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CHARACTERIZATION OF ANTINEOPLASTIC PROPERTIES OF NOVEL ANDROSTANE-BASED SYNTHETIC STEROIDS

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1 INTRODUCTION

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. Furthermore, cancer has become the leading cause of death in developed countries as opposed to developing countries where it is still the second one.

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.

Steroids are an important group of biologically active agents. Owing to their diverse biological effects, they play essential roles in various physiological processes. Besides endogenous hormones, exogenous steroid exposure exerts versatile effects on several biological processes. Therefore, a wide range of steroidal agents are utilized in the medicine and serve as therapeutic tools for hormone replacement therapy and contraception, also for men and women. Thanks to their miscellaneous biological effects, a high number of steroids are utilized in the medication of cachexia, gynecological disorders, hormone dependent cancers and inflammatory diseases. Their immune suppressive properties play key role in organ transplantation, treatment of autoimmune diseases, allergic reactions and chronic inflammatory processes.

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. 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. 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.

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.

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. Some 19-nortestosterone derivatives have been reported with noticeable antiproliferative action against several cancer cell lines.

Hybridization of steroids with other molecules is often accompanied by alterations in biological activity of the original compounds, which can be favourable in the case of appearance of advantageous properties. Besides steroidal compounds, some quinoline derivatives have also been reported as potent anticancer agents. Since advantageous biological properties of synthetic steroid-quinoline hybrids have been revealed, design and evaluation of novel similar compounds for antitumor activities became to an important goal of anticancer research.

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 means 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

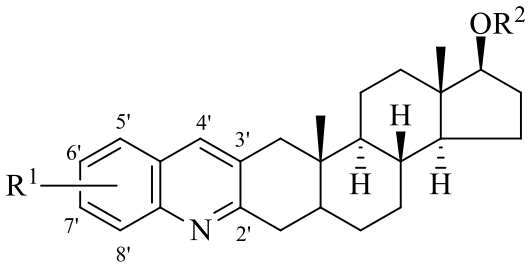
3.1 Chemicals

3.1.1 Test compounds

All the test compounds were synthetized at the Department of Organic Chemistry, University of Szeged, Hungary.

3.1.1.1 Steroid-quinoline hybrid molecules

A set of novel A-ring-fused quinolines in the 5α-androstane series were prepared (Figure 1).

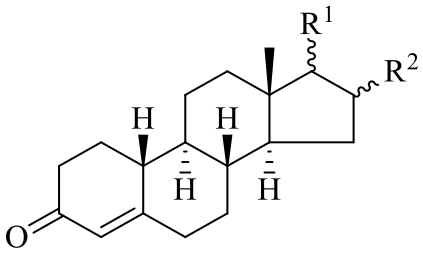


Compound	R_2	R_1	Position of R_1
SQH1	Ac	H	-
SQH2	Ac	Me	6'
SQH3	Ac	MeO	6'
SQH4	Ac	Cl	6'
SQH5	Ac	Br	6'
SQH6	Ac	Cl	7'
SQH7	H	H	-
SQH8	H	Me	6'
SQH9	H	MeO	6'
SQH10	H	Cl	8'
SQH11	H	Cl	6'
SQH12	H	Br	6'
SQH13	H	Cl	7'
SQH14	H	Cl	5'
SQH15	H	Me	8'

Figure 1. *Chemical structures of the tested steroid-quinoline hybrids (SQH1-15).*

3.1.1.2 19-nortestosterone derivatives

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 analogs (Figure 2).



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 2. *Chemical structure of the tested 19-nortestosterone analogs, including the structurally analogous reference agent, nandrolone* (NAN).*

3.2 Cell lines and culture conditions

Gynecological cancer cell lines, including ovarian (A2780), cervical (HeLa, SiHa and C33A) and breast cancer cell lines (MCF7, T47D, MDA-MB-231 and MDA-MB-361) were used for antiproliferative screening. Intact fibroblast cells (MRC-5) and noncancerous epithelial (hTERT-HME1) cells were utilized for determination of cancer selectivity. With the exception of hTERT-HME1, all cells were maintained in tissue culture flasks containing Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids and 1% antibiotic-antimycotic mixture. hTERT-HME1 cells were cultured in serum-free mammary epithelial cell growth medium completed with human epidermal growth factor, insulin, hydrocortisone, bovine pituitary extract and an antibiotic-antimycotic mixture. The cells were maintained at 37°C in humidified air containing 5% CO_2.

