterone Analogs Without Substantial Androgenic Activity. *Frontiers in Pharmacology* 9:825. (2018)

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ADDITIONAL PUBLICATIONS

I. Mótyán G, Kovács F, Wölfling J, **Gyovai A**, Zupkó I, Frank É: Microwave-assisted stereoselective approach to novel steroidal ring D-fused 2-pyrazolines and an evaluation of their cell-growth inhibitory effects in vitro. *Steroids* 112:36-46. (2016)

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II. Vollár M, **Gyovai A**, Szűcs P, Zupkó I, Marschall M, Csupor-Löffler B, Bérdi P, Vecsernyés A, Csorba A, Liktor-Busa E, Urbán E, Csupor D: Antiproliferative and Antimicrobial Activities of Selected Bryophytes. *Molecules* 23:1520 (2018)

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III. Kiss A, Wölfling J, Mernyák E, Frank É, **Gyovai A**, Kulmány Á, Zupkó I, Schneider Gy: Stereoselective synthesis of new type steroid hybrid molecules and their antiproliferative activities. *Steroids* (accepted for publication, 2019)

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LIST OF ABBREVIATIONS

AAS	anabolic androgenic steroids
ADT	androgen deprivation therapy
bFGF	basic fibroblast growth factor
CIS	cisplatin (reference compound)
CPRG	chlorophenol red-β-D-galactopyranoside
CS	cardiotonic steroids
DHT	5α-dihydrotestosterone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC_{50}	half maximal effective concentration
EDT	estrogen deprivation therapy
EMEM	Eagle's Minimum Essential Medium
EMT	epithelial-mesenchymal transition
FBS	fetal bovine serum
HER2	human epidermal growth factor receptor 2
HPV	human papillomavirus
HRT	hormone replacement therapy
IC_{50}	concentration eliciting 50% inhibition
LDH	lactate dehydrogenase
MDR	multidrug resistance
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAN	nandrolone (19-nortestosterone, reference compound)
NEAA	non-essential amino acids
NHL	non-Hodgkins lymphoma
PAC	paclitaxel (reference compound)
PBS	phosphate-buffered saline
PI	propidium iodide
***p*NA**	*p*-nitroaniline
ROS	reactive oxygen species
SEM	standard error of mean
V_{max}	maximal rate of tubulin polymerization

1 INTRODUCTION

1.1 Overview of cancer epidemiology

The global burden of cancer is still an unsolved problem in public health. Following cardiovascular diseases, cancerous disorders are the second leading cause of death and were responsible for 8.7 million deaths in 2015. More than 17 million cancer cases were registered worldwide in the same year, reported by a global epidemiological study [1]. Furthermore, cancer has become the leading cause of death in developed countries as opposed to developing countries where it is still the second [2].

On the basis of estimated data, the global cancer burden will considerably rise in the future, the number of new cases is expected to increase above 22 million by 2030 [3]. The annual number of cancer death will be 17 million and 75 million patients will suffer from diagnosed cancer. The risk of cancer raises to 78% in developed countries and to 58% in developing countries among the population over 55 years of age, respectively [2].

The ten most prevalent cancers in males worldwide include lung, prostate, colorectal, stomach, liver, bladder, esophagus, non-Hodgkins lymphoma, kidney and leukemia [4], while breast, colorectal, lung, cervix, stomach, uterus, ovary, thyroid, liver and non-Hodgkins lymphoma are the most common among women [5]. Based on gender inequalities there are differences also in mortality between males and females (Fig. 1).

1.1.1 Current situation of gynecological cancers in global cancer burden

In 2012, approximately 6.7 million new cancer cases and 3.5 million deaths occured among females all over the world [6]. Gynecological cancers accounted for about 40% of the cancer burden and were responsible for approximately 29% of cancer death among women worldwide [4]. The incidence and mortality of these malignancies are expected to raise [5].

Worldwide the most common cancer among females was breast cancer with 1.7 million new cases, it was responsible for 521,900 deaths in the same year. The disease alone accounts for 25% of all cases and 15% of all deaths among women with cancer. However, the mortality rate of breast cancer are higher in developing countries due to delayed diagnosis and restricted access to treatment, even in countries where its incidence is lower [6].

There were about 530,000 new cervical cancer cases and approximately 266,000 deaths in the world in 2012. It is the fourth most frequently diagnosed cancer and fourth leading cause of cancer death among women. Nevertheless, cancer of cervix is the second most commonly diagnosed malignancy after breast cancer in developing countries, where it is the third leading

cause of cancer death following breast and lung cancers. Consequently, nearly 90% of cervical cancer deaths are registered in these less developed countries [6].

Uterine corpus cancer, the vast majority of wich is endometrial cancer, is the sixth most commonly identified cancer affecting females worldwide with estimated 320,000 new cases. It is 14th in the case of mortality and was responsible for more than 76,000 death [6].

Ovarian cancer, a considerable type of gynecological malignancies is the 7th most commonly detected cancer in females, with an estimated 239,000 cases and approximately 152,000 deaths occurring in 2012. It was the 8th leading cause of cancer death among women [6].

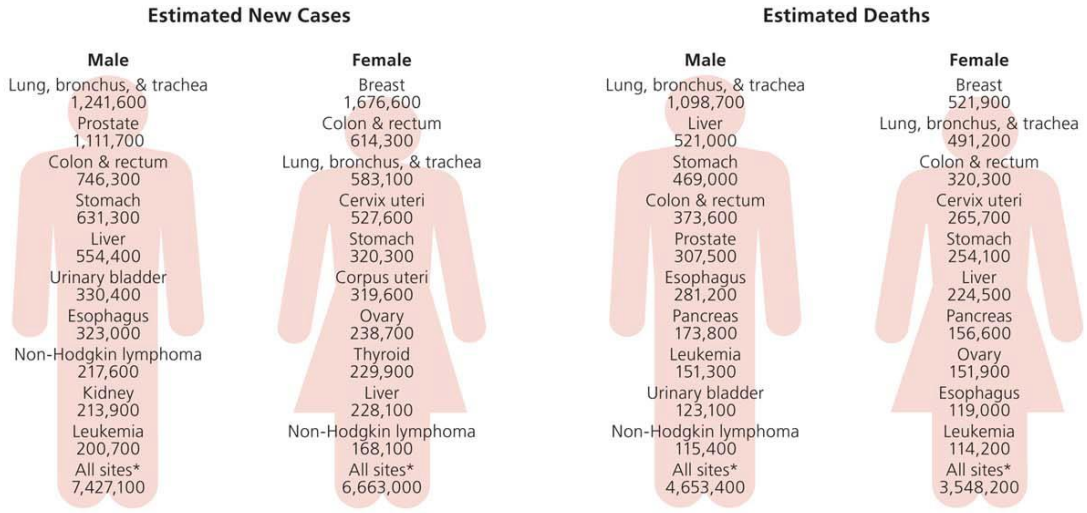


Figure 1. *Estimated new cancer cases and deaths worldwide by sex (*excluding non-melanoma skin cancers)[4].*

The importance of gynecological cancers is reflected by that breast cancer is the most frequent cause of cancer death in 103 countries, while cervical cancer in 43 countries among women. Indeed, the situation of malignancies of women remaines maleficient principally in low income countries, where treatments and some preventable strategy like screening or vaccination are poorly available due to limited resource and logistics barriers [6].

Despite the newfangled developments in personalized medicine, extensive preventive strategies and recent treatment approaches such as immunetherapy, the number of new cancer cases continues to grow owing to population growth, aging population and increasing age-specific incidence rates [1]. The increasing incidence and prevalence of cancer will require remarkable efforts and changes in health care systems, more intensive prevention and new perspectives in the field of early diagnosis and drug discovery. The development of novel chemotherapeutic agents is still a mainstay of anticancer therapy and one of the most important missions due to the continuously expanding global burden of cancer.

1.2 Steroids as biologically active compounds

Steroids are an important group of biologically active agents. Owing to their diverse biological effects, they play essential roles in various physiological processes. A set of endogenic hormones of the human body belong to the class of steroid compounds, they participate in some phases of ontogenesis, contribute to growth, differentiation and reproduction. Their influences are exerted on molecular level from participation in several signalizing pathways to regulation of gene expression, furthermore manifested equally at cellular and organzation levels.

Hormones of glucocorticoid subclass (e.g., cortisol) are crucial regulators of immune system and inflammatory processes. They possess fundamental roles in metabolism, moreover their functions in the response to physical and psychological stress are indispensable [7].

Mineralocorticoids (e.g., aldosterone) regulate electrolyte and water balances, therefore also have strong effect on control of blood pressure and plasma volume [8].

Sex hormones (e.g., testosterone, progesterone and estradiol) are responsible for the development of secondary sex characteristics, the growth and function of the reproductive organs. They exert a wide range of biological effects on some tissues and biological processes from the regulation of metabolism and menstrual cycle to the maintenance of pregnancy [9]. Furthermore, recent studies revealed that these endocrine compounds are substantial regulators of immune system [10].

Bile acids (e.g., cholic acid, glycocholic acid and taurocholic acid) facilitates the digestion of dietary fats and oils by their emulsifying properties. Besides the support of intestinal nutrient absorption, they possess several important physiological functions, such as regulation of lipid and glucose metabolism or hepatobiliary secretion of various toxic endobiotics and xenobiotics [11].

Neurosteroids (e.g., androstanediol, pregnenolone sulfate and allopregnanolone) possess outstanding importance in the central nervous system, such as modulation of neuronal excitability *via* interaction with membrane receptors and ion channels. Some of them contribute to age-related memory and learning. They have significant therapeutic potential although their roles are still poorly understood [12].

Besides endogenous hormones, exogenous steroid exposure exerts versatile effects on several physiological processes. Therefore, a wide range of steroidal agents are utilized in the medicine and serve as therapeutic tools for hormone replacement therapy (HRT) and contraception, also for men and women [13-16]. Thanks to their miscellaneous biological effects, a high number of steroids are utilized in the medication of cachexia [17], gynecological disorders (e.g., benign myomas or endometriosis) [18, 19], hormone dependent cancers [20, 21] and inflammatory

diseases. Their immune suppressive properties play key role in organ transplantation [22, 23], treatment of autoimmune diseases, allergic reactions and chronic inflammatory processes (eg.: asthma, chronic obstructive pulmonary disease, inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, allergic skin diseases, etc.) [22-27].

Anabolic androgenic steroids (AAS) are a generally known group of exogenous agents, that contains an arsenal of anabolic compounds (e.g., methyltestosterone, oxymetholone or stanozolol). Although the medicinal practice has utilized them for decades for treatment of some disorders (e.g., severe burns, short stature, osteoporosis and anemia) and testosterone replacement therapy for androgen deficiency syndrome, they are unsuitable for long-term usage due to their inappropriate steroid receptor selectivity and unfavorable pharmacokinetic properties [28]. Furthermore, owing to their varied and nonselective biological activities, most AAS display serious adverse effects, such as prostate enlargement, virilization, sexual dysfunction, hepatotoxicity, altered blood lipid profile and cardiovascular risks, they even elicit neuropsychiatric symptoms, like hostile behavior, aggression and depression [28, 29].

1.2.1 Steroids with anticancer properties

In spite of the fact that numerous steroidal agents display a broad spectrum of bioactivity, the oncological practice mainly utilizes them in the therapy of hormone dependent malignancies of the reproductive system exploiting their endocrine effects. Antiestrogens (e.g., fulvestrant) play essential roles in estrogen deprivation therapy (EDT) related to breast cancer treatment [30]. They compete with endogenous estrogens for binding to their receptors and thus inhibit estrogen-maintained proliferation of the hormone sensitive tissue. Steroidal aromatase inhibitors (e.g., formestane, exemestane) contribute to EDT preventing the production of estrogens from androgens via irreversible inactivation of aromatase [31].

The utilization of steroidal agents in androgen deprivation therapy (ADT) provides efficacious medications for therapy of prostate cancer. Besides the numerous non-steroidal compounds, steroidal antiandrogens and enzyme inhibitors are important tools of ADT. The antiandrogene cyproterone inhibits the action of androgens by means of competitive interaction with androgen receptor. Furthermore, it decreases the testicular secretion of androgens owing to its progestogenic activity [32]. Abiraterone and galeterone, two steroidal CYP17A1 enzyme inhibitors, hinder physiological androgen production that required for growth of hormone dependent prostate cells [33, 34].

Although an elevated number of steroidal compounds with anticancer effect have been identified in the last few decades, the utilization of steroidal agents in oncological usage is

relatively untapped. Numerous steroidal agents eliciting antineoplastic activity in a hormone-independent manner have been described. These compounds exert their effects through non-hormonal targets, such as microtubules or topoisomerases [31]. There are several molecules with notable therapeutic value among them, which are equally synthetic or originate from natural source. These observations have made the steroidal molecules a focus of attention.

1.2.1.1 Anticancer steroids from natural source

Natural antiproliferative compounds (e.g., taxanes, epipodophyllotoxins, camptothecin, Vinca alkaloids, etc.) are utilized in cancer chemotherapy for a long time [35-37]. The widespread natural occurrence of steroidal agents reflects their diversified biological activities including antiproliferative effect and offers exploitable feedstock for the development of novel anticancer agents. A great deal of plant steroids possess antineoplastic properties, moreover similar molecules in some animals and even in humans have been identified.

Some phytosterols (e.g. β-sitosterol, campesterol or stigmasterol) possess anticarcinogenic and anticancer properties. Cereal grains, legumes, nuts and vegetable oils contain large quantities of plant sterols, their dietary intake may contribute to the prevention of cancer. Association beetween phytosterol consumption and reduced risk of various cancerous disorders have been revealed by case-control studies. Certain phytosterols exhibit antineoplastic effect against lung, stomach and breast cancers. Some of them possess preventive actions against reactive oxygen species (ROS), β-sitosterol is able to increase the activities of antioxidant enzymes. An elevated number of investigations evinced the antiproliferative and apoptosis iducing effect of this steroid alcohol in different cancerous breast, prostate, colon and leukaemia cells *in vitro* [38-40]. Noticeable anticancer properties of an other phytosterol, guggulsterone have been explored by recent investigations. The compound derives from *Commiphora mukul* and has been used for thousands of years to cure distinct diseases, like inflammatory and cardiovascular disorders [41]. A number of studies demonstrated its antiproliferative action against a variety of cell lines, such as leukemia, breast, prostate cancer, pancreatic, esophageal, liver and colorectal cancer cells. Additionally, apoptosis induction in cancerous liver and cholangiocarcinoma cells by guggulsterone through different signaling pathways have been observed [41, 42].

Steroidal saponins, biologically active secondary metabolites of various plants (Sapindaceae, Ranunculaceae, Asparagaceae, etc.), consist of sugar moieties and their aglycon forms, sapogenins. Besides their cardiovascular and immunestimulatory effects, anticancer properties of numerous saponins have been disclosed. Several saponins stem from *Dioscorea*

zingiberensis exerted outstanding growth inhibitory and apoptotic inducing effects in cancerous ovarian, lung, liver and melanoma cell lines [43]. Deltonin expressed more pronounced cytotoxic activity against colon cancer than 5-fluorouracil, furthermore it arrested the cell cycle in G2/M phase and induced apoptosis *in vitro* and *in vivo* [44]. Diosgenin, occurs in *Trigonella foenum graecum*, is a potent proliferation inhibitor of different tumor cells, such as breast, liver, leukemia or osteosarcoma. Besides antiproliferative action, diosgenin inhibited the migration and invasion of PC-3 cells by reducing the mRNA level of matrix metalloproteinases, furthermore suppressed angiogenesis *via* termination of vascular endothelial growth factor expression [45].

Solanaceae family are important vegetable source of steroidal compounds with anticancer properties. Solanidines, a group of promising glycoalkaloids and their aglycone forms have already demonstrated beneficial effects against a high number of cancer cells under experimental conditions. In many cases *Solanum* glycosides display more pronounced antineoplastic activity than their aglycone forms, moreover some of them proved to be more effective against liver cancer than anticancer drugs doxorubicin and camptothecin. Solanidine and its trisaccharide glycosides α-chaconine and α-solanine from potato expressed potent toxicity against cervical, liver, lymphoma and stomach cancer cells, while their impact seemed to be limited on normal liver cells. Antimetastatic and antiangiogenic properties of α-chaconine have also been described. *In vitro* studies revealed the potent antiproliferative activity of the eggplant glycoalkaloids α-solamargine and α-solasonine and their common aglycone solasodine on human colon carcinoma, liver carcinoma, glioblastoma, breast and cervical adenocarcinoma. Solamargine inhibits growth of lung cancer cells and is also effective against multidrug resistant leukemia cells. This steroidal alkaloid evokes cell cycle arrest and initiates programmed cell death in liver cancer cells. Glycosylated forms of tomatidin, such as dehydrotomatine and α-tomatine have been described as promising anticancer agents of tomato. Their antiproliferative effects are proven against cancerous colon, liver, breast and prostate cell lines. Furthermore, antimigratory and proapoptotic function of α-tomatine have been reported *in vitro* and *in vivo*, respectively [46].

Withanolides (e.g., withanone, withanolide D) a substantial group of steroidal lactones from Solanaceae, exhibits powerful antitumor effects against a wide variety of tumor cells *in vitro* and even in animal models, like colon, pancreas, liver, breast, prostate and many others. The most potent withanolide withaferin A, isolated in *Withania somnifera*, demonstrates a wide range of anticancer effect, such as inhibition of tumor cell growth, induction of apoptosis and destruction of cancer stem cells. Furthermore, withaferin A exhibits strong antimetastatic

effects including inhibition of epithelial-mesenchymal transition (EMT), repression of angiogenesis and invasion [47, 48].

Cardiotonic steroids (CS) are one of the largest divisions of naturally occured steroidal agents, some of them have been in use for a long time for the treatment of heart failure [49, 50]. Two classes of compounds belong to CS including bufadienolides (e.g., hellebrin and amabufotalin) and cardenolides (e.g., digoxin, ouabain) [51]. Their cardiovascular effects are mediated by inhibition of Na^+/K^+-ATPase. Since epidemiological studies revealed the substantially lower mortality rates among cancer patients treated with CS, these molecules have gained a considerable attention. Additionally, Na^+/K^+-ATPase has been become to a novel target for anticancer therapy [50]. In addition to pump inhibition, regulation of some signaling pathway by CS also contributes to their antineoplastic activity [52].

Recent studies demonstrated that some bufadienolides originated from *Helleborus purpurascens* like hellebrin and its aglycon hellebrigenin exhibit pronounced growth inhibitory effects against a set of human cancer cell lines (glioma, melanoma, breast, prostate and colon) in nanomolar range. Furthermore the compounds are able to overcome apoptosis resistance in cancer cells and the multidrug resistance phenotype. Gamabufotalin is also considered as a potent antiproliferative agent [51].

Cardiac glycosides of *Digitalis species* like digoxin, digitoxin, gitoxin and their aglycon forms have been reported as powerful growth inhibitors in several cancer cell lines at very low concentrations *in vitro*. Their microtubule suppressing, cell cycle arresting and antimigratory properties elicited in various cancer cells have been revealed by *in vitro* and *in vivo* studies. Ouabain, a similar cardenolide described in *Strophanthus gratus* possesses multifarious anticancer actions including antiproliferative activity, autophagy induction and inhibition of migration indicated in liver, lung and breast cancer cells [51, 52].

In addition to plants, several cardiac glycosides occur in insects (e.g., *Danaus gilippus* and *Syntomeida epilais*) and amphibians (e.g., *Bufo rubescens, Bufo marinus*), such as digoxin, ouabain, bufalin, and telecinobufagin. Surprisingly, endogenous CS like digoxin, ouabain, marinobufagenin and telecinobufagin have been identified even in the human body, mainly in blood, adrenal gland and brain. In addition to their influence on different physiological process, they can contribute to distinct abnormalities like heart failure or renal disease [52].

Several steroidal compound have been identified in the marine fauna, including polyoxygenated marine steroids. Nautical wildlife can serve as prominent sources of biologically active molecules, this relatively untapped area of steroidal compounds may

contribute the development of new anticancer agents. Marine steroids possess unique structure with unusual residues, oxygenation patterns and functionalizations [53].

Cephalostatins, a group of bis-steroidal molecules explored in marine tube worm *Cephalodiscus gilchristi*, have been recognized as the most cytotoxic substances in the nature. Their emergent antiproliferative actions against 60 different cancer cell lines have been reported by a comprehensive invetigation of National Cancer Institute. Cephalostatin 1 and 2 proved to be efficacious at extremely low concentrations, in nano and subnanomolar ranges. The first compound is also potent inhibitor of melanoma, sarcoma and leukemia in xenograft models, furthermore induce apoptosis in an unconventional way [53, 54].

Cortistatins, a group of steroidal alkaloids discovered in marine sponge *Corticium simplex*, have been described as antiproliferative agents against human oral carcinoma and leukemia cells. Some of them possess potent antiangiogenic activity. Cortistatin A inhibits proliferation and migration of human umbilical vein endothelial cells induced by vascular endothelial growth factor, furthermore hampered bFGF-induced tubular formation [55, 56].

Further marine steroids with anticancer activity have been described. Squalamine, a novel steroidal agent discovered in dogfish shark *Squalus acanthias*, have exhibited significant antiangiogenic and antitumor activities. In clinical trials it proved to be safe and increased the cytotoxic effects of other chemotherapeutic agents [57]. Additionally a wide range of sponge and coral steroids have been decribed as antiproliferative agents. Moreover, some multidrug resistance (MDR) modulator exists among them, like the steroidal agosterol A which is able to suppress MDR mediated by P-glycoprotein 1 or multidrug resistance-associated protein 1 [53].

Besides the numerous compounds occured in amphibians and marine organisms, some steroidal agents with antineoplastic activity have been observed in mammals inculing humans. The endogenous mammalian metabolite of estradiol, 2-methoxyestradiol have attracted scientific attention because of its miscellaneous anticancer activity. The antiproliferative activity of this estradiol derivative have been reported against several cancer cell lines (e.g., ovarian, lung, prostate, colorectal etc.) *in vitro* and *in vivo*. The compound elicits antitumor effects in hormone independent manner including antitubulin, antiangiogenic, ROS induction and proapoptotic properties [58, 59].

Surprisingly, some endogenous bile acids have been recognized as potent anticancer agent. Cholic acid and deoxycholic acid proved to be potent inhibitors of cell growth and inducers of programmed cell death in esophageal cancer cells [60]. Deoxycholic acid and chenodeoxycholic acid demonstrated potent antiproliferative activity and induced apoptosis in ovarian and colon cancer cells [61, 62].

1.2.1.2 Synthetic anticancer steroids

Since sterane skeleton is in the spotlight due to its metabolically stable and functionalizable structure, steroids are proved to be an intensively investigated area in pharmacology, organic and medicinal chemistry. The core scaffold offers various possibilities for chemical modifications which may result altered biological activity.

Some synthetic solanidin analogs with antiproliferative activity against human leukemia cells have been reported. The most potent agent among them exerted cell cycle arresting and apopotosis induction in examined cells [63].

A potent digitoxin derivative, β-D-Digitoxose was examined for its anticancer effect. The compound displayed strong cytotoxic activity and apoptosis initiating impact on different cancer cell lines at nanomolar concentrations. This synthetic analog elicited disturbances in cell cycle and hampered the migration of cancer cells [64].

Recent analogs of estradiol have been described as potent antiproliferative agents against triple-negative breast cancer cells. Apoptosis inducing and cell cycle arresting compounds were identified among them, which also were able to suppress migration and invasive properties on the studied cell line [65].

An elevated number of publications demonstrated the growth inhibitory and proapoptotic effect of newly synthesized bile acid derivatives and their conjugates against several cancerous cell line. Some of them elicited membrane alterations, oxidative stress or prevented angiogenesis [53].

The abundance of synthetic steroids with anticancer activities indicates that this branch of medicinal chemistry is generally considered as perspective.

1.2.1.2.1 Androstanes as antineoplastic agents

The androstane scaffold provides easily editable parent compounds for establishement of novel steroid-based anticancer agents, a large amount of experience in chemical construction and editing of androstanes has already been accumulated. Increasing number of literature reports reflects the importance of these compounds in anticancer research, numerous androstane analogs with antiproliferative activity against several cancer cell lines (e.g., colon, renal, prostate, breast, melanoma, and leukemia) have been reported [66-68].

1.2.1.2.2 19-nortestosterone derivatives

This class of androstanes contain compounds with typically pronounced anabolic effect which made this group therapeutically important and also there are riports on their illegal use in professional sports. Therefore, such norsteroids became a vigorously investigated area of organic chemistry and pharmacology. They have been in use for many decades in treatment of several disorders such as osteoporosis, anemia, male hypogonadism endometriosis and HRT [18, 28, 69-71]. Owing to their progestagenic effect, some 19-nortestosterone derivatives contributed to the establishement of an arsenal of oral contraceptive drugs (eg. norethisterone, lynestrenol, levonorgestrel, norgestimate, gestoden, dienogest). Usually they possess antigonadotropic and antiestrogenic effects, often androgenic activity too, in contrast estrogen activity occurs occasionally among them. Consequently, these compounds are considered as the basis for the success of hormonal contraception [72].

Since the removal of 19-C methyl group is accompanied by reduced androgenic activity, 19-norsteroids provide attractive skeleton for development novel therapeutic agents without collateral endocrine effect. These compounds have also been discovered by anticancer research. *In vitro* and *in vivo* studies revealed the outstanding anticancer effects of gestodene, 3-ketodesogestrel and tibolone against some breast cancer cells [73, 74].

Dienogest, a well known progestagenic derivative used in the medication of endometriosis and as oral contraceptive, exhibited unique anticancer effect against endometrial and breast cancer cell lines. Moreover, its effective dose proved to be lower than that of tamoxifen or medroxyprogesterone [75].

Mibolerone, another non-metabolizable analog with androgenic activity have been recognized as potent growth inhibitor of breast cancer cells *in vitro* in nanomolar concentration [76]. Some newly synthesized 19-nortestosterone derivatives have also been reported with noticeable antiproliferative action against several cancer cell lines (e.g., renal, brain, prostate cells) [77].

Besides the few compounds described with promising antiproliferative activity, this family of androstanes are a very untapped area in anticancer research. Althoug the antineoplastic effect of these compounds is often the consequence of their hormonal effects, they can be considered as excellent parent molecules for the development of novel antitumor agents with atypical mechanism of action.

1.2.1.2.3 Steroidal hybrid compounds

The combination of biologically active agents with different chemical structures may result changes in mechanism of action and biological target. Hybridization of steroids with other

molecules is often accompanied by alterations in biological activity of the original compounds, which can be favorable in the case of appearance of advantageous properties.

Novel steroid-therapeutic drug hybrids have been described, which allowed the targeted drug delivery and potent antitumor action in breast cancer targeting estrogen receptor [78]. Androgen-linked alkylating agents have also been created and examined *in vivo*. Some of them demonstrated a remarkable antineoplastic activity in induced rat mammary carcinoma [79].

A recent study investigated the potent growth inhibitory effect of newly synthesized pregnenolone-2-cyanoacryloyl conjugates, which are proved to be effective against lung, breast, oral squamous cell carcinoma and leukemia cell lines [80]. These observations offer new perspectives in steroid chemistry and drug designing.

1.2.1.2.4 Androstane-quinoline hybrids

Quinolins are intensively examined compounds in medicinal chemistry due to their versatile biological effects. Besides steroidal compounds, some quinoline derivatives have also been reported as potent anticancer agents. These molecules exhibited a powerful antiproliferative action against cervical, stomach, liver and lung cancer cell lines, futhermore they arrested the cell cycle and induced programmed cell death [81, 82].

Although the quinoline incorporation into steroid backbone is relatively rarely utilized, some A and B-ring fused steroidal quinolines have been published and evaluated for their antimicrobial activities. Some compounds exhibited potent antibacterial and antifungal effect [83]. Since advantageous biological properties of synthetic steroid-quinoline hybrids have been revealed, preparation and evaluation of novel similar compounds for antitumor activities became to an important goal of anticancer research. Whereas a set of steroidal hybrids and quinolie derivates with antineoplastic effect have been described, the possibility of attachement of quinolines to the androstane scaffold may provide novel molecules with exploitable biological actions for anticancer therapy.

1.3 Chemical modifications of androstane skeleton for improvement of therapeutic value

The conventional skeleton of androstanes offers various opportunities for a wide range of chemical modifications which have a remarkable influence on their pharmacological activities. Direct chemical editing of their scaffold allows the establishement of novel engineered steroids with favorable effects. In addition to preservation of beneficial properties, unfavorable adverse reactions can be reduced by using an adequate modification.

Until recently, a set of structure-activity relationship have been revealed by several previous investigations related to androstanes. Consequently, a considerable amount of information is available concerning the structural motifs which are influencing factors of hormonal, anabolic activity and bioavailability.

Since hydrogen-bonding plays a crucial role in the interaction between natural hormones and their receptors, every modification of the apolar sterane scaffold or the polar functional groups at C-3 and C-17 positions may affect the binding affinity of the molecule, thereby may raise or reduce receptor mediated hormonal activity [9]. Roles of C-3 and C-17 positions are undisputed in the case of interacting with the proper amino acid residues of the hormone binding pocket of androgen receptor. 3-Keto group in the A-ring, 17β-OH in the D-ring or the double bond between C-4 and C-5 are cardinal structural elements which ensure the androgenic activity of androstane skeleton. All the modifications affecting these chemical entities diminish androgenic properties. The removal of C-17 oxygen function results in the loss of androgenic activity. 17α-hydroxyl group instead of β position results in two order of magnitude lower activity in the case of testosterone [84].

5α-steroidal framework enhances affinity to androgen receptor, reflected by the androgenic difference between 5α and 5β-DHT. Such as 11-oxo substitution, installation of bulky groups at the 7α position decrease binding affinity, while small substituents increased the hormonal activity. The absence of methyl group at C-19 also results in diminished androgenic activity [84].

Some modifications alter the anabolic efficiency of androgens, thus alkylation at C-1 and C-2 or removal of C-19 contribute to anabolic effects. 17α-alkylation of androstanes results in prolonged anabolic activity owing to increase resistance to enzymatic oxidation of the D-ring. These 17α-alkylated androgens (e.g., methyltestosterone, methandienone, norethandrolone) undergo a significantly slower hepatic inactivation supporting longer and increased anabolic effects. Most of orally active synthetic androgenes are 17α-alkylated. Such as 5α-framework, this modification is even unfavorable for aromatization of the A-ring to estrogens [84, 85].

Esterification of the 17-hydroxy group with long-chain acids decelerates transformation to ketosteroids and contributes to a longer effect. These esters are suitable for only parenteral administration. Surprisingly, the size of the hydrocarbon part at C-17 correlates with the duration of anabolic effect, thus short-chain esters exert only a short action [85].

In many cases, exchange of carbon atoms to heteroatoms in the androstane skeleton eventuate in the alteration of biological activity. Some examples show that oxygen or sulfur atom at C-2

position (e.g., oxandrolone) results in compounds with substantial myotropic activity and modest androgenicity, while C-4 substitution leads to loss of efficiency [84].

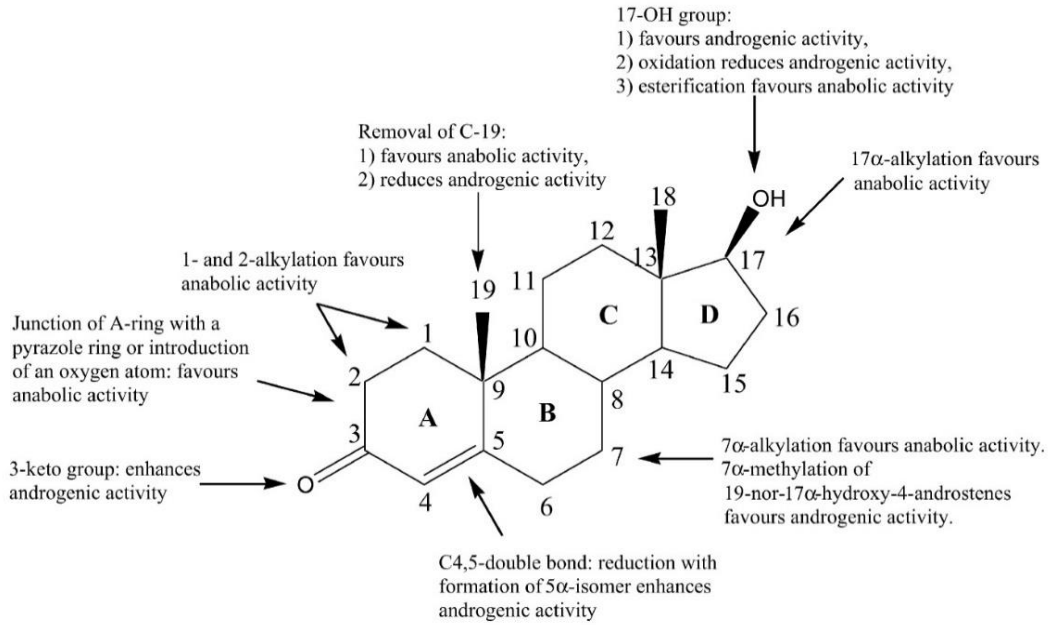


Figure 2. *Substantial modificable moieties of testosterone [84].*

Extensive possibilities of structural modifications provide opportunities for development of novel non or less androgenic androstane-based compounds with satisfying bioavailability and favorable biological properties. Furthermore, the elimination of undesired adverse effects such as endocrine activity can considerably increase the therapeutic value of the compounds. Though a high number of modified steroids with anticancer action have been synthesised and several chemical modifications resulting an increase in cytotoxic activity have already been reported, fundamental correlations between structure and anticancer properties are still missing. One of the greates challenges is still to discover further structure-activity relationships and appropriate modifications which are favorable and contribute to the development of more selective and cytotoxic compounds. The structure-based drug design may result in promising androstane-based drug candidates for cancer therapy in the nearly future.

2 SPECIFIC AIMS

The basic aim of the presented works was to characterize the antiproliferative properties of recently synthesized steroid-quinoline hybrids and 19-nortestosterone derivatives. The specific examinations of the study aimed at to recognize the possible mechanism of action of the effective compounds. The aims of the implemented investigations were the followings:

- Examination of antiproliferative activity of androstane-based test substances against human gynecological cancer cell lines. Determination of IC_{50} values, cytotoxicity and cancer selectivity in the case of the most potent compounds.
- Investigation of alterations in cell cycle by means of flow cytometry.
- Characterization of apoptosis inducing effect elicited by the most effective molecules by dint of cell cycle analysis, fluorescent microscopy and examination of inducing effect on various caspase enzymes.
- Description of their direct influence on microtubular system by means of tubulin polymerization assay.
- Determination of the androgen activity of the selected agents using endocrine disruptor test.
- Recognition of structure-activity relationships based on the experimental results.

3 MATERIALS AND METHODS

3.1 Chemicals

All the test compounds were synthetized at the Department of Organic Chemistry, University of Szeged, Hungary. 10 mM stock solutions of the tested agents were prepared with dimethyl sulfoxide (DMSO) for all *in vitro* experiments. The highest applied DMSO concentration of the medium (0.3 %) did not have a notable effect on the cell proliferation. All other chemicals and kits, if otherwise not specified, were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary).

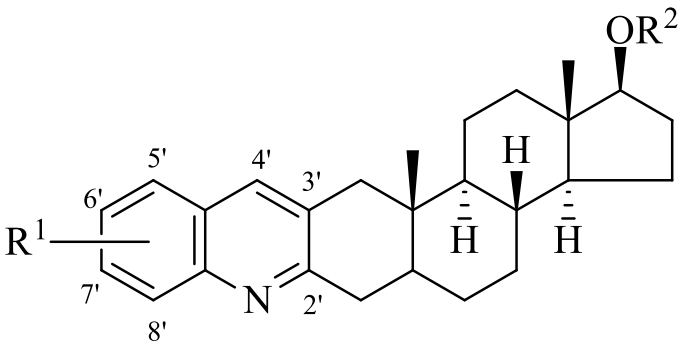
3.1.1 Test compounds

3.1.1.1 Steroid-quinoline hybrid molecules

A set of novel A-ring-fused quinolines in the 5-α-androstane series were prepared as described previously [86][Appendix I]. The six-membered fused ring system offer opportunities for a wide range of chemical modifications, attachment of several substituents to various positions resulted a new series of double-substituted steroid-quinoline hybrid agents (Figure 3).

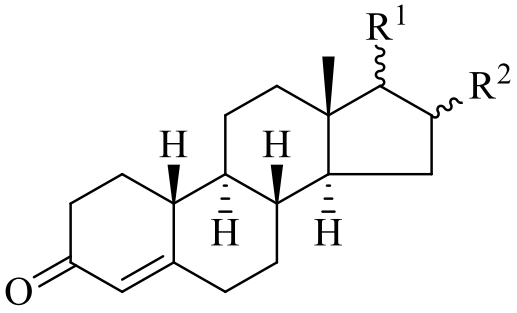
3.1.1.2 19-nortestosterone derivatives

A series of novel 19-nortestosterone derivatives [Appendix I] were synthesized as described previously [87, 88][Appendix II, III]. Installation of various substituents at the C-16 and C-17 positions led to the production of a set of structurally related compounds of 19-nortestosterone and its 17α-epimers (Figure 4).



