Final report of the 109293 OTKA project entitled
"Investigation of anti-breast cancer activity of newly synthesized nitrogen-containing steroids *in vitro* and *in vivo*"

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The aim of the supported project was the identification of steroidal original molecules with steroidal skeleton exhibiting potent anticancer properties. During the project a reasonably high number of compounds (approximately 800 molecules) were screened for antiproliferative action against a panel of human breast cancer cell lines (MCF7, T47D, MDA-MB-231, MDA-MB-361) by means of viability assay (MTT test). The most promising agents exhibiting antiproliferative properties in nanomolar concentration range were further investigated in order to determine the cancer selectivity of the effect using human fibroblasts or immortalized mammary cell line (hTERT-HME1). A variety of additional cell-based assays were performed with these selected agents to characterize the mechanism of the action. The investigations of the first potent and selected compounds have been completed mostly on cervical Hela cells instead of breast cancer cells because in many cases this cell line exhibited high selectivity towards the tested agent.

The results of the project were disseminated as a considerable number of research papers in leading international journals (45 papers) and presentations in conferences (19 presentations). Besides these publications the results of the project constituted the crucial part of several doctoral (Ph.D.) theses prepared at the Doctoral School of Pharmaceutical Sciences, University of Szeged: Judit Molnár (defended in 2015), Noémi Bózsity (defended in 2018), András Gyovai (thesis submitted) and Izabella Sinka (thesis under finalization). The reported OTKA project played an essential role in the doctoral thesis of the principal investigator which was submitted to the Hungarian Academy of Sciences in 2016 and defended in 2018.

The most relevant findings of the project are summarized in the followings. A set of androstane-based solanidine analogs were tested on human adherent cells six of them elicited the accumulation of a hypodiploid population of HeLa cells, indicating their apoptosis-inducing character, and a further one resulted in a cell cycle arrest at the G2/M phase. The most effective agents inhibited the activity of topoisomerase I, as evidenced by plasmid supercoil relaxation assays. One of the most potent analogs down-regulated the expression of cell-cycle related genes at the mRNA level (e.g. tumor necrosis factor alpha). The multidrug resistance reverting capacity of the molecules were additionally tested and some of the investigated compounds inhibited the ABCB1 transporter and caused rhodamine-123 accumulation in murine lymphoma cells transfected by human MDR1 gene, expressing the efflux pump. One of the most active agents from this aspect potentiated the antiproliferative action of doxorubicin without substantial intrinsic cytostatic capacity. The current results indicate that the modified solanidine skeleton is suitable for the rational design and synthesis of further innovative drug candidates with anticancer activities. The investigation of a similar set of solanodine analogs is in progress on breast cancer cell line panel (Zupkó et al. *Molecules*, 2014).

A molecular library containing D-secooxime derivatives of estrone were tested for antiproliferative action of adherent cell lines. It was concluded that the oxime function is needed for the action and 13-β configuration is favored. Some of the compounds exhibited activities with submicromolar IC_{50} values, better than that of the reference agent cisplatin. The most potent agent was investigated by flow cytometry. A cell cycle disturbance was revealed even at 3 μM after 24 hours of incubation and a longer exposure (1 μM for 48 h) resulted in an increased hypodiploid population indicating the proapoptotic property of the selected agent (Mernyák et al. *Steroids*, 2014a).

A set of D-homoestrone derivatives were tested and reasonable IC_{50} values (comparable to those of reference agent cisplatin) were obtained against adherent cell lines. The cancer selectivity of the most potent member was evidenced by means of MTT assay performed on noncancerous human fibroblast cells. According to in silico docking studies the selected molecule exhibits remarkable affinity to the colchicine binding site of tubulin. The finding was confirmed by in vitro tubulin polymerization assay: it increased the rate of tubulin polymerization similarly to reference agent paclitaxel (Mernyák et al. *Steroids*, 2014b).

A library of 26 estrone derivatives containing substituted triazoles at position 16 was screened for cell growth inhibitory action on a panel of human adherent cell lines. The most effective analogs were submitted to additional experiments in order to characterize their antiproliferative properties. As evidenced by flow cytometry, the selected steroids induced a disturbance in the HeLa cell cycle in a concentration- and exposure-dependent manner, through an increase of the hypodiploid population (subG1) and a cell cycle arrest in the G2/M phase. A noncancerous human fibroblast cell line (MRC5) was used to determine the selectivities of these compounds. Fluorescent microscopy after Hoechst 33258 - propidium iodide (HOPI) double staining revealed nuclear condensation and disturbed cell membrane integrity. The enhanced activities of caspase-3 and caspase-9 without activation of caspase-8 in the treated cells indicated the activation of the intrinsic pathway of apoptosis. The levels of cell cycle regulators (CDK1, cyclin B1/B2 and cdc25B) were decreased and the ratio Bax/Bcl-2 was increased 24 h after the treatment of HeLa cells (determined at an mRNA level by means of an RT-PCR technique). Under the same conditions, two agents elicited substantially increased degrees of phosphorylation of stathmin, as evidenced by Western blotting. The presented results demonstrate that these steroids can be regarded as appropriate structural scaffolds for the design and synthesis of further steroid analogs as innovative drug candidates with good efficacy (Minorics et al. *J Cell Mol Med*, 2015).

