Final Report

1. Aims

Identification of selectively acting antitumoral therapeutics is one of the most important objectives of the modern drug development. Compounds inhibiting the biosynthesis or transport of hormones may serve as good candidates for the treatment of hormone-dependent tumors. The biosynthesis of estrogens is often inhibited by estrone-based compounds, which usually do not reach clinical therapy due to their retained estrogenic activity. The availability of selectively acting inhibitors without hormonal behavior would be of particular interest. **The aim of our research was the synthesis of novel selective intracrine modulators based on D-seco- or 13α-estrane core**. **The uniqueness of our research is its starting point, namely the use of the hormonally inactive core-modified estrone derivatives as basic compounds for the modifications. Syntheses of certain 13β-estrone derivatives were also designed with the aim of preparing the 13β-counterparts for comparative investigations.** Our research group has great experience in the transformations of estrone, but here we payed attention to green technologies (microwave-assisted synthesis and fluorescent labeling). The investigation of inhibitory action of the newly synthesized compounds on human aromatase, STS, 17β-HSD1, 17β-HSD2 or AKR1C1–3 activity was planned. The determination of the OATP2B1modulatory activity was subsequently performed. OATP-selectivity investigations were intended on OATP1B1, OATP1B3 and OATP2B1 with certain test compounds. Evaluation of *in vitro* antiproliferative action of the newly synthesized compounds against certain human reproductive cancer cell lines was also planned, including mechanistic analyses. The biochemical investigations have been complemented by molecular mechanistic and dynamic studies.

2. Results and discussion

2.1. Ring A halogenated estrone derivatives

2.1.1. Chemistry

Ring A halogenated 17-keto-, 17-deoxy-, D-seco-13α- and -13β-estrone derivatives with different groups at position 3 (**3-OH, 3-OMe, 3-OBn, 3-O-(*N*-benzyltriazolylmethyl)**) were synthesized [1–4, D1]. *N*-halosuccinimides (NCS, NBS, NIS) were used as electrophile triggers. The ***ortho*-substitutions** occurred at positions C-2 and/or C-4. ***Mono*-substituted and *bis*-substituted derivatives** were formed. **2,4-Disubstituted** compounds that included **different halogens** were also synthesized. The order of the halogen introduction had to be defined, whereby the smaller halogen had to be introduced first. The D-seconitrile, the 13α- and 13β-estrone and their 17-deoxy counterparts were additionally transformed by **Selectfluor** as an oxidating and fluorinating agent. The regio- and chemoselectivity of the fluorination greatly depended on the reaction conditions applied. **10β-Fluoro-1,4-dien-3-one derivative** was formed exclusively in acetonitrile at room temperature. Microwave assisted reaction at 50–60 ^{o}C lead to 2- and 4-monofluorinated aromatic product. **10β-Chloro derivatives** were also synthesized *via* reaction of the appropriate starting compound with *N*-chlorosuccinimide (NCS) in acetonitrile using catal. amount of (trifluoroacetic acid, TFA). Mechanistic studies suggest that reactions in acetonitrile occur via **SET**, while halogenations in TFA follow a different mechanism.

2.1.2. Biochemical investigations and computational simulations

The results obtained from the **aromatase assay** suggest that in estrane-based aromatase inhibitors, the presence of the **small, β-oriented halogen at C-10 and the 1,4-diene-3,17-dione moiety are advantageous** and might lead to potent derivatives with submicromolar inhibitory potential [2]. Docking calculations reinforced that the stereocenter in the 13 position has an important role in the binding affinity. **The D-secooxime, the 13α- and the 17-deoxyestrone core proved to be disadvantageous in this sense.** Our results might contribute not only to the research field of type I steroidal inhibitors of aromatase, but also to the development of biologically active compounds bearing substituted phenol moieties, by improving their biological potency *via* halogenations.

2-Halogenated 13α-estrone derivatives (3-OH) proved to be effective **17β-HSD1** inhibitors, however the **C-4 regioisomers** showed marked affinity to **STS** enzyme [1]. The newly synthesized halogenated derivatives had **no impact on the function of 17β-HSD2** enzyme. In summary, potent nanomolar or low micromolar **17β-HSD1 and/or STS** inhibitors were found with occasional dual or multiple inhibitory properties. Valuable structure–activity relationships were established from the comparison of the inhibitory data obtained. **Kinetic experiments** performed with selected compounds revealed **competitive reversible inhibition mechanisms against 17β-HSD1** and **competitive irreversible** manner in the inhibition of the **STS enzyme**.