Compound	R_2	R_1	Position of R_1
SQH1	Ac	H	-
SQH2	Ac	Me	6'
SQH3	Ac	MeO	6'
SQH4	Ac	Cl	6'
SQH5	Ac	Br	6'
SQH6	Ac	Cl	7'
SQH7	H	H	-
SQH8	H	Me	6'
SQH9	H	MeO	6'
SQH10	H	Cl	8'
SQH11	H	Cl	6'
SQH12	H	Br	6'
SQH13	H	Cl	7'
SQH14	H	Cl	5'
SQH15	H	Me	8'

Figure 3. *Chemical structures of the tested steroid-quinoline hybrids (SQH1-15). The compounds possess various substituents in R_1 and R_2 positions.*



Compound	R_1	R_2
NTD1	β-OH	β-CH_2OH
NTD2	β-OH	α-CH_2OH
NTD3	α-OH	β-CH_2OH
NTD4	α-OH	α-CH_2OH
NTD5	α-OH	H
NTD6	α-OAc	H
NTD7	α-OAcPh	H
NTD8	α-Cl	H
NTD9	α-OBz	H
NTD10	α-O-4-toluoyl	H
NTD11	α-O-4-OMe-Bz	H
NTD12	α-O-4-Br-Bz	H
NTD13	α-O-4-NO_2-Bz	H
NTD14	α-O-2,4-NO_2-Bz	H
NTD15	α-O-3,5-NO_2-Bz	H
NTD16	α-O-2,4,6-Me-Bz	H
NTD17	α-O-2-I-Bz	H
NTD18	β-OAc	β -CH_2OAc
NTD19	α-OAc	α-CH_2OAc
NTD20	α-Br	H
NTD21	α-I	H
NAN*	β-OH	H

Figure 4. *Chemical structure of the tested 19-nortestosterone analogs, including the structurally analogous reference agent, nandrolone* (NAN). The compounds possess various substituents in R_1 and R_2 positions.*

3.2 Cell lines and culture conditions

Gynecological cancer cell lines, including ovarian (A2780), cervical (HeLa) and breast cancer cell lines (MCF7, T47D, MDA-MB-231 and MDA-MB-361) were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). Two additional cervical cell lines (SiHa and C33A) were purchased from American Tissue Culture Collection (ATCC, LGC Standards GmbH, Wesel, Germany). Intact fibroblast cells (MRC-5) were also obtained from ECACC, while noncancerous epithelial (hTERT-HME1) cell line dereived from ATCC. With the exception of hTERT-HME1, all cells were maintained in tissue culture flasks containing Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA) and 1% antibiotic-antimycotic mixture (Penicillin, Streptomycin and Amphotericin B). hTERT-HME1 cells were cultured in serum-free mammary epithelial cell growth medium (MEGM) completed with human epidermal growth factor, insulin, hydrocortisone, bovine pituitary extract, and an antibiotic-antimycotic mixture. The flasks were stored at 37°C in humidified air containing 5% CO_2 in order to provide appropriate environment for cells growth. During subculturing process, washing step with phosphate-buffered saline (PBS) and trypsinization were performed. PBS, EMEM and supplements were purchased from Lonza Group Ltd. (Basel, Switzerland), cell dissociation reagent derived from Life Technologies Magyarország Ltd. (Budapest, Hungary).

3.3 Determination of antiproliferative effect

The antiproliferative properties of the compounds have been determined by MTT assay. For screening measurements, cells were generally seeded onto 96-well plates at a density of 5,000 cells/well, 10,000 cells/well in the case of MDA-MB-361 and C33A cells. After an overnight repose, fresh medium supplemented with the tested compounds (at 10 or 30 μM) was added and cells were incubated again for 72 h under culture conditions. Thereafter, the medium was completed with 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution. During 4-h contanct peroid, MTT was converted to purple formazan crystals due to the enzymatic reactions of the living cells. The precipitated crystals were measured by means of spectrophotometry at 545 nm using microplate reader after dissolving in DMSO. The measured absorbance is directly proportional to the number of viable cells and inversely proportional to growth-inhibitory effect of the compounds. Untreated cells were regarded as control sapmles. In the case of compounds with remarkable activity, the assay was repeated with a series of dilutions (0.1 – 30 μM) and IC_{50} values were determined using sigmoidal concentration-response curves fitting to measured data (GraphPad Prism 5.0, GraphPad

Software, San Diego, CA, United States). Cisplatin (Ebewe Pharma GmbH, Unterach, Austria) served as reference agent routinely used in the therapy of some malignancy, while NAN was utilized as reference compounds with analogous chemical structure. In order to confirm the reliability of this method, each test was implemented in two independent measurements.

To obtain information about tumor selectivity of the potent compounds, the assay was also performed on noncancerous cells under the same experimental conditions.

3.4 Detection of cytotoxic effect

Release of intracellular enzymes from cells reflects the extent of membrane damage and cytotoxic effect elicited by chemicals. The direct cytotoxicity of the tested agents has been determined by means of a lactate dehydrogenase (LDH) detection assay.

HeLa cells were seeded onto 96-well plate at a density of 5000 cells/well. After an overnight repose, the medium containing the appropriate concentrations of tested compounds were added, then cells were incubated under culture conditions for 24 h. Thereafter the activity of released LDH from treated cells was determined using a commercially available colorimetric kit following the instructions of the manufacturer (Roche Magyarország Ltd., Budapest, Hungary). CIS was used as reference agent. Untreated cells served as control, while detergent (Triton X-100) was regarded as positive control, triggering a maximal LDH release from the cells.

3.5 Flow cytometric analysis of cell cycle and apoptosis

Changes in distribution of cells in the different cell-cycle phases (G0/G1, S and G2/M) were detected via measuring cellular deoxyribonucleic acid (DNA) content by dint of flow cytometry after staining with fluorescent intercalating dye, propidium iodide (PI). This method also allowed to identify hypodiploid populations (subG1), indicating apoptotic fragmentation [89].

The cells were plated onto 6-well plates at a density of 2.5 or 3 x 10^5 cells/well and allowed to stand for an overnight before the treatment with the selected compounds for 24 h, 48 h or 72 h. After the incubation time, the cells were harvested by trypsinization, washed with PBS and fixed in ice cold 70% EtOH. After one hour storage at –20ºC, each sample were supplemented with DNA staining solution (containing distilled water, 0.1 mg/ml PI, 0.3 % v/v Triton X-100, 1.0 mg/ml sodium citrate and 0.02 mg/ml ribonuclease-A) and stored in the dark at room temperature for an hour. The samples were analyzed by flow cytometry (Partec CyFlow, Partec GmbH, Munster) and their measured data were evaluated using ModFit LT 3.3.11 software (Verity Software House, Topsham, ME, USA). Approximately 20,000 events were recorded in each analysis and three parallel measurements were carried out in each conditions.

3.6 Morphological studies by fluorescence microscopy

In view of the previous results, fluorescent staining with DNA-specific dyes was performed in order to detect morphological changes and induction of programmed cell death exhibited by the selected compounds using fluorescent microscopy. For this purpose, cells were seeded onto 96-well plate at a density of 3000 or 5000 cells/well and allow them in repose for an overnight under culture conditions. The cells were treated with increasing concentrations of the selected compounds for 24 h or 48 h. After the treatment, cells were incubated with fluorescent staining solution (containing Hoechst 33258 and PI, 5 μg/mL and 3 μg/mL, respectively) in dark for an hour. The stained cells were analyzed under fluorescence microscope (Nikon ECLIPSE 146 TS100, Nikon Instruments Europe, Amstelveen, The Netherlands) using appropriate optical filters corresponded with the applied dyes. At least three fields were recorded with an attached QCapture CCD camera in case of all conditions.

This examination provides opportunity to distinguish intact, early apoptotic and necrotic morphology of cells due to their diverse morphological appearance and different membrane integrity after the double staining. Hoechst 33258 dye is able to permeate through the cellular membranes, therefore nuclei of intact cells express a pale and homogeneous blue staining. Intense blue fluorescence alone and inhomogeneous morhpology of nuclei indicate the chromosome condensation, a characteristic step of the apoptotic process. PI can penetrate into the cells only after membrane damage therefore its red fluorescence displays necrotic cells. The intact, early apoptotic and necrotic cells were numerated and then were analyzed statistically.

3.7 Determination of caspase activities

Programmed cell death is a strictly regulated process with various biochemical and morphological changes which can be initiated through two molecular pathways. Both of *intrinsic* (mitochondrial) and *extrinsic* (death receptor) pathways are mediated by a sequential activation of cysteine proteases, also known as caspase cascade. Several iniciator and effector enzymes are involved in these overlapping pathways [90].

To evidence apoptosis inducing effect of the selected compounds, the activation of a common effector protease, caspase-3 was analysed by means of colorimetric assay in accordance with the instructions of the manufacturer (Abnova Corporation, Taipei, Taiwan). For the decision about which pathway of the apoptosis is induced due to the treatment, the activities of two iniciator caspases which are participating in different pathways were determined.

For this purpose, measurements of enzyme activity were performed using colorimetric assays in the case of caspase-8 (Abnova Corporation) and caspase-9 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions, respectively.

In all cases cells were seeded onto cell culture flasks at a density of approximately 12 million cells/flask and allowed in repose for an overnight. The adhered cells were exposed to the proper concentrations of the compounds for 24 h, 48 h or 72 h. After the treatment, the cells were collected with scraping, then lysed and centrifuged. Supernatants containing the enzyme were collected and enzyme activities were determined by means of spectophotometric way. In these measurements, specific oligopeptid linked *p*-nitroaniline (*p*NA) compounds served as substrates for the corresponding caspases. The chromophore *p*NA after cleavage from the labeled substrate was quantified at an absorbance wavelength of 405 nm using microplate reader. Elevation in absorbance of free *p*NA indicates increase of caspase activity. Results were expressed in fold increase of enzyme activity compared with the untreated control.

3.8 Tubulin polymerization assay

This method provides opportunity for determination of incidental influence of the selected molecules on microtubular system under a cell free condition. In view of the former results, *in vitro* tubulin polymerization assay was carried out using a commercially available kit (Cytoskeleton Inc., Denver, CO, USA) following the instructions of the manufacturer. The assay reactions were performed on pre-warmed (37°C) UV-transparent microplate and measured by pre-warmed (37°C) UV-spectrophotometer. 10 µL of the test compound solutions at proper concentrations were loaded into the wells. All the wells were supplemented with 2 mM $MgCl_2$, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 1 mM guanosine triphosphate (GTP) and 10.2% glycerol. 10 µL of general tubulin buffer served as untreated control. 10 µL of 10 µM paclitaxel (PAC) served as reference agent. The polymerization reaction was started with addition of 100 µL 3.0 mg/ml tubulin (in 80 mM PIPES, pH 6.9) to each sample. The absorbance of the samples was measured per minute at 340 nm by means of a 60-min kinetic protocol. Each sample was prepared in two parallels.

The maximal difference between the absorbances which are belonging to two consecutive timepoints was regarded as V_{max} (Δabsorbance/min) of the polymerization reaction. V_{max} values of the reactions were determined in all conditions and analyzed statistically. The effect of the selected compounds on polymerization were compared with the polymerization rate of the untreated control (basal tubulin polymerization) and the reference agent.

3.9 Determination of hormone effect

For detection of incidental androgenic activity of the selected agents, endocrine bioassay kit was purchased (Xenometrix AG, Allschwil, Switzerland). Genetically modified *Saccharomyces cerevisiae* strain, containing DNA sequences of human androgen receptor, androgen responsive element and reporten gene (lacZ) served as a basis of the examination. The expression of reporter gene due to androgenic effect causes β-galactosidase production in the cells. The enzyme secretes into the medium and converts its yellow CPRG (chlorophenol red-β-D-galactopyranoside) substrate into red product which can be quantified by dint of spectrophotometry. The amount of the formed red product correlates with the agonistic effect, while the decrease of the 5α-dihydrotestosterone (DHT) induced product forming correlates with the antagonistic effect.

Briefly, yeast cells were cultured in humidified air at 31°C with agitation. The examinations were performed on 96-well microplate containing the cells in accordance with the provider's instructions. The cells were exposed to the proper concentrations of the tested agents for 48 h in the persence of CPRG substrate solution. For antagonistic examinations, all conditions were supplemented with DHT solution. After 48 h incubation at 31°C, the absorbances were measured at 570 nm using microplate reader and analyzed statistically, the agonistic and antagonistic properties of selected molecules were determined. Each sample was prepared in two parallels. NAN was used as positive control in agonistic measurements. Flutamide served as reference compound in antagonistic examinations.

3.10 Statistical analysis

In all examination, statistical evaluation of the experimental results was performed by one-way analysis of variance using GraphPad Prism 5 software. Results were expressed as mean ± SEM (standard error of mean). Dunnett posttest was used to estimate the significance of differences in comparisons. P-value of <0.05 was regarded as statistically significant.

4 RESULTS

4.1 Antiproliferative properties of steroid-quinoline hybrid molecules

4.1.1 Antiproliferative effect of steroid-quinoline hybrids

The antiproliferative activities of the prepared compounds were determined by means of MTT assay using cervical and breast cancer cell lines. 15 steroid-quinoline hybrids were examined in screening measurements for their antiproliferative properties. In the case of compounds with growth inhibitory effect higher than 50% at 30 μM, the assay was performed on the appropriate cell lines using a series of dilutions and the IC_{50} values were calculated (Table 1).

All the compounds exhibited a moderate growth inhibitory effect on one or a few of the applied cell lines with the exception of SQH1, however their effect is not comparable to the action of reference agent in most cases. With the exception of SQH5 and SQH6 none of the tested agents elicited a substantial effect on the HPV+ cell lines, HeLa and Siha. Most of the tested agents triggered growth inhibitory action on the HPV- cell line C33A, however their IC_{50} values are with an order of magnitude higher than that of the reference agent CIS.

Only three compdounds (SQH3, 10 and 13) exhibited antiproliferative action on MDA-MB-231 cell line, which is considered as comparable to the effect of reference agent. Nevertheless, IC_{50} values with approximately 20 μM are regarded as relatively high among antiproliferative agents. Five of the tested agents displayed a remarkable growth inhibition against MDA-MB-361 cells. Although SQH15 possesses the most pronounced effect (10.5 μM) on this cell line, it is significantly weaker than that of CIS (3.7 μM).

Although many of the tested hybrids demonstrated a moderate antiproliferative action against MCF-7 and T47D cell lines, most of them have an order of magnitude higher IC_{50} values on both cell types compared to the reference compound. However, there are two molecules among them, SQH7 and SQH9 with pronounced growth inhibitory action (12.4 and 10.2 μM, respectively) on T47D cells, their effects are regarded as comparable to the impact of CIS (9.8 μM).

Among the tested hybrids, SQH9 is proved to be the most potent compound with promising antiproliferative effect on some cancerous cell lines. Although this molecule elicited only a moderate action on MCF-7, its IC_{50} values on C33A and T47D are slightly higher than 10 μM. On T47D, this growth inhibitory action of SQH9 (10.2 μM) is considered as comparable with the effect of the reference agent (9.8 μM). Based on these findings, the most potent compound

was selected for additional investigations in order to reveal some further details about its possible mechanism of action. All the additional experiments with SQH9 were performed on T47D cells.

Comp.	IC_{50} values (μM)						
	HeLa	SiHa	C33A	MCF-7	MDA-MB-231	MDA-MB-361	T47D
SQH1	>30	>30	>30	>30	>30	>30	>30
SQH2	>30	>30	**16.2**	>30	>30	>30	>30
SQH3	>30	>30	>30	>30	**21.5**	>30	>30
SQH4	>30	>30	>30	>30	>30	**12.6**	>30
SQH5	>30	**23.6**	**26.5**	**27.9**	>30	**16.8**	**20.8**
SQH6	**20.3**	>30	>30	**17.6**	>30	**16.8**	>30
SQH7	>30	>30	**11.8**	**18.7**	>30	>30	**12.4**
SQH8	>30	>30	**20.1**	>30	>30	>30	>30
SQH9	>30	>30	**10.2**	**19.1**	>30	>30	**10.2**
SQH10	>30	>30	**15.4**	**29.9**	**18.7**	**12.8**	**27.1**
SQH11	>30	>30	**12.2**	**24.9**	>30	>30	**23.4**
SQH12	>30	>30	**12.3**	**23.3**	>30	>30	**18.5**
SQH13	>30	>30	**18.6**	**24.2**	**22.4**	>30	>30
SQH14	>30	>30	**20.5**	**16.4**	>30	>30	**24.0**
SQH15	>30	>30	**20.0**	>30	>30	**10.5**	>30
CIS	**12.4**	**7.8**	**1.8**	**5.8**	**19.1**	**3.7**	**9.8**

Table 1. *Calculated IC_{50} values of the tested steroid-quinoline hybrids and cisplatin, measured by MTT assay after incubation for 72 h on the applied cancer cell lines. n.d. indicates not determined.*

4.1.2 Effects of the selected steroidal quinoline on cell cycle

According to the antiproliferative capacity of the tested hybrids, the influence of the most potent compound on the distribution of T47D cell cycle phases was determined by means of flow cytometry after 48 h exposure. Remarkable changes were occured owing to treatment with 10 and 30 μM of SQH9 (Figure 5), such as the concentration-dependent increase of hypodiploid cells (subG1 phase), which can be regarded as an apoptotic population. Furthermore, incubation with SQH9 at the higher concentration significantly increased the proportion of cells in G1 phase. In contrast, a considerable reduction was observed in the ratio of cells in both of S and G2/M phases due to 30 μM treatment.

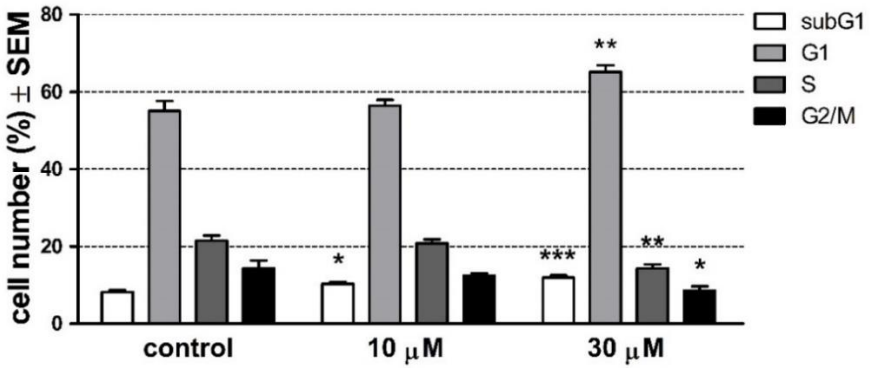


Figure 5. *Effect of SQH9 on T47D cell cycle phase distribution determined by flow cytometry after treatment for 48 h. *, ** and *** indicate p<0.05, p<0.01 and p<0.001 as compared with the untreated control cells.*

4.1.3 Morphological changes and apoptosis induction

On the basis of cell cycle changes elicited by tested compound, morphological features of T47D cells were examined by means of fluorescent double staining under fluorescent microscopy after 48 h incubation with 10 or 30 μM of the selected hybrid (Figure 6).

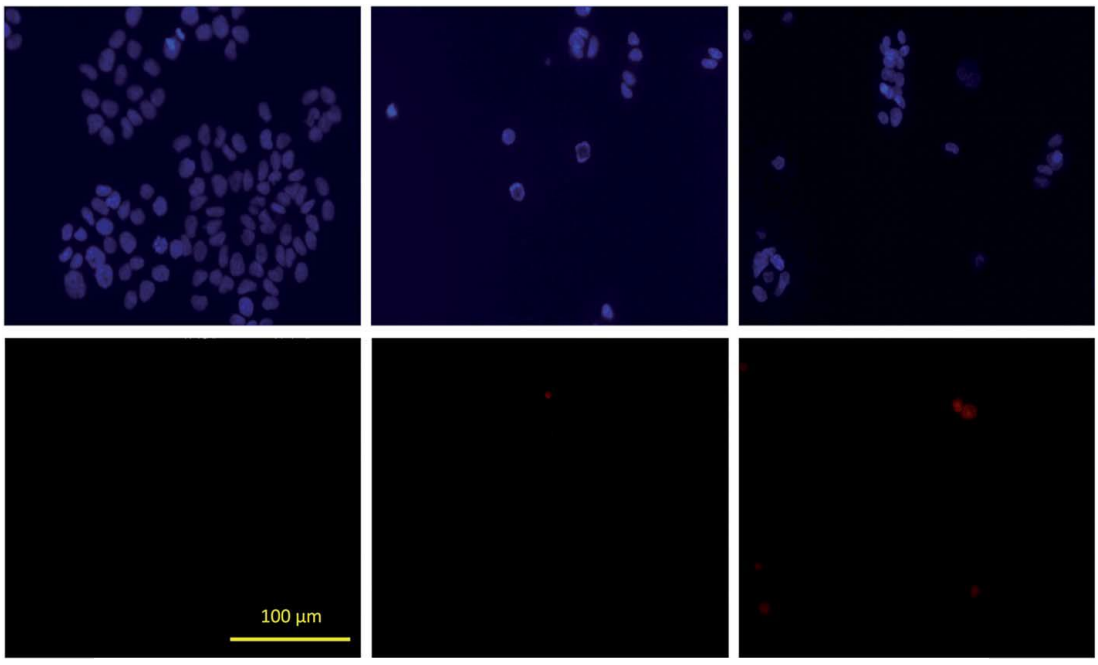


Figure 6. *Representative picures from fluorescent staining of T47D cells exposed to 10 or 30 μM of SQH9. Blue (upper panels) and red (lower panels) fluorescence indicate Hoechst 33258 and PI uptake, respectively.*

Treatment with SQH9 increased the number of apoptotic cells, which are emitting powerful blue light due to chromatin condensation. The concentration-dependent emergence of

membrane damaged cells with red fluorescence indicated secondary necrosis, a possible consequence of *in vitro* conditions.

For a quantitative analysis, cells with different morphology were numerated in all conditions and the ratio of intact, apoptotic and necrotic cells were determined. The proportion of apoptotic cells exhibited a concentration-dependent increase due to the treatment at the expense of intact cells (Figure 7). All of these changes proved to be statistically significant. Additionally, the ratio of the cells with necrotic morphology increased significantly only after treatment with the higher concentration of SQH9.

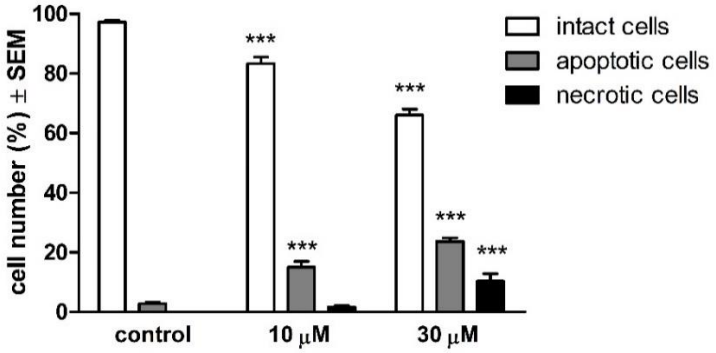


Figure 7. *Quantitative evaluation of fluorescent double staining of T47D cells after 48 h incubation with 10 or 30 μM SQH9. *** indicates p<0.001 as compared with the untreated control cells.*

4.1.4 Influence of steroid-quinoline hybrids on caspase-3 activity

In accordance with the results of cell cycle analysis and fluorescent double staining, the effect of the selected compound on caspase-3 activity was determined in order to confirm its apoptosis inducing effect. Treatment with SQH9 for 48 h increased the activity of the enzyme in a concentration-dependent manner in treated T47D cells, significantly at the higher concentration (Figure 8).

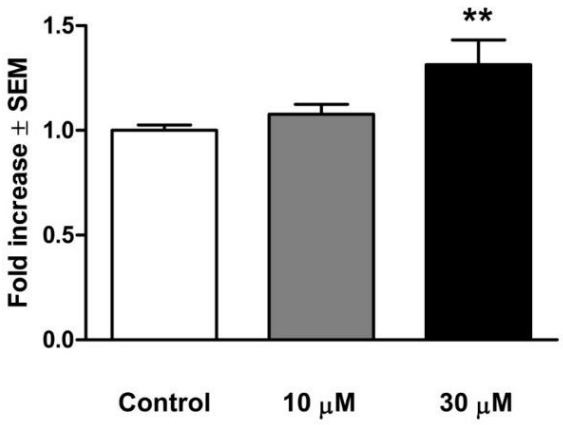


Figure 8. *Induction of caspase-3 activity after 48 h treatment with 10 or 30 μM SQH9. ** indicates p<0.01 as compared with the untreated control.*

4.2 Antiproliferative properties of 19-nortestosterone derivatives

4.2.1 Antiproliferative effect of 19-nortestosterone derivatives

The antiproliferative properties of the synthesized 19-nortestosterone derivatives were determined by means of MTT assay using ovarian, cervical and breast cancer cell lines. 21 compounds were investigated in a screening step for their growth inhibitory activities. Based on the results of the screening phase, the potent analogs were exposed to a more detailed examination in order to determine their effective concentration range. In the case of test compounds that triggered growth inhibition of at least 50% at 30 μM, the assay was repeated on the proper cell lines using a series of dilutions and the IC_{50} values were calculated (Table 2). As mentioned above, compounds with IC_{50} value higher than 30 μM are regarded as ineffective.

Comp.	IC_{50} values (μM)							
	HeLa	SiHa	C33A	A2780	MCF-7	MDA-MB-231	MDA-MB-361	T47D
NTD1	>30	>30	>30	>30	>30	>30	>30	>30
NTD2	>30	>30	>30	>30	>30	>30	>30	>30
NTD3	>30	>30	>30	>30	>30	>30	>30	>30
NTD4	>30	>30	>30	>30	>30	>30	>30	>30
NTD5	>30	>30	>30	>30	>30	>30	>30	>30
NTD6	**16.7**	>30	>30	>30	>30	>30	>30	>30
NTD7	>30	>30	>30	>30	>30	>30	>30	>30
NTD8	**1.2**	>30	**28.4**	>30	>30	>30	>30	>30
NTD9	**14.7**	>30	>30	**11.4**	**14.0**	>30	>30	>30
NTD10	**21.0**	>30	>30	**14.6**	**22.2**	>30	>30	>30
NTD11	>30	>30	**21.1**	**10.3**	**27.0**	>30	**28.7**	**25.1**
NTD12	**17.4**	>30	>30	**10.3**	**14.1**	>30	**26.2**	**15.7**
NTD13	>30	>30	>30	**17.0**	>30	>30	>30	>30
NTD14	>30	>30	>30	>30	>30	>30	>30	>30
NTD15	>30	>30	>30	**15.1**	>30	>30	>30	>30
NTD16	>30	>30	**15.3**	**12.4**	**20.3**	>30	>30	>30
NTD17	**19.8**	>30	**13.6**	**12.4**	**24.4**	>30	>30	**29.5**
NTD18	>30	>30	>30	>30	>30	>30	>30	>30
NTD19	>30	>30	>30	>30	>30	>30	>30	>30
NTD20	**1.7**	>30	>30	**26.7**	>30	>30	>30	>30
NTD21	**1.5**	>30	>30	**20.9**	>30	>30	>30	>30
NAN	**0.7**	>30	>30	>30	>30	>30	>30	>30
CIS	**12.4**	**7.8**	**1.8**	**1.3**	**5.8**	**19.1**	**3.7**	**9.8**

Table 2. *Calculated IC_{50} values of the tested 19-nortestosterone analogs and cisplatin, measured by MTT assay after incubation for 72 h on the applied cancer cell lines.*

The results indicated that nine of the tested agents (NTD1-5, 7, 14, 18 and 19) exerted no substantial growth inhibitory effect against the gynecological cancer cell lines. None of the compounds elicited a remarkable impact on both of the HPV16+ SiHa and the triple negative MDA-MB-231 cell lines. Except the weak effect of NTD17, two of the compounds (NTD11-12) were able to inhibit noticeably but not substantially the ploriferation of T47D and MDA-MB-361 cells. Although MCF-7 possess the same receptor status as T47D, this cell line was proved to be more sensitive especially against most of the 17α substituted analogs containing a benzoyl moiety, such as NTD9-12, 16 and 17. With the exception of NTD14, derivatives with these bulky groups (NTD9-17) exhibited an explicit antiproliferative action on the ovarian cell line. Furthermore, three of them (NTD11, 16 and 17) hampered substantially the growth of HPV- C33A cells. The 17α-OAc analog (NTD6) and four derivatives containing benzoyl moiety at the position C-17α (NTD9, 10, 12 and 17) displayed a moderate inhibitory effect on the proliferation of HeLa cells. In spite of the fact that many of the examined 19-nortestosterone analog possessed pronounced antiproliferative activity, their efficacy was much weaker compared to that of the reference agent. Nevertheless, three of the tested compounds (NTD8, 20 and 21) demonstrated an outstanding growth inhibitory action selectively against HPV18+ HeLa cells, while their effect was only modest or negligible against the other cell lines. The IC_{50} values (1.2-1.7 μM) of these promising halogenated agents on HeLa cells were comparable to that of NAN (0.7 μM) and proved to be much lower than that of CIS (12.4 μM).

Although comparative *in vitro* examinations using intact cells can not be considered as an alternative to *in vivo* toxicity determination, implementation of viability assays on noncancerous cells may provide some details about cancer selectivity of the tested compounds. Therefore antiproliferative properties of the potent analogs were determined by means of MTT assay on intact fibroblast and immortalized epithelial cell lines, dereived from fetal lung and adult mammary gland, respectively (Table 3). None of the tested agents were able to exert a substantial antiproliferative action on fibroblasts except for CIS, which exhibited considerable growth inhibitory effect on both intact cell lines IC_{50} values in the low micromolar range. Although hTERT-HME1 cells were proved to be more sensitive against the compounds than fibroblasts, NAN demonstrated only a modest action against them even at the higher concentration. Although NTD8 possessed a negligible effect on fibroblasts, it demostrated a robust action on intact epithelial cells. However this effect of NTD8 (4.6 μM) was less pronounced than that of CIS (2.5 μM).

Comp.	Conc. (μM)	Growth inhibition (%) and IC_{50} values [μM]	
		MRC-5	hTERT-HME1
NTD8	10	4.3 ± 3.7	76.2 ± 0.5
	30	8.0 ± 2.3	99.9 ± 0.1 [4.6]
NTD20	10	11.9 ± 2.3	n.d.
	30	20.4 ± 2.1	
NTD21	10	13.1 ± 1.9	n.d.
	30	13.8 ± 2.0	
NAN	10	5.4 ± 1.2	16.9 ± 1.2
	30	11.3 ± 0.5	37.1 ± 1.0
CIS	10	60.3 ± 3.3	97.9 ± 0.3
	30	61.9 ± 1.0 [6.2]	99.1 ± 0.3 [2.5]

Table 3. *Antiproliferative effect of the potent 19-nortestosterone analogs and the reference agents on noncancerous cell lines, measured by MTT assay after incubation for 72 h. n.d. indicates not determined.*

Among the tested 19-nortestosterone analogs, NTD8 was proved to be the most potent compound against HPV18+ cervical cancer cells (IC_{50} = 1.2 μM) with considerable cancer selectivity. According to these findings, NTD8 was selected for additional examinations in order to identify some further details about its mechanism of action. Since NAN possessed a selective antiproliferative action against HeLa cells, its influences were also examined within the framework of additional investigations. With the exception of cell free systems and hormone assessment, the additional experiments were performed on HeLa cells.

4.2.2 Cytotoxic effect of 19-nortestosterone derivatives

To obtain information about the direct toxic effect of the compounds, changes in membrane integrity triggered by the selected agents was examined by means of LDH assay on HeLa cells. All the compounds exhibited a concentration-dependent release of the intracellular enzyme due to loss of membrane integrity after 24 h treatment (Figure 9). NAN significantly increased the detectable enzyme activity at the concentration of 1.5 μM, while a remarkable membrane damaging effect of NTD8 was appeared at the concentration of 3.0 μM and above. The damaging concentrations of these compounds accompanied by a significant elevation of enzyme activity were at least two fold higher than their IC_{50} values, while CIS demonstrated a notable cytotoxic action under its IC_{50} values at 10 μM. In contrast, these effects were poved to be considerably weaker than the detergent (Triton X-100) mediated maximal LDH release.

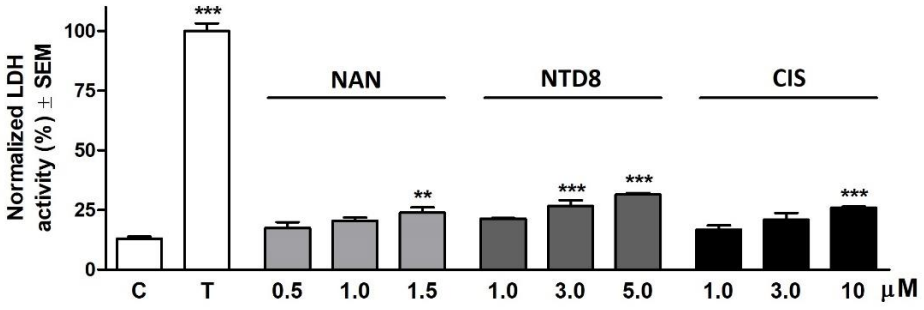


Figure 9. *Cytotoxic effects of NTD8, NAN and CIS on HeLa cells after 24 h incubation. The effect of Triton X-100 (T) was regarded as 100%. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ as compared with the untreated control (C).*

4.2.3 Effects of 19-nortestosterone derivatives on cell cycle

To reveal how the selected agents influence the cell cycle, flow cytometric analysis of treated HeLa cells were performed. Incubation with NAN for 24 h resulted in a concentration-dependent increase of cell number in G2/M phase at the expense of G1 phase significantly at the concentration of 1.0 and 1.5 μM (Figure 10). These effects became more pronounced after 48 h. The proportion of cells in the synthetic phase was slightly elevated in the persence of 1.0 and 1.5 μM of the compound, however it was significant at 1.5 μM after 48 h. Incubation for 72 h with NAN caused a reversed tendency in S and G2/M phases as a result of the robust, concentration-dependens increase of hypodiploid population (subG1 phase). With the exception of S phase at the concentration of 0.5 μM, the ratio of the cells in all cell cycle phases was significantly decreased in favor of subG1 phase after 72 h treatment with NAN, reflecting the high level of apoptotic fragmentations. There were only negligible changes in the cell cycle due to 24 h incubation with NTD8, with a modest elevation of cell number in S and G2/M phases, while G1 phase exhibited a weak reduction. However, 48 h exposure lead to a concentration-dependent decrease in the ratio of G1 phase in favor of subG1 cells, significantly at all concentration. The considerably increased hypodiploid population indicated the appearance of apoptotic bodies in the persence of 2.0 and 3.0 μM NTD8.

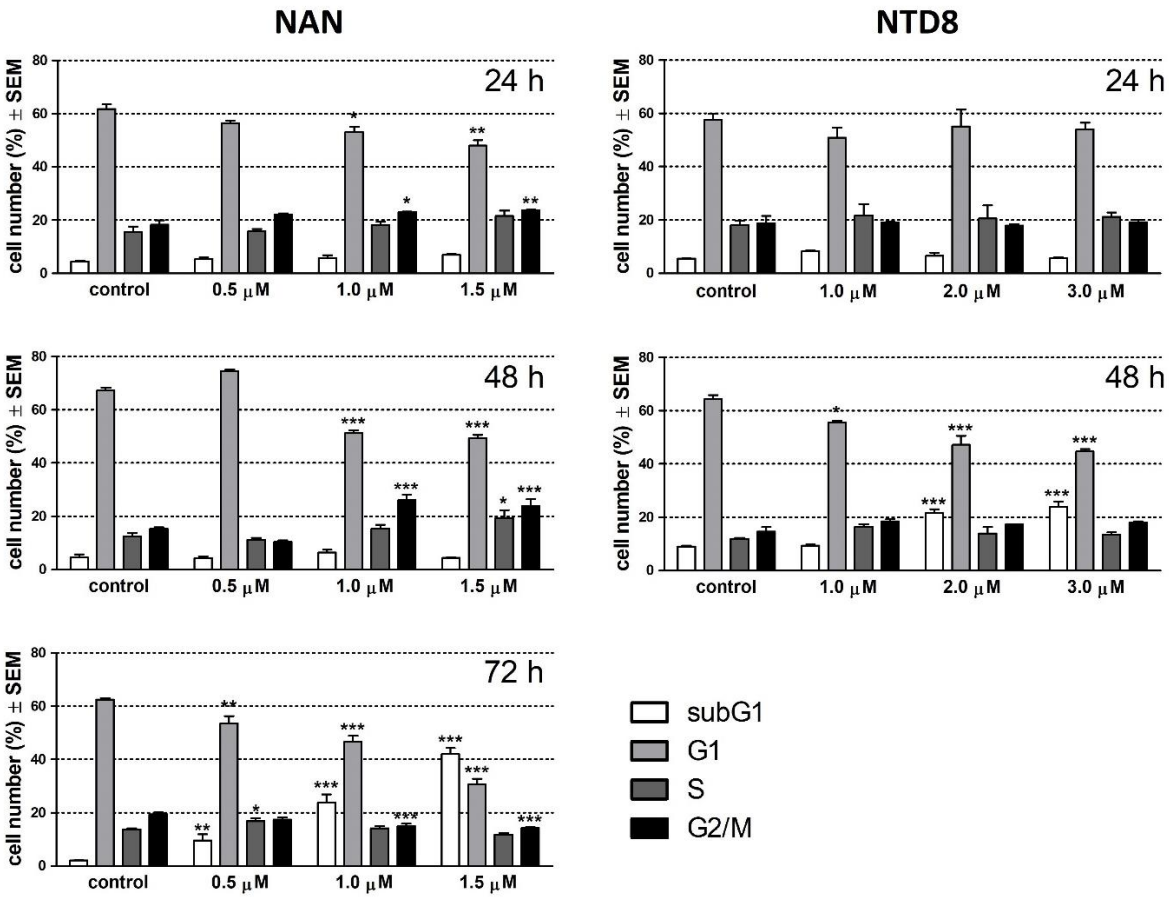


Figure 10. *Effects of NAN and NTD8 on cell cycle phase distribution of HeLa cells determined by flow cytometry after incubation for 24, 48, or 72 h. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ as compared with the untreated control.*

4.2.4 Morphological changes and apoptosis induction

On the basis of cell cycle changes triggered by tested compounds, morphological characteristics of HeLa cells were examined by means of fluorescent microscopy after 24 h incubation with NAN or NTD8 at the concentration of 1.0, 3.0, or 5.0 μM (Figure 11).

Blue fluorescence and homogeneously stained nuclei were observed in the case of untreated control cells without PI uptake. After treatment with the selected compounds, cells with apoptotic morphology were appeared even at the lowest concentrations. Bright blue fluorescence of the cells indicated the advanced chromatin condensation in case of all conditions. Cells with necrotic features (red fluorescence) were detected due to treatment with NAN or NTD8, more frequently at higher concentrations. The loss of membrane integrity indicated by PI uptake may be considered as a consequence of secondary necrosis.