A set of androstenes containing 2-pyrazolines condensed on ring D investigated against four human breast and three cervical cell lines. The most promising agent exhibited IC_{50} values of 3.7 μM on MDA-MB-361 cells as determined by means of MTT assay. In order to obtain further information concerning the antiproliferative properties of this analog flow cytometric cycle analyses were performed. The compound elicited a marked and significant accumulation of MDA-MB-361 cells in the G2/M phase of the expense of the G1 population, which is consistent with a cell cycle blockade at the G2–M transition (Mótyán et al. *Steroids*, 2016).

A further set of compounds contained D- and A-ring-fused quinolines in the estrone and andostane series were initially investigated against the same seven human cell lines. One of them exhibited potency similar to that of reference agent cisplatin against estrogen-dependent breast cancer T47D (its IC50: 10.3 μM). The compound resulted in changes of the cell cycle distribution after 48 h of treatment. The selected compound induced concentration-dependent increase of subdiploid (SubG1) cells which was significant at both applied concentrations (10 and 30 μM) and suggested apoptotic fragmentation and appearance of apoptotic bodies. Besides this a significant decrease in the cell number of S and G2/M phases on the expense of G0/G1 population was also evidenced at the higher concentration. Based on this cell cycle behavior the inhibition of DNA synthesis can be suggested as an explanation of the antiproliferative property of the compound. Hoechst 33258 – propidium iodide fluorescent double staining was performed with T47D cells to provide morphological proof for the apoptotic potential of the selected agent. The treatment with 10 μM for 48 h resulted in appearance of higher proportion of apoptotic cells with chromatin condensation while 30 μM elicited necrotic feature too. To gain further evidence about the apoptosis-inducing effect of compound caspase-3 activity was determined from T47D cells after treatment for 48 h. The agent induced the enzyme activity in a concentration-dependent manner. Based on these data quinoline-fused steroids can be regarded as promising hit compounds for development of anti-breast cancer drug (Baji et al. *RSC Adv*, 2016).

A set of 22 compounds estrone-16-triazole basic structure has been tested on the cancer cell panel including 4 breast cell lines. The most potent agent exhibited low IC_{50} values (2.4-2.9 μM) on cancer cell but only a limited action on intact fibroblasts. Since HeLa cells proved to be the most sensitive the further experiments were carried out on this cervical cell line. After treatment of the cells 3 and 10 microM for 48 h the agent activated caspase-3 and caspase-9 without influencing caspase-8, confirming the induction of apoptosis via the intrinsic pathway. Also, it increased the mRNA expression of tumor suppressor p21 in a concentration- and time-dependent way. Morphological approach (Hoechst 33258 – propidium iodide fluorescent staining) was additionally used to confirm the proapoptotic potential of the selected molecule (Mernyák et al. *J Steroid Biochem Mol Biol*, 2015).

A set of C13 epimeric D-secoestrones containing substituted triazolyl group on ring A has been tested on 4 breast cancer cell lines, 3 cervical cell lines and A2780 (ovarian cancer cell line). The structure-activity relationships determining the antiproliferative potencies have been described. Briefly, the introduction of triazolyl function into ring A seems advantageous, especially with unsubstituted phenyl group. Concerning the C13 epimeric pairs beta epimeric compounds are generally more potent that alpha analogs. The most potent compound exhibited outstandingly low IC_{50} on some of the cells. The lowest IC_{50} was detected against HeLa cells (1.1 μM) and therefore this cell line was used for mechanistic studies. No substantial cytotoxic effect was evidenced up to 30 μM, therefore the agent can be considered cancer selective. The proapoptotic property of the compound has been evidenced by means of flow cytometry (concentration-increased increase of hypodiploid population), and colorimetric caspase-3 assay after 24 hours of incubation. Moreover, and fluorescent Hoechst 33258 – propidium iodide staining indicated the dominance of apoptosis over necrosis. Since the results of cell cycle

analysis indicated cell cycle blockade at G2/M the distinction between G2 and M populations seemed rational. Therefore 9-histone H3 was of the treated cells was labeled and the accumulation of cells at M phase was evidenced (similarly to reference agent paclitaxel). The direct action on the compound on the tubulin polymerization in a cell-free system was assayed and the results indicated the increase of the rate of polymerization. Therefore, the mechanism of our selected compound seems similar to paclitaxel. Additionally, to the original aim of the project the antimetastatic potential of the selected agent has been investigated within a framework of an international dual Ph.D. program. Migration of cancer cells was investigated using the wound healing assay. A cell-free strip on the confluent monolayer was prepared by a specific assay chamber infiltration was recorded by a CCD camera after 24 hours. Our compound significantly and concentration dependently inhibited the migration of cancer cells into the cell-free strip. The action on the invasion of the treated cells was tested by means of Boyden chamber assay. The invasion capacity of the cancer cells was substantially decreased by even 0.3 μM of the tested agent (Szabó et al. *Molecules*, 2016; Bózsity et al. *J Steroid Biochem Mol Biol*, 2017).