3.3 Determination of antiproliferative effect

The antiproliferative properties of the compounds have been determined by MTT assay. Cells were seeded onto 96-well plates, after an overnight repose, fresh medium supplemented with the tested compounds (10 or 30 μM) was added. After 72 h incubation, the medium was completed with MTT solution. After 4 h contact peroid, the precipitated formazan crystals were solubilized and the absorbance was measured by means of spectrophotometry. 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. 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

HeLa cells were seeded onto 96-well plate, after an overnight repose, the medium containing the tested compounds were added, then cells were incubated under culture conditions for 24 h. Thereafter the activity of released lactate dehydrogenase from treated cells was determined using a commercially available colorimetric kit.

3.5 Flow cytometric analysis of cell cycle and apoptosis

Changes in distribution of cells in the different cell-cycle phases (subG1, G0/G1, S and G2/M) were detected via measuring cellular DNA content after staining with propidium iodide (PI). The cells were plated onto 6-well plates and treated with the selected compounds for 24 h, 48 h or 72 h. After the treatment, the cells were harvested, fixed and stained with PI dye solution. The samples were analyzed by flow cytometry.

3.6 Morphological studies by fluorescence microscopy

Fluorescent staining with DNA-specific dyes was performed in order to detect morphological changes and induction of apoptosis exhibited by the selected compounds. Cells were seeded onto 96-well plate. After treatment with the selected compounds, cells were incubated with staining solution then analyzed under fluorescence microscope. The intact, early apoptotic and necrotic cells were numerated and then analyzed statistically.

3.7 Determination of caspase activities

To evidence apoptosis inducing effect of the selected compounds, the activation of caspase-3 was examined by means of colorimetric assay. For the decision about which pathway of the apoptosis is induced due to the treatment, the activities of caspase-8 and -9 were determined. In all cases cells were seeded onto cell culture flasks and exposed to the compounds for 24 h, 48 h or 72 h. After the treatment, the cells were harvested and lysed. Supernatants containing the enzyme were collected and a labeled substrate was added. The chromophore after enzyme mediated cleavage from the substrate was quantified by means of spectrophotometry.

3.8 Tubulin polymerization assay

The direct effect of the test compound on microtubular system was tested in a cell free system. The assay reactions were performed on UV-transparent microplate using tubulin solution completed with the test compound. The polymerization reaction was determined by means of spectrophotometry using a 60-min kinetic protocol and kinetic curves were recorded. The maximal rate of polymerization reaction (V_{max}) was determined.

3.9 Determination of hormone effect

The residual hormonal activity of the test compound was determined using a yeast-based reporter assay. Yeast cells were plated onto 96-well plates and exposed to the test compound in the presence of chromogenic substrate. The production of converted substrate due to the compound mediated reporter gene expression was quantified by means of spectrophotometry. The measured data were analyzed statistically, the agonistic and antagonistic properties of selected molecules were determined.

3.10 Statistical analysis

In all examinations, 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

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). SQH9 is proved to be the most potent compound with promising antiproliferative effect on some cancerous cell lines. On T47D cell line, its growth inhibitory action (10.2 μM) is considered as comparable with the effect of the reference agent (9.8 μM). Based on these findings, SQH9 was selected for additional investigations.

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.*

4.1.2 Effects of the selected steroidal quinoline on cell cycle

The influence of SQH9 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 3), such as the concentration-dependent increase of hypodiploid cells (subG1). 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.

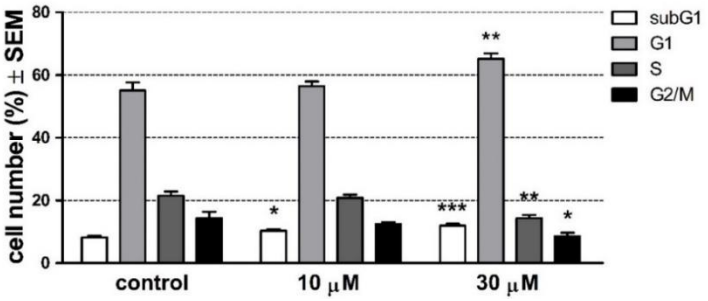


Figure 3. *Effect of SQH9 on T47D cell cycle phase distribution determined by flow cytometry after treatment for 48 h. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ as compared with the untreated control cells.*

4.1.3 Morphological changes and apoptosis induction

Morphological features of T47D cells were examined by means of fluorescent double staining under fluorescent microscopy after 48 h incubation with the selected hybrid. The proportion of apoptotic cells exhibited a concentration-dependent increase due to the treatment at the expense of intact cells (Figure 4). Additionally, the ratio of the cells with necrotic morphology increased significantly only after treatment with the higher concentration of SQH9.