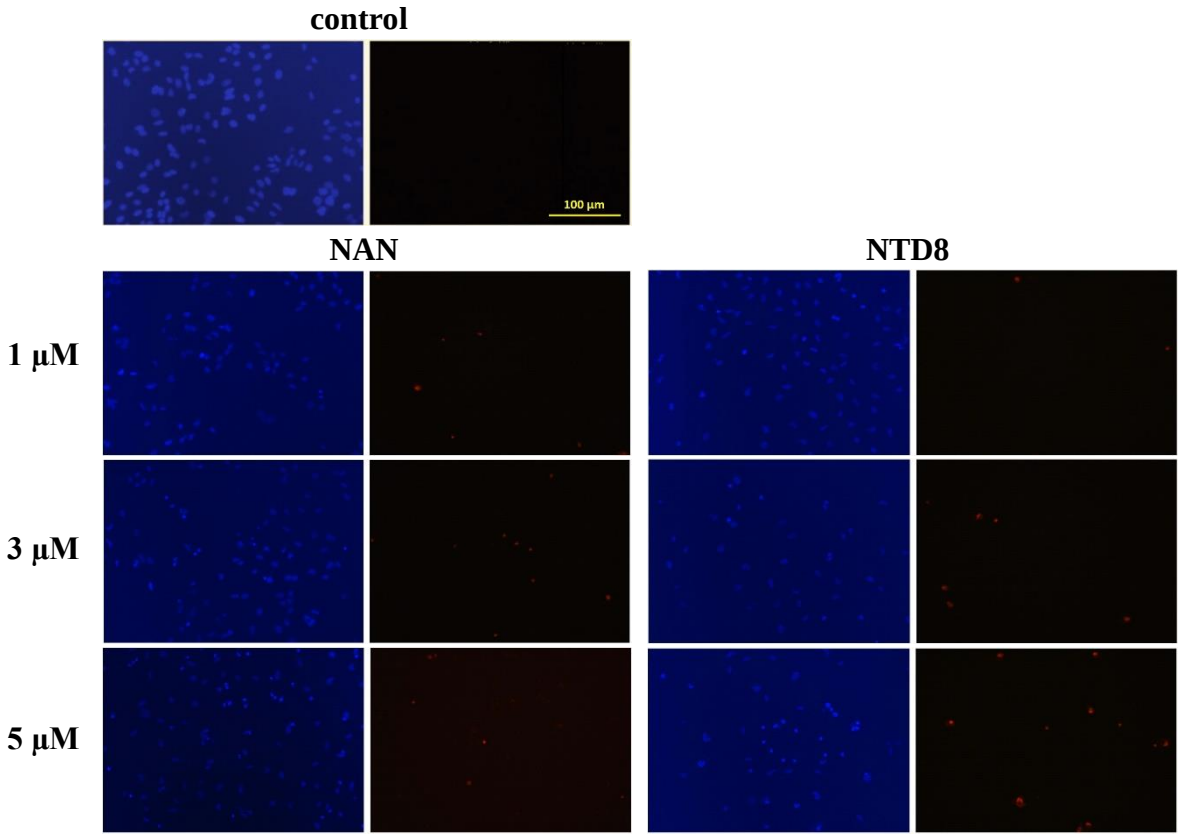


Figure 11. *Comparative representation of fluorescent double stained HeLa cells after 24 h treatment with NAN or NTD8. Blue (left panels) and red (right panels) fluorescence indicate Hoechst 33258 and PI uptake, respectively.*

In order to perform a quantitative analysis, intact, apoptotic and necrotic cells were counted in all conditions, then the ratio of cells with different morphologies were determined and analyzed statistically. After treatment with NAN, the proportion of apoptotic cells was substantially increased at the expense of intact cells, significantly at all the applied

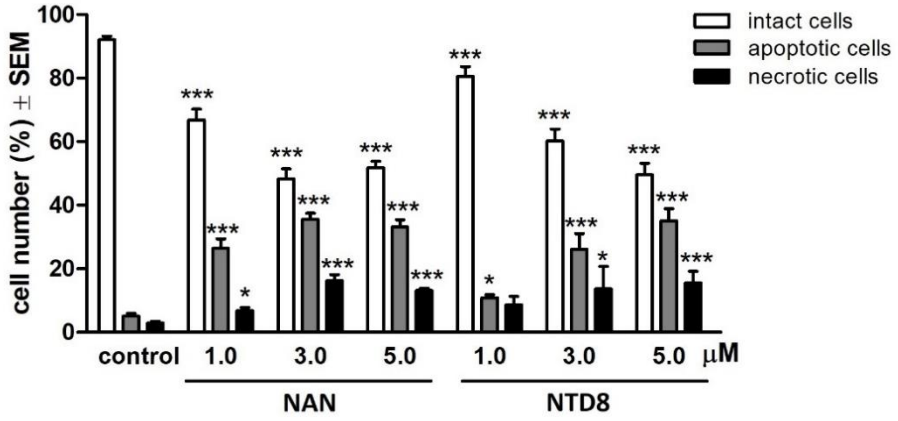


Figure 12. *Quantitative evaluation of fluorescent double staining of HeLa cells after 24 h incubation with NAN and NTD8. * and *** indicate $p<0.05$ and $p<0.001$ as compared with the untreated control.*

concentrations (Figure 12). The compound elicited remarkable elevation of the ratio of necrotic cells, significantly even at the lowest concentration. Incubation with NTD8 resulted in a considerable and concentration-dependent increase in the proportion of apoptotic cells followed by a simultaneous reduction in ratio of intact cells. Remarkably elevated number of necrotic cells was detected in the persence of 3.0 or 5.0 μM of NTD8, though its effect was less pronounced at the lowest concentration.

4.2.5 Influence of 19-nortestosterone analogs on activities of caspase-3, -8 and -9

To obtain further evidence about the apoptosis-inducing capacity of the selected 19-nortestosterone derivatives, activities of three different apoptotic enzymes were determined from HeLa cells after incubation with the selected compounds. Treatment with NAN for 72 h resulted in a concentration-dependent increase of caspase-3 activity (Figure 13). The activities of initiator caspases were also substantially elevated due to 72 h exposure, though caspase-8 activation was less explicit. A concentration-dependent induction of caspase-3 was observed due to treatment with NTD8 for 24 h, significantly only at the higher concentration. While NTD8 exhibited a considerable caspase-9 induction, there were no detectable changes in caspase-8 activity in the same experimental conditions.

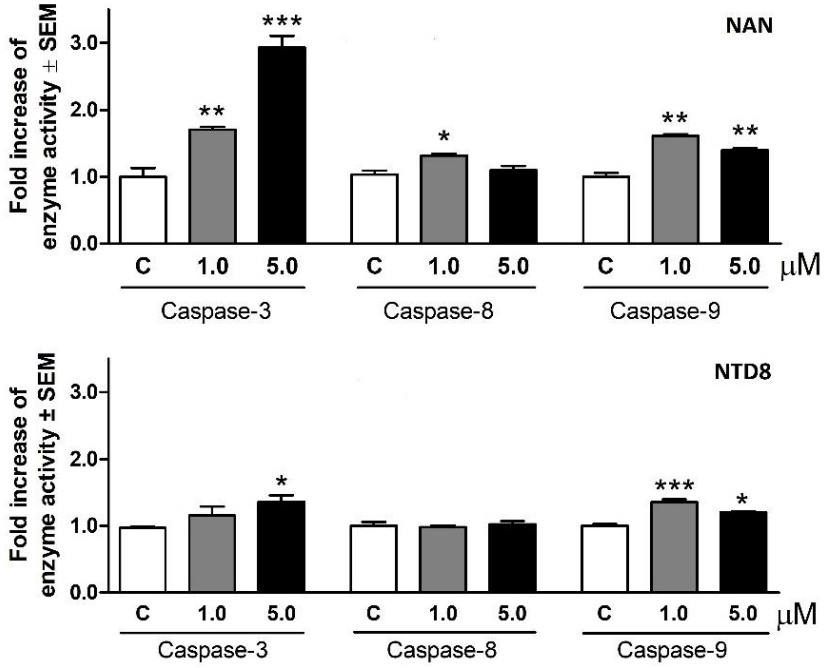


Figure 13. *Activation of apoptotic enzymes in HeLa cells after incubation with NAN (upper panel) and NTD8 (lower panel) for 72 or 24 h, respectively. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ as compared with the untreated control.*

4.2.6 Direct effects of 19-nortestosterone derivatives on tubulin polymerization

In order to reveal the influence of the selected agents on the microtubular system the direct effect of NAN and NTD8 on tubulin polymerization was determined by a photometric-based kinetic assay under cell-free experimental conditions. Pactlitaxel, a well known microtubule stabilizer was utilized as positive control. The applied concentrations of the compounds were selected on the basis of their IC_{50} values on HeLa cell line.

Representative kinetic curves fitted to the measured points (Figure 14) reflect the acceleration of the polymerization reaction in the growth phase in the persence of NAN or NTD8. These effects were more pronounced at higher concentrations of the compounds, and were less explicit compared to influence of PAC.

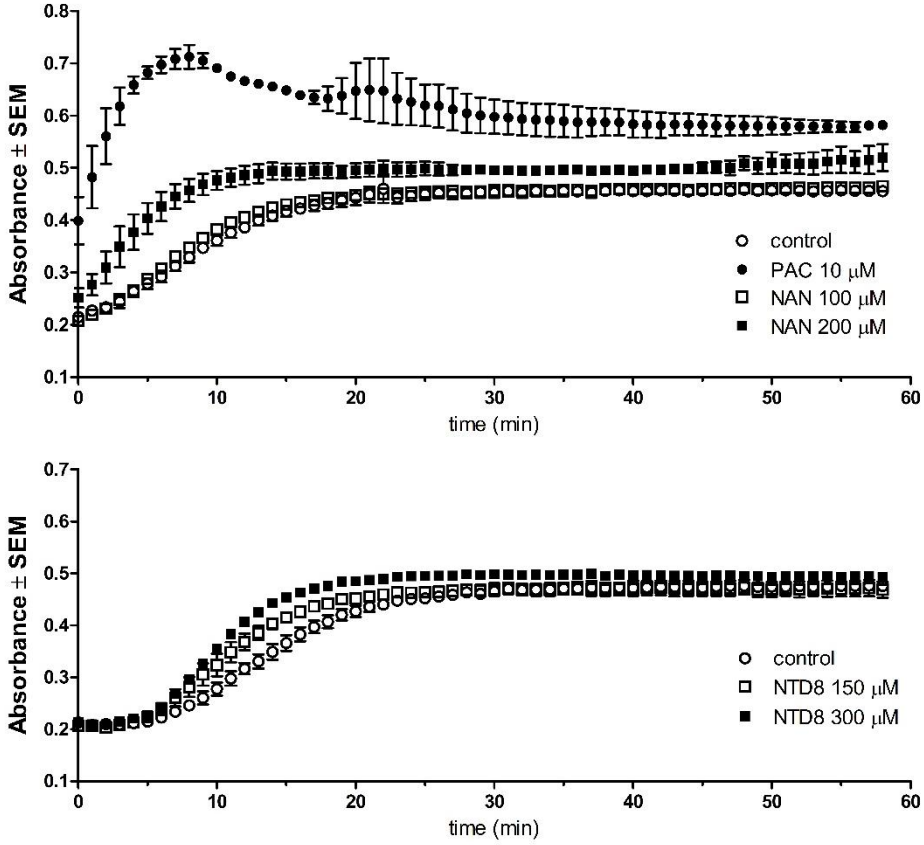


Figure 14. *Direct effect of the test compounds on tubulin polymerization determined by means of a photometry-based kinetic assay in a cell-free system in the persence of NAN, NTD8 or PAC.*

For a statistical evaluation, the maximal rate of tubulin polymerization was calculated in all conditions and analyzed statistically. Both of the selected 19-nortestosterone analogs increased a maximal rate of microtubule formation (V_{max}), significantly at the higher concentrations

compared to the untreated controll (Figure 15). However, the effect of the compounds was proved to be similar but milder than that of the positive control.

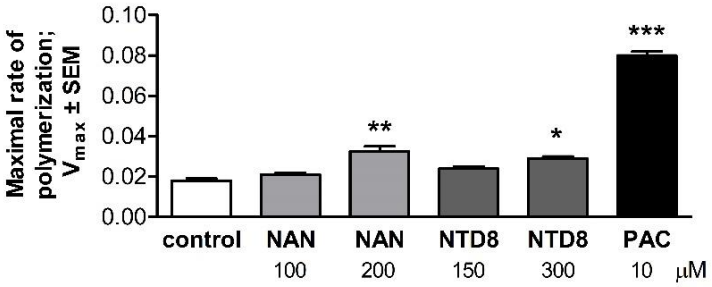


Figure 15. *Effect of the test compounds on the maximal rate of polymerization reaction. *, ** and *** indicate p<0.05, p<0.01 and p<0.001 as compared with the untreated control.*

4.2.7 Androgenic activity of the selected 19-nortestosterones

Hormonal property of a steroidal agent with potent hormone-unrelated action is essential factor in the case of its utilization, hence the residual androgenic effect of the most active analog was examined in a colorimetric way by means of a yeast-based reporter gene system.

Since NAN is an acknowledged androgen demonstrating a potent affinity for androgen receptor similarly to DHT [91], it was utilized as a reference compound. With the exception of extremely high concentrations, NTD8 elicited a substantially lower β-galactosidase production compared to NAN (Figure 16), indicating its considerably weaker androgenicity.

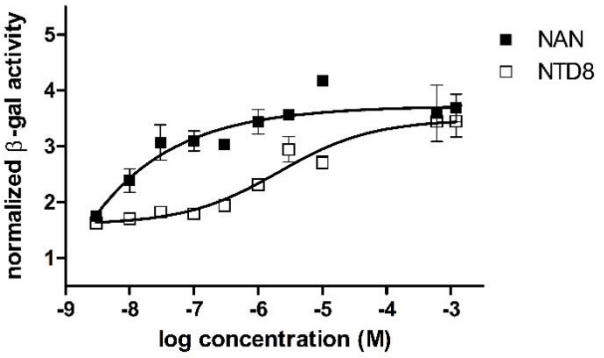


Figure 16. *Effect of the test compounds on reporter gene expression in a yeast-based experimental system.*

The effective concentration range of the selected compounds was determined and their EC_{50} values were calculated. The two orders of magnitude difference between EC_{50} of NAN (34.3 nM) and NTD8 (3.8 μM) reflect the negligible hormonal activity of the most promising test compound. In the same experimental conditions NTD8 express no detectable antagonistic property in the persence of DHT (data not presented).

5 DISCUSSION

Since cancerous disorders are the second leading cause of death worldwide and the global cancer burden is expected to raise, the development of novel efficacious agents for the therapy is one of the greatest challenges in medicinal chemistry and pharmacology [2-6]. Steroids are a well-known group of diversified compounds, many of them possess different physiological functions, their widespread natural occurrence indicates their huge biological potential.

Although a serial of natural steroids and synthetic derivatives have been described as antineoplastic agents, only few compounds have been used in cancer therapy. The oncological practice utilized them mainly for their hormone-related activity [92, 93].

In recent studies, some steroidal compounds have been identified as antiproliferative agent with hormone-independent mechanism of action. Some of them arrest cell cycle, possess proapoptotic functions or influence cytoskeletal system. The sterane skeleton provides possibility for a wide range of chemical modifications which contribute to the exploitation of these beneficial properties and also allow the reduction of unfavorable features. The large amount of experience and accumulated knowledge about chemical structure and biological effects of steroidal agents facilitates the planning and establishment of new steroid-based anticancer drug candidates [66-68, 94-96].

Although a high number of steroid compounds with anticancer properties have been described, androstane skeleton possessing agents are particularly rare among them [97]. The aim of the present study was the pharmacological characterization of newly synthesized androstane-based antiproliferative molecules including androstane-quinoline hybrids and 19-nortestosterone derivatives. Additional purpose of the research was to describe structure-activity relationships.

15 steroid-quinoline hybrids were investigated for their antiproliferative effect. The compounds proved to be effective in the screening phase were subjected to a more detailed analysis in order to determine their effective concentration range. Although there is no officially determined threshold for efficacy, compounds with IC_{50} value higher than 30 μM are considered as ineffective. While compounds with 17-OAc group generally exhibited a modest growth inhibitory action, 17-OH analogs hampered cell proliferation more efficiently with the exception of HPV+ cell lines, HeLa and SiHa. Although SQH1 is proved to be ineffective, the action of the other unsubstitued quinoline containing SQH7 was comparable to that of some effective hybrids containing substitued quinoline moiety. Thus, these findings confirm that the character of the substituent on the quinoline part do not play essential role in the growth

inhibition. In contrast, the location of the substituent contributed to the antiproliferative action, together with the character of the substituent at the C-17 position of the sterane skeleton.

Compounds substitued at the position 5' and 7' on the qiunoline moiety exhibited a moderate activity, while substitution at position 6' generally seems to be favorable. Substitution at the position 8' promoted the marked growth inhibition especially on MDA-MB-361 cells. Halogen substitution at the 6' position of the quinoline parts of 17-OH analogs appeared advantageous against HPV- C33A cells. While incorporation of bromine at the 6' position of a 17-OAc analog contributed to a modest and non-selective action, integration of chlorine at the same position supported the growth inhibition on MDA-MB-361 cells. The most potent hybrid SQH9, a 17-OH analog containing a 6' methoxy substituted quinoline moiety, demonstrated a considerable antiproliferative action on C33A and T47D cell lines, while its effect on MCF-7 was less pronounced. Since the efficacy of SQH9 on T47D cells was similar to that of the reference compound, this hybrid was subjected to further investigations in order to determine the mechanism of its action.

The analysis of cell cycle provided notable informations about the possible mechanism of action of the selected compound. Examinations of treated T47D cells by flow cytometry revealed the cell cycle arresting effect of SQH9. The accumulation of the cells in G1 phase at the expense of S and G2/M phase indicated the hindered G1-S transition during the cell cycle. The elevated number of subG1 cells reflected the concentration-dependent apoptosis inducing effect of the test compound [89].

The programmed cell death inducing capability are one of the expectations of current medicines in anticancer therapy [98]. Reinforcement of proapoptotic pathways or targeted inhibition of antiapoptotic proteins promotes cell death or decrease the treshold for its initiation facilitating tumor suppression [99]. In order to confirm the proapoptotic functions of SQH9, apoptosis inducing effect of the compound was examined by fluorescent microscopy using Hoechst 33258-PI double staining. After 48 h treatment, changes in morphology of treated cells indicated substantial and concentration-dependent apoptosis induction even at the lower dose. The elevation of the concentration resulted in an increased proportion of necrotic cells reflecting the appearence of secondary necrosis due to loss of membrane integrity.

On the other hand, the biochemical examination of the main apoptosis executor enzyme in treated T47D cells also confirmed the proapoptotic property of the test compound. After 48 h incubation, a concentration-dependent elevation in caspase-3 activity was observed, which was more pronounced in the persence of 30 μM SQH9. These results corroborate the capability of SQH9 to induce programmed cell death in a concentration-dependent manner.

Beyond the hybrid compounds, 21 19-nortestosterone analogs with different substitutions were tested for their antiproliferative properties. It has been concluded that persence of substituent at the C-16 position is absolutely unfavorable in terms of antiproliferative activity. Furthermore, 17α-OH substitution did not seem to be necessary for growth inhibition. Although the compound with 17α-OAc group and no other substitution exhibited a moderate antiproliferative effect on HeLa cells, incorporation of acetoxy-phenyl moiety instead of acetoxy group was accompanied by loss of effectivity. With the exception of NTD14, compounds with bulky substitution containing a benzoyl group (NTD9-17) demonstrated moderate but not considerable activities against some cell lines in a broad spectrum in more cases reflecting their non-selective effect. Apart from the modest effect on C33A or A2780 cells, 17α-halogenated compounds exerted outstanding antiproliferative action against HeLa cells. Moreover, their IC_{50} values were an order of magnitude lower than that of CIS. NAN also inhibited the growth of HeLa cells in a selective manner at the same concentration range. These findings suggests that besides the 17β-OH substitution, integration of halogen group at the position C-17α of the 19-nortestosterone scaffold results in a dramatically increased antiproliferative activity against HPV18+ cervical cancer cells.

Tumor selectivity plays pivotal rule in the destiny of antineoplastic drug candidates. In this regards, the growth inhibitory effect of the promising analogs were determined on intact cell lines. In general, halogen substituted derivatives exerted substantially weaker impact on noncancerous cell lines than against cervical cancer cells. Besides NTD8 is proved to be the most potent compound with outstanding antiproliferative effect on HeLa cells, its cancer selectivity exceeded the effect of CIS on intact epithelial cells. Furthermore it elicited an order of magnitude lower growth inhibiton of fibroblast cells than that of the reference agent. Based on these findings, the most potent agent was selected for additional investigations in order to reveal some details about the mechanism of its action. Since NAN demostrated a prominent inhibitory action on the proliferation of HeLa cell, it also involved in the additional examinations as a reference compound with analogous chemical structure. Beyond the antiproliferative activity, the most effective 19-nortestosterone analog and NAN demonstrated a moderate cytotoxic effect, that were less pronounced than that of CIS.

The flow cytometric analysis of treated HeLa cells disclosed the cell cycle arresting effect of the tested compounds. Both of the compounds exerted a similar enhancer effect on the appearance of hypodiploid population at the expense of G1 phase. Since the hypodiploid cells indicate nuclear fragmentation and appearance of apoptotic bodies, this examinations evidenced the proapoptotic properties of the test compounds. While NTD8 triggered a fast and direct

reduction of G1 phase in favor of subG1 phase, NAN exerted a slower effect accompanied by an early and pronounced elevation of G2/M ratio that finally led to a late transformation into hypodiploid population.

Since exploration of apoptotic characteristics are a fundamental feature of our study, the programmed cell death inducing effect of the selected derivatives was confirmed by means of fluorescent microscopy. Fast appearance of apoptotic morphilogies such as chromatin condensation of treated cells was observed after incubation with the tested 19-nortestosterones in a concentration dependent manner. Another evidence of proapoptotic functions is the activation of the common executor enzyme caspase-3, that was found to be significantly elevated by both of NTD8 and NAN. Examinations of initiator caspases ensured insight into the molecular process of apoptosis induction and provides details about signaling mechanisms. While caspase-8 involved in the extrinsic pathway of apoptosis, caspase-9 is a crucial element of intrinsic signalization [100]. Both of NTD8 and NAN provoked a considerable increase in caspase-9 activity that suggested the induction of mitochondrial pathway of the programmed cell death. The concentration dependency was different from expected, probably it was a consequence of *in vitro* late necrotizing effect of the compounds. In contrast, the significant but less explicit increase in caspase-8 activity by NAN at the lower concentration was presumably not satisfactory for the induction of extrinsic pathway.

Direct action of some antiproliferative steroidal compounds on microtubular system has been described, probably their interaction with colchicine binding site contributes their cell cycle arresting effect [101, 102]. Investigation of NTD8 and NAN in a cell-free experimental system evinced their direct influence on formation of microtubuli. The tested compounds significantly increased the maximal rate of tubulin polymerization and exhibited a stabilizer effect like PAC. According to these results, the intervention of the tested 19-nortestosterone derivatives in microtubular processes probably contributes to the disruption of cell cycle and may be a crucial element of the mechanism of action.

Since the tested compounds contain an adrostane backbone, their interaction with androgen receptors may participate in the mechanism of action and can serve as a potential source of undesired adverse reactions. Based on well-established structure-activity relationships [84] and steric structure of the compounds, androgen receptor mediated effect of steroid-quinoline hybrides can be excluded. In contrast, chemical structure of 19-nortestosterone derivatives may allows their bindig affinity for nuclear receptors. Accordingly, androgenic activity of the most potent derivative was determined by means of a yeast-based reporter assay. As described above, NAN have a relatively high affinity for androgen receptor and possesses a similar hormonal

action compared to DHT. Therefore NAN was utilized as an androgenic reference agent with analog structure. According to our results, NTD8 had no substantial hormonal action in this experimental system, its action was modest, i.e. two orders of magnitude lower than that of NAN at relevant concentrations. Repeated examinations in the persence of DHT did not reveal antagonistic propertiy of the tested analog. These findings suggest that the most potent agent has negligible affinity for androgen receptor. While the β configuration of C-17 position contributes to receptor binding, 17α substitution seems unfavorable. In this regard, integration of 17α-halogen into 19-nortestosterone skeleton is probably responsible for the loss of androgenic action [84]. Although endocrine effects are mediated mainly through nuclear receptors, the possibility of hormonal action mediated by membrane-associated androgen receptors can not be excluded without additional investigations.

The presented results indicate that A-ring fused androstanes provides suitable parent scaffold for design of further antiproliferative compounds. Incorporation of a quinoline moiety can allow a wide range of chemical modifications and may contribute to a broad growth inhibitory spectrum. In addition to steroid-hybrid molecules, the 19-nortestosterone skeleton with 17α-halogen substituents can be considered as an optimal backbone for development of more efficient anticancer agents without substantial androgenicity.

6 SUMMARY

On the basis of our current scientific results, the following statements can be declared:

- 14 hybrid compounds from the 15 prepared androstane-quinoline hybrids demonstrated modest or pronounced antiproliferative action on different breast and cervival cancer cell lines *in vitro*.
- The most potent hybrid SQH9 exerts an explicit growth inhibitory effect on HPV-cervival cells (C33A) and two breast cell lines (MCF-7 and T47D), though its action is less pronounced on MCF-7. The antiproliferative effect of the compound on T47D cell line is proved to be comparable to that of cisplatin, a widely utilized chemotherapeutic agent in treatment of different malignancies.
- The flow cytometric analysis of T47D cells revaled the cell cycle arresting effect of the compound mediated by accumulation of cells in G1 phase. SQH9 exhibits apoptosis inducing effect in the susceptible cells as evidenced by both of flow cytometry and fluorescent microscopy, furthermore it results in caspase-3 activation.
- 12 of the 21 newly synthesized 19-nortestosterone derivatives exert moderate or intense antiproliferative action on different gynecological cancer cell lines.
- Three of the tested agents demonstrate outstanding growth inhibitory effect on the HPV18+ cell line HeLa similarly to NAN, the androstane reference compound. These potent agents bear 17α-halogen substitution and exhibit an order of magnitude lower IC_{50} value than that of CIS. The results of examinations on non-cancerous intact cell lines reflects that the tested compounds possess higher tumor selectivity than that of CIS.
- The most potent compound NTD8 and the NAN exert a moderate cytotoxic effect on HeLa cells and provoke cell cycle arrest accompanied by appearance of apoptotic bodies. Their proapoptotic function is confirmed by fluorescent microscopy and caspase-3 activation. The powerful caspase-9 activation by the compounds indicates the induction of mitochondrial pathway of programmed cell death. In addition, these two nortestosterone analogs have a direct influence on the formation of microtubules increasing the rate of tubulin polymerization.
- NTD8 elicits only a weak androgen receptor mediated androgenic effect compared to NAN, the reference compound with considerable androgenicity.

In summary, the presented steroids can be considered as prototypes of new promising antiproliferative agents without substantial androgenic activity. Our described results and the observed structure-activity relationships can be utilized in the development of novel androstane-based anticancer drugs without undesired hormonal effect.

In conclusion, the androstane scaffold is proved to be a suitable basis for development of novel drug candidates. This sterane backbone provides a set of opportunities for chemicals modifications, which allow the optimization of the biological functions. Futhermore, utilization of hybrid structures can increase the number of possibilities. The exploitation of beneficial properties and simultaneous reduction of unfavorable features by targeted chemical editing of the androstane skeleton is a potential approach to establish further innovative compounds for cancer therapy.

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APPENDIX

I.

RSC Advances

PAPER

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Microwave-assisted one-pot synthesis of steroid–quinoline hybrids and an evaluation of their antiproliferative activities on gynecological cancer cell lines†

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Novel D- and A-ring-fused quinolines in the estrone and 5α-androstane series were efficiently synthesized from the corresponding β-chlorovinyl aldehydes with different arylamines in DMF under microwave irradiation. The rates of the one-pot catalyst-free syntheses and the yields of the desired products were found to be affected significantly by the electronic and steric character of the substituents on the anilines and the different reactivities of rings D and A of the sterane skeleton. All the synthesized compounds were tested *in vitro* on human cervical (C33A, HeLa and SiHa) and breast (MCF-7, MDA-MB-231, MDA-MB-361 and T47D) cancer cell lines in order to investigate their antiproliferative activities *in vitro*. Evidence of cell cycle blockade and apoptosis induction was obtained for the most effective compound **14c** by means of flow cytometry, caspase-3 activity determination and microscopic techniques.

1. Introduction

Hybrid compounds, which combine two or more structural entities in a single molecule, at least one part being a biologically active natural product, hold great promise for the design of novel pharmaceutical agents with better selectivity and diminished toxicity.[1] Steroids are attractive building blocks for such hybrids in view of their widespread natural occurrence, their rigid sterane skeleton with a range of functionalization, their broad spectrum of bioactivity and their specific ability to penetrate cell membranes.[2] Several molecular hybrids or 'chimeras' derived from steroids and other molecules through the domain integration of key functional elements or *via* covalent linkages were reported earlier.[3] Among these strategies, the introduction of different heterocyclic systems either connected to or condensed with the sterane core offer an excellent possibility through which to modify the physicochemical properties of the parent compound[4] and to optimize certain pharmacokinetic features. Hybridization of steroids often allows modulation of the binding ability and therefore the biological activity by altering the effect of the original molecule through action on another biological target.[5] Consequently, in recent years considerable attention has been devoted to the synthesis and pharmacological evaluation of different ring A- and D-fused steroidal heterocycles containing pyrazol(in)e,[6] (is)oxazol(id)ine,[7] thiazole,[8] pyri(mi)dine[9] or triazole[10] building blocks, a number of which have been reported to possess cell-growth-inhibitory, antibacterial, anti-inflammatory and other beneficial effects.

In spite of the fact that quinolines and their derivatives are known to be very common structural motifs in various natural products, pharmacologically active synthetic compounds and clinically relevant drugs (Fig. 1),[11] the incorporation of these moieties into the steroid backbone is rather rare[12] and the pharmacological effects of these derivatives have not been deeply investigated. In this respect, Boruah *et al.* recently reported the synthesis of some quinolines condensed to ring D of the androstane and ring A of the cholestane skeleton, and tested their antimicrobial activities.[13]

Since a number of sex-hormone-derived heterocyclic steroids exert cell-growth-inhibitory effects on malignant cell lines of diverse origins by disturbing the normal cell cycle and inducing apoptosis,[8,14] and since a number of natural and synthetic quinoline derivatives are also well-known anticancer agents,[15] integration of the quinoline and the sterane scaffolds may be of interest in a search for novel hybrid molecules with antiproliferative activity. In view of the absence of sufficient information concerning the exact mode of action of steroidal heterocycles due to the complexity of the apoptotic mechanisms and the different targets that may be affected, random searches

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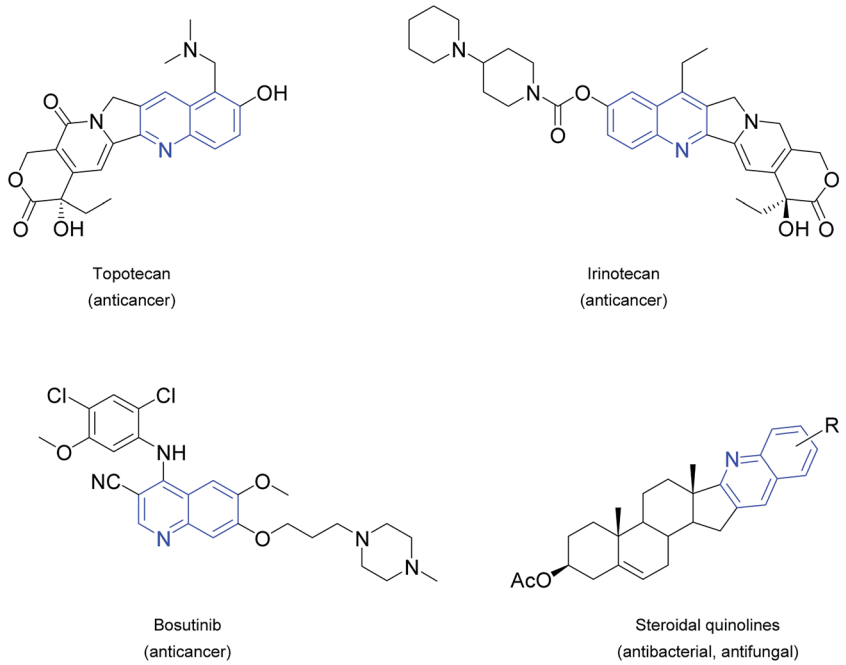


Fig. 1 Structures of some quinoline-based pharmacologically active compounds.

still remain effective for the identification of new lead compounds.

Quinolines can be obtained by various synthetic routes, including the Skraup, Combes, Conrad–Limpach, Doebner–Miller, Friedlander, Povarov, Camps or Niementowski methods, all of which involve an initial and intermolecular reaction of an aniline derivative with a carbonyl-containing compound or a precursor thereof.[16] Despite their versatility, these reactions suffer from certain disadvantages, such as a multistep character, or the need for harsh conditions, large amounts of promoters, expensive additives or difficult work-up procedures. Accordingly, there have been continuous efforts to develop clean and rapid novel protocols for the construction of quinoline-based structures. In recent years, considerable attention has been focused on the application of β-halovinyl aldehydes, which are easily accessible from α-methylene ketones by use of the Vilsmeier–Haack reagent, as useful synthons for the synthesis of polycyclic azaarenes.[17] As regards the regioselective access to 2,3-disubstituted quinolines, β-halovinyl aldehydes react with 2 equivalents of anilines to afford *N*-aryleneaminoimine hydrohalogenide intermediates, which then undergo thermolysis in solution[18] or under solvent-free conditions.[13] On the other hand, the selective arylamination of β-chloro- or β-bromovinyl aldehydes can occur at position β on use of an equimolar amount of aniline derivative in the presence of a palladium catalyst in basic medium without any trace of imine formation.[19] These results led several groups to develop a two-step procedure for the synthesis of *N*-arylaminovinyl aldehydes through a cross-coupling reaction and subsequent acid-catalyzed cyclization to quinolines. The one-pot version of the metal-catalyzed reaction has also been investigated, but a long reaction time proved to be needed for sufficient conversion under conventional heating,[20] or by-products were formed under microwave conditions.[21] Imines, readily available from β-halovinyl aldehydes under modified reaction conditions, were also found to be cyclized to quinolines.[18,20] Despite the great potential of β-halovinyl aldehydes as organic synthons, their application in steroid chemistry is yet to be investigated.

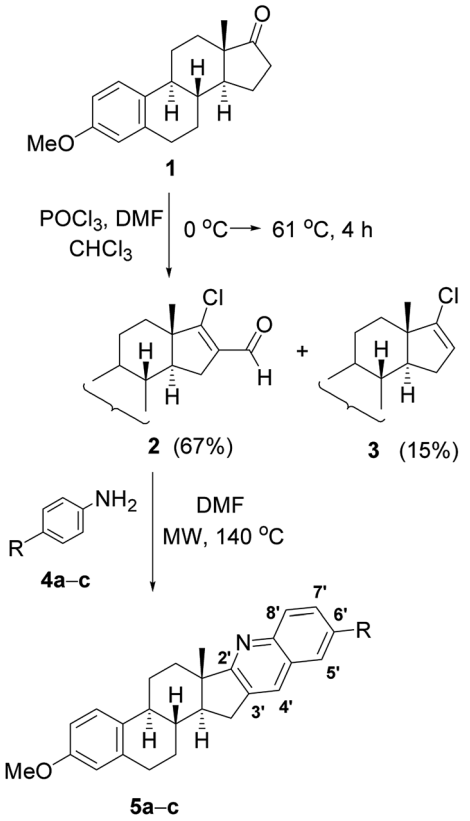
In a continuation of our research on the synthesis of novel sex-hormone-derived heterocyclic steroids with potential antiproliferative activity, we now report a microwave-assisted catalyst-free one-pot approach to novel molecular hybrids integrating steroidal and substituted quinoline structural elements. All of the synthesized compounds were tested *in vitro* on three cervical and four breast cancer cell lines in order to determine their cell-growth-inhibitory potency. The most effective compound was subjected to additional *in vitro* experiments on T47D cells, including flow cytometric cell cycle analysis and fluorescence microscopy, in order to characterize the mechanism of its action.

2. Results and discussion

2.1. Synthetic studies

For the preparation of ring D-fused quinoline derivatives, estrone 3-methyl ether (**1**) was used as starting material. The simultaneous addition of $POCl_3$ and DMF to an ice-cold solution of **1** in $CHCl_3$ and subsequent reflux for 4 h afforded a β-chlorovinyl aldehyde (**2**), together with a 17-chloro derivative (**3**)[23] as by-product (Table 1). The microwave-assisted solvent-free reaction of **2** with aniline (**4a**, 2 equiv.) at 140 °C for 10 min was first attempted, according to the method reported for the synthesis of similar ring D-fused quinolines in the

Table 1 Synthesis of steroidal ring D-fused quinolines



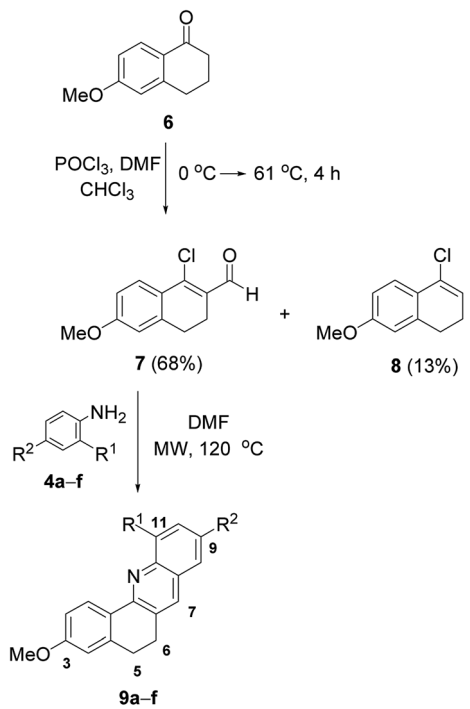
Entry	Aniline[a]	R	Reaction time (min)	Product	Yield[b] (%)
1	**4a**	H	20	**5a**	53
2	**4b**	CH_3	10	**5b**	54
3	**4c**	OMe	10	**5c**	47

[a] Equimolar amount; in case of *ortho* substituent or EWG in any position, the reaction failed. [b] After purification by column chromatography.

androstane series by Boruah *et al.*,[13] but the TLC control of the reaction indicated the presence of several compounds in the mixture, and the purification procedure proved extremely difficult. Consequently, the reaction was repeated with 1 equiv. of **4a** in DMF, which was earlier found to be a suitable solvent for such reactions,[18,22] and the solution was irradiated at 140 °C for 10 min. In this case, a significant conversion of **2** was observed by TLC, but an additional 10 min was needed for the disappearance of the starting material. Besides the main spot, however, the presence of a considerable amount of polar compounds could be noticed, and these were not transformed on further lengthening of the reaction time. After chromatographic purification, the desired ring D-fused quinoline **5a** was obtained in a yield of 53% (Table 1, entry 1), which could not be improved either by the application of **4a** in a 2-fold excess in solution or by prolonging the reaction time. Similar transformations of **2** with aniline **4b** or **4c** containing an electron-donating group (EDG) in the *para* position on the aromatic moiety likewise furnished the corresponding product **5b** or **5c** in moderate yield, but within 10 min (Table 1, entries 2 and 3). Interestingly, *o*-toluidine was not sufficiently reactive even at a longer (30 min) irradiation time, which suggested the steric hindrance of the *ortho*-CH_3 substituent on the ring-closure reaction. At the same time, similar transformations also failed when anilines with an electron-withdrawing group (EWG), such as a halogen or nitro substituent, were used and only certain intermediates could be detected by TLC, which underwent decomposition during the purification process.