Certain **potent AKR1C1–3 inhibitors** were identified, with occasional **dual or triple** inhibitory properties [4]. **Selective compounds** were also found against each of these three enzymes. These halogenated estrones represent a **new class of potent and selective AKR1C inhibitors**, and thus have the potential for development of new antitumor agents against **chemoresistant tumors**.

Data obtained from the **OATP–selectivity** investigations provide valuable structure-activity relationships [5, 6]. **C-2 Halogenated 13α-estrones** proved to be **selective** submicromolar **inhibitors of OATP2B1**. However, the **C-4 regioisomers**, bearing large halogens had great influence on the transport function of **both OATP1B1 and OATP1B3**. These results might help in the design of more potent, selective, estrone-based OATP inhibitors. The newly identified inhibitors and the structure–activity relationships specified here promote our understanding about **drug recognition of OATPs**.

The results obtained from the ***in vitro* antiproliferative assays** reveal that **the cell growth-inhibitory potential** of the newly synthesized halogenated estrone derivatives greatly **depends on** their **regiochemistry** and the **nature of the substituent at C-3** [3]. **The most potent compound, the 3-*O*-(*N*-benzyltriazolylmethyl)-4-bromo-13α-estrone derivative** exerted substantial selective cell growth-inhibitory activity against A2780 cell line with a submicromolar IC_{50} value. Computational calculations reveal **strong interactions** of the 4-bromo derivative **with both colchicine and taxoid binding sites of tubulin**. Disturbance of tubulin function has been confirmed by photometric polymerization assay. **C-2 halogenated 3-hydroxy 13α-estrones** had a **selective OATP2B1-mediated cell growth inhibitory effect**. In order to demonstrate that increased sensitization can be attributed to OATP2B1-mediated cellular uptake, **tritium labeled 2-bromo-13α-estrone** was synthesized for direct transport measurements [7]. These experiments revealed **increased accumulation of [^{3}H]2-bromo-13α-estrone due to OATP2B1 function**. Our results indicate that C-2 halogenated 13α-estrones are good candidates in the design of anticancer drugs targeting OATP2B1.

2.2. Pd- or Ni-Catalyzed cross-coupling and C–H activation reactions on ring A

2.2.1. Chemistry

Pd-Catalyzed cross couplings at the **C-2 or C-4** position of the **13α-estrane core** [8–10, D1] were carried out. Starting from steroidal aryl halides, **microwave-assisted C–C, C–N or C–P couplings** were performed. **Suzuki–Miyaura** reactions were carried out using substituted phenylboronic acids as reagents leading to biphenyl derivatives [10]. **Buchwald–Hartwig** aminations of the bromoarene substrates with aniline derivatives led to phenylamino compounds [8]. 2-Amino-13α-estrone was also synthesized in a two-step protocol including an amination of 2-bromo-13α-estrone 3-benzyl ether with benzophenone imine and subsequent hydrogenolysis. **Hirao** couplings facilitated the functionalization of the ring A with substituents differing in size and polarity [9]. Bromo regioisomers (2- or 4-) of 13α-estrone and its 3-benzyl or 3-methyl ether were reacted with diethyl phosphite or diphenylphosphine oxide using $Pd(PPh_3)_4$ as catalyst under microwave irradiation. **Steroidal phosphonates and tertiary phosphine oxide derivatives** were formed. In addition to positions C-2 or C-4, the **3-OH group was also modified** by synthesizing 3-*O*-alkyl, 3-*O*-aralkyl or sulphate derivatives.

Compound **(3-benzyloxy-13α-estra-1,3,5(10)-trien-17-on-2-yl)-diethylphosphonate** served as the initial compound for further slight modifications [12]. This compound exhibited **the highest affinity as a 13α-estrone-based OATP2B1 inhibitor** [9] and it offered excellent possibilities for reasonable structural modifications. **Diversification of structure** included indirect modification of the diethylphosphonate moiety, reduction or removal of the C-17 keto function, fluorescent labeling or switching to the 13β-estrone series.