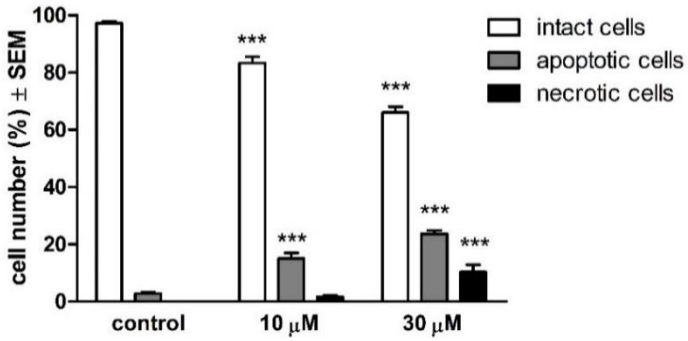


Figure 4. *Quantitative evaluation of fluorescent double staining of T47D cells after 48 h incubation with 10 or 30 μM SQH9. *** indicates $p<0.001$ as compared with the untreated control cells.*

4.1.4 Influence of the selected steroid-quinoline hybrid on caspase-3 activity

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 T47D cells, significantly at the higher concentration (Figure 5).

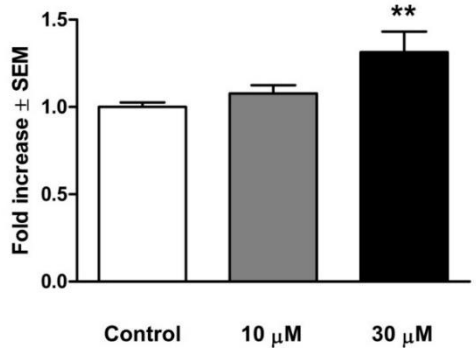


Figure 5. *Induction of caspase-3 activity after 48 h treatment with 10 or 30 μM SQH9. ** indicates $p<0.01$ as compared with the untreated control.*

4.2 Antiproliferative properties of 19-nortestosterone derivatives

4.2.1 Antiproliferative effect of 19-nortestosterone derivatives

21 compounds were investigated in a screening step for their growth inhibitory activities. In the case of potent compounds, IC_{50} values were calculated (Table 2). Three of the tested compounds (NTD8, 20 and 21) demonstrated an outstanding antiproliferative action selectively against HeLa cells, while their effect was only modest 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 cisplatin (CIS, 12.4 μM).

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.*

In order to characterize the cancer selectivity of the potent analogs, their antiproliferative effect were determined on intact fibroblast and immortalized epithelial cell lines (Table 3). None of the tested agents were able to exert a substantial antiproliferative action on fibroblasts except for CIS. Although NTD8 possessed only 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). 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 the mechanism of its action. Since NAN possessed a selective antiproliferative action against HeLa cells, its influences were also examined within the framework of additional investigations.

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.*

4.2.2 Cytotoxic effect of 19-nortestosterone derivatives

All the tested compounds exhibited a concentration-dependent release of the lactate dehydrogenase due to loss of membrane integrity after 24 h treatment (Figure 6). NAN significantly increased the 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.