The different behavior of partially unsaturated rings A and D of the sterane core under identical reaction conditions has often been observed previously, which may be attributed to the higher rigidity and sterically more hindered character of the five-membered ring D, mainly due to the presence of the angular methyl group on C-13, as compared with the more flexible six-membered ring A.[24] 6-Methoxy-1-tetralone (**6**) was therefore used for further experiments as a model of the six-membered ring A (Table 2). β-Chlorovinyl aldehyde[25] **7** was prepared similarly to described for the synthesis of **2** with the Vilsmeier–

Table 2 Model reaction for the preparation of benzacridines



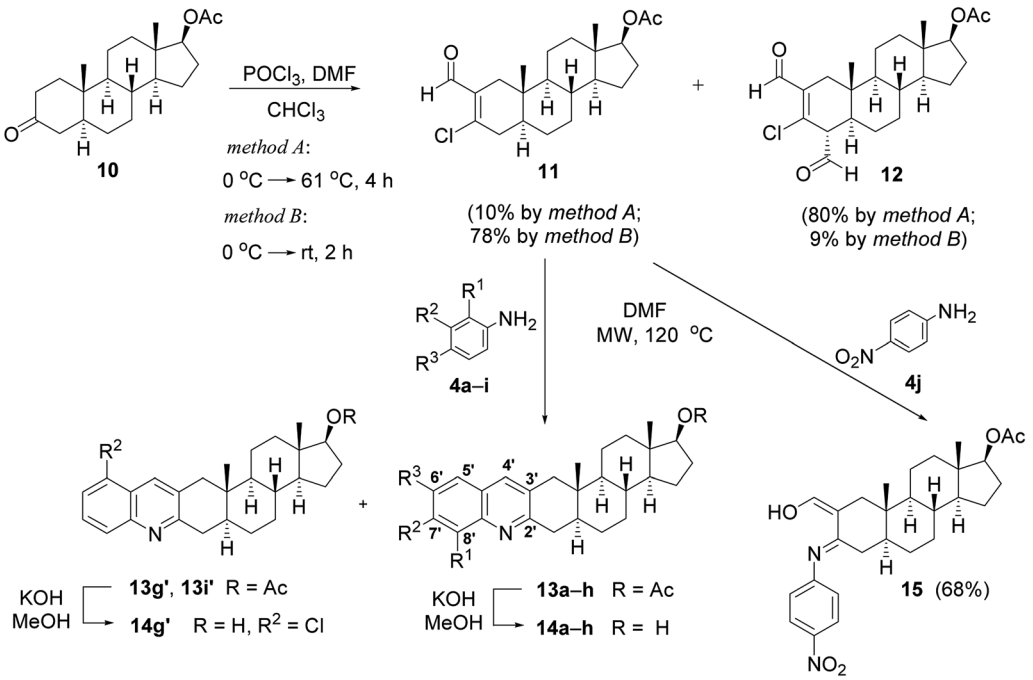
Entry	Aniline[a]	R^1	R^2	Reaction time (min)	Product	Yield[b] (%)
1	**4a**	H	H	5	**9a**	76
2	**4b**	H	CH_3	2	**9b**	90
3	**4c**	H	OMe	2	**9c**	89
4	**4d**	Cl	H	20	**9d**	35
5	**4e**	H	Cl	10	**9e**	54
6	**4f**	H	Br	10	**9f**	62

[a] Equimolar amount. [b] After purification by column chromatography.

Haack reagent, and was separated from the 4-chloro-1,2-dihydro-7-methoxynaphthalene by-product (**8**).[26] The reactions of **7** with anilines (**4a–f**) containing either EDGs or EWGs resulted in the formation of benz[*c*]acridine derivatives (**9a**,[13] **9b** [13] and **9c–f**) in varying yields, depending on the steric and electronic character of the substituents on the aromatic moiety. The possibility of the application of a lower temperature (120 °C) and a shorter reaction time to achieve the complete conversion of **7** indicated the higher reactivity of **7** relative to that of steroidal β-chlorovinyl aldehyde **2**. Moreover, the substituent effect was found to be more pronounced in these cases. Thus, EDG-substituted anilines (**4b** and **4c**) favored the reaction in comparison with unsubstituted **4a**, and the corresponding products **9b** and **9c** were obtained in high yields after purification when a 2 min irradiation time was used (Table 2, entries 2 and 3). The isolated yields of the desired products (**9e** and **9f**), however, were only moderate, even when the heating period was extended to 10 min when anilines containing electron-withdrawing halogens (**4e** and **4f**) were reacted with **7** (Table 2, entries 5 and 6). During the reaction of **7** with 2-chloroaniline **4d**, the additive electron-withdrawing and steric effects of the *ortho* substituent hampered the formation of **9d**, resulting in a yield of only 35% within longer reaction time (Table 2, entry 4).

These results led us next to investigate similar reactions on ring A of the sterane skeleton. For this purpose, 17β-acetoxy-5α-dihydrotestosterone (**10**) was reacted with the Vilsmeier–Haack reagent in refluxing $CHCl_3$ for 4 h, but bis-formylation occurred under these conditions to afford compound **12** [27] as main product (Table 3, *method A*), together with the minor formation of the desired β-chlorovinyl aldehyde **11**.[28] However, this latter compound could be obtained in 78% yield by performing the reaction at 25 °C for 2 h (Table 3, *method B*), and its reactions with differently substituted aniline derivatives (**4a–i**) were then carried out. The outcomes of the microwave-assisted transformations were quite similar to those observed for the reactions of **7**. Anilines with EDGs (**4b**, **4c** and **4i**) readily underwent

Table 3 Synthesis of steroidal ring A-fused quinolines

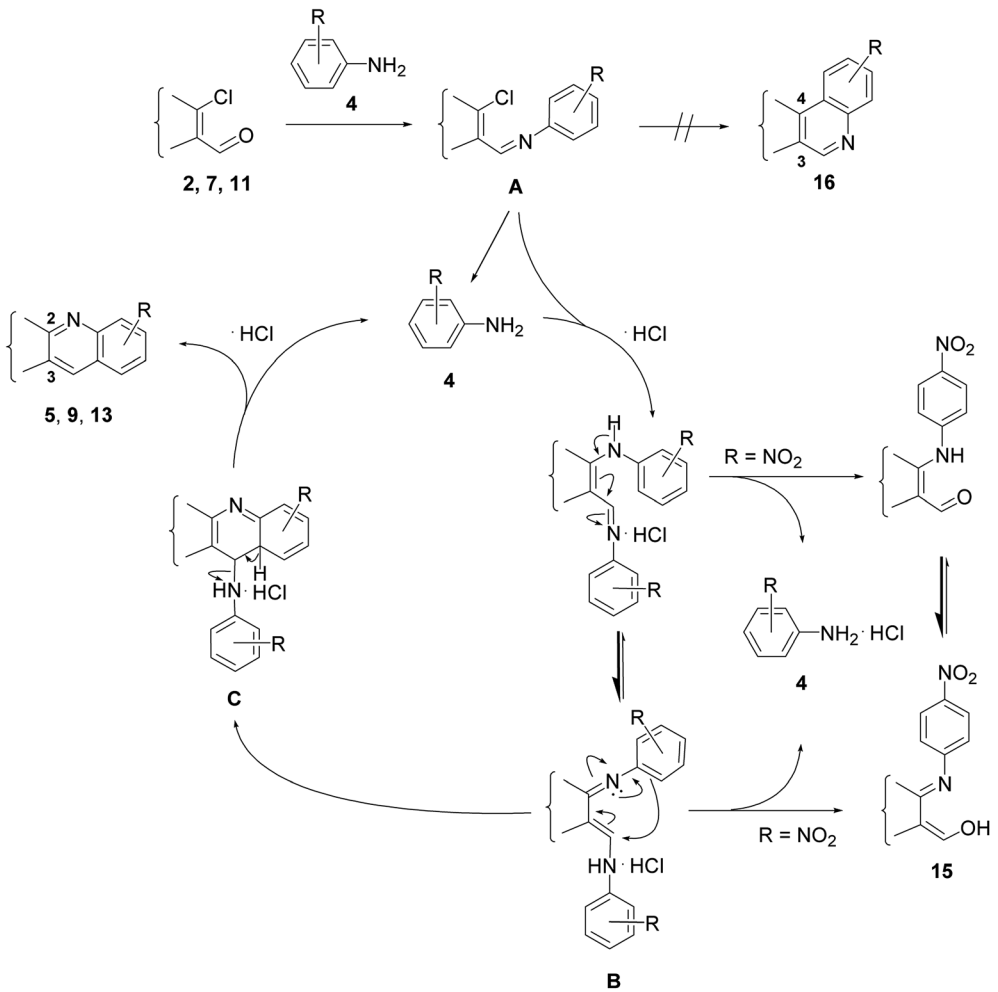


Entry	Aniline[a]	R^1	R^2	R^3	Reaction time (min)	Product(s)	Yield[b] (%)
1	**4a**	H	H	H	5	**13a**	76
2	**4b**	H	H	CH_3	2	**13b**	91
3	**4c**	H	H	OMe	2	**13c**	92
4	**4d**	Cl	H	H	20	**13d**	43
5	**4e**	H	H	Cl	10	**13e**	59
6	**4f**	H	H	Br	10	**13f**	60
7	**4g**	H	Cl	H	10	**13g** + **13g′**	46 + 16
8	**4h**	CH_3	H	H	5	**13h**	73
9	**4i**	H	CH_3	H	2	**13i** + **13i′**	90[c]

[a] Equimolar amount. [b] After purification by column chromatography. [c] The 3 : 1 mixture of **13i** and **13i′** determined by ^{1}H NMR could not be separated by column chromatography.

cyclization with **11** within 2 min to furnish ring A-fused quinolines (**13b**, **13c** and **13i** + **13i′**) in excellent yields (Table 3, entries 2, 3 and 9), although the *ortho* position of the substituent in **4h** had a certain steric effect resulting in a somewhat diminished yield of the corresponding quinoline **13h**. The presence of EWGs on the aromatic moiety in **4e–g** led to moderate yields of **13e–g** (Table 3, entries 5–7), and for **4d** with an *ortho*-Cl substituent the yield of **13d** was further reduced (entry 4). Furthermore, two regioisomeric quinolines (**13g** and **13g′** or **13i** and **13i′**) were found to be produced in a ratio of about 3 : 1 when *m*-chloroaniline **4g** or *m*-toluidine **4i** was applied (Table 3, entries 7 and 9). The failure of quinoline formation was observed in the attempted reaction of 4-nitroaniline **4j**, containing a strong EWG, with **11**, and the isolated product was identified by NMR spectroscopy as being the imino–enol form of a β-arylaminovinyl aldehyde **15** (Table 3). A similar result was reported previously for the reaction between 3-nitroaniline and 3-chloro-2-methylbut-2-enal.[29] In order to find evidence for the advantage of microwave irradiation, the reaction of **11** with **4b** (1 equiv.) under conventional heating (120 °C, DMF) was also carried out. However, this latter method required a longer time (4 h instead of 2 min) for the formation of the corresponding quinoline **13b**, with a lower yield (75% instead of 91%). The ring A-fused quinolines (**13a–h** and **13g′**) were finally deacetylated in alkaline MeOH to give the 17-OH analogs (**14a–h** and **14g′**), with the exception of **13i** and **13i′**, which could not be separated by column chromatography (Table 3). The structures of all the synthesized compounds were confirmed by ^{1}H and ^{13}C NMR measurements indicating the presence of the characteristic signals of the newly-formed quinoline ring in the aromatic range of the related spectra as compared with the corresponding β-chlorovinyl aldehydes.

The reactions of β-chlorovinyl aldehydes such as **2**, **7**, or **11** with aniline derivative **4** always lead to 2,3-disubstituted quinolines such as **5**, **9** or **13** instead of the 3,4-disubstituted regioisomers **16** (Scheme 1), regardless of the intermediately formed species which depend on the applied conditions.[18,29] Thus, transformations with 1 equiv. of aniline were earlier proposed to proceed through 1-chlorovinyl(*N*-aryl)imine **A** *via* a rather complex intermolecular cyclization process involving tetrahydropyrimidine intermediates to afford 2,3-disubstituted quinolines.[22] However, imine **A** is more likely to undergo initial hydrolysis due to the inadvertent presence of moisture in the DMF, to generate a small amount of the aromatic primary



Scheme 1 Explanation of substituent effects on the basis of the proposed mechanisms.

Table 4 Antiproliferative effects of the synthesized compounds on human gynecological cancer cell lines

		Growth inhibition% ± SEM [calculated IC_{50} value (μM)]						
Compound	Conc. (μM)	C33A	HeLa	SiHa	MCF7	MDA-MB-231	MDA-MB-361	T47D
5a	10	—[a]	30.8 ± 1.2	—	26.0 ± 1.4	—	—	—
	30	47.7 ± 0.5	59.5 ± 1.1, [21.2]	—	51.2 ± 1.7, [29.6]	37.3 ± 2.1	29.9 ± 1.9	37.2 ± 0.7
5b	10	—	—	—	21.9 ± 0.4	—	25.4 ± 1.3	32.9 ± 2.9
	30	35.8 ± 1.0	46.5 ± 0.8	—	46.1 ± 0.6	29.5 ± 2.1	41.1 ± 0.7	48.1 ± 1.8
5c	10	—	28.3 ± 1.5	—	27.1 ± 1.9	—	—	32.0 ± 1.9
	30	40.6 ± 1.0	43.9 ± 0.8	24.6 ± 1.7	57.0 ± 1.5, [24.1]	45.0 ± 1.8	47.2 ± 3.7	63.0 ± 2.2, [18.9]
9a	10	—	—	—	—	—	—	—
	30	—	—	—	31.5 ± 2.2	—	—	—
9b	10	—	—	24.7 ± 1.4	—	—	—	—
	30	—	—	—	—	—	—	—
9c	10	—	—	—	—	—	—	—
	30	—	26.7 ± 1.9	—	35.6 ± 1.1	—	—	—
9d	10	—	—	—	—	—	—	—
	30	—	—	25.6 ± 1.0	21.3 ± 2.5	—	—	—
9e	10	—	25.9 ± 2.3	—	35.3 ± 2.2	—	—	—
	30	—	96.6 ± 0.4, [12.3]	—	40.2 ± 2.1	—	—	—
9f	10	—	—	—	—	—	—	—
	30	—	89.4 ± 0.7, [13.1]	25.0 ± 2.6	26.4 ± 1.7	—	21.1 ± 2.8	—
13a	10	—	—	—	—	—	—	—
	30	—	28.1 ± 1.2	—	38.6 ± 1.4	—	—	—
13b	10	37.1 ± 2.5	—	—	—	—	—	—
	30	61.0 ± 1.8, [16.2]	—	—	38.7 ± 2.8	—	27.5 ± 1.9	—
13c	10	—	—	—	—	—	—	—
	30	38.9 ± 1.3	42.5 ± 1.1	35.2 ± 0.7	47.5 ± 0.7	71.2 ± 0.9, [21.5]	28.5 ± 1.2	49.9 ± 1.1
13d	10	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	30	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
13e	10	—	38.5 ± 0.7	28.0 ± 0.8	25.9 ± 0.9	—	45.2 ± 0.8	—
	30	32.0 ± 2.5	34.0 ± 0.9	43.0 ± 1.5	37.0 ± 0.7	24.4 ± 2.7	66.0 ± 0.9, [12.6]	48.2 ± 1.1
13f	10	—	—	—	28.3 ± 1.7	—	33.7 ± 1.7	—
	30	57.9 ± 2.4, [26.5]	48.0 ± 2.7	65.9 ± 0.5, [23.6]	51.5 ± 2.2, [27.9]	48.0 ± 1.1	68.2 ± 0.5, [16.8]	57.0 ± 0.8, [20.8]
13g	10	37.9 ± 1.7	46.9 ± 0.7	—	36.1 ± 2.4	—	—	—
	30	46.1 ± 1.7	51.6 ± 0.6, [20.3]	31.3 ± 2.0	62.9 ± 0.9, [17.6]	30.3 ± 0.9	59.9 ± 1.5, [16.8]	48.6 ± 1.5
13g′	10	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	30	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
13h	10	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	30	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
14a	10	24.3 ± 1.2	—	—	—	—	—	24.1 ± 0.6
	30	93.6 ± 0.2, [11.8]	—	47.8 ± 1.0	91.3 ± 0.2, [18.7]	44.2 ± 0.9	48.0 ± 1.6	89.5 ± 0.6, [12.4]
14b	10	43.2 ± 0.5	—	—	—	—	—	—
	30	71.0 ± 1.0, [20.1]	—	49.4 ± 1.5	—	37.9 ± 3.1	47.7 ± 1.6	31.5 ± 1.9
14c	10	44.8 ± 1.8	—	—	20.3 ± 1.4	—	—	47.3 ± 1.4
	30	78.8 ± 0.9, [10.2]	—	35.3 ± 2.4	73.6 ± 0.7, [19.1]	41.3 ± 1.0	45.7 ± 1.1	80.4 ± 0.4, [10.2]

Table 4 (*Contd.*)

Compound	Conc. (μM)	Growth inhibition% ± SEM [calculated IC_{50} value (μM)]						
		C33A	HeLa	SiHa	MCF7	MDA-MB-231	MDA-MB-361	T47D
14d	10	—	—	—	—	26.6 ± 1.8	42.7 ± 1.4	—
	30	53.6 ± 0.7, [15.4]	48.6 ± 1.6	35.4 ± 0.7	51.8 ± 0.6, [29.9]	63.0 ± 2.0, [18.7]	73.1 ± 0.3, [12.8]	59.7 ± 1.3, [27.1]
14e	10	27.1 ± 0.5	—	—	—	—	29.6 ± 1.5	22.4 ± 1.6
	30	86.4 ± 0.6, [12.2]	—	47.5 ± 2.1	79.8 ± 1.6, [24.9]	43.7 ± 1.0	47.1 ± 0.7	61.0 ± 1.1, [23.4]
14f	10	48.6 ± 2.5	—	—	—	—	23.1 ± 2.1	—
	30	86.9 ± 2.5, [12.3]	—	38.1 ± 0.6	64.7 ± 1.0, [23.3]	31.9 ± 1.9	40.1 ± 1.5	62.2 ± 0.4, [18.5]
14g	10	—	—	—	—	—	—	—
	30	74.7 ± 0.7, [18.6]	—	39.9 ± 2.3	62.0 ± 1.3, [24.2]	76.0 ± 0.8, [22.4]	40.8 ± 1.9	48.8 ± 0.9
14g′	10	—	—	—	—	—	—	—
	30	80.4 ± 0.4, [20.5]	—	43.5 ± 1.2	58.8 ± 1.0, [16.4]	44.2 ± 0.4	47.9 ± 1.4	65.5 ± 1.4, [24.0]
14h	10	24.0 ± 0.7	22.9 ± 2.0	—	—	26.5 ± 1.5	46.2 ± 0.7	—
	30	63.5 ± 0.3, [20.0]	31.31 ± 0.3	30.7 ± 0.2	42.5 ± 0.9	34.4 ± 1.9	75.2 ± 0.9, [10.5]	46.8 ± 1.4
Cisplatin	10	43.5 ± 1.8	42.6 ± 2.3	88.6 ± 0.5	66.9 ± 1.8	—	67.5 ± 1.0	51.0 ± 2.0
	30	74.0 ± 2.3, [3.69]	99.9 ± 0.3, [12.43]	90.2 ± 1.8, [7.84]	96.8 ± 0.4, [5.78]	71.5 ± 1.2, [19.13]	87.8 ± 1.1, [3.74]	55.0 ± 1.5, [9.78]

[a] Compounds eliciting less than 20% inhibition of proliferation were considered ineffective and the exact results are not given, for simplicity. n.d.: not determined.

amine **4**, which in a subsequent reaction with **A** leads to the formation of *N*-arylenaminoimine hydrochloride **B**.[18] This latter compound can cyclize through intermediate **C** to give the heterocyclic product **5**, **9** or **13** in varying yield depending on the steric and electronic character of the substituent R in the aromatic moiety. Thus, EDGs facilitate the reaction of **B** to **C**, while the presence of EWGs is unfavorable from the aspect of the ring-closure step. The strong electron-withdrawing effect of the NO_2 group disables the cyclization and the *N*-arylenaminoimine intermediate **B** is rather converted to aldehyde **15**, which is more stable in its tautomeric imino–enol form.[29] The presence of an *ortho* substituent on the aromatic amine and the vicinity of functional groups to the reaction center as well as the conformational flexibility of the affected sterane ring may exert significant influence on the quinoline formation.

2.2. Pharmacological studies

2.2.1. Antiproliferative properties of the synthesized compounds. The synthesized steroid–quinoline hybrids were subjected to *in vitro* pharmacological studies. Their antiproliferative activities were determined by means of the MTT assay[30] on a panel of adherent gynecological cancer cell lines after treatment for 72 h (Table 4). A screening step was first performed with 10 or 30 μM of each agent; then, in the cases of promising molecules that elicited growth inhibition of at least 50% at 30 μM, IC_{50} values were determined by using a set of dilutions. The results indicated that ring D-fused quinolines **5a–c** exhibited weak or modest antiproliferative properties, typically eliciting 30–50% growth inhibition at 30 μM. The cytostatic activities of benz[*c*]acridine derivatives **9a–f** were even weaker, except for **9e** and **9f**, which blocked the proliferation of HeLa cells selectively with IC_{50} values comparable to that of the reference agent cisplatin. Ring A-fused quinolines generally inhibited cellular growth more efficiently, and compounds with a 17-OH group (**14a–c** and **14e–g**) tended to display more pronounced action than the corresponding 17-OAc analogs (**13a–c** and **13e–g**). Since the efficacy of analog **14a** containing an unsubstituted quinoline moiety was similar to those of **14b–h**, the character of the substituent on the quinoline does not seem to be crucial for the antiproliferative actions. However, substitution at position 6′ (**14c**, **14e** and **14f**) appeared favorable. The efficacy of **14c** against T47D cells was comparable to that of the reference agent cisplatin, and **14c** was therefore selected for additional investigations to characterize the mechanism of its action.

2.2.2. Cell cycle analysis. Treatment with 10 or 30 μM **14c** for 48 h resulted in characteristic changes in the cell cycle phase distribution of T47D cells (Fig. 2). **14c** induced a concentration-dependent increase of the subdiploid cells ($subG_1$), which may be considered an indicator of apoptotic fragmentation and the appearance of apoptotic bodies.[31] On the other hand, at 30 μM there was a substantial increase in the cell number of the G_0/G_1 phase at the expense of the S and G_2/M phases. The accumulation of cells in the G_0/G_1 state can be a consequence of disturbed G_1–S transition during the cell cycle; similar behavior has been experienced during the investigation of highly effective D-homoestrone analogs.[32]

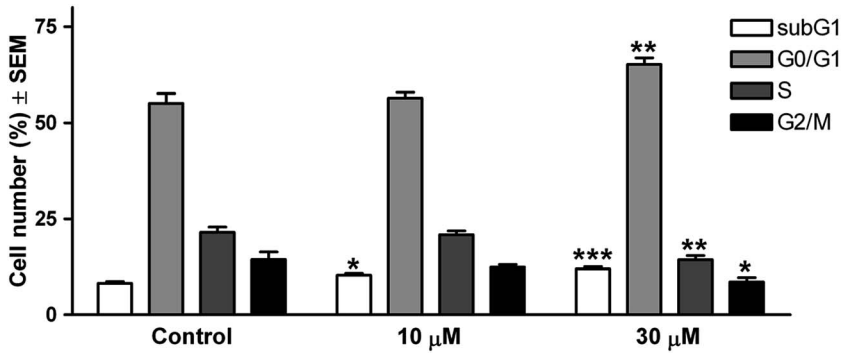


Fig. 2 Effects of **14c** on the cell cycle phase distribution of T47D cells determined by flow cytometry after incubation for 48 h. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ as compared with the untreated control cells. $N = 6$.

2.2.3. Morphological changes. In view of the former results, T47D cells were treated with 10 and 30 μM of **14c** and investigated by fluorescent microscopy after staining with Hoechst 33258 and propidium iodide (PI) dyes. Treatment for 48 h resulted in the appearance of a higher proportion of apoptotic cells with chromatin condensation, reflected by intense blue fluorescence (Fig. 3). The appearance of cells stained with PI (red fluorescence) indicated membrane damage and an increased membrane permeability, presumably as a consequence of *in vitro* secondary necrosis.

For a quantitative description, intact, apoptotic and necrotic cells were counted in all conditions. The ratio of intact cells demonstrated a concentration-dependent decrease after the treatment, and the proportion of apoptotic cells was increased significantly even at the lower concentration (Fig. 4). The tendency was more pronounced in the presence of 30 μM **14c** and the ratio of the necrotic cells was also significantly elevated. These findings suggest that **14c** has the capacity to induce apoptosis in a concentration-dependent manner.

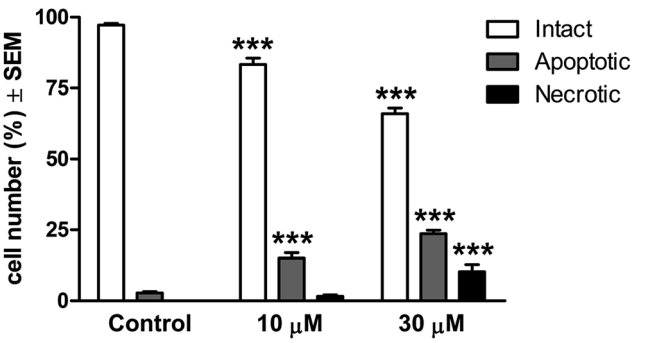


Fig. 4 Qualitative evaluation of fluorescent double staining of T47D cells treated with 10 or 30 μM **14c**. *** indicates $p < 0.001$ as compared with the untreated control cells.

2.2.4. Caspase-3 induction. To obtain further evidence about the apoptosis-inducing ability of **14c**, the caspase-3 activity was determined from T47D cells after treatment for 48

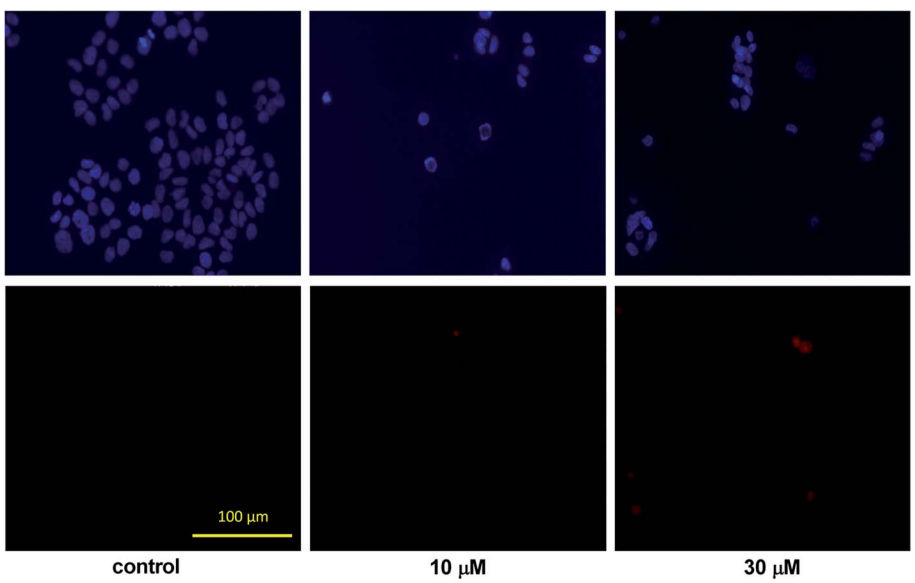


Fig. 3 Fluorescent double-stained T47D cells treated with 10 or 30 μM **14c**. The upper and lower panels indicate the fluorescence of Hoechst 33258 and PI, respectively.

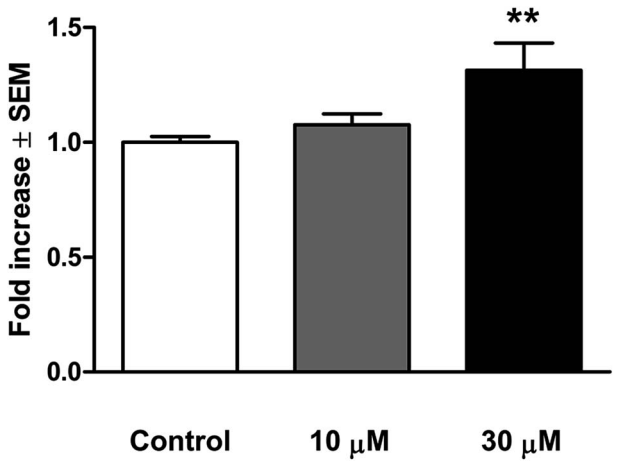


Fig. 5 Activation of caspase-3 after 48 h incubation with **14c**. ** indicates $p < 0.01$ as compared with the untreated control cells.

h. The activity of the main apoptosis executor enzyme increased significantly in the presence of 30 μM **14c** (Fig. 5).

3. Conclusions

In summary, novel ring D- and A-modified steroids containing a fused quinoline scaffold were prepared from β-chlorovinyl aldehydes of estrone 3-methyl ether and 17β-acetoxy-5α-dihydrotestosterone, respectively, with differently substituted anilines under microwave conditions. The yields of the heterocyclic products were observed to depend on the substitution pattern of the arylamines and also on the different number of members of rings D and A. Thus, a shorter reaction time and a lower temperature were sufficient for the conversion of 17β-acetoxy-3-chloro-5α-androst-2-ene-2-carbaldehyde than for that of 17-chloro-3-methoxyestra-1,3,5(10),16-tetraene-16-carbaldehyde especially with anilines containing EDGs, indicating the higher reactivity of the six-membered ring A as compared with that of the five-membered ring D. The presence of EWGs and/or *ortho* substituents was found to hamper the cyclization step of the reactions, resulting in sluggish or moderate yields of the desired quinolines. The experimental findings can be explained by assuming that the reaction occurs through an *N*-arylenaminoimine intermediate, the ring-closure of which can be affected by electronic and steric factors. As concerns the pharmacological profile of the presented molecules, most of them exerted weak or modest antiproliferative activities against the utilized panel of human adherent cell lines. The most potent agents, some of the ring A-fused quinolines, exhibited IC_{50} values in the low micromolar range, *i.e.* comparable to those of the reference agent cisplatin. The apoptosis-inducing capacity of a selected molecule was evidenced by morphological and biochemical approaches. The described results and the structure–activity relationships can be utilized to design further anticancer drug candidates with steroid skeletons.

4. Experimental

4.1. General

All solvents were distilled prior to use. Reagents and materials were obtained from commercial suppliers and were used without purification. The reactions under microwave irradiation were carried out with a CEM Corporation Focused Microwave System, Model Discover SP. The reactions were monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick). The spots were detected by spraying with 5% phosphomolybdic acid in 50% aqueous phosphoric acid. Flash chromatography: Merck silica gel 60, 40–63 μm. Melting points (mp) were determined on an SMS Optimelt digital apparatus. Elementary analysis data were determined with a PerkinElmer CHN analyzer model 2400. NMR spectra were obtained at room temperature with a Bruker DRX 500 instrument. Chemical shifts are reported in ppm (δ scale), and coupling constants (J) in Hz. For the determination of multiplicities, the *J*-MOD pulse sequence was used. Automated flow injection analyses were performed by using an HPLC/MSD system. The system comprised an Agilent 1100 micro vacuum degasser, a quaternary pump, a micro-well plate autoinjector and a 1946A MSD equipped with an electrospray ion source (ESI) operated in positive ion mode. The ESI parameters were: nebulizing gas N_2, at 35 psi; drying gas N_2, at 350 °C and 12 L min^{-1}; capillary voltage (V_{Cap}) 3000 V; fragmentor voltage 70 V. The MSD was operated in scan mode with a mass range of m/z 60–620. Samples (0.2 μL) with automated needle wash were injected directly into the solvent flow (0.3 mL min^{-1}) of CH_3CN/H_2O 70 : 30 (v/v) supplemented with 0.1% formic acid. The system was controlled by Agilent LC/MSD Chemstation software.

4.2. General procedure for the preparation of β-chlorovinyl aldehydes 2, 7 and 11

To an ice-cold solution of estrone 3-methyl ether (**1**, 5.69 g, 20.0 mmol), 6-methoxy-1-tetralone (**6**, 3.52 g, 20.0 mmol) or 17β-acetoxy-5α-dihydrotestosterone (**10**, 6.64 g, 20.0 mmol) in $CHCl_3$ (120 mL), $POCl_3$ (30 mL) and DMF (30 mL) were simultaneously added dropwise and the solution was stirred for 10 min at 0 °C. The mixture was allowed to reach room temperature and then refluxed for 4 h (for **1**, **6** and **10**, *method A*) or stirred at 25 °C for 2 h (in the case of **10**, *method B*). After completion of the reaction, the mixture was poured into ice and extracted with CH_2Cl_2 (3 × 50 mL), and the organic phase was washed with brine. The combined organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The crude product was purified by column chromatography.

4.2.1. 17-Chloro-3-methoxyestra-1,3,5(10),16-tetraene-16-carbaldehyde (2)

*Substrate: **1**.* After purification with hexane/CH_2Cl_2 (25 : 75) as eluent, **2** (4.43 g, 67%) and **3** (ref. 23) (0.91 g, 15%) were obtained as white solids.

2. Mp 129–131 °C; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta_H = 1.00$ (s, 3H, 18-CH_3), 1.44 (m, 1H), 1.57–1.68 (overlapping m, 3H), 1.74 (m, 1H), 1.95 (m, 1H), 2.00 (m, 1H), 2.16 (m, 1H), 2.32 (m, 1H), 2.46 (m, 1H), 2.65 (dd, 1H, $J = 14.7$ Hz, $J = 6.4$ Hz), 2.90 (m, 2H,

6-CH_2), 3.78 (s, 3H, 3-OMe), 6.65 (d, 1H, J = 2.1 Hz, 4-H), 6.73 (dd, 1H, J = 8.5 Hz, J = 2.1 Hz, 2-H), 7.19 (d, 1H, J = 8.5 Hz, 1-H), 10.02 (s, 1H, 16-CHO); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 15.2 (C-18), 25.9 (CH_2), 27.0 (CH_2), 28.2 (CH_2), 29.5 (CH_2), 33.0 (CH_2), 37.2 (CH), 44.0 (CH), 51.0 (C-13), 53.0 (CH), 55.2 (3-OMe), 111.6 (C-2), 113.8 (C-4), 125.9 (C-1), 131.8 (C-10), 136.4 (C-16), 137.7 (C-5), 157.6 (C-3), 162.4 (C-17), 188.0 (16-CHO); ESI-MS: 331 $[M + H]^+$; anal. calcd for $C_{20}H_{23}ClO_2$ C, 72.61; H, 7.01. Found: C, 72.80; H, 7.12.

4.3. General procedure for the preparation of steroidal quinolines (5 and 13) and benz[*c*]acridines (9)

Steroidal or nonsteroidal β-chlorovinyl aldehyde (**2**, **11** or **7**, 1.00 mmol) and (susbstituted)aniline (1.00 mmol) were dissolved in DMF (3 mL) and the solution was irradiated in a closed vessel at 140 °C or 120 °C for the appropriate time. After completion of the reaction, the mixture was poured into water (10 mL) and the precipitate was filtered off. The crude product was purified by column chromatography.

4.3.1. 3-Methoxyestra-1,3,5(10),16-tetraeno[16,17:3′,2′]quinoline (5a). Steroidal β-chlorovinyl aldehyde (**2**) and aniline **4a** (0.091 mL) were used for the reaction. Temperature: 140 °C; reaction time: 20 min. Eluent: CH_2Cl_2. Yield: 196 mg (white solid); mp 179–181 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 1.10 (s, 3H, 18-CH_3), 1.55 (m, 1H), 1.74–1.94 (overlapping m, 4H), 2.08 (m, 1H), 2.40 (m, 1H), 2.55 (m, 2H), 2.74 (m, 1H), 2.98 (m, 3H), 3.80 (s, 3H, 3-OMe), 6.69 (d, 1H, J = 2.4 Hz, 4-H), 6.76 (dd, 1H, J = 8.5 Hz, J = 2.4 Hz, 2-H), 7.28 (d, 1H, J = 8.5 Hz, 1-H), 7.46 (t-like m, 1H, 6′-H), 7.63 (t-like m, 1H, 7′-H), 7.74 (d, 1H, J = 7.9 Hz, 5′-H), 7.91 (s, 1H, 4′-H), 8.10 (d, 1H, J = 8.4 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 17.7 (C-18), 26.4 (CH_2), 27.5 (CH_2), 29.7 (CH_2), 30.0 (CH_2), 33.8 (CH_2), 37.9 (CH), 44.3 (CH), 46.1 (C-13), 54.6 (CH), 55.2 (3-OMe), 111.5 (C-2), 113.9 (C-4), 125.4 (C-6′), 126.2 (C-1), 127.5 (C-4a′), 127.6, 128.2 and 128.9 (C-5′, C-7′ and C-8′), 131.0 (C-4′), 132.5 (C-10), 134.9 (C-3′), 137.7 (C-5), 147.3 (C-8a′), 157.5 (C-3), 174.4 (C-2′); ESI-MS: 370 $[M + H]^+$; anal. calcd for $C_{26}H_{27}NO$ C, 84.51; H, 7.37. Found: C, 84.42; H, 7.45.