Based on our previous works oxime function may be considered as a pharmacophore when built on a steroidal skeleton. Therefore, a set of 12 oxime containing steroids have been prepared and investigated antiproliferative action and additionally for chemo-sensitizing activity. Four of them exhibited growth inhibiting activities against triple negative MDA-MB-231 breast cancer cells comparable to that of reference agent cisplatin. Though MCF7 cells exhibited generally less sensitivity many some of the compounds were active against cervical cancer cell lines (HeLa and SiHa). Their action on ABCB-mediated multidrug resistance was assayed by means of rhodamine 123 accumulation assay on transfected murine lymphoma cell line L5178. Four of the tested steroids substantially (above 50%) inhibited the activity of the transporter even at 2 μM and potentiated the antiproliferative action of doxorubicin on the resistant leukemia cell line (Vágvölgyi et al. *Eur J Med Chem*, 2018).

A previously tested pyrazole-containing androstane has been selected for additional mechanistic studies based on its low IC_{50} (below 3 μM) on our breast cancer panel. The cancer selectivity of the compound was determined by murine fibroblast cells (NIH/3T3) and its growth inhibiting action was less pronounced than on malignant cells. Cancer cells exhibited increased apoptotic and necrotic population after 24 h of treatment as evidenced by Hoechst-propidium fluorescent staining. The compound significantly increased the hypodiploid (subG1) population in treated MDA-MB-231 cells. Additionally, the activity of caspase-3 was increased after 24, 48 or 72 h treatment. The apoptosis was elicited by the activation of the intrinsic pathway evidenced by JC-1 mitochondrial staining. Finally, the treatment with the compound resulted in an approximately 50% decrease in tumor burden in a 4T1 model in mice. The publication of the results is still in progress.

As a continuation of our previous studies with 19-nortestosterone analogs the most promising agent was selected for mechanistic investigation. The growth inhibition was more pronounced against cervical and ovarian cell lines. The cell cycle analyses and tubulin polymerization data have been completed with lactate dehydrogenase (LDH) assay indicating the modest cytotoxic property of the tested agent. The proapoptotic effects of the tested steroids

were confirmed by fluorescence microscopy and caspase-3 activity determination. The treatment-related caspase-9 activation without substantial change in caspase-8 activity indicated the induction of intrinsic apoptotic pathway. Yeast-based reporter gene assay revealed that these examined 19-nortestosterones exhibit an order of magnitude weaker androgenic activity than 5α-dihydrotestosterone (DHT) (Gyovai et al. *Front Pharmacol*, 2018).

A set of newly prepared conjugates of estrogen and artemisinin (terpenoid) derivatives were recently included in our lead-finding project. Our aim was to investigate the antiproliferative properties of our original compounds, combinations of them and their new conjugates on four different breast cancer cell line (MDA-MB-231, MDA-MB-361, MCF7, T47D). Our data demonstrated that IC_{50} values of these new molecules were one order of magnitude lower than that of parent compounds which means a more potent antitumor effect. Morphological signs of apoptosis were detected after 24h incubation with our most potent conjugate on the most sensitive cell line (MDA-MB-361) by Hoechst-Propidium iodide fluorescent staining. Cell cycle analysis recorded by flow cytometry evidenced the increase of the ratio of MDA-MB-231 cells in G0/G1 phase followed by a decrease of the ratio of cells in S phase after 24h and 48h incubation indicating the inhibition of cell division. This study provides experimental evidence that chemical conjugation of artemisinin derivatives and estrogens results in new molecules with more potent antiproliferative properties than their parent compounds. These new compounds can be considered as promising new candidates in the field of anticancer research (Kulmány et al. *Acta Pharm Hung*, 2017).

Estrane analogs without hormonal activities (e.g. those with modified in D-ring) have been recently proposed as basic structure for innovative antiproliferative and antimetastatic drug candidates. A set of 16-hydroxymethyl-estranes was investigated for their anticancer properties on MCF-7, T47D, MDA-MB-231 and MDA-MB-361 cells. The most potent agent was selected for additional in vitro investigation in order to describe the mechanism of the action as well as to test its action on the metastatic ability of cancer cells. The most promising agents (IC_{50} values below 5 μM) exhibited modest cancer selectivity as determined using fibroblasts. The selected molecules elicited a cell cycle disturbance with increased G1 and decreased S and G2/M population after 24 h incubation. The same compounds blocked the cell migration at subantiproliferative concentration and inhibited the activity of matrix metallopeptidase 9 evidenced by wound healing assay and gelatin zymography, respectively. Estrane skeleton with modified D-ring can be regarded as a promising structure for design lead molecules with direct antiproliferative as well as antimetastatic capacity (Sinka et al. *Eur J Pharm Sci* 2017).