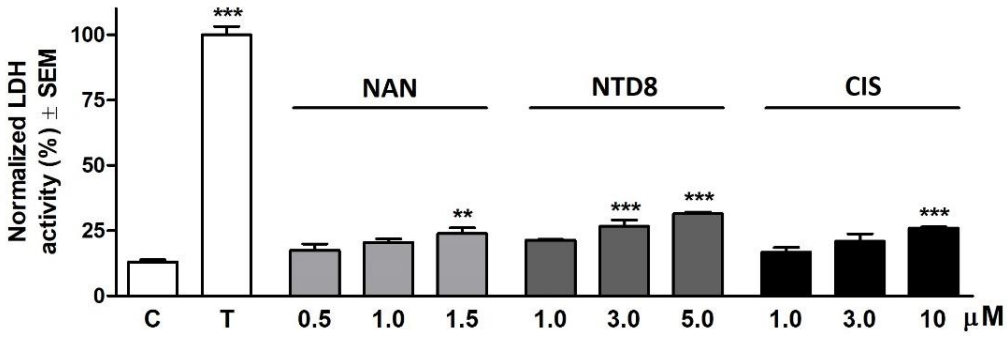


Figure 6. *Cytotoxic effects of NTD8, NAN and CIS on HeLa cells after 24 h incubation. The effect of Triton X-100 (T) was regarded as 100%. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ as compared with the untreated control (C).*

4.2.3 Effects of 19-nortestosterone derivatives on cell cycle

Incubation with NAN for 24 h resulted in a concentration-dependent increase of the number of HeLa cells in G2/M phase at the expense of G1 phase significantly at the concentration of 1.0 and 1.5 μM (Figure 7). After 48 h, these effects became more pronounced and the proportion of cells in the S phase was significantly elevated in the persence of 1.5 μM of the compound. Incubation for 72 h with NAN caused a reversed tendency in S and G2/M phases as a result of the concentration-dependent increase of hypodiploid population. 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 concentrations. The considerably increased hypodiploid population indicated the appearance of apoptotic bodies in the persence of 2.0 and 3.0 μM NTD8.