4.3.2. 3-Methoxyestra-6′-methyl-1,3,5(10),16-tetraeno[16,17:3′,2′]quinoline (5b). Steroidal β-chlorovinyl aldehyde (**2**) and *p*-toluidine **4b** (107 mg) were used for the reaction. Temperature: 140 °C; reaction time: 10 min. Eluent: CH_2Cl_2. Yield: 207 mg (white solid); mp 201–204 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 1.09 (s, 3H, 18-CH_3), 1.54 (m, 1H), 1.72–1.92 (overlapping m, 4H), 2.06 (m, 1H), 2.38 (m, 1H), 2.51 (s, 3H, 6′-CH_3), 2.53 (m, 1H), 2.58 (m, 1H), 2.72 (m, 1H), 2.97 (m, 3H), 3.80 (s, 3H, 3-OMe), 6.68 (d, 1H, J = 2.4 Hz, 4-H), 6.75 (dd, 1H, J = 8.4 Hz, J = 2.4 Hz, 2-H), 7.28 (d, 1H, J = 8.5 Hz, 1-H), 7.46 (d, 1H, J = 8.3 Hz, 7′-H), 7.50 (s, 1H, 5′-H), 7.83 (s, 1H, 4′-H), 8.03 (d, 1H, J = 8.3 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 17.6 (C-18), 21.4 (6′-CH_3), 26.4 (CH_2), 27.4 (CH_2), 29.7 (CH_2), 29.9 (CH_2), 33.8 (CH_2), 37.8 (CH), 44.3 (CH), 46.0 (C-13), 54.6 (CH), 55.2 (3-OMe), 111.5 (C-2), 113.9 (C-4), 126.2 (C-1), 126.5 (C-5′), 127.6 (C-4a′), 128.2, 130.5 and 130.8 (C-4′, C-7′ and C-8′), 132.5 (C-10), 134.9 (2C, C-3′ and C-6′), 135.9 (C-8a′), 137.7 (C-5), 157.5 (C-3), 174.4 (C-2′); ESI-MS: 384 $[M + H]^+$; anal. calcd for $C_{27}H_{29}NO$ C, 84.55; H, 7.62. Found: C, 84.40; H, 7.76.

4.3.3. 3-Methoxyestra-6′-methoxy-1,3,5(10),16-tetraeno[16,17:3′,2′]quinoline (5c). Steroidal β-chlorovinyl aldehyde (**2**) and *p*-anisidine **4c** (123 mg) were used for the reaction. Temperature: 140 °C; reaction time: 10 min. Eluent: CH_2Cl_2. Yield: 188 mg (white solid); mp 203–205 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 1.08 (s, 3H, 18-CH_3), 1.54 (m, 1H), 1.72–1.91 (overlapping m, 4H), 2.06 (m, 1H), 2.38 (m, 1H), 2.55 (m, 2H), 2.72 (m, 1H), 2.96 (m, 3H), 3.79 (s, 3H, 3-OMe), 3.91 (s, 3H, 6′-OMe), 6.68 (d, 1H, J = 2.4 Hz, 4-H), 6.75 (dd, 1H, J = 8.4 Hz, J = 2.4 Hz, 2-H), 7.03 (s, 1H, 4′-H), 7.28 (d, 1H, J = 8.5 Hz, 1-H), 7.28 (dd, 1H, J = 8.7 Hz, J = 2.6 Hz, 7′-H), 7.83 (s, 1H, 5′-H), 8.03 (d, 1H, J = 8.7 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 17.7 (C-18), 26.3 (CH_2), 27.4 (CH_2), 29.7 (CH_2), 30.0 (CH_2), 33.8 (CH_2), 37.8 (CH), 44.3 (CH), 45.9 (C-13), 54.7 (CH), 55.2 (3-OMe), 55.5 (6′-OMe), 105.9 (C-5′), 111.4 (C-2), 113.9 (C-4), 120.2 (C-7′), 126.2 (C-1), 128.5 (2C, C-3′ and C-4a′), 129.9 and 130.4 (C-4′ and C-8′), 132.5 (C-10), 135.3 (C-8a′), 137.7 (C-5), 157.1 (C-3), 157.5 (C-6′), 171.9 (C-2′); ESI-MS: 400 $[M + H]^+$; anal. calcd for $C_{27}H_{29}NO_2$ C, 81.17; H, 7.32. Found: C, 81.30; H, 7.51.

4.3.4. 5,6-Dihydro-3,9-dimethoxybenz[*c*]acridine (9c). Nonsteroidal β-chlorovinyl aldehyde (**7**) and *p*-anisidine **4c** (123 mg) were used for the reaction. Temperature: 120 °C; reaction time: 2 min. Eluent: CH_2Cl_2. Yield: 298 mg (yellowish solid); mp 135–137 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 2.96 (m, 2H) and 3.08 (m, 2H): 5-CH_2 and 6-CH_2, 3.87 (s, 3H, 3-OMe), 3.91 (s, 3H, 9-OMe), 6.78 (d, 1H, J = 2.4 Hz, 4-H), 6.96 (dd, 1H, J = 8.6 Hz, J = 2.4 Hz, 2-H), 7.00 (d, 1H, J = 2.7 Hz, 8-H), 7.29 (dd, 1H, J = 9.1 Hz, J = 2.7 Hz, 10-H), 7.77 (s, 1H, 7-H), 8.01 (d, 1H, J = 9.1 Hz, 11-H), 8.47 (d, 1H, J = 8.6 Hz, 1-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 28.7 and 28.9 (C-5 and C-6), 55.3 (3-OMe), 55.5 (9-OMe), 104.8 (C-8), 112.9 (2C, C-2 and C-4), 121.0 (C-10), 127.3 (C-1), 128.3 (C-7a), 130.3 (C-6a), 130.4 (C-11), 132.5 (C-7), 134.5 (C-12), 140.8 (C-4a), 148.8 (C-11a), 151.1 (C-12a), 157.3 (C-9), 160.6 (C-3); ESI-MS: 400 $[M + H]^+$; anal. calcd for $C_{19}H_{17}NO_2$ C, 78.33; H, 5.88. Found: C, 78.17; H, 6.01.

4.3.5. 11-Chloro-5,6-dihydro-3-methoxybenz[*c*]acridine (9d). Nonsteroidal β-chlorovinyl aldehyde (**7**) and *o*-chloroaniline **4d** (0.1 mL) were used for the reaction. Temperature: 120 °C; reaction time: 20 min. Eluent: hexane/CH_2Cl_2 = 70 : 30. Yield: 104 mg (yellowish solid); mp 143–145 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.98 (m, 2H) and 3.11 (m, 2H): 5-CH_2 and 6-CH_2, 3.88 (s, 3H, 3-OMe), 6.78 (d, 1H, J = 2.4 Hz, 4-H), 6.97 (dd, 1H, J = 8.6 Hz, J = 2.4 Hz, 2-H), 7.33 (t, 1H, J = 7.6 Hz, 9-H), 7.62 (d, 1H, J = 7.6 Hz, 8-H), 7.74 (d, 1H, J = 7.6 Hz, 10-H), 7.86 (s, 1H, 7-H), 8.63 (d, 1H, J = 8.6 Hz, 1-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 28.5 and 28.6 (C-5 and C-6), 55.3 (3-OMe), 112.8 and 113.2: C-2 and C-4, 125.4 and 125.9: C-8 and C-9, 127.5 (C-7a), 128.4 and 128.7: C-1 and C-10, 128.7 (C-12), 130.8 (C-6a), 133.3 (C-11), 133.8 (C-7), 141.2 (C-4a), 143.6 (C-11a), 153.9 (C-12a), 161.2 (C-3); ESI-MS: 296 $[M + H]^+$; anal. calcd for $C_{18}H_{14}ClNO$ C, 73.10; H, 4.77. Found: C, 73.25; H, 4.86.

4.3.6. 9-Chloro-5,6-dihydro-3-methoxybenz[*c*]acridine (9e). Nonsteroidal β-chlorovinyl aldehyde (**7**) and *p*-chloroaniline **4e** (128 mg) were used for the reaction. Temperature: 120 °C; reaction time: 10 min. Eluent: EtOAc/hexane = 10 : 90. Yield: 160 mg (yellowish solid); mp 122–124 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 2.96 (m, 2H) and 3.08 (m, 2H): 5-CH_2 and 6-CH_2,

3.87 (s, 3H, 3-OMe), 6.79 (d, 1H, J = 2.4 Hz, 4-H), 6.96 (dd, 1H, J = 8.6 Hz, J = 2.4 Hz, 2-H), 7.55 (dd, 1H, J = 8.9 Hz, J = 2.2 Hz, 10-H), 7.67 (d, 1H, J = 2.2 Hz, 8-H), 7.76 (s, 1H, 7-H), 8.02 (d, 1H, J = 8.9 Hz, 11-H), 8.48 (d, 1H, J = 8.6 Hz, 1-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 28.5 and 28.8 (C-5 and C-6), 55.3 (3-OMe), 112.9 and 113.1 (C-2 and C-4), 125.5 (C-8), 127.3 (C-7a), 127.8 (C-1), 128.0 (C-6a), 129.4 (C-10), 130.6 (C-11), 131.0 and 131.1 (C-9 and C-12), 132.5 (C-7), 141.2 (C-4a), 148.4 (C-11a), 153.6 (C-12a), 161.1 (C-3); ESI-MS: 296 $[M + H]^+$; anal. calcd for $C_{18}H_{14}ClNO$ C, 73.10; H, 4.77. Found: C, 72.98; H, 4.69.

4.3.7. 9-Bromo-5,6-dihydro-3-methoxybenz[*c*]acridine (9f). Nonsteroidal β-chlorovinyl aldehyde (**7**) and *p*-bromoaniline **4f** (172 mg) were used for the reaction. Temperature: 120 °C; reaction time: 10 min. Eluent: hexane/CH_2Cl_2 = 20 : 80. Yield: 211 mg (orange solid); mp 122–124 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 2.96 (m, 2H) and 3.09 (m, 2H): 5-CH_2 and 6-CH_2, 3.87 (s, 3H, 3-OMe), 6.78 (d, 1H, J = 2.3 Hz, 4-H), 6.96 (dd, 1H, J = 8.6 Hz, J = 2.3 Hz, 2-H), 7.68 (dd, 1H, J = 8.9 Hz, J = 1.9 Hz, 10-H), 7.75 (s, 1H, 7-H), 7.85 (d, 1H, J = 1.9 Hz, 8-H), 7.96 (d, 1H, J = 8.9 Hz, 11-H), 8.48 (d, 1H, J = 8.6 Hz, 1-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 28.2 and 28.4 (C-5 and C-6), 55.0 (3-OMe), 112.6 and 112.8 (C-2 and C-4), 118.9 (C-9), 127.5 (C-1), 128.2 (C-7a), 128.5 (C-8), 130.3 (C-11), 130.6 (C-6a), 131.6 (C-10), 132.1 (C-7), 134.5 (C-12), 140.9 (C-4a), 147.1 (C-11a), 153.4 (C-12a), 160.8 (C-3); ESI-MS: 340 $[M + H]^+$; anal. calcd for $C_{18}H_{14}BrNO$ C, 63.55; H, 4.15. Found: C, 63.75; H, 4.10.

4.3.8. 17β-Acetoxy-5α-androstano[2,3:3′,2′]quinoline (13a). Steroidal β-chlorovinyl aldehyde (**11**) and aniline **4a** (0.091 mL) were used for the reaction. Temperature: 120 °C; reaction time: 5 min. Eluent: EtOAc/CH_2Cl_2 = 10 : 90. Yield: 317 mg (white solid); mp 226–228 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.80 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3), 0.88 (m, 1H), 0.98 (m, 1H), 1.07 (m, 1H), 1.23 (m, 1H), 1.29–1.55 (overlapping m, 5H), 1.66–1.82 (overlapping m, 6H), 2.05 (s, 3H, Ac-CH_3), 2.17 (m, 1H), 2.60 (d, 1H, J = 16.0 Hz, one of 1-H_2), 2.79 (dd, 1H, J = 18.5 Hz, J = 12.5 Hz, one of 4-H_2), 2.97 (d, 1H, J = 16.0 Hz, the other of 1-H_2), 3.08 (dd, 1H, J = 18.5 Hz, J = 5.5 Hz, the other of 4-H_2), 4.62 (t, 1H, J = 8.4 Hz, 17-H), 7.42 (t-like m, 1H, 6′-H), 7.60 (t-like m, 1H, 7′-H), 7.69 (d, 1H, J = 8.0 Hz, 5′-H), 7.79 (s, 1H, 4′-H), 7.97 (d, 1H, J = 8.4 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.6 (C-19), 12.0 (C-18), 20.8 (CH_2), 21.2 (Ac-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.6 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.3 (CH), 36.9 (CH_2), 37.3 (CH_2), 42.2 (CH), 42.5 (C-13), 43.5 (CH_2), 50.7 (CH), 53.5 (CH), 82.8 (C-17), 125.5 (C-6′), 126.8 (C-5′), 127.2 (C-4a′), 128.2 and 128.5 (C-7′ and C-8′), 130.0 (C-3′), 135.8 (C-4′), 146.6 (C-8a′), 158.3 (C-2′), 171.2 (Ac-CO); ESI-MS: 418 $[M + H]^+$; anal. calcd for $C_{28}H_{35}NO_2$ C, 80.53; H, 8.45. Found: C, 80.34; H, 8.30.

4.3.9. 17β-Acetoxy-6′-methyl-5α-androstano[2,3:3′,2′]quinoline (13b). Steroidal β-chlorovinyl aldehyde (**11**) and *p*-toluidine **4b** (107 mg) were used for the reaction. Temperature: 120 °C; reaction time: 2 min. Eluent: EtOAc/CH_2Cl_2 = 5 : 95. Yield: 393 mg (white solid); mp 247–249 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.79 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3), 0.86 (m, 1H), 0.97 (m, 1H), 1.05 (m, 1H), 1.22 (m, 1H), 1.28–1.54 (overlapping m, 5H), 1.64–1.82 (overlapping m, 6H), 2.05 (s, 3H, Ac-CH_3), 2.17 (m, 1H), 2.49 (s, 3H, 6′-CH_3), 2.58 (d, 1H, J = 16.0 Hz, one of 1-H_2), 2.77 (dd, 1H, J = 18.3 Hz, J = 12.8 Hz, one of 4-H_2), 2.95 (d, 1H, J = 16.0 Hz, the other of 1-H_2), 3.06 (dd, 1H, J = 18.3 Hz, J = 4.7 Hz, the other of 4-H_2), 4.62 (t-like m, 1H, 17-H), 7.44 (d, 1H, J = 8.2 Hz, 7′-H), 7.44 (s, 1H, 5′-H), 7.71 (s, 1H, 4′-H), 7.88 (d, 1H, J = 8.2 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.6 (C-19), 12.0 (C-18), 20.8 (CH_2), 21.2 (Ac-CH_3), 21.5 (6′-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.6 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.3 (CH), 36.9 (CH_2), 37.1 (CH_2), 42.2 (CH), 42.5 (C-13), 43.5 (CH_2), 50.7 (CH), 53.5 (CH), 82.8 (C-17), 125.6 (C-5′), 127.2 (C-4a′), 127.7 (C-8′), 130.0 (C-3′), 131.0 (C-7′), 135.3 (C-6′), 135.4 (C-4′), 146.4 (C-8a′), 157.2 (C-2′), 171.2 (Ac-CO); ESI-MS: 432 $[M + H]^+$; anal. calcd for $C_{29}H_{37}NO_2$ C, 80.70; H, 8.64. Found: C, 80.55; H, 8.82.

4.3.10. 17β-Acetoxy-6′-methoxy-5α-androstano[2,3:3′,2′]quinoline (13c). Steroidal β-chlorovinyl aldehyde (**11**) and *p*-anisidine **4c** (123 mg) were used for the reaction. Temperature: 120 °C; reaction time: 2 min. Eluent: EtOAc/CH_2Cl_2 = 5 : 95. Yield: 412 mg (white solid); mp 255–257 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.79 (s, 3H, 18-CH_3), 0.81 (s, 3H, 19-CH_3), 0.86 (m, 1H), 0.96 (m, 1H), 1.06 (m, 1H), 1.21 (m, 1H), 1.28–1.54 (overlapping m, 5H), 1.64–1.82 (overlapping m, 6H), 2.05 (s, 3H, Ac-CH_3), 2.17 (m, 1H), 2.57 (d, 1H, J = 16.0 Hz, one of 1-H_2), 2.74 (dd, 1H, J = 18.2 Hz, J = 12.7 Hz, one of 4-H_2), 2.93 (d, 1H, J = 16.0 Hz, the other of 1-H_2), 3.05 (dd, 1H, J = 18.2 Hz, J = 5.4 Hz, the other of 4-H_2), 3.89 (s, 3H, 6′-OMe), 4.62 (t, 1H, J = 8.7 Hz, 17-H), 6.95 (d, 1H, J = 2.6 Hz, 5′-H), 7.26 (dd, 1H, J = 9.1 Hz, J = 2.6 Hz, 7′-H), 7.68 (s, 1H, 4′-H), 7.86 (d, 1H, J = 9.1 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.6 (C-19), 12.0 (C-18), 20.8 (CH_2), 21.2 (Ac-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.6 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.3 (CH), 36.9 (CH_2), 37.0 (CH_2), 42.2 (CH), 42.5 (C-13), 43.5 (CH_2), 50.7 (CH), 53.5 (CH), 55.4 (6′-OMe), 82.8 (C-17), 104.3 (C-5′), 121.3 (C-7′), 128.0 (C-4a′), 129.6 (C-8′), 130.3 (C-3′), 134.7 (C-4′), 146.5 (C-8a′), 155.5 and 157.1 (C-2′ and C-6′), 171.2 (Ac-CO); ESI-MS: 448 $[M + H]^+$; anal. calcd for $C_{29}H_{37}NO_3$ C, 77.82; H, 8.33. Found: C, 77.65; H, 8.47.

4.3.11. 17β-Acetoxy-8′-chloro-5α-androstano[2,3:3′,2′]quinoline (13d). Steroidal β-chlorovinyl aldehyde (**11**) and *o*-chloroaniline **4d** (0.1 mL) were used for the reaction. Temperature: 120 °C; reaction time: 20 min. Eluent: CH_2Cl_2. Yield: 194 mg (white solid); mp > 310 °C (decomp.); ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = δ 0.79 (s, 3H, 18-CH_3), 0.81 (s, 3H, 19-CH_3), 0.87 (m, 1H), 0.98 (m, 1H), 1.06 (m, 1H), 1.22 (m, 1H), 1.28–1.55 (overlapping m, 5H), 1.65–1.82 (overlapping m, 6H), 2.05 (s, 3H, Ac-CH_3), 2.17 (m, 1H), 2.58 (d, 1H, J = 16.1 Hz, one of 1-H_2), 2.87 (dd, 1H, J = 18.6 Hz, J = 12.8 Hz, one of 4-H_2), 2.98 (d, 1H, J = 16.1 Hz, the other of 1-H_2), 3.21 (dd, 1H, J = 18.6 Hz, J = 5.3 Hz, the other of 4-H_2), 4.62 (t, 1H, J = 8.6 Hz, 17-H), 7.33 (t-like m, 1H, 6′-H), 7.61 (d, 1H, J = 8.1 Hz, 5′-H), 7.71 (d, 1H, J = 7.3 Hz, 7′-H), 7.80 (s, 1H, 4′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.7 (C-19), 12.0 (C-18), 20.8 (CH_2), 21.2 (Ac-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.4 (CH_2), 31.1 (CH_2), 35.2 (C-10), 35.3 (CH), 36.9 (CH_2), 37.5 (CH_2), 42.1 (CH), 42.5 (C-13), 43.3 (CH_2), 50.7 (CH), 53.4 (CH), 82.7 (C-17), 125.3 and 126.0 (C-5′ and C-6′), 128.5 (C-4a′), 128.5 (C-7′), 131.1 (C-3′), 132.3 (C-8′), 136.1 (C-4′), 142.8 (C-8a′), 159.6 (C-2′), 171.2 (Ac-CO); ESI-MS: 453 $[M + H]^+$; anal. calcd for $C_{28}H_{34}ClNO_2$ C, 74.40; H, 7.58. Found: C, 74.61; H, 7.39.

4.3.12. 17β-Acetoxy-6′-chloro-5α-androstano[2,3:3′,2′]quinoline (13e). Steroidal β-chlorovinyl aldehyde (**11**) and *p*-chloroaniline **4e** (128 mg) were used for the reaction. Temperature:

120 °C; reaction time: 10 min. Eluent: EtOAc/CH_2Cl_2 = 5 : 95. Yield: 267 mg (white solid); mp 288–291 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.79 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3), 0.88 (m, 1H), 0.98 (m, 1H), 1.08 (m, 1H), 1.23 (m, 1H), 1.29–1.55 (overlapping m, 5H), 1.64–1.82 (overlapping m, 6H), 2.05 (s, 3H, Ac-CH_3), 2.17 (m, 1H), 2.59 (d, 1H, J = 16.2 Hz, one of 1-H_2), 2.76 (dd, 1H, J = 18.4 Hz, J = 12.9 Hz, one of 4-H_2), 2.97 (d, 1H, J = 16.2 Hz, the other of 1-H_2), 3.05 (dd, 1H, J = 18.4 Hz, J = 5.2 Hz, the other of 4-H_2), 4.62 (t, 1H, J = 8.5 Hz, 17-H), 7.53 (dd, 1H, J = 8.9 Hz, J = 1.9 Hz, 7′-H), 7.66 (d, 1H, J = 1.9 Hz, 5′-H), 7.70 (s, 1H, 4′-H), 7.90 (d, 1H, J = 8.9 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.8 (C-19), 12.0 (C-18), 20.8 (CH_2), 21.2 (Ac-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.5 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.3 (CH), 36.9 (CH_2), 37.2 (CH_2), 42.1 (CH), 42.5 (C-13), 43.5 (CH_2), 50.7 (CH), 53.4 (CH), 82.7 (C-17), 125.4 (C-5′), 127.8 (C-4a′), 129.4 and 129.8 (C-7′ and C-8′), 131.1 (2C, C-6′ and C-3′), 134.8 (C-4′), 144.9 (C-8a′), 158.7 (C-2′), 171.2 (Ac-CO); ESI-MS: 453 $[M + H]^+$; anal. calcd for $C_{28}H_{34}ClNO_2$ C, 74.40; H, 7.58. Found: C, 74.25; H, 7.43.

4.3.13. 17β-Acetoxy-6′-bromo-5α-androstano[2,3:3′,2′]quinoline (13f). Steroidal β-chlorovinyl aldehyde (**11**) and *p*-bromoaniline **4f** (172 mg) were used for the reaction. Temperature: 120 °C; reaction time: 10 min. Eluent: EtOAc/CH_2Cl_2 = 5 : 95. Yield: 298 mg (white solid); mp 295–297 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.79 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3), 0.88 (m, 1H), 0.98 (m, 1H), 1.08 (m, 1H), 1.22 (m, 1H), 1.28–1.55 (overlapping m, 5H), 1.64–1.82 (overlapping m, 6H), 2.05 (s, 3H, Ac-CH_3), 2.17 (m, 1H), 2.59 (d, 1H, J = 16.2 Hz, one of 1-H_2), 2.75 (dd, 1H, J = 18.5 Hz, J = 12.7 Hz, one of 4-H_2), 2.97 (d, 1H, J = 16.2 Hz, the other of 1-H_2), 3.04 (dd, 1H, J = 18.5 Hz, J = 5.4 Hz, the other of 4-H_2), 4.62 (t-like m, 1H, 17-H), 7.65 (dd, 1H, J = 9.0 Hz, J = 2.0 Hz, 7′-H), 7.69 (s, 1H, 5′-H), 7.82 (d, 1H, J = 9.0 Hz, 8′-H), 7.83 (s, 1H, 4′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.7 (C-19), 12.1 (C-18), 20.8 (CH_2), 21.2 (Ac-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.5 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.3 (CH), 36.9 (CH_2), 37.3 (CH_2), 42.1 (CH), 42.5 (C-13), 43.5 (CH_2), 50.7 (CH), 53.4 (CH), 82.7 (C-17), 119.2 (C-6′), 128.3 (C-4a′), 128.8 (C-5′), 130.0 (C-8′), 131.1 (C-3′), 131.9 (C-7′), 134.7 (C-4′), 145.2 (C-8a′), 158.9 (C-2′), 171.2 (Ac-CO); ESI-MS: 498 $[M + H]^+$; anal. calcd for $C_{28}H_{34}BrNO_2$ C, 67.74; H, 6.90. Found: C, 67.89; H, 6.75.

4.3.14. 17β-Acetoxy-7′-chloro-5α-androstano[2,3:3′,2′]quinoline (13g) and 17β-acetoxy-5′-chloro-5α-androstano[2,3:3′,2′]quinoline (13g′). Steroidal β-chlorovinyl aldehyde (**11**) and *m*-chloroaniline **4g** (0.1 mL) were used for the reaction. Temperature: 120 °C; reaction time: 10 min. Eluent: EtOAc/CH_2Cl_2 = 2 : 98. Yields: 208 mg (**13g**) and 72 mg (**13g′**) as white solids. **13g**: mp 258–260 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.78 (s, 3H, 18-CH_3), 0.81 (s, 3H, 19-CH_3), 0.86 (m, 1H), 0.96 (m, 1H), 1.06 (m, 1H), 1.22 (m, 1H), 1.29–1.55 (overlapping m, 5H), 1.64–1.82 (overlapping m, 6H), 2.05 (s, 3H, Ac-CH_3), 2.17 (m, 1H), 2.56 (d, 1H, J = 16.1 Hz, one of 1-H_2), 2.76 (dd, 1H, J = 18.5 Hz, J = 12.7 Hz, one of 4-H_2), 2.94 (d, 1H, J = 16.1 Hz, the other of 1-H_2), 3.05 (dd, 1H, J = 18.5 Hz, J = 5.4 Hz, the other of 4-H_2), 4.62 (t, 1H, J = 8.6 Hz, 17-H), 7.37 (dd, 1H, J = 8.7 Hz, J = 1.8 Hz, 6′-H), 7.61 (d, 1H, J = 8.7 Hz, 5′-H), 7.76 (s, 1H, 4′-H), 7.96 (dd, 1H, J = 1.8 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.7 (C-19), 12.0 (C-18), 20.8 (CH_2), 21.2 (Ac-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.5 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.3 (CH), 36.9 (CH_2), 37.3 (CH_2), 42.1 (CH), 42.5 (C-13), 43.4 (CH_2), 50.7 (CH), 53.4 (CH), 82.7 (C-17), 125.5 (C-4a′), 126.5 (C-6′), 127.3 (C-8′), 128.0 (C-5′), 130.4 (C-3′), 134.2 (C-7′), 135.6 (C-4′), 146.9 (C-8a′), 159.5 (C-2′), 171.2 (Ac-CO); ESI-MS: 453 $[M + H]^+$; anal. calcd for $C_{28}H_{34}ClNO_2$ C, 74.40; H, 7.58. Found: C, 74.24; H, 7.65. **13g′**: mp 262–265 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.80 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3), 0.89 (m, 1H), 0.97 (m, 1H), 1.08 (m, 1H), 1.23 (m, 1H), 1.28–1.55 (overlapping m, 5H), 1.66–1.83 (overlapping m, 6H), 2.05 (s, 3H, Ac-CH_3), 2.18 (m, 1H), 2.63 (d, 1H, J = 16.2 Hz, one of 1-H_2), 2.78 (dd, 1H, J = 18.5 Hz, J = 12.7 Hz, one of 4-H_2), 3.06 (d, 1H, J = 16.2 Hz, the other of 1-H_2), 3.10 (dd, 1H, J = 18.5 Hz, J = 5.4 Hz, the other of 4-H_2), 4.63 (t, 1H, J = 8.6 Hz, 17-H), 7.50 (m, 2H, 6′-H and 7′-H), 7.90 (dd, 1H, J = 5.9 Hz, J = 3.1 Hz, 8′-H), 8.20 (s, 1H, 4′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.7 (C-19), 12.1 (C-18), 20.8 (CH_2), 21.2 (Ac-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.5 (CH_2), 31.1 (CH_2), 35.3 (CH), 35.3 (C-10), 36.9 (CH_2), 37.1 (CH_2), 42.1 (CH), 42.5 (C-13), 43.6 (CH_2), 50.7 (CH), 53.4 (CH), 82.7 (C-17), 125.3 (C-4a′), 125.5, 127.5 and 128.2 (C-6′, C-7′ and C-8′), 130.4 (C-5′), 131.3 (C-3′), 132.6 (C-4′), 147.2 (C-8a′), 159.2 (C-2′), 171.2 (Ac-CO); ESI-MS: 453 $[M + H]^+$; anal. calcd for $C_{28}H_{34}ClNO_2$ C, 74.40; H, 7.58. Found: C, 74.58; H, 7.70.

4.3.15. 17β-Acetoxy-8′-methyl-5α-androstano[2,3:3′,2′]quinoline (13h). Steroidal β-chlorovinyl aldehyde (**11**) and *o*-toluidine **4h** (0.1 mL) were used for the reaction. Temperature: 120 °C; reaction time: 5 min. Eluent: EtOAc/CH_2Cl_2 = 5 : 95. Yield: 393 mg (white solid); mp 262–265 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.81 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3), 0.87 (m, 1H), 0.98 (m, 1H), 1.06 (m, 1H), 1.22 (m, 1H), 1.29–1.55 (overlapping m, 5H), 1.66–1.82 (overlapping m, 6H), 2.06 (s, 3H, Ac-CH_3), 2.18 (m, 1H), 2.58 (d, 1H, J = 16.0 Hz, one of 1-H_2), 2.78 (s, 3H, 8′-CH_3), 2.82 (dd, 1H, J = 18.2 Hz, J = 12.7 Hz, one of 4-H_2), 2.97 (d, 1H, J = 16.0 Hz, the other of 1-H_2), 3.12 (dd, 1H, J = 18.2 Hz, J = 3.8 Hz, the other of 4-H_2), 4.62 (t-like m, 1H, 17-H), 7.31 (t-like m, 1H, 6′-H), 7.45 (d, 1H, J = 6.8 Hz, 7′-H), 7.54 (d, 1H, J = 8.1 Hz, 5′-H), 7.75 (s, 1H, 4′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.7 (C-19), 12.0 (C-18), 18.0 (8′-CH_3), 20.8 (CH_2), 21.2 (Ac-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.6 (CH_2), 31.2 (CH_2), 35.2 (C-10), 35.4 (CH), 36.9 (CH_2), 37.6 (CH_2), 42.3 (CH), 42.5 (C-13), 43.5 (CH_2), 50.7 (CH), 53.5 (CH), 82.8 (C-17), 124.9 and 125.2 (C-5′ and C-6′), 127.1 (C-4a′), 128.5 (C-7′), 129.6 (C-3′), 132.1 (C-8′), 135.9 (C-4′), 136.0 (C-8a′), 157.2 (C-2′), 171.2 (Ac-CO); ESI-MS: 432 $[M + H]^+$; anal. calcd for $C_{29}H_{37}NO_2$ C, 80.70; H, 8.64. Found: C, 80.87; H, 8.52.

4.3.16. 17β-Acetoxy-7′-methyl-5α-androstano[2,3:3′,2′]quinoline (13i) and 17β-acetoxy-5′-methyl-5α-androstano[2,3:3′,2′]quinoline (13i′). Steroidal β-chlorovinyl aldehyde (**11**) and *m*-toluidine **4i** (0.1 mL) were used for the reaction. Temperature: 120 °C; reaction time: 2 min. The 3 : 1 mixture of **13i** and **13i′** could not be separated by column chromatography. Yield: 388 mg (white solid). **13i** (assigned from the spectra of the regioisomeric mixture); ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.77 (s, 3H, 18-CH_3), 0.81 (s, 3H, 19-CH_3), 0.85 (m, 1H), 0.96 (m, 1H), 1.04 (m, 1H), 1.22 (m, 1H), 1.29–1.55 (overlapping m, 5H), 1.64–1.80 (overlapping m, 6H), 2.04 (s, 3H, Ac-CH_3), 2.15 (m, 1H), 2.51 (s, 3H, 7′-CH_3), 2.55 (d, 1H, J = 16.0 Hz, one of 1-H_2), 2.75 (dd, 1H, J = 18.3 Hz, J = 12.3 Hz, one of 4-H_2), 2.93 (d, 1H, J = 16.0

Hz, the other of 1-H_2), 3.05 (dd, 1H, J = 18.5 Hz, J = 5.8 Hz, the other of 4-H_2), 4.61 (t-like m, 1H, 17-H), 7.25 (d, 1H, J = 8.0 Hz, 6′-H), 7.57 (d, 1H, J = 8.0 Hz, 5′-H), 7.73 (s, 1H) and 7.75 (s, 1H): 4′-H and 8′-H; ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.6 (C-19), 12.0 (C-18), 20.8 (CH_2), 21.2 (Ac-CH_3), 21.8 (7′-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.6 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.3 (CH), 36.9 (CH_2), 37.3 (CH_2), 42.1 (CH), 42.5 (C-13), 43.4 (CH_2), 50.7 (CH), 53.4 (CH), 82.8 (C-17), 125.3 (C-4a′), 126.4 (C-6′), 127.2 (C-8′), 127.8 (C-5′), 129.1 (C-3′), 135.5 (C-4′), 138.6 (C-7′), 146.9 (C-8a′), 158.1 (C-2′), 171.2 (Ac-CO); ESI-MS: 432 $[M + H]^+$; anal. calcd for $C_{29}H_{37}NO_2$ C, 80.70; H, 8.64. Found: C, 80.59; H, 8.74.

4.3.17. 17β-Acetoxy-3-[4′-nitrophenyl]amino-5α-androst-2-ene-2-carbaldehyde (15). Steroidal β-chlorovinyl aldehyde (**11**) and *p*-nitroaniline **4j** (138 mg) were used for the reaction according to the general procedure 4.2. After irradiation of the reaction mixture at 120 °C for 40 min, no conversion was observed by TLC. After stirring of the mixture at room temperature for 3 days, an orange precipitate was formed and filtered off. The crude product was purified by column chromatography with EtOAc/CH_2Cl_2 = 2 : 98. Yield: 326 mg (orange solid); >270 °C (decomp.); ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.81 (s, 3H), 0.82 (s + m, 4H): 18-H_3, 19-H_3 and 1H, 0.94 (m, 1H), 1.06 (m, 1H), 1.14–1.25 (overlapping m, 2H), 1.32 (m, 1H), 1.41 (m, 2H), 1.46–1.54 (overlapping m, 2H), 1.58–1.68 (overlapping m, 3H), 1.72 (m, 1H), 1.79 (m, 1H), 2.04 (s, 3H, Ac-CH_3), 2.09–2.18 (overlapping m, 2H, one of 4-H_2 and 1H), 2.22 (d, 1H, J = 14.7 Hz, one of 1-H_2), 2.35 (dd, 1H, J = 19.2 Hz, J = 5.6 Hz, the other of 4-H_2), 2.42 (d, 1H, J = 14.7 Hz, the other of 1-H_2), 4.61 (t-like m, 1H, 17-H), 7.03 (d, 2H, J = 9.1 Hz, 2′-H and 6′-H), 7.09 (d, 1H, J = 11.3 Hz, CH –OH), 8.18 (d, 2H, J = 9.1 Hz, 3′-H and 5′-H), 11.98 (d, 1H, J = 11.3 Hz, OH); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.5 (C-19), 12.0 (C-18), 20.8 (CH_2), 21.2 (Ac-CH_3), 23.5 (CH_2), 27.5 (CH_2), 28.2 (CH_2), 31.0 (CH_2), 35.3 (CH), 35.7 (C-10), 36.8 (CH_2), 41.7 (CH), 42.3 (C-13), 42.4 (C-4), 42.5 (C-1), 50.7 (CH), 53.1 (CH), 82.7 (C-17), 108.2 (C-2), 112.5 (C-3), 114.7 (2C, C-2′ and C-6′), 126.0 (2C, C-3′ and C-5′), 139.4 (CHOH), 142.2 (C-1′), 146.2 (C-4′), 171.2 (Ac-CO); ESI-MS: 503 $[M + Na]^+$; anal. calcd for $C_{28}H_{36}N_2O_5$ C, 69.98; H, 7.55. Found: C, 70.14; H, 7.69.

4.4. General procedure for the synthesis of 17-deacetylated ring A-fused quinolines (14)

The 17β-acetoxy derivative (**13**, 0.30 mmol) was dissolved in MeOH (10 mL), and KOH (56 mg, 1.00 mmol) was added. The mixture was stirred for 2 h at room temperature, and then diluted with water. The resulting precipitate was filtered off, washed with water and dried.

4.4.1. 17β-Hydroxy-5α-androstano[2,3:3′,2′]quinoline (14a). Compound **13a** (125 mg) was used for the synthesis. Yield: 101 mg (90%, white solid); mp 232–235 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.77 (s, 3H, 18-CH_3), 0.79 (s, 3H, 19-CH_3), 0.85 (m, 1H), 0.96 (m, 1H), 1.12 (m, 1H), 1.24–1.48 (overlapping m, 5H), 1.63–1.75 (overlapping m, 6H), 1.87 (m, 1H), 2.06 (m, 1H), 2.58 (d, 1H, J = 16.0 Hz, one of 1-H_2), 2.79 (dd, 1H, J = 18.1 Hz, J = 14.1 Hz, one of 4-H_2), 2.98 (d, 1H, J = 16.0 Hz, the other of 1-H_2), 3.08 (dd, 1H, J = 18.1 Hz, J = 4.1 Hz, the other of 4-H_2), 3.66 (t, 1H, J = 7.5 Hz, 17-H), 7.42 (t-like m, 1H, 6′-H), 7.60 (t-like m, 1H, 7′-H), 7.69 (d, 1H, J = 7.8 Hz, 5′-H), 7.79 (s, 1H, 4′-H), 7.97 (d, 1H, J = 8.1 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.1 (C-19), 11.7 (C-18), 20.9 (CH_2), 23.4 (CH_2), 28.6 (CH_2), 30.5 (CH_2), 31.2 (CH_2), 35.3 (C-10), 35.6 (CH), 36.7 (CH_2), 37.3 (CH_2), 42.3 (CH), 42.9 (C-13), 43.5 (CH_2), 50.9 (CH), 53.7 (CH), 81.8 (C-17), 125.5 (C-6′), 126.8 (C-5′), 127.2 (C-4a′), 128.1 and 128.6 (C-7′ and C-8′), 130.1 (C-3′), 135.8 (C-4′), 146.5 (C-8a′), 158.3 (C-2′); ESI-MS: 476 $[M + H]^+$; anal. calcd for $C_{26}H_{33}NO$ C, 83.15; H, 8.86. Found: C, 82.99; H, 8.76.

4.4.2. 17β-Hydroxy-6′-methyl-5α-androstano[2,3:3′,2′]quinoline (14b). Compound **13b** (129 mg) was used for the synthesis. Yield: 106 mg (91%, white solid); mp 228–230 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.77 (s, 3H, 18-CH_3), 0.79 (s, 3H, 19-CH_3), 0.85 (m, 1H), 0.97 (m, 1H), 1.12 (m, 1H), 1.25–1.50 (overlapping m, 5H), 1.60–1.73 (overlapping m, 6H), 1.87 (m, 1H), 2.07 (m, 1H), 2.49 (s, 3H, 6′-CH_3), 2.57 (d, 1H, J = 16.0 Hz, one of 1-H_2), 2.77 (dd, 1H, J = 18.1 Hz, J = 13.5 Hz, one of 4-H_2), 2.96 (d, 1H, J = 16.0 Hz, the other of 1-H_2), 3.06 (dd, 1H, J = 18.1 Hz, J = 4.4 Hz, the other of 4-H_2), 3.67 (t, 1H, J = 8.0 Hz, 17-H), 7.44 (d, 1H, J = 8.1 Hz, 7′-H), 7.44 (s, 1H, 5′-H), 7.70 (s, 1H, 4′-H), 7.88 (d, 1H, J = 8.1 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.1 (C-19), 11.6 (C-18), 20.9 (CH_2), 21.5 (6′-CH_3), 23.4 (CH_2), 28.6 (CH_2), 30.5 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.6 (CH), 36.7 (CH_2), 37.1 (CH_2), 42.2 (CH), 42.8 (C-13), 43.5 (CH_2), 50.9 (CH), 53.6 (CH), 81.8 (C-17), 125.6 (C-5′), 127.2 (C-4a′), 127.6 (C-8′), 130.0 (C-5′), 131.0 (C-7′), 135.3 (C-3′), 135.4 (C-4′), 144.9 (C-8a′), 157.2 (C-2′); ESI-MS: 390 $[M + H]^+$; anal. calcd for $C_{27}H_{35}NO$ C, 83.24; H, 9.06. Found: C, 83.39; H, 8.95.

4.4.3. 17β-Hydroxy-6′-methoxy-5α-androstano[2,3:3′,2′]quinoline (14c). Compound **13c** (134 mg) was used for the synthesis. Yield: 112 mg (92%, white solid); mp 193–195 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.63 (m, 1H), 0.73 (s, 3H) and 0.74 (s, 3H): 18-CH_3 and 19-CH_3, 0.81 (m, 2H), 1.04 (m, 1H), 1.22–1.56 (overlapping m, 9H), 1.67 (m, 2H), 1.86 (m, 1H), 1.98 (m, 1H), 2.43 (d, 1H, J = 16.0 Hz, one of 1-H_2), 2.66 (dd, 1H, J = 17.2 Hz, J = 13.2 Hz, one of 4-H_2), 2.93 (m, 2H, the other of 1-H_2, the other of 4-H_2), 3.55 (t, 1H, J = 7.7 Hz, 17-H), 3.90 (s, 3H, 6′-OMe), 7.14 (s, 1H, 5′-H), 7.29 (d, 1H, J = 8.8 Hz, 7′-H), 7.78 (d, 1H, J = 8.8 Hz, 8′-H), 7.88 (s, 1H, 4′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.6 (C-19), 11.9 (C-18), 22.0 (CH_2), 24.3 (CH_2), 29.7 (CH_2), 30.7 (CH_2), 32.2 (CH_2), 36.3 (C-10), 36.9 (CH), 37.5 (CH_2), 38.0 (CH_2), 43.3 (CH), 44.0 (C-13), 44.4 (CH_2), 52.2 (CH), 55.0 (CH), 56.1 (6′-OMe), 82.5 (C-17), 105.8 (C-5′), 123.1 (C-7′), 129.2 (C-8′), 130.0 (C-4a′), 132.1 (C-3′), 137.2 (C-4′), 143.2 (C-8a′), 156.5 and 159.0 (C-2′ and C-6′); ESI-MS: 406 $[M + H]^+$; anal. calcd for $C_{27}H_{35}NO_2$ C, 79.96; H, 8.70. Found: C, 80.12; H, 8.57.

4.4.4. 17β-Hydroxy-8′-chloro-5α-androstano[2,3:3′,2′]quinoline (14d). Compound **13d** (136 mg) was used for the synthesis. Yield: 112 mg (91%, white solid); mp > 300 °C (decomp.); ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.77 (s, 3H, 18-CH_3), 0.80 (s, 3H, 19-CH_3), 0.86 (m, 1H), 0.97 (m, 1H), 1.12 (m, 1H), 1.25–1.52 (overlapping m, 5H), 1.60–1.77 (overlapping m, 6H), 1.88 (m, 1H), 2.08 (m, 1H), 2.59 (d, 1H, J = 16.1 Hz, one of 1-H_2), 2.87 (dd, 1H, J = 18.6 Hz, J = 12.8 Hz, one of 4-H_2), 2.99 (d, 1H, J = 16.1 Hz, the other of 1-H_2), 3.20 (dd, 1H, J = 18.6 Hz, J = 5.4 Hz, the other of 4-H_2), 3.66 (t, 1H, J = 8.5 Hz, 17-H), 7.32 (t-like m, 1H, 6′-H), 7.61 (d, 1H, J = 8.1 Hz, 5′-H), 7.71 (d, 1H, J = 7.4 Hz, 7′-H),

7.79 (s, 1H, 4′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.1 (C-19), 11.7 (C-18), 20.9 (CH_2), 23.4 (CH_2), 28.5 (CH_2), 30.5 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.6 (CH), 36.7 (CH_2), 37.6 (CH_2), 42.2 (CH), 42.8 (C-13), 43.4 (CH_2), 50.9 (CH), 53.6 (CH), 81.9 (C-17), 124.9 and 125.3 (C-5′ and C-6′), 128.5 (C-4a′), 128.5 (C-7′), 131.2 (C-3′), 132.4 (C-8′), 136.1 (C-4′), 142.9 (C-8a′), 159.7 (C-2′); ESI-MS: 410 $[M + H]^+$; anal. calcd for $C_{26}H_{32}ClNO$ C, 76.17; H, 7.87. Found: C, 76.29; H, 7.96.

4.4.5. 17β-Hydroxy-6′-chloro-5α-androstano[2,3:3′,2′]quinoline (14e). Compound **13e** (136 mg) was used for the synthesis. Yield: 111 mg (90%, white solid); mp 242–244 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.78 (s, 3H, 18-CH_3), 0.79 (s, 3H, 19-CH_3), 0.86 (m, 1H), 0.98 (m, 1H), 1.13 (m, 1H), 1.26–1.51 (overlapping m, 5H), 1.57–1.73 (overlapping m, 6H), 1.87 (m, 1H), 2.06 (m, 1H), 2.58 (d, 1H, J = 16.1 Hz, one of 1-H_2), 2.76 (dd, 1H, J = 18.1 Hz, J = 13.4 Hz, one of 4-H_2), 2.98 (d, 1H, J = 16.1 Hz, the other of 1-H_2), 3.05 (dd, 1H, J = 18.1 Hz, J = 4.4 Hz, the other of 4-H_2), 3.67 (t, 1H, J = 7.5 Hz, 17-H), 7.53 (d, 1H, J = 8.5 Hz, 7′-H), 7.66 (s, 1H, 5′-H), 7.69 (s, 1H, 4′-H), 7.90 (d, 1H, J = 8.7 Hz, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.1 (C-19), 11.7 (C-18), 20.9 (CH_2), 23.4 (CH_2), 28.6 (CH_2), 30.5 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.6 (CH), 36.7 (CH_2), 37.3 (CH_2), 42.2 (CH), 42.9 (C-13), 43.5 (CH_2), 50.9 (CH), 53.6 (CH), 81.8 (C-17), 125.4 (C-5′), 127.8 (C-4a′), 129.4 and 129.8 (C-7′ and C-8′), 131.1 (C-6′), 131.2 (C-3′), 134.8 (C-4′), 144.9 (C-8a′), 158.8 (C-2′); ESI-MS: 410 $[M + H]^+$; anal. calcd for $C_{26}H_{32}ClNO$ C, 76.17; H, 7.87. Found: C, 76.03; H, 7.65.

4.4.6. 17β-Hydroxy-6′-bromo-5α-androstano[2,3:3′,2′]quinoline (14f). Compound **13f** (149 mg) was used for the synthesis. Yield: 125 mg (92%, white solid); mp 247–249 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.77 (s, 3H, 18-CH_3), 0.79 (s, 3H, 19-CH_3), 0.86 (m, 1H), 0.97 (m, 1H), 1.12 (m, 1H), 1.24–1.51 (overlapping m, 5H), 1.60–1.76 (overlapping m, 6H), 1.88 (m, 1H), 2.07 (m, 1H), 2.58 (d, 1H, J = 16.2 Hz, one of 1-H_2), 2.75 (dd, 1H, J = 18.4 Hz, J = 12.8 Hz, one of 4-H_2), 2.97 (d, 1H, J = 16.2 Hz, the other of 1-H_2), 3.04 (dd, 1H, J = 18.4 Hz, J = 5.3 Hz, the other of 4-H_2), 3.67 (t, 1H, J = 8.4 Hz, 17-H), 7.65 (d, 1H, J = 9.0 Hz, 7′-H), 7.69 (s, 1H, 5′-H), 7.83 (d, 1H, J = 9.0 Hz, 8′-H), 7.84 (s, 1H, 4′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.1 (C-19), 11.7 (C-18), 20.9 (CH_2), 23.4 (CH_2), 28.5 (CH_2), 30.5 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.6 (CH), 36.7 (CH_2), 37.2 (CH_2), 42.1 (CH), 42.8 (C-13), 43.5 (CH_2), 50.9 (CH), 53.6 (CH), 81.8 (C-17), 119.2 (C-6′), 128.3 (C-4a′), 128.8 (C-5′), 129.9 (C-8′), 131.2 (C-3′), 132.0 (C-7′), 134.7 (C-4′), 145.1 (C-8a′), 158.9 (C-2′); ESI-MS: 454 $[M + H]^+$; anal. calcd for $C_{26}H_{32}BrNO$ C, 68.72; H, 7.10. Found: C, 68.89; H, 7.23.

4.4.7. 17β-Hydroxy-7′-chloro-5α-androstano[2,3:3′,2′]quinoline (14g). Compound **13g** (136 mg) was used for the synthesis. Yield: 111 mg (90%, white solid); mp 229–232 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.77 (s, 3H, 18-CH_3), 0.79 (s, 3H, 19-CH_3), 0.85 (m, 1H), 0.98 (m, 1H), 1.12 (m, 1H), 1.24–1.51 (overlapping m, 5H), 1.60–1.77 (overlapping m, 6H), 1.87 (m, 1H), 2.07 (m, 1H), 2.56 (d, 1H, J = 16.1 Hz, one of 1-H_2), 2.75 (dd, 1H, J = 18.4 Hz, J = 12.7 Hz, one of 4-H_2), 2.96 (d, 1H, J = 16.1 Hz, the other of 1-H_2), 3.05 (dd, 1H, J = 18.4 Hz, J = 5.3 Hz, the other of 4-H_2), 3.67 (t, 1H, J = 8.4 Hz, 17-H), 7.37 (d, 1H, J = 8.7 Hz, 6′-H), 7.61 (d, 1H, J = 8.7 Hz, 5′-H), 7.76 (s, 1H, 4′-H), 7.97 (s, 1H, 8′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.1 (C-19), 11.7 (C-18), 20.9 (CH_2), 23.4 (CH_2), 28.5 (CH_2), 30.5 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.6 (CH), 36.7 (CH_2), 37.3 (CH_2), 42.1 (CH), 42.8 (C-13), 43.5 (CH_2), 50.9 (CH), 53.6 (CH), 81.8 (C-17), 125.5 (C-4a′), 126.6 (C-6′), 127.2 (C-8′), 128.0 (C-5′), 130.5 (C-3′), 134.2 (C-7′), 135.6 (C-4′), 146.8 (C-8a′), 159.6 (C-2′); ESI-MS: 410 $[M + H]^+$; anal. calcd for $C_{26}H_{32}ClNO$ C, 76.17; H, 7.87. Found: C, 76.32; H, 7.93.

4.4.8. 17β-Hydroxy-5′-chloro-5α-androstano[2,3:3′,2′]quinoline (14g′). Compound **13g′** (136 mg) was used for the synthesis. Yield: 112 mg (91%, white solid); mp 140–142 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.78 (s, 3H, 18-CH_3), 0.80 (s, 3H, 19-CH_3), 0.87 (m, 1H), 0.98 (m, 1H), 1.13 (m, 1H), 1.25–1.52 (overlapping m, 5H), 1.59–1.77 (overlapping m, 6H), 1.88 (m, 1H), 2.07 (m, 1H), 2.63 (d, 1H, J = 16.2 Hz, one of 1-H_2), 2.79 (dd, 1H, J = 18.4 Hz, J = 13.0 Hz, one of 4-H_2), 3.07 (d, 1H, J = 16.2 Hz, the other of 1-H_2), 3.08 (dd, 1H, J = 18.4 Hz, J = 5.6 Hz, the other of 4-H_2), 3.67 (t, 1H, J = 7.4 Hz, 17-H), 7.50 (m, 2H, 6′-H and 7′-H), 7.90 (m, 1H, 8′-H), 8.20 (s, 1H, 4′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.1 (C-19), 11.7 (C-18), 20.9 (CH_2), 23.4 (CH_2), 28.5 (CH_2), 30.5 (CH_2), 31.1 (CH_2), 35.3 (C-10), 35.6 (CH), 36.7 (CH_2), 37.1 (CH_2), 42.2 (CH), 42.9 (C-13), 43.7 (CH_2), 50.9 (CH), 53.6 (CH), 81.8 (C-17), 125.3 (C-4a′), 125.5, 127.4 and 128.2 (C-6′, C-7′ and C-8′), 130.4 (C-5′), 131.3 (C-3′), 132.6 (C-4′), 147.2 (C-8a′), 159.3 (C-2′); ESI-MS: 410 $[M + H]^+$; anal. calcd for $C_{26}H_{32}ClNO$ C, 76.17; H, 7.87. Found: C, 76.03; H, 7.72.

4.4.9. 17β-Hydroxy-8′-methyl-5α-androstano[2,3:3′,2′]quinoline (14h). Compound **13h** (129 mg) was used for the synthesis. Yield: 105 mg (90%, white solid); mp 233–235 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 0.77 (s, 3H, 18-CH_3), 0.81 (s, 3H, 19-CH_3), 0.87 (m, 1H), 0.98 (m, 1H), 1.13 (m, 1H), 1.25–1.51 (overlapping m, 6H), 1.60–1.77 (overlapping m, 5H), 1.87 (m, 1H), 2.08 (m, 1H), 2.58 (d, 1H, J = 16.0 Hz, one of 1-H_2), 2.79 (s, 3H, 8′-CH_3), 2.83 (dd, 1H, J = 18.2 Hz, J = 12.7 Hz, one of 4-H_2), 2.97 (d, 1H, J = 16.0 Hz, the other of 1-H_2), 3.14 (m, 1H, the other of 4-H_2), 3.65 (m, 1H, 17-H), 7.32 (t-like m, 1H, 6′-H), 7.45 (d, 1H, J = 6.8 Hz, 7′-H), 7.54 (d, 1H, J = 8.0 Hz, 5′-H), 7.77 (s, 1H, 4′-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ_C = 11.1 (C-19), 11.7 (C-18), 18.1 (8′-CH_3), 20.9 (CH_2), 23.4 (CH_2), 28.6 (CH_2), 29.7 (CH_2), 30.5 (CH_2), 31.2 (CH_2), 35.3 (C-10), 35.6 (CH), 36.7 (CH_2), 42.3 (CH), 42.8 (C-13), 43.4 (CH_2), 50.9 (CH), 53.6 (CH), 81.9 (C-17), 124.9 and 125.3 (C-5′ and C-6′), 127.1 (C-4a′), 128.7 (C-7′), 129.7 (C-3′), 132.1 (C-8′), 135.8 (C-4′), 136.0 (C-8a′), 157.3 (C-2′); ESI-MS: 390 $[M + H]^+$; anal. calcd for $C_{27}H_{35}NO$ C, 83.24; H, 9.06. Found: C, 83.07; H, 9.17.

4.5. Determination of antiproliferative activities

Antiproliferative effects were determined *in vitro* against a panel of human cell lines of gynecological origin, including three cervical (HeLa, SiHa and C33A) and four breast cancer cell lines (MCF7, T47D, MDA-MB-361 and MDA-MB-231). All these cell lines were purchased from the European Collection of Cell Cultures (ECCAC, Salisbury, UK) with the exceptions of SiHa and C33A, which were obtained from LGC Standards GmbH, Wesel, Germany. The cells were cultivated in minimal essential medium (Lonza Ltd, Basel, Switzerland) supplemented with 10% fetal bovine serum, 1% non-essential amino acids and an antibiotic–antimycotic mixture. Near-confluent cancer cells were seeded onto a 96-well microplate (5000 cells per well except

in the cases of C33A and MDA-MB-361, which were seeded at 10 000 per well) and, after overnight standing, new medium (200 μL) containing the tested compound at 10 or 30 μM was added. After incubation for 72 h at 37 °C in humidified air containing 5% CO_2, the living cells were assayed by the addition of 5 mg mL^{-1} MTT solution (20 μL). MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a 4 h contact period. The medium was then removed and the precipitated formazan crystals were dissolved in DMSO (100 μL) during a 60 min period of shaking at 25 °C. Finally, the reduced MTT was assayed at 545 nm, using a microplate reader; wells with untreated cells were utilized as controls.[30] When compounds elicited at least 50% growth inhibition at 30 μM, the assays were repeated with a set of dilutions, sigmoidal dose–response curves were fitted to the determined data and the IC_{50} values (the concentration at which the extent of cell proliferation was half that of the untreated control) were calculated by means of GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). All *in vitro* experiments were carried out on two microplates with at least five parallel wells. Stock solutions of the tested substances (10 mM) were prepared in DMSO. The highest DMSO content of the medium (0.3%) did not have any substantial effect on the cell proliferation. Cisplatin (Ebewe Pharma GmbH, Unterach, Austria) was used as reference agent.

4.6. Cell cycle analysis

The subsequent experiments were carried out only with one of the most effective compounds, **14c**. In order to characterize the cell cycle phase distribution, the cellular DNA content was determined by means of flow cytometry. T47D cells were seeded onto six-well plates (250 000 cells per well) and treated with the selected compound (10 μM or 30 μM) for 48 h. Untreated cells served as control. After the incubation, the medium was removed and the cells were washed in phosphate-buffered saline (PBS) and harvested using trypsin. The collected cells were washed in PBS and the pellets were resuspended in ice-cold 70% EtOH. The fixed cells were stained with PI solution (1 mL of DNA staining buffer containing PI, ribonuclease-A, Triton-X and sodium citrate) for 1 h at room temperature in the dark, and then analyzed by flow cytometry (Partec CyFlow, Partec GmbH, Münster). Cells belonging in the various phases of the cell cycle (subG_1, G_1, S and G_2/M) were determined by ModFit LT 3.3.11 software (Verity Software House, Topsham, ME, USA).

4.7. Morphological changes and double staining

Morphological changes and extents of apoptosis induction by the tested compound were detected by Hoechst 33258 and PI double staining using fluorescent microscopy. T47D cells were seeded onto 96-well plates (5000 cells per well) and treated with two concentrations of **14c** for 48 h. Untreated cells were used as control. After the treatment, fluorescent dye solution (containing 5 and 3 μg mL^{-1} Hoechst 33258 and PI, respectively) were added to each well and the plate was stored in humidified air for 1 h. The cells were then examined with a fluorescent microscope (Nikon ECLIPSE 146 TS100, Nikon Instruments Europe, Amstelveen, The Netherlands) equipped with a Digital Sight Camera System, including appropriate filters for Hoechst 33258 and PI.[33] At least six fields were recorded in the case of each condition and the cells were counted. The ratio of intact, early apoptotic and late apoptotic/necrotic cells were determined and analyzed statistically.

4.8. Determination of caspase-3 activity

In order to evince apoptosis, the activity of caspase-3 was determined by colorimetric assay (Sigma-Aldrich Ltd, Budapest, Hungary) according to the manufacturer's instructions. Briefly, T47D cells were treated with two concentrations (10 μM or 30 μM) of **14c** for 48 h in cell culture flasks. After incubation, the cells were scraped, washed, lysed and centrifuged, and the supernatants were then transferred into new tubes. Lysates were diluted in order to achieve equal protein concentrations, determined by the BCA method. The reactions were performed on 96-well plates; the samples of lysates and substrate were added into wells in the presence or the absence of caspase-3 inhibitor. After the incubation, the activity of caspase-3 was measured at 405 nm by using ELISA reader (Stat Fax-2100, Awareness Technologies Inc., Palm City, FL, USA). Measured data were evaluated as fold increase compared with the caspase-3 activity of control samples.

4.9. Statistical analysis

Statistical evaluation of the *in vitro* results was performed by one-way analysis of variance followed by the Dunnett posttest, using GraphPad Prism 4 (GraphPad Software; San Diego, CA, USA) software. The mean values and standard deviation were calculated in all experiments.

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Stereocontrolled synthesis of the four 16-hydroxymethyl-19-nortestosterone isomers and their antiproliferative activities

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ABSTRACT

Novel 16-hydroxymethyl-19-nortestosterone diastereomers were prepared by Birch reduction from the corresponding 3-methoxy-16-hydroxymethylestra-1,3,5(10)-trien-17-ol isomers with known configurations. The synthesized compounds are 16α- and 16β-hydroxymethyl-substituted 19-nortestosterone and their 17α-epimers. To prepare 17α-19-nortestosterone, the Mitsunobu inversion reaction of 19-nortestosterone with different alkyl and aryl carboxylic acids was chosen. Deacylation of the new compounds by the Zemplén method yielded the required 17α-19-nortestosterone.

The antiproliferative activities of the structurally related compounds were determined *in vitro* through microculture tetrazolium assays on a panel of human adherent cervical (HeLa, SiHa and C33A), breast (MCF-7, MDA-MB-231, MDA-MB-361 and T47D) and ovarian (A2780) cell lines. The 17α epimer of 19-nortestosterone demonstrated considerable activity, selectively for HeLa cells, with a calculated IC_{50} of 0.65 μM. The reference compound, cisplatin, displayed an order of magnitude higher IC_{50} (12.4 μM). The cancer selectivity of 17α-19-nortestosterone was tested by MTT assay performed with noncancerous human fibroblast cell line MRC-5. The results indicated that 17α-19-nortestosterone selectively disturbs the viability of HeLa cells without greatly affecting other cancer cell types and intact fibroblasts.

1. Introduction

There is evidence to suggest that the administration of anabolic androgenic steroids, which act via the androgen receptor, can upregulate levels of the androgen receptor, stimulate satellite cell proliferation, and promote gains in skeletal muscle [1,2]. The androgenic/anabolic ratio of androstanes is mainly determined by the substituents on the steroid skeleton [3]. As concerns the therapy of androgen-dependent cancers, it is widely known that certain testosterone derivatives inhibit cell proliferation [4]. The development of testosterone-based anticancer drugs lacking hormonal activity is one of the major challenges in the medicinal chemistry of steroids.

Nandrolone (19-nortestosterone or 17β-hydroxyestra-4-en-3-one) (**1**) is one of the most widely distributed androgenic anabolic steroids. An early study of the function of androgens in the growth of the MCF-7 cell line revealed that certain derivatives of nortestosterone can inhibit cell proliferation *in vitro* [5]. To study the intracellular trafficking and localization of **1**, Drašar et al. recently prepared fluorescent nandrolone-bodipy derivatives and examined their effects on the LNCaP, PC-3, MCF-7 HeLa and HEC-293T cell lines [6]. Derivatives of trenbolone (17β-hydroxyestra-4,9,11-trien-3-one) (**2**), a potent anabolic steroid with reduced androgenic and estrogenic activity, have binding affinity for the androgen receptor up to five times that of testosterone [7]. Metribolone (17α-methyl-17β-hydroxyestra-4,9,11-trien-3-one) (**3**) is the most potent synthetic anabolic steroid. It was first used to treat advanced breast cancer, but although successful, was too aggressive against the body [8]. Mibolerone (7α,17α-dimethyl-17β-hydroxyestra-4-en-3-one) (**4**) a synthetic, nonmetabolizable agonist for the androgen receptor, has a significant antiproliferative effect on the T47D breast cancer cell line [9]. From the aspect of the diagnostic value of estrogenic and androgenic receptors, they are also expressed in a substantial proportion of breast cancer cases and their presence is related to a better prognosis [10] (Scheme 1).

Dihydrotestosterone has been reported to inhibit both the basal and the estrogen-stimulated proliferation of estrogen-positive breast cancer cells, independently of the presence of estrogen,

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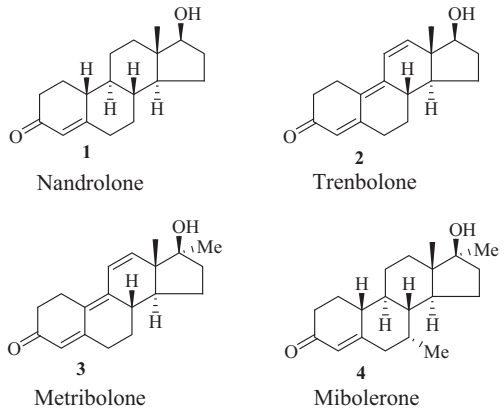


Scheme 1. Anabolic androgen steroids.

and also to induce a cell cycle disturbance in treated MCF7 cells [11]. The similar growth-inhibition action of the synthetic androgen **4** on T47D cells was explained by its androgenic and progestogenic properties [12].

A common feature of **1**–**4** is the β-orientation of the hydroxy group on C-17. Reduction of the 17-ketone function in steroids normally yields 17β-hydroxy derivatives [13–15], though there are a few exceptions [16–18]. Further, alkylation through the Grignard reaction produces 17α-alkyl-17β-hydroxy compounds. Formation of the 17β-hydroxy group is explained by the nucleophilic attack of a hydride ion or an alkyl group on the sterically slightly shielded α site [13,14].

We set out here to synthesize the 16α- and 16β-hydroxymethyl-substituted 19-nortestosterones by Birch reduction from the corresponding 3-methoxy-16-hydroxymethylestra-1,3,5(10)-trien-17-ol isomers. We planned to produce the 17α-hydroxy counterparts by Mitsonobu inversion reaction on 19-nortestosterones with different alkyl and aryl carboxylic acids. Zemplen deacylation of the 17α-esters furnished the 17α-nortestosterone. The antiproliferative activities of the synthesized compounds were determined *in vitro* through MTT assays on a panel human adherent cervical (HeLa, SiHa and C33A), breast (MCF-7, MDA-MB-231, MDA-MB-361 and T47D) and ovarian (A2780) cell lines.

We set out to obtain answers to the following questions: (1) How do the antiproliferative activities in the C-17 epimer series differ? (2) How are the antiproliferative activities influenced by the relative steric positions of the 16-hydroxymethyl and 17-hydroxy groups?

2. Experimental

2.1. General

Melting points (mp) were determined on a Kofler block and are uncorrected. Specific rotations were measured in $CHCl_3$ (*c* 1) at 20 °C with a POLAMAT-A (Zeiss-Jena) polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. Elementary analysis data were determined with a PerkinElmer CHN analyzer model 2400. The reactions were monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick); solvent system (ss): (A) ethylacetate, (B) acetone/toluene/hexane (30:35:35 v/v). The spots were detected by spraying with 5% phosphomolybdenic acid in 50% aqueous H_3PO_4. The R_f values were determined for the spots observed by illumination at 254 and 365 nm. Flash chromatography: silica gel 60, 40–63 μm. All solvents were distilled immediately prior to use. NMR spectra were recorded on a Bruker DRX 500 instrument at 500 (^{1}H NMR) or 125 MHz (^{13}C NMR). Chemical shifts are reported in ppm (δ scale), and coupling constants (*J*) in Hz. For the determination of multiplicities, the *J*-MOD pulse sequence was used.

2.2. General procedure for the synthesis of 16-hydroxymethyl-17-hydroxyestra-4-en-3-ones (**11**–**14**)

A solution of 1.20 g (3 mmol) of 16-acetoxymethyl-3-methoxyestra-1,3,5(10)-triene-17-acetate isomer (**7a**, **8a**, **9a** or **10a**) [20,21] in anhydrous THF (15 ml) was added to a mixture of *t* BuOH (15 ml) and liq. NH_3 (150 ml). 760 mg (33 mmol) of Na was than added in small portions at −50 °C. After a reaction time of 1 h, the NH_3 was evaporated off in a slow stream of N_2. The crystals obtained were dissolved in a mixture of acetone (4 ml), MeOH (20 ml) and H_2O (3 ml). 3 ml of conc. HCl was than added dropwise to the stirred solution. After 30 min, the reaction mixture was poured into H_2O (50 ml), and neutralized with $NaHCO_3$. The organic solvents were evaporated off, and the mixture was extracted with CH_2Cl_2 (3 × 25 ml). The extract was concentrated *in vacuo*, and the residue was purified by flash chromatography, using CH_2Cl_2/EtOAc (90:10 v/v).

2.2.1. 16β-Hydroxymethyl-17β-hydroxyestra-4-en-3-one (**11**)

Compound **7a** [20] (1.20 g, 3 mmol) was used for the synthesis as described in Section 2.2. **11** (820 mg, 89%), mp 214–215 °C; R_f = 0.45 (ss A); $[\alpha]_D^{20}$ + 23 (*c* 1 in $CHCl_3$). ($C_{19}H_{28}O_3$: calcd. C, 74.96; H, 9.27. Found C, 74.82; H, 9.44%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.86 (s, 3H, 18-H_3); 3.64 (d, 1H, *J* = 4.5 Hz, 16-CH_2); 3.82 (t, 1H, *J* = 10.0 Hz, 17-H); 3.87 (dd, 1H, *J* = 9.5 Hz, *J* = 3.3 Hz, 16-CH_2); 5.82 (s, 1H, 4-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 12.2 (C-18); 42.5 (C-13); 64.6 (16-CH_2); 82.8 (C-17); 124.5 (C-4); 166.6 (C-5); 200.0 (C-3).

2.2.2. 16α-Hydroxymethyl-17β-hydroxyestra-4-en-3-one (**12**)

Compound **8a** [20] (1.20 g, 3 mmol) was used for the synthesis as described in Section 2.2. **12** (805 mg, 88%), mp 183–185 °C; R_f = 0.25 (ss A); $[\alpha]_D^{20}$ + 27 (*c* 1 in $CHCl_3$). ($C_{19}H_{28}O_3$: calcd. C, 74.96; H, 9.27. Found C, 74.68; H, 9.36%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.87 (s, 3H, 18-H_3); 3.45 (d, 1H, *J* = 6.5 Hz, 16-CH_2); 3.63 (t, 1H, *J* = 9.0 Hz, 17-H); 3.77 (s, 1H, 16-CH_2); 5.82 (s, 1H, 4-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 11.9 (C-18); 42.5 (C-13); 66.8 (16-CH_2); 85.8 (C-17); 124.6 (C-4); 166.5 (C-5); 200.0 (C-3).

2.2.3. 16β-Hydroxymethyl-17α-hydroxyestra-4-en-3-one (**13**)

Compound **9a** [20] (1.20 g, 3 mmol) was used for the synthesis as described in Section 2.2. **13** (826 mg, 90%), mp 133–135 °C; R_f = 0.20 (ss A); $[\alpha]_D^{20}$ + 22 (*c* 1 in $CHCl_3$). ($C_{19}H_{28}O_3$: calcd. C, 74.96; H, 9.27. Found C, 75.06; H, 9.15%). ^{1}H NMR (δ, ppm, DMSO): 0.67 (s, 3H, 18-H_3), 4.30 (d, 1H, *J* = 4.0 Hz, 17-H); 4.48 (brs, 1H, OH); 5.72 (s, 1H, 4-H). ^{13}C NMR (δ, ppm, DMSO): 17.4 (C-18); 43.9 (C-13); 64.6 (16-CH_2); 80.4 (C-17); 123.6 (C-4); 168.8 (C-5); 198.35 (C-3).

2.2.4. 16α-Hydroxymethyl-17α-hydroxyestra-4-en-3-one (**14**)

Compound **10a** [21] (1.20 g, 3 mmol) was used for the synthesis as described in Section 2.2. **14** (790 mg, 86%), mp 139–140 °C; R_f = 0.50 (ss A); $[\alpha]_D^{20}$ + 39 (*c* 1 in $CHCl_3$). ($C_{19}H_{28}O_3$: calcd. C, 74.96; H, 9.27. Found C, 74.83; H, 9.35%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.77 (s, 3H, 18-H_3); 3.68 (t, 1H, *J* = 8.5 Hz, 16-CH_2); 3.83 (dd, 1H, *J* = 11.0 Hz, *J* = 4.0 Hz, 16-CH_2); 3.87 (d, 1H, *J* = 5.5 Hz, 17-H); 5.81 (s, 1H, 4-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 17.2 (C-18); 46.0 (C-13); 63.5 (16-CH_2); 81.5 (C-17); 124.4 (C-4); 167.0 (C-5); 200.1 (C-3).

2.3. General procedure for the synthesis of 17-acetoxy-16-acetoxymethylestra-4-en-3-one (**11a**, **12a**, **13a**, **14a**)

Compound **11**, **12**, **13** or **14** (304 mg, 1 mmol) was dissolved in a mixture of pyridine (2 ml) and Ac_2O (2 ml) and the solution was allowed to stand at room temperature for 12 h. The mixture was then diluted with water and the precipitate was collected by filtration and recrystallized from MeOH.

11a (370 mg, 95%), mp 130–133 °C; R_f = 0.70 (ss B). ($C_{23}H_{32}O_5$: calcd. C, 71.11; H, 8.30. Found C, 69.98; H, 8.43%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.87 (s, 3H, 18-H_3); 2.01 (s, 3H, OAc); 2.05 (s, 3H, OAc); 3.98 (dd, 1H, J = 11.0 Hz, J = 7.5 Hz, 16-CH_2); 4.09 (dd, 1H, J = 11.5 Hz, J = 7.0 Hz, 16-CH_2); 4.81 (d, 1H, J = 10.0 Hz, 17-H); 5.82 (s, 1H, 4-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 12.8 (C-18); 43.3 (C-13); 65.1 (16-CH_2); 81.4 (C-17); 124.7 (C-4); 160.0 (C-5); 170.8 and 170.9 (acetyl CH_3); 199.7 (C-3).

12a (363 mg, 94%), mp 142–144 °C; R_f = 0.65 (ss B). ($C_{23}H_{32}O_5$: calcd. C, 71.11; H, 8.30. Found C, 71.34; H, 8.22%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.86 (s, 3H, 18-H_3); 2.02 (s, 3H, OAc); 2.05 (s, 3H, OAc); 4.01 (dd, 1H, J = 11.0 Hz, J = 7.0 Hz, 16-CH_2); 4.08 (dd, 1H, J = 11.0 Hz, J = 6.0 Hz, 16-CH_2); 4.65 (d, 1H, J = 8.0 Hz, 17-H); 5.81 (s, 1H, 4-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 12.6 (C-18); 20.9 and 21.0 (acetyl CH_3); 44.0 (C-13); 66.5 (16-CH_2); 83.5 (C-17); 124.7 (C-4); 166.0 (C-5); 170.7 and 171.0 (acetyl C═O), 199.7 (C-3).

13a (375 mg, 96%), an oil; R_f = 0.60 (ss B). ($C_{23}H_{32}O_5$: calcd. C, 71.11; H, 8.30. Found C, 71.02; H, 8.36%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.87 (s, 1H, 18-H_3); 2.02 and 2.04 (s, 3H, OAc); 4.13 (dd, 1H, J = 11.0 Hz, J = 7.5 Hz, 16-CH_2); 4.19 (dd, 1H, J = 11.0 Hz, J = 7.5 Hz, 16-CH_2); 4.66 (d, 1H, J = 2.0 Hz, 17-H); 5.81 (s, 1H, 4-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 16.9 (C-18); 20.9 and 21.0 (acetyl CH_3); 44.3 (C-13); 66.4 (16-CH_2); 83.2 (C-17); 124.6 (C-4); 166.2 (C-5); 170.3 and 171.0 (acetyl C═O); 199.8 (C-3).

14a (368 mg, 94%), mp 118–120 °C; R_f = 0.75 (ss B). ($C_{23}H_{32}O_5$: calcd. C, 71.11; H, 8.30. Found C, 71.28; H, 8.42%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.85 (s, 3H, 18-H_3); 1.97 and 2.02 (s, 3H, acetyl CH_3); 4.01 (m, 2H, 16-CH_2); 5.03 (d, 1H, J = 6.0 Hz, 17-H); 5.80 (s, 1H, 4-H).

^{13}C NMR (δ, ppm, $CDCl_3$): 16.6 (C-18); 20.7 and 20.8 (acetyl CH_3); 45.7 (C-13); 63.9 (16-CH_2); 80.3 (C-17); 124.6 (C-4); 166.1 (C-5); 170.1 and 170.9 (acetyl C═O); 199.7 (C-3).

2.4. General procedure for Mitsunobu inversion esterification of 19-nortestosterone (**1**)

Compound **1** (548 mg, 2 mmol), Ph_3P (786 mg, 3 mmol), and AcOH, phenylacetic acid, benzoic acid or substituted benzoic acid (3 mmol) were suspended in dry toluene (25 ml). Diethyl azodicarboxylate (523 mg, 3 mmol) in toluene (5 ml) was added dropwise at room temperature. The suspension cleared to give a yellow-orange solution and became slightly warm. The reaction mixture was kept at 80 °C for 1.5 h, after which the solvent was removed *in vacuo*, and the residue was subjected to chromatographic separation on silica gel with CH_2Cl_2/hexane (1:3 v/v).

2.4.1. 17α-Acetoxyestra-4-en-3-one (**15a**)

15a (180 mg, 28%), mp 89–91 °C; R_f = 0.68 (ss B); $[\alpha]_D^{20}$ + 24 (*c* 1 in $CHCl_3$). ($C_{20}H_{28}O_3$: calcd. C, 75.91; H, 8.92. Found C, 76.08; H, 8.76%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.80 (s, 3H, 18-H3); 2.02 (s, 3H, 2′-H_3); 4.83 (d, 1H, J = 6.0 Hz, 17-H); 5.83 (s, 1H, 4-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 16.6 (C-18); 21.2 (2′-CH_3); 44.7 (C-13); 81.6 (C-17); 124.6 (C-4); 166.5 (C-1′); 199.8 (C-3).

2.4.2. 17α-Phenylacetoxyestra-4-en-3-one (**15b**)

15b (310 mg, 39%), mp 67–70 °C; R_f = 0.60 (ss B); $[\alpha]_D^{20}$ + 9 (*c* 1 in $CHCl_3$). ($C_{26}H_{32}O_3$: calcd. C, 79.56; H, 8.22. Found C, 79.32; H, 8.42%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.77 (s, 3H,18-H_3); 3.59 (s, 2H, 2′-CH_2); 4.82 (d, 1H, J = 5.0 Hz, 17-H); 5.84 (s, 1H, 4-H); 7.28 (m, 5H, 2″-, 3″-, 4″-,5″- and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 16.5 (C-18); 44.6 (C-13); 62.1 (C-2′); 82.00 (C-17); 166.6 (C-1′); 199.9 (C-3).