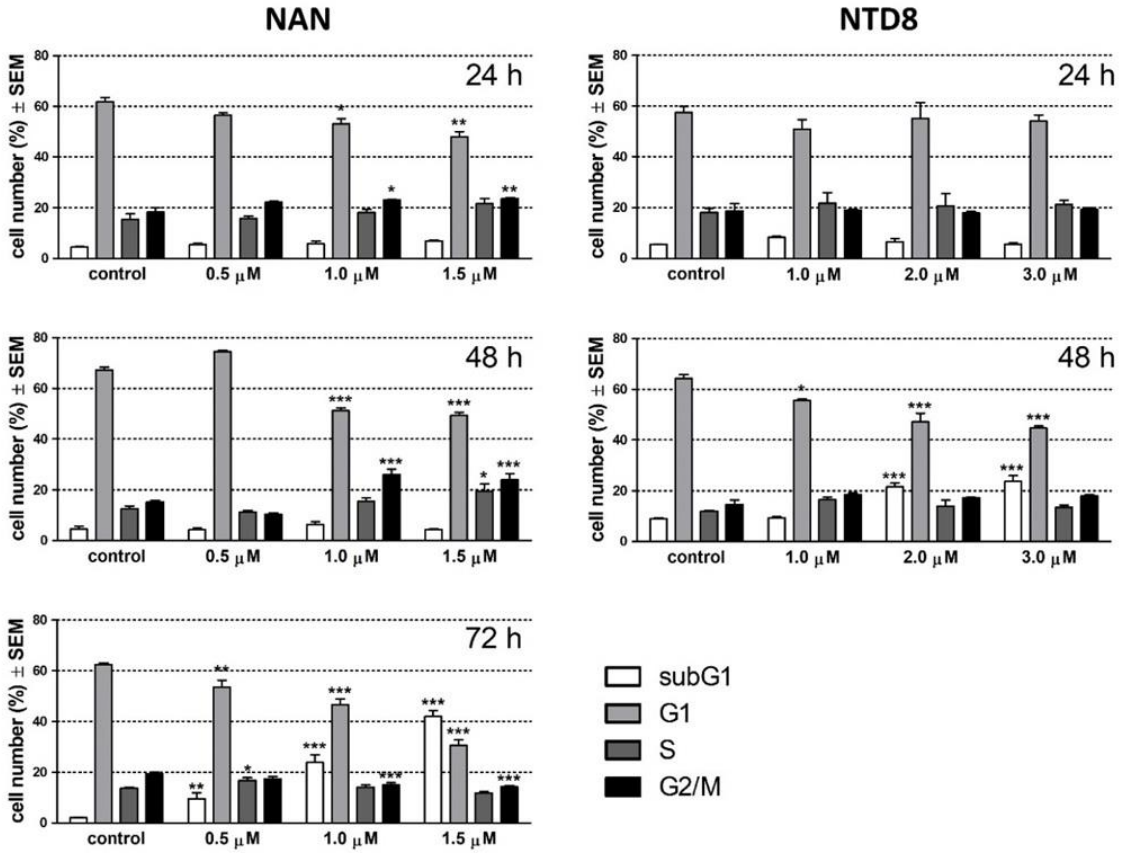


Figure 7. *Effects of NAN and NTD8 on cell cycle phase distribution of HeLa cells determined by flow cytometry after incubation for 24, 48, or 72 h. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ as compared with the untreated control.*

4.2.4 Morphological changes and apoptosis induction

After 24 h treatment with NAN, the proportion of apoptotic cells was substantially increased at the expense of intact cells, significantly at all the applied concentrations. 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 of intact cells. Remarkably elevated number of necrotic cells was detected in the persence of 3.0 or 5.0 μM.

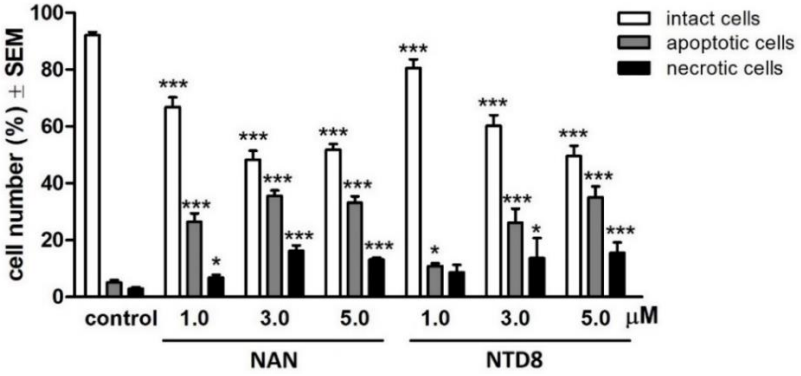


Figure 8. *Quantitative evaluation of fluorescent double staining of HeLa cells after 24 h incubation with NAN and NTD8. * and *** indicate $p<0.05$ and $p<0.001$ as compared with the untreated control.*

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

Activities of three different apoptotic enzymes were determined in 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 9).

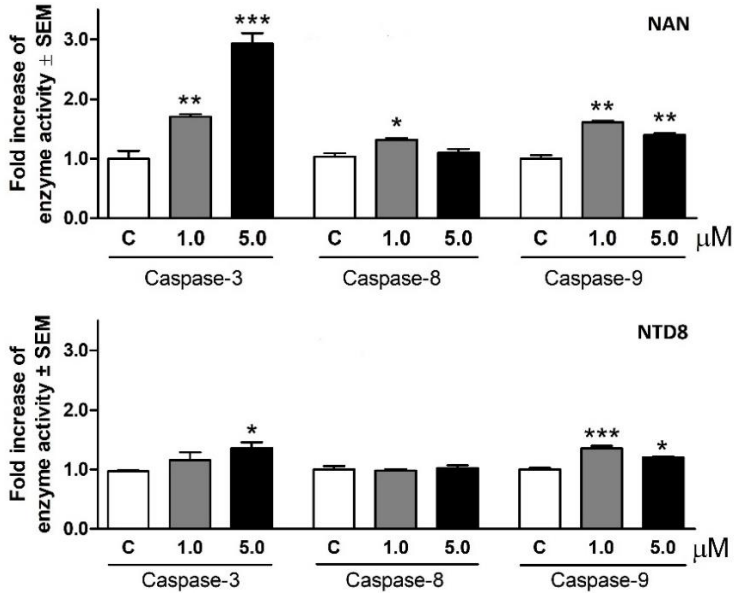


Figure 9. *Activation of apoptotic enzymes in HeLa cells after incubation with NAN (upper panel) and NTD8 (lower panel) for 72 or 24 h, respectively. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ as compared with the untreated control.*

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. While NTD8 exhibited a considerable caspase-9 induction, there were no detectable changes in caspase-8 activity in the same experimental conditions.