2.4.3. 17α-Benzoyloxyestra-4-en-3-one (**15c**)

15c (510 mg, 67%), mp 177–178 °C; R_f = 0.54 (ss B); $[\alpha]_D^{20}$ − 45 (*c* 1 in $CHCl_3$). ($C_{25}H_{30}O_3$: calcd. C, 79.33; H, 7.99. Found C, 79.45; H, 8.13%). ^{1}H NMR (δ, ppm, $CDCl_3$,): 0.88 (s, 3H, 18-H_3); 5.08 (d, 1H, J = 6.5 Hz, 17-H); 5.84 (s, 1H, 4-H); 7.44 (t, 2H, J = 15.5 Hz, 3″- and 5″-H); 7.55 (t, 1H, J = 15.0 Hz, 4″-H); 8.20 d, 2H, J = 7.5 Hz, 2″- and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$):16.7 (C-18); 45.1 (C-13); 82.2 (C-17); 124.6 (C-4); 130.7 (C-1″); 132.8 (C-4″); 166.0 (C-5); 166.4 (C-1′); 199.8 (C-3).

2.4.4. 17α-p-Toluoyloxyestra-4-en-3-one (**15d**)

15d (490 mg, 62%), mp 120–122 °C; R_f = 0.55 (ss B); $[\alpha]_D^{20}$ - 48 (*c* 1 in $CHCl_3$). ($C_{26}H_{32}O_3$: calcd. C, 79.56; H, 8.22. Found C, 79.68; H, 8.41%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.87 (s, 3H, 18-H_3); 2.39 (s, 3H, 4″-H_3); 5.06 (d, 1H, J = 5.5 Hz, 17-H); 5.84 (s, 1H, 4-H); 7.22 (d, 2H, J = 7.5 Hz, 3″- and 5″-H); 7.89 (d, 2H, J = 8.0 Hz, 2″- and 6″-H). ^{13}C NMR (125 MHz, δ, ppm, $CDCl_3$): 16.7 (C-18); 21.6 (*p*-CH_3); 45.1 (C-13); 82.0 (C-17); 124.5 (C-4); 143.4 (C-4″); 166.0 (C-5); 166.5 (C-1′); 199.8 (C-3).

2.4.5. 17α-p-Methoxybenzoyloxyestra-4-en-3-one (**15e**)

15e (456 mg, 55%), mp 145–147 °C; R_f = 0.50 (ss B); $[\alpha]_D^{20}$ − 66 (*c* 1 in $CHCl_3$). ($C_{26}H_{32}O_4$: calcd. C, 76.44; H, 7.90. Found C, 76.62; H, 8.14%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.87 (s, 3H, –OCH_3); 5.05 (d, 1H, J = 6.0 Hz, 17-H); 5.84 (s, 1H, 4-H); 6.92 (d, 2H, J = 9.0 Hz, 3″- and 5″-H); 7.96 (d, 2H, J = 8.5 Hz, 2″- and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$): 16.7 (C-18); 45.1 (C-13); 81.9 (C-17); 113.6 (C-3″ and -5″); 123.1 (-OCH_3); 124.5 (C-4); 166.5 (C-1′); 199.9 (C-3).

2.4.6. 17α-p-Bromobenzoyloxyestra-4-en-3-one (**15f**)

15f (572 mg, 62%), mp 140–143 °C; R_f = 0.56 (ss B); $[\alpha]_D^{20}$ − 57 (*c* 1 in $CHCl_3$). ($C_{25}H_{29}BrO_3$: calcd. C, 65.65; H, 6.39. Found C, 65.43; H, 6.52%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.88 (s, 3H, 18-H_3); 5.05 (d, 1H, J = 6.0 Hz, 17-H); 5.84 (s, 1H, 4-H); 7.56–7.87 (m, 5H, aromatic protons). ^{13}C NMR (δ, ppm, $CDCl_3$): 16.7 (C-18); 45.2 (C-13); 82.6 (C-17); 124.6 (C-4); 166.4 (C-1′); 199.8 (C-3).

2.4.7. 17α-p-Nitrobenzoyloxyestra-4-en-3-one (**15g**)

15g (720 mg, 85%), mp 184–185 °C; R_f = 0.54 (ss B); $[\alpha]_D^{20}$ − 51 (*c* 1 in $CHCl_3$). ($C_{25}H_{29}NO_5$: calcd. C, 70.90; H, 6.90. Found C, 71.14; H, 6.75%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.90 (s, 3H, 18-H3); 5.11 (d, 1H, J = 6.0 Hz, 17-H); 5.84 (s, 1H, 4-H); 8.16–8.29 (m, 5H, aromatic protons). ^{13}C NMR (δ, ppm, $CDCl_3$): 16.7 (C-17); 45.2 (C-13); 83.5 (C-17); 124.66 (C-4); 164.1 (C-1′); 166.1 (C-5); 199.7 (C-3).

2.4.8. 17α-2,4,6-Trimethylbenzoyloxyestra-4-en-3-one (**15h**)

15h (265 mg, 31%), mp 205–207 °C; R_f = 0.60 (ss B); $[\alpha]_D^{20}$ − 48 (*c* 1 in $CHCl_3$). ($C_{28}H_{36}O_3$: calcd. C, 79.96; H, 8.63. Found C, 79.82; H, 8.76%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.87 (s, 3H, 18-H_3); 2.28 (d, 12H, J = 6.5 Hz, 2″-, 4″-, 6″-H_3); 5.10 (d, 1H, J = 5.0 Hz, 17-H); 5.81 (s, 1H, 4-H). ^{13}C NMR (δ, (ppm, $CDCl_3$): 24.8 (C-18); 25.9 (C-4′); 45.0 (C-13); 82.5 (C-17); 124. 6 (C-4); 128.4 (C-3″ and -5″); 169.8 (C-5); 199.8 (C-3).

2.4.9. 17α-2-Iodobenzoyloxyestra-4-en-3-one (**15i**)

15i (218 mg, 21%), mp 135–137 °C; R_f = 0.56 (ss B); $[\alpha]_D^{20}$ − 76 (*c* 1 in $CHCl_3$). ($C_{25}H_{29}IO_3$: calcd. C, 59.53; H, 5.80. Found C, 59.72; H, 5.68%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.88 (s, 3H, 18-H3); 5.10 (d, 1H, J = 6.0 Hz, 17-H); 5.82 (s, 1H, 4-H); 7.13–7.40 (m, 2H, 4″- and 5″-H); 7.73–7.98 (m, 2H, 3″- and 6″-H). ^{13}C NMR (δ, ppm, $CDCl_3$):

16.6 (C-18); 45.0 (C-13); 83.4 (C-17); 93.9 (C-2″); 124.6 (C-4); 135.6 (C-1″); 166.1 (C-5); 166.4 (C-1′); 199.8 (C-3).

2.4.10. 17α-19-nortestosterone (**15**)

Compounds **15a–i** (1 mmol) were dissolved in MeOH (25 ml) containing NaOMe (54 mg, 1 mmol) and the solution was allowed to stand for 24 h. It was then diluted with H_2O, and the white precipitate was collected by filtration and recrystallized from acetone/hexane to obtain **15** (0.50–0.75 mmol). Mp 149–150 °C (lit [24] mp 146–149 °C); R_f = 0.45 (ss B); $[\alpha]_D^{20}$ + 23 (*c* 1 in $CHCl_3$). ($C_{18}H_{26}O_2$: calcd. C, 78.79; H, 9.55. Found C, 78.86; H, 9.43%). ^{1}H NMR (δ, ppm, $CDCl_3$): 0.71 (s, 3H, 18-H_3); 3.75 (d, 1H, *J* = 5.5 Hz, 17-H); 5.81 (s, 1H, 4-H).^{13}C NMR (δ, ppm, $CDCl_3$): 11.0 (C-18); 42.0 (C-13); 81.6 (C-17); 124.5 (C-4); 166.7 (C-5); 199.9 (C-3).

2.5. Determination of antiproliferative activities

The antiproliferative properties of the prepared 16-hydroxymethyl-19-nortestosterones (**11**–**14**), 19-nortestosterone (**1**) and 17α-19-nortestosterone (**15**) were determined on a panel of human adherent gynecological cancer cell lines. MCF-7, MDA-MB-231, MDA-MB-361 and T47D were isolated from breast cancers differing in biochemical background, while HeLa, SiHa and C33A cells were from cervical cancers of various pathological histories, and A2780 cells were isolated from ovarian cancer. All cell lines were purchased from European Collection of Cell Cultures (ECCAC, Salisbury, UK). Cells were cultivated in minimal essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids and an antibiotic–antimycotic mixture. All media and supplements were obtained from Lonza Group Ltd., Basel, Switzerland. Near-confluent cancer cells were seeded onto a 96-well microplate (5000 or 10,000 cells/well) and, after overnight standing, 200 μl new medium, containing the tested compounds at 10 and 30 μM, was added. After incubation for 72 h at 37 °C in humidified air containing 5% CO_2, the living cells were assayed by the addition of 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. MTT was converted by intact mitochondrial reductase and precipitated as purple crystals during a 4-h contact period. The medium was next removed and the precipitated formazan crystals were dissolved in 100 μl of DMSO during a 60-min period of shaking at 37 °C.

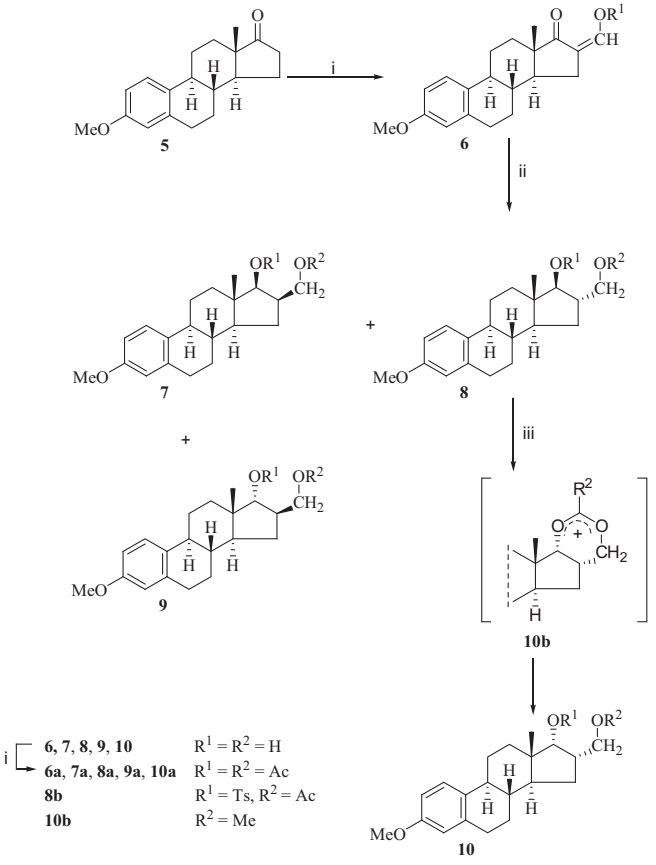
Finally, the reduced MTT was assayed at 545 nm, using a microplate reader; wells with untreated cells served as control [19]. In the case of the most active compounds, the assays were repeated with a set of dilutions, sigmoidal dose–response curves were fitted to the determined data and the IC_{50} values (the concentration at which the extent of cell proliferation was half that of the untreated control) were calculated by means of GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). All *in vitro* experiments were carried out on two microplates with at least five parallel wells. Stock solutions of the tested substances (10 mM) were prepared in DMSO. The highest DMSO content of the medium (0.3%) did not have any substantial effect on the cell proliferation. Cisplatin was used as positive control. The clinically used infusion concentrate (Ebewe Pharma GmbH, Unterach, Austria) was diluted with medium and this solution contained no DMSO. The subsequent analysis was carried out only with the most effective compound, **15**. To obtain further information on the selectivity of its cytotoxic effect, **15** was subjected to the MTT assay with noncancerous human lung fibroblast cells (MRC-5) under the same experimental conditions. Similarly as in the determination of the antiproliferative activities of the tested compounds, cisplatin was used as positive control in this assay.

3. Results and discussion

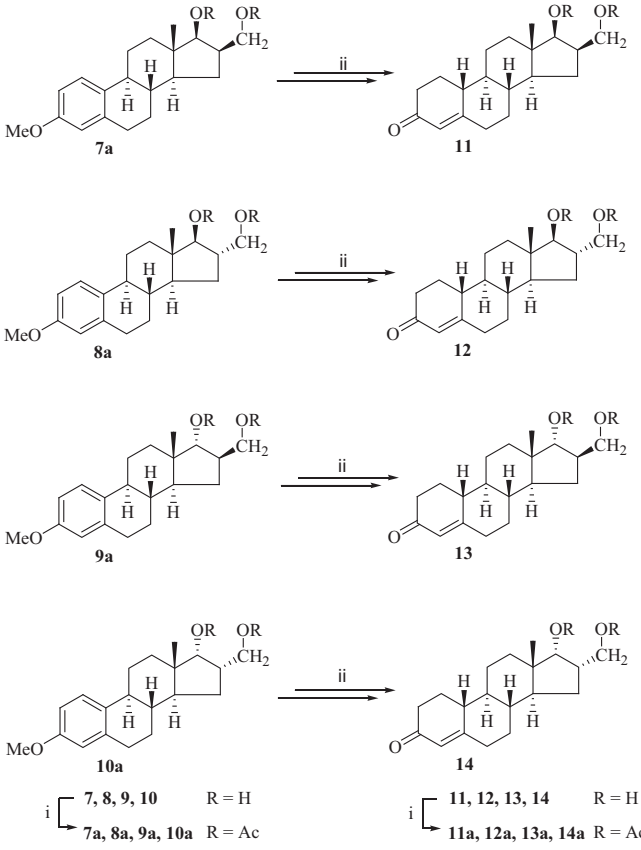
3.1. Synthetic studies

We earlier reported the preparation of the four possible isomers of the 3-methoxy-16-hydroxmethylestra-1,3,5(10)-trien-17-ol (**7**–**10**) [20]. Treatment of 3-methoxyestra-1,3,5(10)-trien-17-one (**5**) with NaOMe and ethyl formate gave 3-methoxy-16-hydroxymethylidenestra-1,3,5(10)-trien-17-one (**6**). Its acetyl derivative (**6a**) was obtained quantitatively by the reaction of **6** with Ac_2O in pyridine, and was reduced with KBH_4 in ethanol, yielding a mixture of three (**7**, **8** and **9**) of the four possible isomers of 3-methoxy-16-hydroxymethylestra-1,3,5(10)-trien-17-ol in a ratio of 50:45:5, in 94% yield. The 16β-hydroxymethylestra-1,3,5(10)-trien-17β-ol (**7**) with *cis*-orientation of the 16,17 groups, could easily be separated from the *trans* isomers **8** and **9** by flash chromatography on aluminium oxide. Acetylation of **8** and **9** yielded the diacetates **8a** and **9a**. From the methanolic solution, **8a** crystallized as a hard crystalline product, while **9a** remained as oil in the mother liquor [20]. The fourth isomer, **10**, was prepared from 16α-acetoxymethyl-3-methoxyestra-1,3,5(10)-trien-17β-*p*-tolylsulfonate (**8b**) by neighboring group participation during solvolysis in aqueous AcOH. The structures of the isomers were confirmed unambiguously by their IR, ^{1}H and ^{13}C NMR spectra [20,21] (Scheme 2).

Novel 16-hydroxymethyl-19-nortestosterones (**11**–**14**) were prepared from 3-methoxy-16-hydroxymethylestra-1,3,5(10)-trien-17-ols (**7a**–**10a**) starting compounds with known steric structures [20,21]. The strategy for the syntheses of the 16-hydroxymethyl-19-nortestosterones is illustrated in Scheme 3. Acetylation of **7**–**10** with Ac_2O in the presence of pyridine yielded



Scheme 2. Reagents and conditions: (i) toluene, ethyl formate, NaOMe.



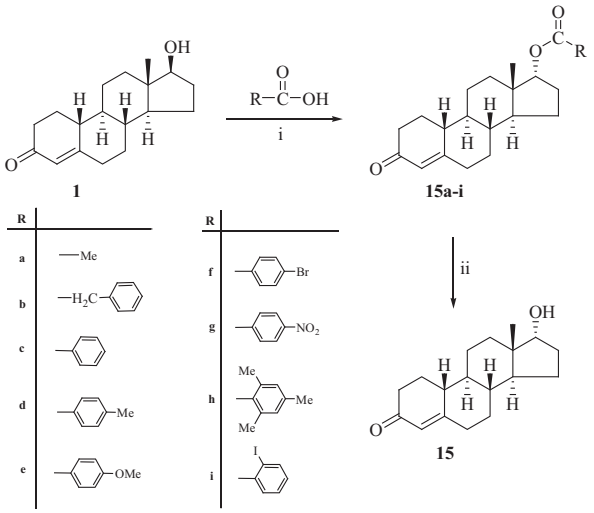
Scheme 3. Reagents and conditions: (i) Ac_2O, pyridine, rt.; (ii) liquid NH_3, *t*-BuOH, Na, −50 °C; acetone, MeOH, H_2O, conc. HCl.

the corresponding diacetates **7a**, **8a**, **9a** and **10a**, Birch reduction of which in liquid NH_3 at −50 °C in the presence of *t*-BuOH and Na and subsequent acidic hydrolysis furnished the corresponding 16-hydroxymethyl-19-nortestosterones **11**–**14** [22]. As the chemical reactions did not affect the chiral centers C-16 and C-17, the configurations of the products **11**–**14** followed from the exact configurations of the starting compounds [23].

11 and **12** are the 16β- and the 16α-hydroxymethylated 19-nortestosterone with a 17β-hydroxy function, while **13** and **14** are the 16β- and the 16α-hydroxymethylated 17α-hydroxy-19-nortestosterone. **1** and **15** are well known; they were synthesized by Robinson et al. [24], and are metabolites of androgenic/anabolic steroids in mammal serum [25].

Compound **15** was prepared by the Mitsunobu inversion esterification [26,27] of **1** with different alkyl and aryl carboxylic acids. Since both the carboxylic acid and the solvent used for the Mitsunobu inversion process considerably influence the outcome of the reactions of sterically hindered hydroxy functions, we performed the inversion of **1** with various carboxylic acids in toluene. There appears to be a relationship between the dissociation constant of the electron-withdrawing substituent on the aryl acid and the overall effectiveness of the reaction, with more acidic species generally providing a higher yield of inverted product [27]. The best result was achieved with 4-nitrobenzoic acid. The traditionally used relatively weak benzoic, acetic and phenylacetic acids were the least attractive coupling partners from the aspect of the yields of the reactions (Scheme 4).

The subsequent transesterification of **15a–i** by the Zemplén [28] gave the basic compound **15**.



Scheme 4. Reagents and conditions: (i) Ph_3P, alkyl or aryl acids, diethyl azodicarboxylate, toluene; (ii) MeOH, NaOMe.

3.2. Determination of the antiproliferative properties of the 19-nortestosterone derivatives

The aim of our present study included an evaluation of the direct antiproliferative capacities of the newly synthesized diastereomers (**11–14**) and their epimeric basic compounds (**1** and **15**). The impact of various structural modifications on the *in vitro* antiproliferative activity of the presented derivatives was determined using on a panel of human adherent cervical (HeLa, SiHa and C33A), breast (MCF-7, MDA-MB-231, MDA-MB-361 and T47D) and ovarian (A2780) cell lines.

Compound **1** displayed only moderate growth-inhibitory potential, as its inhibitions were under 50% at both applied concentrations against all the tested cell lines. The inversion of C-17 configuration resulted in significant improvement of the antiproliferative effect. 17α-19-nortestosterone (**15**) selectively inhibited the growth of HeLa cells with a calculated submicromolar IC_{50} of 0.65 μM. Introduction of a 16-hydroxymethyl side chain into compound **1** led to retained activities for compounds **11** or **12**. The orientation of the 16-substituent did not have an appreciable influence of the antiproliferative effects. Both of the 17α,16-diols (**13**, **14**) displayed limited growth inhibition in comparison with their analog **15**. The results of the MTT assays of the 17α-esters (**15a–i**) revealed their substantially lower activities than those of the compound **15**. Concerning the inhibitions against HeLa, only two ester derivatives (**15a** and **15c**) exceeded 50%, applied in 30 μM. The benzoate and its substituted derivatives (**15c–i**) proved to be more potent against A2780 cell line, exerting nearly or more than 80% inhibitions at 30 μM. The benzoate (**15c**) and the brominated compound (**15f**) inhibited the growth of MCF-7 cells with IC_{50} value of 14 μM (Table 1).

The cancer selectivity of the most potent compound, 17α-19-nortestosterone (**15**), was tested on the noncancerous human fibroblast cell line MRC-5 by MTT assay. While the reference agent cisplatin elicited substantial and concentration-related inhibition of the growth of fibroblasts at 3, 10 and 30 μM, treatment with **15** resulted in less than 15% inhibition even at the highest applied concentration (Table 2).

These results suggest that **15** has the capacity to disturb the viability of HeLa cells selectively, without affecting other cancer cell types and intact fibroblast. HeLa is known as a human papillomavirus HPV-18 positive cervical cancer cell line [29]. High percent

Table 1
Antiproliferative activities of compounds **1**, **11**–**15**.

Compd.	Conc. (μM)	Growth inhibition; % ± SEM [calculated IC_{50} value; μM]							
		Hela	SiHa	C33A	A2780	MCF-7	MDA-MB-231	MDA-MB-361	T47D
11	10	44.8 ± 2.7	–*	–	–	–	–	–	–
	30	59.6 ± 0.9	–	–	–	–	–	–	–
12	10	26.7 ± 2.8	–	–	–	–	–	–	–
	30	47.9 ± 1.9	–	–	–	–	20.1 ± 1.3	–	–
13	10	–	–	–	–	–	–	–	–
	30	30.9 ± 2.3	–	–	–	–	–	–	–
14	10	28.4 ± 1.7	–	–	–	–	–	–	–
	30	48.2 ± 2.3	–	–	–	21.7 ± 3.7	–	–	–
1	10	28.1 ± 4.0	–	–	–	–	–	–	–
	30	37.9 ± 3.4	–	–	–	20.4 ± 1.9	–	–	–
15	10	99.5 ± 0.2	–	–	–	–	–	–	–
	30	99.5 ± 0.3 [0.65]#	20.7 ± 2.6	25.2 ± 1.7	–	–	21.4 ± 1.2	–	–
15a	10	35.7 ± 2.9	–	–	–	28.7 ± 1.6	–	–	–
	30	86.9 ± 1.1 [16.66]	–	38.2 ± 0.9	31.2 ± 1.2	28.1 ± 0.8	–	–	20.0 ± 0.7
15b	10	–	–	–	–	–	–	–	–
	30	30.0 ± 2.1	–	46.3 ± 1.5	39.2 ± 1.4	32.3 ± 0.9	29.8 ± 2.8	22.2 ± 0.5	31.7 ± 1.4
15c	10	27.5 ± 1.4	–	–	25.2 ± 1.7	21.0 ± 2.6	–	–	–
	30	93.7 ± 0.7 [14.66]	29.7 ± 1.3	41.3 ± 2.5	92.7 ± 0.6 [11.38]	84.6 ± 1.5 [13.98]	58.7 ± 1.4	52.0 ± 0.5	70.4 ± 0.6
15d	10	–	–	–	30.2 ± 2.2	–	–	–	–
	30	71.0 ± 1.6	30.1 ± 2.03	41.7 ± 1.2	86.9 ± 0.5 [14.60]	69.3 ± 1.9	57.0 ± 1.2	44.1 ± 2.0	65.9 ± 1.4
15e	10	–	–	–	43.5 ± 2.9	20.3 ± 2.8	–	–	–
	30	48.8 ± 2.7	32.6 ± 1.1	50.9 ± 0.9	96.3 ± 0.2 [10.25]	54.2 ± 1.5	71.0 ± 1.1	57.2 ± 1.1	66.9 ± 1.1
15f	10	26.3 ± 2.1	–	–	46.4 ± 1.7	34.0 ± 2.4	–	–	–
	30	77.7 ± 1.1 [17.35]	32.3 ± 0.4	47.9 ± 4.5	92.8 ± 0.8 [10.27]	83.7 ± 0.1 [14.07]	53.5 ± 1.7	64. 6 ± 1.1	54.5 ± 1.2
15g	10	27.8 ± 2.7	–	–	28.7 ± 2.3	31.7 ± 2.0	–	–	–
	30	41.4 ± 1.4	29.2 ± 2.8	36.8 ± 2.8	79.6 ± 1.7 [16.98]	47.2 ± 1.4	47.3 ± 1.6	43.7 ± 2.1	51.1 ± 2.4
15h	10	–	–	–	36.3 ± 2.0	–	–	20.0 ± 1.9	–
	30	51.0 ± 1.5	50.9 ± 2.3	65.5 ± 2.6	85.7 ± 1.0 [12.44]	73.3 ± 1.9	23.4 ± 1.8	42.2 ± 2.1	57.4 ± 2.3
15i	10	23.2 ± 2.8	–	–	36.6 ± 1.7	–	–	–	–
	30	69.8 ± 1.8	46.4 ± 1.1	86.2 ± 0.5 [18.90]	92.4 ± 0.6 [12.35]	76.5 ± 2.5 [24.41]	35.1 ± 2.4	49.5 ± 2.8	63.0 ± 2.9
11a	10	–	–	–	–	–	–	–	–
	30	25.5 ± 1.4	–	23.6 ± 1.6	–	–	–	–	–
14a	10	–	–	–	–	–	–	–	–
	30	20.4 ± 1.7	–	–	–	–	–	21.5 ± 1.5	–
CIS	10	42.6 ± 2.3	88.6 ± 0.5	43.5 ± 1.8	83.6 ± 1.2	66.9 ± 1.8	–	67.5 ± 1.0	51.0 ± 2.0
	30	99.9 ± 0.3 [12.43]	90.2 ± 1.8 [7.84]	74.0 ± 2.3 [3.69]	95.0 ± 0.3 [1.30]	96.8 ± 0.4 [5.78]	71.5 ± 1.2 [19.13]	87.8 ± 1.1 [3.74]	55.0 ± 1.5 [9.78]

* Growth inhibition values of <20%, which are regarded as negligible.
\# IC_{50} values were calculated if the growth inhibition of the compound at 30 μM was >75%.

Table 2
Effects of cisplatin and compound **15** on the proliferation of human fibroblast MRC-5 cells.

Compd.	Inhibition (%) ± SEM		
	3 μM	10 μM	30 μM
15	6.2 ± 2.6	5.4 ± 1.2	11.3 ± 0.5
Cisplatin	33.0 ± 0.4	72.9 ± 1.9	69.5 ± 1.5

of cervical carcinomas are derived from high-risk HPV infection of the epithelial layer of the cervix, including HPV-16, -18, -31 and -35 among others [30]. Although our other investigated cervical cancer cell line, SiHa, is infected with HPV-16, **15** is not able to significantly inhibit its proliferation. This might refer to an essential difference between the mechanisms of malignant transformation of the cell lines caused by HPV-16 or -18. This pathological difference may be the basis of HeLa selectivity of recently reported novel antiproliferative compounds [31–33].

Since the aim of the present work was the development of a nortestosterone-based selective antiproliferative compound lacking hormonal activity, one of the most crucial questions concerns the potential androgenic or estrogenic activity of **15**. Its endogenous production is well-established in many animal tissues and fluids, including horses and sheep, and also in humans [34,35]. Most publications deal with analyses of the compound, and its origin and physiological role have not been fully elucidated, but its possible androgenic activity appears evident [36]. However, interaction of the 17β-hydroxy function in natural androgens with amino acid residues Asn705 and Thr877 is a basic requirement of signal transduction at the androgen receptor. Any modification of the 17β-hydroxy function decreases the affinity of the compound for the androgen receptor. The 17-epimers of the natural androgens therefore do not exhibit relevant androgenic activities [37]. Besides the typical estrogens, some nonaromatic steroids with hydroxy functions at positions C-3 and/or C-17 may interact with estrogen receptors. One of the first-described nonaromatic agonists for estrogenic receptors was 5α-androstane-3β,17β-diol [38]. These findings led Wang et al. to test a large set of nonaromatic steroids for binding to estrogen receptors. Six out of the sixty tested agents exhibited considerable affinity for both isoforms of the human estrogen receptors, but 17α-19-nortestosterone (**15**) proved to be free of such properties [39]. In summary, it can be concluded that the most potent compound (**15**) in the present study exerts substantial antiproliferative action in a selective manner, without relevant hormonal action, and the design and synthesis of structural analogues of **15** therefore appears promising.

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Corrigendum

Corrigendum to "Stereocontrolled synthesis of the four 16-hydroxymethyl-19-nortestosterone isomers and their antiproliferative activities" [Steroids 105 (2016) 113–120]

Gyula Schneider [a,*], Anita Kiss [a], Erzsébet Mernyák [a], Zsanett Benke [a], János Wölfling [a], Éva Frank [a], Noémi Bózsity [b], András Gyovai [b], Renáta Minorics [b], István Zupkó [b,*]

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The authors regret that an accidental interchange of the results concerning 19-nortestosterone (nandrolone, **1**) and its epimer pair, 17α-19-nortestosterone (**15**) was made in Section 3.2 entitled "Determination of the antiproliferative properties of the 19-nortestosterone derivatives" and in the corresponding tables (Tables 1 and 2). As a consequence, in the related discussion, statements regarding compound **15** should now be interpreted as statements regarding compound **1**. Nevertheless, it is to be noted that nandrolone (**1**) is a widely known androgenic anabolic steroid.

Moreover, we would like to supplement our findings with the following: (a) the 17α-19-nortestosterone esters (**15a–i**) revealed better antiproliferative activities than that of their parent compound (**15**); (b) due to the steric positions of the functional groups at C17, these compounds are not able to interact with the androgen receptor. The design and synthesis of structural analogues of **15**, therefore, still appear to be promising.

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III.

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Antiproliferative Properties of Newly Synthesized 19-Nortestosterone Analogs Without Substantial Androgenic Activity

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19-Nortestosterone C-17 epimers with prominent antiproliferative properties have been previously described. In our present study, five novel 17α-19-nortestosterones (**3–7**) were synthesized to increase their beneficial biological activities with no associated undesired hormonal effects. The compounds were screened by a viability assay against a panel of human adherent gynecological cancer cell lines. Three of the tested derivatives (**3**–**5**) exhibited a remarkable inhibitory effect on the proliferation of HeLa cells with IC_{50} values lower than that of our reference agent cisplatin (CIS). These three active agents also displayed considerable cancer selectivity as evidenced by their weaker growth inhibitory effect on non-cancerous fibroblast cells compared to CIS. The most potent newly synthesized 17α-chloro derivative (**3**) was selected for additional experiments in order to characterize its mechanism of action. Since nandrolone (19-nortestosterone, **1**) is a structural analog with selective antiproliferative action on cervical carcinoma cells, it was utilized as a positive control in these studies. A lactate dehydrogenase (LDH) assay demonstrated a moderate cytotoxic effect of the test compounds. Cell cycle disturbance and the elevation of the hypodiploid population elicited by the test agents were detected by flow cytometry following propidium staining. The proapoptotic effects of the tested steroids were confirmed by fluorescent microscopy and a caspase-3 activity assay. Treatment-related caspase-9 activation without a substantial change in caspase-8 activity indicates the induction of the intrinsic apoptotic pathway. The selected agents directly influence the rate of tubulin assembly as evidenced by a polymerization assay. Yeast-based reporter gene assay revealed that the androgenic activity of the novel 19-nortestosterone derivative **3** is by multiple orders of magnitude weaker than that of the reference agent **1**. Based on the behavior of the examined compounds it can be concluded that a halogen substitution of the 19-nortestosterone scaffold at the 17α position may produce compounds with unique biological activities. The results of the present study support that structurally modified steroids with negligible hormonal activity are a promising basis for the research and development of novel anticancer agents.

Keywords: 19-nortestosterone analogs, antiproliferative action, HeLa Cells, tubulin polymerization, androgenic activity, cell cycle, caspase

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INTRODUCTION

Cancer is the second leading cause of death globally: in 2015 malignancies were responsible for 8.7 million deaths, and 17.5 million new cancer cases were registered worldwide. Based on incidence estimates the number of new cases is expected to rise by about 70% over the next two decades (Fitzmaurice et al., 2017). Besides numerous preventive strategies and early diagnosis, the research for and development of innovative anticancer agents is one of the most important approaches to decrease global cancer burden.

Steroidal agents used in oncological practice are typically administered for their endocrine disruptor properties (e.g., estrogen antagonists, aromatase inhibitors). Synthetic analogs of naturally occurring steroids are widely utilized in the treatment of cancers of the reproductive system (Lin et al., 2010; Sharifi et al., 2010).

Besides the well-known endocrine disruptors several other steroids have been reported to exert pronounced anticancer effects in a hormone-independent manner. 2-Methoxyestradiol, an endogenous metabolite of estradiol without hormonal activity, exhibits a potent antiproliferative action against various tumor cell lines *in vitro*, and inhibits tumor growth *in vivo* (Fotsis et al., 1994). It is also demonstrated to induce programmed cell death in endothelial cells and suppresses cancer-related angiogenesis (Yue et al., 1997; LaVallee et al., 2002).

Cardiac glycosides are a group of steroidal compounds traditionally utilized in the management of congestive heart failure. Epidemiological studies have revealed that many of them, including digitoxin, oleandrin, bufalin, and calotropin exert a potent anticancer effect against different malignancies via the inhibition of proliferation and apoptosis induction involving complex cell signal transduction mechanisms (Mijatovic et al., 2007; Newman et al., 2008).

Steroidal alkaloids are nitrogen containing secondary metabolites found in many plant families (e.g., Liliaceae, Solanaceae), and many of them are well characterized as potent anticancer agents against human malignant cell lines (Koduru et al., 2007). Solasodine glycosides have been investigated in the clinical setting against basal cell carcinoma, and a locally applied cream was found to be effective in a substantial proportion of patients (Punjabi et al., 2008).

Androstanes and their structural analogs are regarded as a promising skeleton for the development of steroid-based anticancer agents. A large body of evidence indicates the outstanding importance of these compounds and their versatile antitumor effects. A considerable antiproliferative action of several sets of innovative androstane analogs have been reported against a broad variety of cell lines, including prostate, breast, cervix, ovarium, leukemia, melanoma, colon, and gastric cancers (Iványi et al., 2012; Ajdukovic et al., 2013, 2015; Acharya and Bansal, 2014; Cui et al., 2015; Jakimov et al., 2015). 19-Nortestosterone derivatives, e.g., levonorgestrel, desogestrel, and dienogest, an important division of testosterone-derived molecules are widely utilized in hormone replacement therapy (Campagnoli et al., 2005), contraception (Minami et al., 2013; Royer and Jones, 2014), and treatment of endometriosis (Minami et al., 2013; Miyashita et al., 2014). Beyond these well-established clinical applications, several 19-nortestosterone derivatives have recently been reported as potential anticancer agents. Mibolerone (7α,17α-dimethyl-19-nortestosterone), a metabolically stable synthetic member of this class has been demonstrated to effectively inhibit estrogen-stimulated breast cancer cell proliferation *in vitro* (Cops et al., 2008).

Tibolone, a selective regulator of tissue estrogen activity for postmenopausal women, is also known to induce apoptosis in breast cancer cells *in vitro*, and has been demonstrated to suppress tumor growth in animal models (Franke and Vermes, 2003; Erel et al., 2006). Further, 19-nortestosterones, such as gestodene and 3-ketodesogestrel exhibit antitumor activity against several breast cancer cell lines *in vitro*, as well as *in vivo*, in rat model of breast cancer (Kloosterboer et al., 1994). Additional, 19-nortestosterone derivatives as potential proliferation inhibitors in brain, prostate, and renal cancer cell lines have also been described (Mohamed et al., 2015).

Although several analogs truly possess a promising anticancer effect, their actions are mainly mediated by their hormonal activity, hindering a wide-scale utilization of these compounds in cancer therapy.

Since the 17β-hydroxy function of endogen androgens play a crucial role in the molecule's interaction with its hormone receptors, modifications of this group reduce hormonal activity. The lack of the C-19 methyl group also decreases the hormonal properties of such analogs substantially (Fragkaki et al., 2009). In a previous research, we have reported on the synthesis of a series of 17-substituted 19-nortestosterone derivatives and demonstrated their antiproliferative properties against human ovarium, cervix, and breast cancer cell lines. Nandrolone (19-nortestosterone, **1**) was found to exhibit a selective proliferation inhibitory effect against cervical cancer cells (HeLa) at low concentrations (Schneider et al., 2016). As an extension of this previous research a set of novel 19-nortestosterone analogs with various substituents at position C-17 have been synthesized. Recent reports about halogen-substituted androstane-derivatives with an increased *in vitro* anticancer activity encouraged us to introduce halogens in order to enhance the antiproliferative activity (Banday et al., 2010; Iványi et al., 2012). The aims of our current study were to assess the antiproliferative properties of these analogs, including tumor selectivity, as well as to characterize the mechanism of action of the most potent compound. Since, the endocrine actions of steroid-based drug candidates are exceptionally relevant, the androgenic potentials of the compounds were also tested.

MATERIALS AND METHODS

Synthesis and Chemicals

The exact conditions applied for the preparation processes of the synthesized 19-nortestosterone analogs **2–7** and their detailed characterization are provided as Supplementary Material. 10 mM stock solutions of the tested agents were prepared with dimethyl sulfoxide (DMSO) for all *in vitro* experiments. The medium with the highest DMSO concentration (0.3%) did not exert any

notable effect on cell proliferation. Unless otherwise specified, all other chemicals and kits were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary).

Cell Cultures

Gynecological cancer cell lines, including ovarian (A2780), cervical (HeLa), and breast cancer cell lines (MCF7, T47D, MDA-MB-231, and MDA-MB-361) were purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, United Kingdom). Additional cervical cell lines (SiHa and C33A) and a non-cancerous immortalized, mammary gland epithelial cell line (hTERT-HME1) were purchased from LGC Standards GmbH (Wesel, Germany). Non-cancerous fibroblast cells (MRC-5) were also obtained from the ECACC. All cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 1% antibiotic-antimycotic mixture, in humidified air containing 5% CO_2 at 37°C. Immortalized hTERT-HME1 cells were maintained in serum-free mammary epithelial cell growth medium (MEGM) supplemented with insulin, human epidermal growth factor (hEGF), hydrocortisone, bovine pituitary extract, and an antibiotic-antimycotic mixture. All the medium and supplements were purchased from Lonza Group Ltd. (Basel, Switzerland).

Assessing the Antiproliferative Effect

The antiproliferative properties of the compounds were assessed by an MTT assay (Mosmann, 1983). Cells were seeded onto 96-well microplates at a density of 10,000 cells/well (MDA-MB-361 and C33A) or 5,000 cells/well (all other cell lines). After an overnight incubation, fresh medium containing the test compounds (at a concentration of 10 or 30 μM) was added. After incubation for 72 h at 37°C in humidified air, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) solution (5 mg/mL) was added. Purple formazan crystals were formed by the living cells during a 4 h contact period, which were assayed by spectrophotometry after having been dissolved in 100 μL DMSO. Untreated cells served as control, and cisplatin (CIS) (Ebewe Pharma GmbH, Unterach, Austria) was used as a reference compound. When a test agent elicited over 50% growth inhibition at the 30 μM concentration, the assay was repeated with a series of dilutions (0.1–30 μM) and IC_{50} values were calculated (GraphPad Prism 5.0, GraphPad Software, San Diego, CA, United States). Two independent measurements were performed with five parallel wells. To present preliminary data concerning tumor selectivity of the potent compounds, the procedure was repeated on MRC-5 fibroblast and hTERT-HME1 immortalized mammary gland epithelial cells under the same experimental conditions.

Assessing the Cytotoxic Effect

The direct cytotoxic effects of the test agents were determined by a lactate dehydrogenase (LDH) assay. Cells were seeded onto 96-well microplates at a density of 5,000 cells/well and were incubated overnight, after which the medium containing the test compounds at proper concentrations was added. After incubation for 24 h, the activity of LDH released by the treated cells was determined by a commercially available colorimetric kit according to the manufacturer's instructions (Hoffmann-La Roche Ltd., Basel, Switzerland). Untreated cells served as control, while detergent Triton X-100 and CIS were used as reference agents.

Flow Cytometric Analysis of Cell Cycle and Apoptosis

The distribution of cells in different cell cycle phases (subG1, G1, S and G2/M) was analyzed via the measurement of cellular DNA content by flow cytometry. HeLa cells were seeded onto 6-well plates and allowed to stand for an overnight. The cells were treated with the selected compounds for 24, 48, or 72 h. Then cells were harvested, washed and fixed in ice cold 70% ethanol and stored at −20°C at least for an hour. Next, a DNA staining solution (containing distilled water, propidium-iodide, Triton-X100, sodium citrate, and ribonuclease-A) was added to each sample and stored in the dark at room temperature for an hour. Stained cells were analyzed by flow cytometry (Partec CyFlow, Partec GmbH, Munster, Germany) with at least 20,000 cells being evaluated for each analysis. Data processing was performed using the ModFit LT 3.3.11 software (Verity Software House, Topsham, ME, United States).

Morphological Studies Using Fluorescent Microscopy

Fluorescent double staining was performed in order to detect apoptosis induction and morphological changes using fluorescent microscopy. HeLa cells were seeded into a 96-well plate at the density of 3,000–5,000 cells per well. After an overnight incubation, the cells were treated with various concentrations of the test compounds for 24 h. The treated cells were then incubated with a fluorescent staining solution (containing Hoechst 33258 and propidium iodide, 500 and 300 μg/mL, respectively) for an hour. After staining, the cells were analyzed using a fluorescent microscope (Nikon ECLIPSE 146 TS100, Nikon Instruments Europe, Amstelveen, Netherlands) equipped with appropriate optical filters. For all different conditions, at least six fields were recorded with an attached QCapture CCD camera. This way cells with an intact, apoptotic or necrotic morphology can be distinguished based on their different nuclear morphological appearance and distinct membrane integrity.

Caspase Activity Measurements

In order to detect, whether the test compounds induce programmed cell death, the activity of caspase-3 was determined by a colorimetric assay. To elucidate the exact pathway of apoptosis, activities of caspase-8 and caspase-9 were additionally determined by colorimetric kits. In all cases approximately 12 million cells were treated with appropriate concentrations of the compounds for 24 or 72 h. After the treatment, the cells were scraped and the enzyme activities were determined by means of colorimetric assays mentioned above. All kits were purchased from Abnova Corp. (Taipei, Taiwan) and used in accordance with the instructions of the manufacturer.

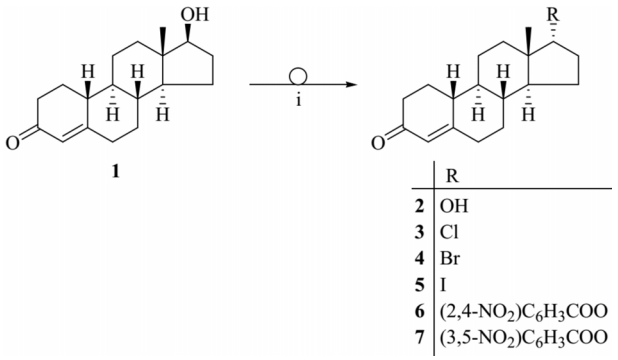

FIGURE 1 | Chemical structures of the synthesized 19-nortestosterone derivatives. Applied reagents and conditions: (i) Ph_3P, aryl acids or alkyl halogenides, diethyl azodicarboxylate, toluene, 80°C.

Tubulin Polymerization Assay

In order to determine the direct action of the test compounds on the microtubular system, an *in vitro* tubulin polymerization assay was performed using a commercially available kit (Cytoskeleton Inc., Denver, CO, United States) in accordance with the provider's instructions. The assay reactions were performed on a pre-warmed (37°C), UV-transparent 96-well microplate. Ten microliters of the test solutions were placed on the wells supplemented with 2 mM $MgCl_2$, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 1 mM guanosine triphosphate (GTP) and 10.2% glycerol. Ten microliters of general tubulin buffer was used as untreated control, and paclitaxel (PAC) served as the reference compound. The polymerization reaction was initiated by adding 100 μL of 3.0 mg/mL tubulin in 80 mM PIPES, pH 6.9, to each sample. Absorbance of the samples was measured per minute, at 340 nm, using a 60-min kinetic measurement protocol. Each sample was prepared in two parallels. To characterize the process, polymerization curves were fitted to the measured data. The highest difference between the absorbances measured at two consecutive time points was regarded as V_{max} (Δabsorbance/min) for the tested compound. A clinically applied reference agent, PAC was used at a relatively high concentration (10 μM) as recommended by the manufacturer. This concentration is approximately 1,000-fold higher than the IC_{50} value of PAC on HeLa cells (Jordan et al., 1996). Since similarly high concentrations of the tested compounds were not possible to be applied because of the limited solubility of the substances in the recommended buffer, we used the highest concentrations reflecting the differences in the efficacies of the tested compounds.

Assessing Hormonal Effect

An endocrine bioassay kit (Xenometrix AG, Allschwil, Switzerland) was used to test for a potential residual androgenic

TABLE 1 | Antiproliferative effects of the synthesized compounds (1–7) on human cell lines.

Comp.	Conc. (μM)	Growth inhibition (%) ± SEM [calculated IC_{50} value (μM)][a]									
		HeLa	SiHa	C33A	A2780	MCF-7	MDA-MB-231	MDA-MB-361	T47D	MRC-5	hTERT-HME1
1[b]	10	99.5 ± 0.2	–[c]	–	–	–	–	–	–	5.4 ± 1.2	16.9 ± 1.2
	30	99.5 ± 0.3 [0.65]	20.7 ± 2.6	25.2 ± 1.7	–	–	21.4 ± 1.2	–	–	11.3 ± 0.5	37.1 + 1.0
2[b]	10	28.1 ± 4.0	–	–	–	–	–	–	–	n.d.[d]	n.d.
	30	37.9 ± 3.4	–	–	–	27.7 ± 3.7	–	–	–		
3	10	95.9 ± 0.3	–	–	–	–	–	–	–	4.3 ± 3.7	76.2 ± 0.5
	30	95.1 ± 0.5 [1.21]	26.0 ± 2.3	61.7 ± 1.7	32.7 ± 1.0	36.9 ± 2.0	–	21.8 ± 2.5	–	8.0 ± 2.3	99.9 ± 0.1 [4.63]
4	10	94.4 ± 0.7	–	–	–	–	–	–	–	11.9 ± 2.3	n.d.
	30	94.1 ± 0.5 [1.69]	–	–	54.4 ± 1.6	–	–	–	–	20.4 ± 2.1	
5	10	95.2 ± 0.4	–	–	27.7 ± 1.3	–	–	–	–	13.1 ± 1.9	n.d.
	30	95.8 ± 0.2 [1.49]	–	–	61.6 ± 1.8	–	–	–	–	13.8 ± 2.0	
6	10	32.0 ± 2.9	–	–	–	–	–	–	–	n.d.	n.d.
	30	26.5 ± 2.3	–	–	31.9 ± 2.6	35.9 ± 1.4	–	–	47.2 ± 2.6		
7	10	26.6 ± 1.8	–	–	49.2 ± 0.9	25.9 ± 3.1	–	–	–	n.d.	n.d.
	30	21.5 ± 1.9	–	–	58.1 ± 2.0	37.7 ± 3.4	–	22.0 ± 1.6	–		
CIS[e]	10	42.6 ± 2.3	88.6 ± 0.5	83.8 ± 0.8	83.6 ± 1.2	66.9 ± 1.8	–	67.5 ± 1.0	51.0 ± 2.0	60.3 ± 3.3	97.7 ± 0.3
	30	99.9 ± 0.3	90.2 ± 1.8	93.9 ± 0.6	95.0 ± 0.3	96.8 ± 0.4	71.5 ± 1.2	87.8 ± 1.1	55.0 ± 1.5	61.9 ± 1.0	99.1 ± 0.3
		12.43	7.84	1.77	1.30	5.78	19.13	3.74	9.78	6.19	[2.45]

[a] IC_{50} values were calculated when the growth inhibition value for a compound exceeded 75% at a concentration of 30 μM. See Supplementary Figure S1 for representative concentration-response curves. [b] Data previously reported (Schneider et al., 2016). [c] Inhibition values less than 20% are not presented. [d] n.d., not determined. [e] Reference agent cisplatin.

activity of the selected agents. Genetically modified yeast cells (*Saccharomyces cerevisiae*) containing the human androgenic receptor gene integrated into a yeast chromosome, as well as an expression plasmid with the sequences of both the androgen responsive element and a lacZ reporter gene were cultured in humidified air at 31°C with agitation for 2 days. The appropriate concentrations of the test compounds and a CPRG substrate solution (chlorophenol red-β-D-galactopyranoside) for β-galactosidase were added into a 96-well microplate according to the instructions of the manufacturer. Androgen agonistic and antagonistic properties of the test compounds were determined by a colorimetric assay. For the antagonistic measurements the medium was supplemented with 5α-dihydrotestosterone (DHT). Once the reporter gene is expressed, β-galactosidase is secreted into the medium, and converts the yellow CPRG substrate into a red product, which can be quantified at 570 nm. Quantities of this red product correlate with the liberation of β-galactosidase, which is increased when an agonistic effect is present, while it is decreased when the test compound exerts an antagonistic effect. For the agonistic and antagonistic assays, nandrolone (**1**) and flutamide were used as reference agents, respectively.

Statistical Analysis

In all experiments, the statistical evaluation of the results was performed by one-way analysis of variance followed by the Dunnett posttest, using the GraphPad Prism 5 software (GraphPad Software; San Diego, CA, United States). Mean values and the SEM were calculated in all cases.

RESULTS

Synthetic Studies

The Mitsunobu reaction is widely employed for the inversion of stereogenic centers of secondary alcohols including steroid alcohols. The reaction allows the conversion of alcohols with alkyl or aryl carboxylic acids in the presence of diethyl azodicarboxylate and triphenylphosphine (Ph_3P). The result is an alkyl- or aryl carboxylic ester of the alcohol with inverted configuration (Mitsunobu, 1981). Here, we describe the Mitsunobu reaction for 19-nortestosterone (**1**) utilizing 2,4-, or 3,5-dinitrobenzoic acid in the presence of diethyl azodicarboxylate and Ph_3P in toluene at 80°C leading to the corresponding 17α-19-nortestosterone-17-yl 2′,4′- or 3′,5′-dinitrobenzoate (**6** or **7**, respectively; **Figure 1**). Reacting compound **1** with isopropyl halides under the same conditions produces the corresponding 17α-chloro-, bromo-, and iodo-19-nortestosterone (**3–5**, respectively). Hydrolyzing compounds **6** or **7** in methanol, in the presence of $NaOCH_3$ yields 17α-19-nortestosterone (**2**).

Antiproliferative Properties of 19-Nortestosterone Derivatives

The antiproliferative activities of the test compounds were determined by MTT assay on a panel of adherent gynecological cancer cell lines (**Table 1**). Nandrolone (**1**) as reported previously,

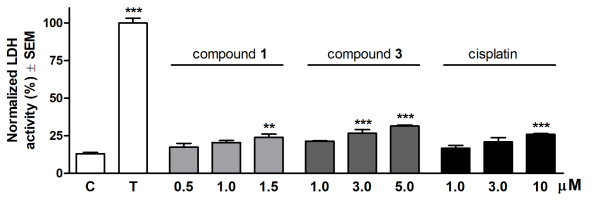

FIGURE 2 | Cytotoxic effects of compounds 1, 3 and cisplatin on HeLa cells after 24 h treatment. The effect of Triton X-100 (T) was considered 100%. Results are mean values ± SEM of the data from two separate measurements, $n = 4$. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to untreated control (C).

exerted a considerable antiproliferative effect against HeLa cells. Compounds **2**, **6**, and **7** exerted no remarkable antiproliferative action against the gynecological cancer cell lines. 17α-Halogen derivatives (**3–5**) exerted a pronounced antiproliferative effect against HeLa cells, while they did not elicit any notable influence on the remaining cell lines including fibroblasts. These derivatives had lower IC_{50} values on HeLa cells than that of the reference agent CIS. Compound **3** proved to be the most potent antiproliferative agent, characterized by an effect size comparable to that of **1**. Cancer selectivity of the potent compounds was determined by the same method using human fibroblasts (MRC-5) and non-cancerous immortalized, mammary gland derived epithelial (hTERT-HME1) cells. None of the test agents exhibited a considerable growth inhibitory effect against intact fibroblasts up to a concentration of 30 μM. Compound **1** had no pronounced action on immortalized epithelial cells, while compound **3** inhibited the growth of these cells with an IC_{50} value approximately four times higher than that obtained on HeLa cells. Compound **3** exerted the most explicit tumor selectivity, showing a substantially weaker effect on non-cancerous cells than CIS. Due to their potent and selective antiproliferative actions, compound **3** and nandrolone (**1**) were selected for further investigations to characterize their mechanism of action and assess their hormonal effect.

Cytotoxic Activity

Cytotoxic properties of the selected compounds were ascertained by measuring LDH activity resulting from cell membrane damage. Each molecule exerted a concentration-dependent increase of LDH activity compared to the untreated control after 24 h treatment (**Figure 2**). Compound **1** elicited a substantial LDH release at a concentration of 1.5 μM. The effect of compound **3** proved to be significant when applied at concentrations above its IC_{50} values (3.0 or 5.0 μM). None of the test agents induced an LDH activity comparable to the maximum LDH release triggered by detergent Triton X-100.

Cell Cycle Analysis

Alterations in cell cycle and apoptotic fragmentations were determined by flow cytometry after treatment with the test compounds for 24 and 48 h. Since **1** elicited no change in the subG1 population at these time points this agent was

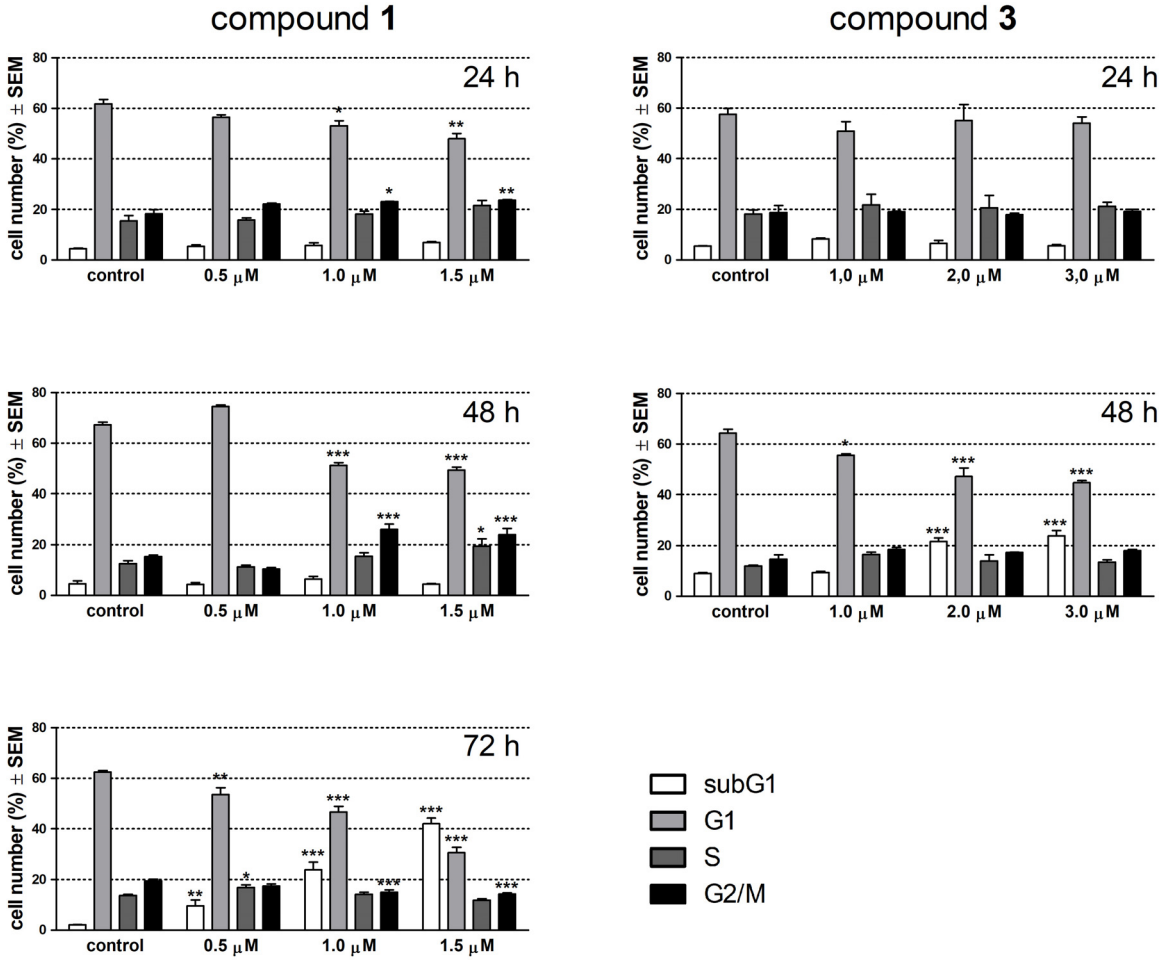

FIGURE 3 | Effects of compounds 1 and 3 on cell cycle phase distribution of HeLa cells determined by flow cytometry after incubation for 24, 48, or 72 h. Results are mean values ± SEM of the data from two independent measurements, $n = 6$. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to untreated control.

re-tested after 72 h incubation. Treatment with **1** for 24 h resulted in a concentration-dependent and significant decrease in the G1 and a moderate but significant increase in the G2/M phase cell population (**Figure 3**). After 48 h of exposure these changes became more pronounced, and completed with the elevated ratio of the S phase cell population in the presence of 1.5 μM of **1**. A longer incubation time (72 h) with **1** elicited a substantial disturbance in the cell phase distribution and a concentration-dependent accumulation of hypodiploid cells indicating apoptotic nuclear fragmentation. Compound **3** did not have any remarkable effect on cell cycle after 24 h exposure, while a longer incubation time (48 h) resulted in a substantially elevated increase of the subG1 population at the expense of G1 cells.

Morphological Changes

To characterize the morphological features of the apoptosis induced by compounds **1** and **3** HeLa cells were examined by fluorescent microscopy after 24 h treatment with three different concentrations (1.0, 3.0, or 5.0 μM) of the test compounds. For the quantitative analysis, cells with intact, apoptotic and necrotic morphological features were labeled, and the ratios of different morphologies were calculated. Treatments with **1** and **3** resulted in a substantial and concentration-dependent increase in both the apoptotic and necrotic cell populations, at the expense of the intact population (**Figure 4**).

Induction of Apoptotic Enzymes

Based on the above results, changes of the activities of caspase-3, caspase-8, and caspase-9 were determined using a colorimetric assay. After treatment with **1** for 72 h, the activity of executive caspase-3 increased significantly and in a concentration-dependent manner (**Figure 5**). Under the same experimental conditions **1** also activated the initiator caspases, although the induction of caspase-8 was less pronounced. Compound **3** enhanced caspase-3 activity at 5.0 μM even after a shorter incubation period (24 h). Caspase-9 activity was also significantly elevated, while there was no change in the function of caspase-8.

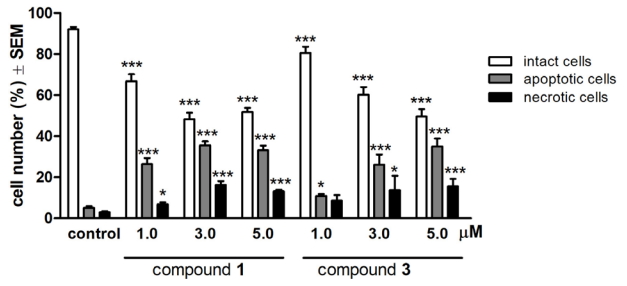

FIGURE 4 | Qualitative evaluation of fluorescent double staining of HeLa cells after 24 h treatment with compounds 1 or 3. $*p < 0.05$ and $***p < 0.001$ compared to untreated control (C). See Supplementary Figure S2 for representative pictures.

Tubulin Polymerization

The direct effect of the test compounds on microtubule formation was determined by a specific photometric assay in a cell-free system. The tested concentrations of the compounds were chosen based on their calculated IC_{50} values according to the recommendation of the manufacturer. Both compounds **1** and **3** induced a significant acceleration of tubulin polymerization compared to untreated control samples (**Figure 6**). Calculated values of maximal rate of tubulin polymerization (V_{max}) were elevated compared to control, although none of these V_{max} values were comparable to that of the reference agent PAC.

Hormonal Effect

Since residual hormonal activity of a potential sterane lead compound is a crucial aspect of further drug development, the androgenic properties of the most promising agents were investigated by a yeast-based reporter assay. As **1** is a well-characterized androgen, it was used as a reference agent (Bergink et al., 1985). According to our results **3** exerts a substantially lower hormonal activity, with no relevant action unless applied in extremely high concentrations (**Figure 7**). Calculated EC_{50} values of **1** and **3** differed by approximately 2 orders of magnitude (3.43×10^{-8} and 3.80×10^{-6} M, respectively). Compound **3** exhibited no antagonistic activity in the assay system (data not shown).

DISCUSSION

Various biological effects of compounds with an androstane skeleton mostly stem from their endocrine disruptor properties, thus the medical use of androgens is mainly limited to androgen replacement and androgen deprivation therapies, including the management of some hormone dependent malignancies (Wadosky and Koochekpour, 2016). Beyond their approved medical applications, numerous androgenic anabolic steroids are utilized illegally to enhance physical performance, a risky use often accompanied by serious adverse effects. Recently, numerous androstanes with anticancer potential have been described, and the importance of androstane compounds and their derivatives in the research and development of steroid-based anticancer agents for hormone-independent malignancies is continuously increasing (Frank and Schneider, 2013; Wadosky and Koochekpour, 2016). Although several 19-nortestosterone derivatives have been identified as potent anticancer agents (e.g., mibolerone, tibolone, gestodene), most of them have pronounced hormonal properties involved in their therapeutic action, as well as in their undesired adverse effects (Saito et al., 2016). In a previous research, we investigated a set of newly synthesized 19-nortestosterone analogs, and reported that some of them exhibited a moderate antiproliferative activity. That study of our research group revealed that the widely known 19-nortestosterone analog **1** (nandrolone) has a potent and selective antiproliferative effect against cervical carcinoma cells positive for type 18 of human papilloma virus (HPV-18) (Schneider et al., 2016).

The aim of our present study was to synthesize and investigate a set of novel 19-nortestosterone derivatives with improved antiproliferative properties and limited hormonal activities. Three of the novel compounds (**3**–**5**) were found to exhibit a pronounced antiproliferative effect against HeLa cells (calculated IC_{50} values: 1.21–1.69 μM), while exerting a negligible or lower impact on other cell lines including intact fibroblasts (MRC-5) and the immortalized mammary gland epithelial cell line (hTERT-HME1). In contrast, dinitrobenzoates (**6** and **7**) appeared to be ineffective in terms of growth inhibition of cancer cells. The most potent compound, **3** was further investigated to describe its possible mechanism of action. A well-known androgen, **1** a with similar antiproliferative capacity was utilized as a steroidal reference agent.

The antiproliferative property of a compound is typically reflected by a disturbance induced in cell cycle distribution. These changes in cell cycle phases inform about the probable mechanism of the antiproliferative action. Both **1** and **3** caused a cell cycle disturbance characterized by the accumulation of hypodiploid (subG1) cells at the expense of the G1 population.

The increase of hypodiploid cell populations can be regarded as an evidence for proapoptotic properties of the test compounds. Alterations in cell cycle during physiological conditions usually lead to induction of programmed cell death. Activation of the apoptotic machinery, selectively in cancer cells without a substantial necrotizing effect is one of the most desirable characteristics of a promising anticancer agent (Tolomeo and Simoni, 2002). Some steroidal compounds with anticancer activity (e.g., 2-methoxyestradiol, D-homoestrone, a D-secoestrone-triazole analog) have been described as efficacious inducers of programmed cell death in cancer cells (Li et al., 2004; Minorics et al., 2015; Bózsity et al., 2017). Therefore, the demonstration of apoptosis induction was a basic feature of our study. We utilized fluorescent microscopy and observed the characteristic features of apoptosis elicited by the test agents in a concentration-dependent manner.

Caspase enzymes are crucial implementers of the apoptotic program executed by downstream effector caspases such as caspase-3. Caspase-9 is the major enzyme involved in the initiation of the intrinsic apoptotic pathway, while caspase-8 plays an essential role in the extrinsic pathway of the

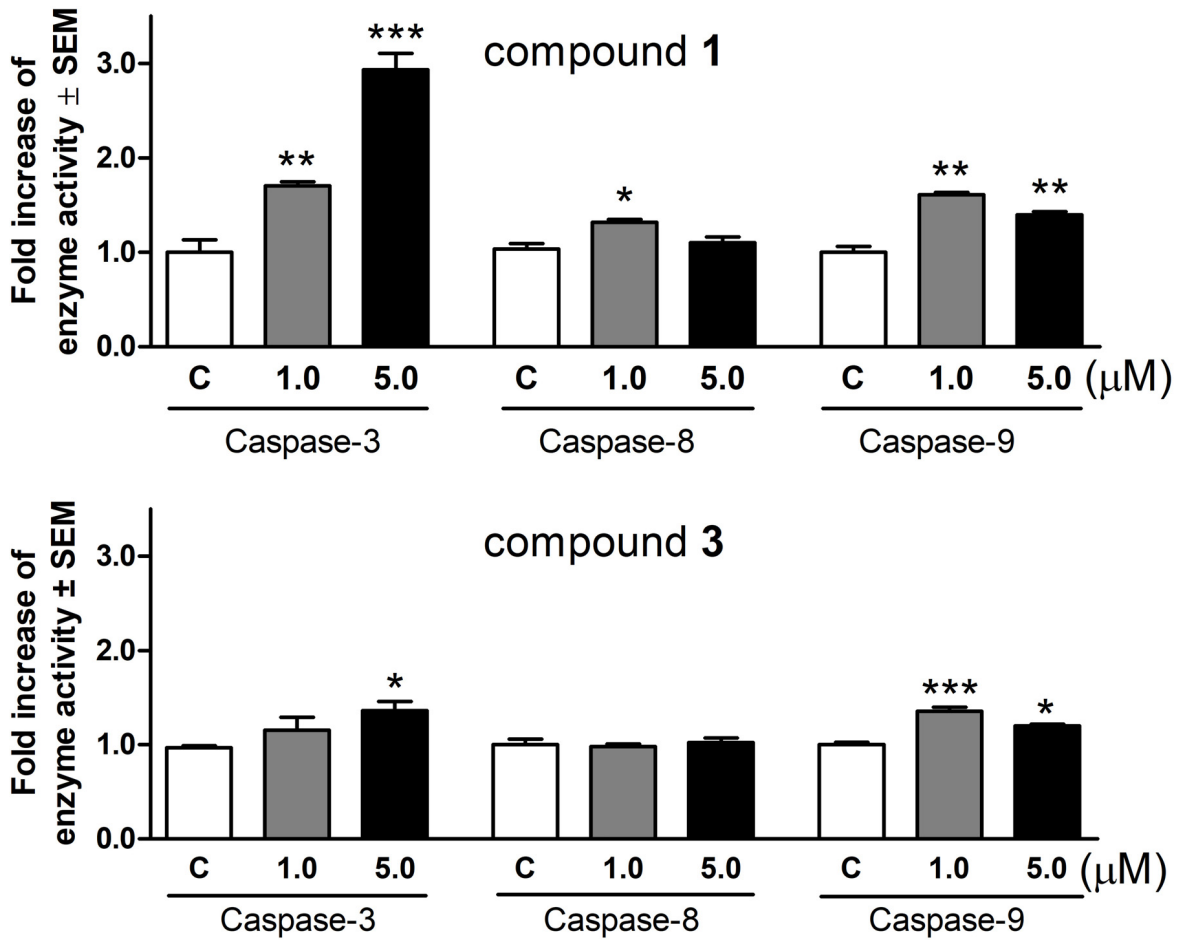

FIGURE 5 | Activation of caspase-3, caspase-8, and caspase-9 enzymes in HeLa cells after incubation with compounds 1 and 3 for 72 or 24 h, respectively. Results are mean values ± SEM of the data from two independent measurements, $n = 6$. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to untreated control.

apoptotic process (Hajra and Liu, 2004). Treatments with the test compounds resulted in a significant elevation in the activity of caspase-3, reflecting the activation of apoptotic cell death. Both agents elicited a considerable increase in activity of caspase-9 at both concentrations tested (1 and 5 μM) without expected concentration-dependency. The exact reason for this is not elucidated, but based on the results of our fluorescent microscopy analysis, necrotic cell death induced by the higher concentration could be a plausible explanation. Based on these findings activation of the intrinsic apoptotic pathway is hypothesized. Although, the activity of caspase-8 was slightly but significantly increased by **1** at a concentration of 1 μM, this limited change seems to be inefficient to indicate a dominant role of the extrinsic apoptotic pathway.

Previous studies revealed that some proapoptotic steroidal compounds induced a pronounced cell cycle arrest via a direct influence on tubulin polymerization during mitosis. The inhibitory effect of 2-methoxyestradiol on microtubule formation resulting from its interaction with the colchicine-binding site of β-tubulin has also been reported (Peyrat et al., 2012). Thus, a possible direct influence of **1** and **3** on the polymerization of tubulin heterodimers in a cell-free system was also investigated.

Both of our test agents elicited a concentration-dependent acceleration of the polymerization reaction as reflected by significantly increased V_{max} values. This molecular behavior indicates a possible PAC-like microtubule stabilizing effect of the test compounds which may contribute to cell cycle arrest and lead to the induction of the apoptotic machinery.

The possible androgenic activity of a novel steroid-based agent may implicate a source of potential adverse reactions limiting its therapeutic value. Receptor binding properties of **1** and 5α-dihydrotestosterone are indistinguishable by a radio ligand assay using androgen receptors prepared from rat prostate and MCF-t cells (Bergink et al., 1985). Therefore, **1** can be utilized as a reference agent when novel compounds with possible androgenic properties are characterized. Compound **3** was detected to possess a substantially lower androgenic activity compared to **1**.

Moreover, **3** had no androgen antagonistic properties when tested in the presence 5α-dihydrotestosterone. This hormonal neutrality of **3** could be explained by the α-position of chlorine

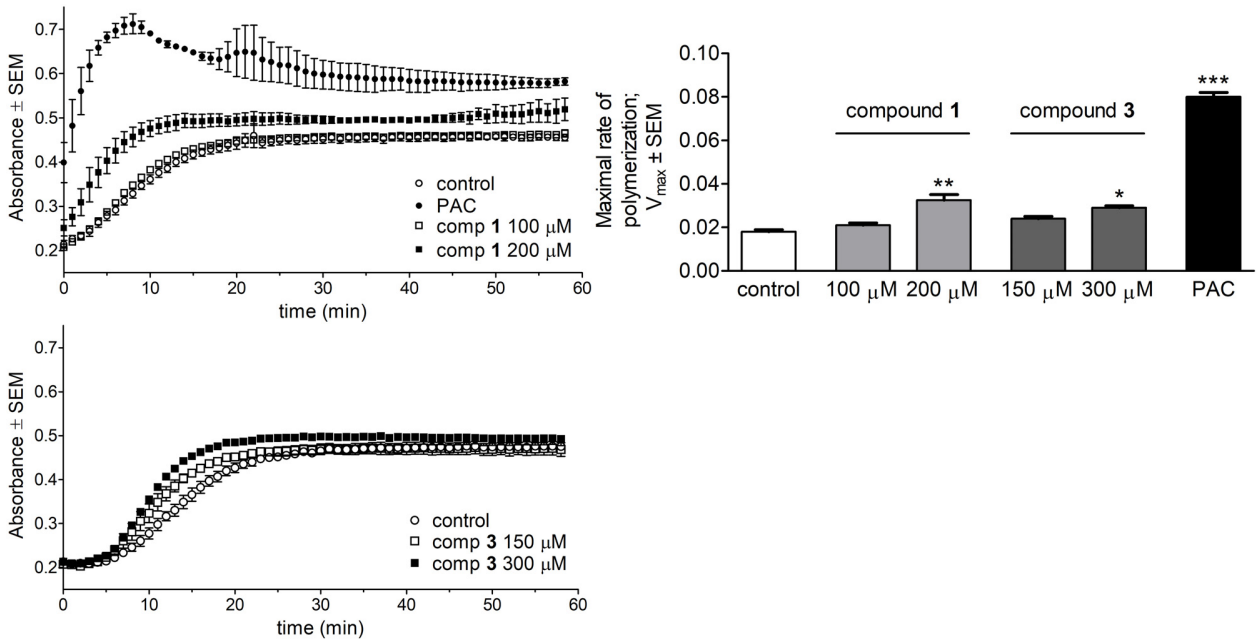

FIGURE 6 | Direct effects of compounds 1 and 3 on tubulin polymerization. Kinetic curves characterizing tubulin polymerization in the presence of 1, 3 or paclitaxel (PAC) were recorded after 58 min kinetic measurements (Left). Direct effects of the test agents on the maximal rate of polymerization were evaluated (Right). Results are mean values ± SEM of the data from two independent measurements, $n = 4$. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to untreated control sample.

on the ring D of the skeleton. The substituent at position 17 determines receptor binding, and only the β configuration is favored. Consequently, 17α-testosterone has virtually no affinity for the receptor (Fragkaki et al., 2009).

Although mediated by the same nuclear receptor, androgenic and anabolic actions of a ligand can be partially dissociated depending on the tissue expression of crucial metabolic enzymes including 5α-reductase. While the androgenic action may be disadvantageous and may limit the development of a drug candidate, an anabolic or "myotropic" property could theoretically be advantageous when a chronic or devastating disease is treated (Tóth and Zakár, 1982).

Beside the well-characterized action of androgens mediated via androgen response elements within the DNA, the presented 19-nortestosterone analogs may also interact with membrane-associated androgen receptors (Kampa et al., 2006). This latter action could be of special importance since the stimulation of these receptors elicits an increase in the intracellular free zinc concentration accompanied by induction of apoptosis in cancer cell (Thomas et al., 2014).Two further membrane-bound proteins have been recently reported as potential non-genomic receptors for androgens and related steroids. One of them is the putative G-protein coupled receptor GPRC6A, an amino acid, calcium, and osteocalcin sensing receptor. The interaction of this receptor with testosterone is reported to result in increased phosphorylation of extracellular signal-regulated kinases in human embryonic kidney cells expressing the GPRC6A protein (Pi et al., 2010).

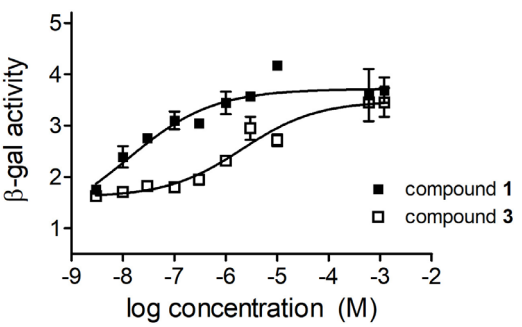

FIGURE 7 | Effects of compounds 1 and 3 in the yeast-based androgenic reporter assay. Results are mean values ± SEM of the data from two independent measurements, $n = 4$.

Oxoeicosanoid receptor 1 (OXER1) is a membrane receptor for the arachidonic acid metabolite 5-oxoeicosatetraenoic acid (5-oxoETE) and serves as a binding site for testosterone in prostate cancer cells. The steroid testosterone is reported to antagonize the action of the natural agonist 5-oxoETE on the intracellular cAMP production, and the interaction between testosterone and OXER1 was confirmed by an *in silico* molecular docking study as well (Kalyvianaki et al., 2017).

Since apoptosis and cell growth are indirectly involved in the signal mechanism of these non-genomic receptors, an action mediated by membrane-bound steroid receptors may contribute to the overall effects of the presented compounds.

In summary, our present results demonstrated that three of a

set of newly synthesized 19-nortestosterone exhibit a pronounced antiproliferative activity against cervical carcinoma cells with lower influence on fibroblasts and a modest action on non-cancerous immortalized epithelial cells. The most potent agent **3** is characterized by a moderate cytotoxic effect, elicits cell cycle disturbance and induces the mitochondrial pathway of apoptosis. As a possible molecular mechanism of these actions, a PAC-like microtubule-stabilizing property is suggested based on its direct effect on the microtubular system.

Based on our present findings, the 19-nortestosterone backbone with a 17α-halogen substitution provides an excellent skeleton for designing novel antiproliferative steroidal compounds with negligible androgenic activity.

AUTHOR CONTRIBUTIONS

AG, AK, and EK performed the experiments. IO and AS analyzed the data. RM, EM, GS, and IZ were involved in experiment planning and supervision. AG, GS, and IZ wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00825/full#supplementary-material

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