4.2.6 Direct effects of 19-nortestosterone derivatives on tubulin polymerization

The acceleration of the polymerization reaction in the growth phase was examined in the presence of NAN or NTD8. The maximal rate of tubulin polymerization (V_{max}) was calculated in all conditions and analyzed statistically. Both of the compounds increased a maximal rate of microtubule formation, significantly at the higher concentrations compared to the untreated control (Figure 10). However, the effect of the compounds was proved to be similar but milder than that of the positive control (PAC).

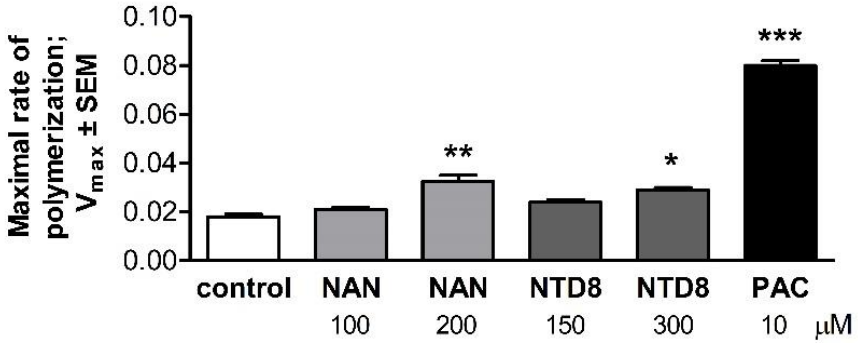


Figure 10. *Effect of the test compounds on the maximal rate of polymerization reaction. *, ** and *** indicate p<0.05, p<0.01 and p<0.001 as compared with the untreated control.*

4.2.7 Androgenic activity of the selected 19-nortestosterones

Since NAN is an acknowledged androgen demonstrating a potent affinity for androgen receptor similarly to DHT, it was utilized as a reference compound. With the exception of extremely high concentrations, NTD8 elicited a substantially lower reporter gene expression compared to NAN, indicating its considerably weaker androgenicity. 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 potent compound. In the same experimental conditions NTD8 express no detectable antagonistic property in the persence of DHT.

5 DISCUSSION

Although a high number of steroid compounds with anticancer properties have been described, androstane skeleton possessing agents are particularly rare among them. The aim of the present study was the pharmacological characterization of newly synthesized androstane-based antiproliferative agents 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 most potent hybrid SQH9, a 17-OH analog containing a 6' methoxy substituted quinoline moiety, demonstrated a considerable antiproliferative action on several cell lines. 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.

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 phases 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.

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. Elevation of the concentration resulted in an increased proportion of necrotic cells reflecting the appearence of secondary necrosis.

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. These results confirmed 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. The most potent compounds (NTD8, 20 and 21) exhibited an outstanding antiproliferative action against HPV18+ cervical cancer cells.

The examinations of tumor selectivity of these halogen substituted derivatives revealed their substantially weaker impact on noncancerous cell lines than against HeLa cells.

Besides NTD8 is proved to be the most potent compound with outstanding antiproliferative effect on HeLa cells, it exhibited substantial cancer selectivity. Based on these findings, NTD8 was selected for additional investigations in order to reveal some details about the mechanism of its action. Since NAN demonstrated a prominent inhibitory action on the proliferation of HeLa cells, it also involved in the additional examinations as a reference compound with

analogous chemical structure. Beyond the antiproliferative activity, the most effective analog and NAN demonstrated a moderate cytotoxic effect, that was 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 cells at the expense of G1 phase. Since the hypodiploid population indicates nuclear fragmentation, this examination evidenced the proapoptotic properties of the test compounds. While NTD8 triggered a fast 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.

Apoptosis inducing effect of the selected compounds was confirmed by means of fluorescent microscopy. Fast appearance of apoptotic morphologies 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 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. 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.

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.

Chemical structure of 19-nortestosterone derivatives may allows their binding affinity for nuclear receptors. According to our results, NTD8 had no substantial hormonal action in yeast-based experimental system. Antagonistic property of the tested analog was not observed in repeated examinations. 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 unfavourable. In this regard, integration of 17α-halogen into 19-nortestosterone skeleton is probably responsible for the loss of androgenic action.

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.

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* 2016; 6:27501-27516.

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* 2016; 105:113-120.

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* 2018; 9:825.

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* 2016; 112:36-46.

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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* 2019 (accepted for publication)