In addition to phenylations *via* Pd-catalyzed cross-coupling reactions of steroidal aryl halides with boronic acid reagents [10], an alternative methodology, based on **C–H activation** was elaborated [11]. The **inconveniences of cross-coupling reactions**, such as halogenation of the substrates and the use of organometallic nucleophilic coupling partners **could be circumvented** by the C–H activation methodology. We introduced ***N*- and/or *O*-containing directing groups** onto the phenolic 3-OH function of 13α-estrone and its 17-deoxy counterpart. The resulting **carbamate, pivalate or sulfamate esters** proved to be suitable for **regioselective *ortho*-arylations *via* Pd-catalyzed C–H activation**. A **mild and efficient microwave-assisted methodology** was elaborated. Arylation of a carbamate or pivalate and the removal of the directing group were achieved *via* a **one-pot, tandem, microwave procedure**. The newly synthesized **phenol esters** were suitable electrophilic substrates in microwave-induced, **Ni-catalyzed Suzuki–Miyaura couplings** with phenylboronic acid as a nucleophilic reagent.

2.2.2. Biochemical investigations and computational simulations

Certain **biphenyl derivatives** displayed **substantial antiproliferative action** against human reproductive cancer cell lines [10, 11]. **2-(4-Chlorophenyl)-13α-estrone** was found to be the most potent compound with low micromolar cell growth inhibitory action against MCF-7 and HeLa cell lines. An important structure–activity relationship was found, since the 3-benzyl ether counterpart proved to be ineffective. Sulfamate derivatives seemed to be superior concerning their substantial antiproliferative potential. **2-(4-Chlorophenyl)-13α-estrone sulfamate** displayed outstanding growth inhibitory action **against the two cervical cancer cell lines** with different HPV-status [11]. **The presence of an *N*,*N*-dimethylsulfamate pharmacophore together with the 2-(4-chlorophenyl) moiety improved the antitumoral action.** Considering that HPV-16 and HPV-18 play a causative role in the majority of cervical cancer cases, newly

identified compound with its hormonally inactive core might be a promising candidate in the design of new anticancer agents acting selectively.
C-2 Phosphonated derivatives proved to be **dual OATP2B1** and **17β-HSD1 inhibitors** [9]. **None** of the newly synthesized organophosphorus derivatives displayed **marked STS inhibitory activity**. 3-Benzyloxy-2- and 4-regioisomers of diethylphosphonates inhibited the transport function of all three investigated OATPs by nano- or low micromolar IC_{50} values [6]. This non-selective inhibitory action is out of accord with that obtained for 2-halogenated compounds. **13β-Derivatives** obtained by slight modification of the 3-benzyloxy C-2 phosphonate displayed outstanding OATP2B1 inhibitory action with IC_{50} values in the **nanomolar range** [12]. The newly identified inhibitors and the structure–activity relationships specified here promote further our understanding about drug recognition of OATP2B1.

2.3. Fluorescent labeling with BODIPY dyes

2.3.1. Chemistry

BODIPY–estrone conjugates were synthesized without transforming the two oxygen functionalities of the steroid and by establishing a chemically and metabolically stabile attaching moiety [13, D1]. Therefore, we chose positions **C-2 or C-15 for labeling**, thereby avoiding covalent modifications at C-3 and C-17. At first we prepared the building elements for the couplings. That is, the BODIPY derivatives bearing terminal alkyne or azide functions and the estrone derivatives possessing the complementer functions were synthesized. BODIPY-alkyne was synthesized using aldehyde–pyrrole condensation strategy, however the parent BODIPY derivative bearing azide function was synthesized using acyl chloride–pyrrole condensation strategy. Estrone azide and –alkyne were synthesized starting from the α,β-unsaturated ketone intermediate by Michael addition as the key step. The alkyne function was introduced not only indirectly onto the C-15, but also directly onto the C-2 position *via* **Sonogashira reaction**. With alkyne and azide complementers in hand, **Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reactions** were carried out in order to synthesize the desired fluorescent estrone conjugates, labeled at C-2 or C-15.
Next, novel **BODIPY–estrone** or **BODIPY–estradiol** conjugates have been synthesized by selecting positions **C-2 or C-3-*O* for labeling** [C1, C2]. The conjugation strategy was based on **CuAAC** or **Pd-catalyzed coupling**. Estradiol derivatives used as azide partners bearing ω-azidoalkyl function through **C_4–C_8-long linkers** have been prepared. CuAAC reactions of estradiol azides with BODIPY alkynes furnished fluorescent 3-*O*-labeled conjugates bearing triazole ring as a coupling moiety. Pd-Catalyzed couplings of 3-*O*-(ω-bromoalkyl)-17β-estradiol derivatives with BODIPY alkyne resulted in labeled conjugates connected with an ether moiety. Labeling of 2-(4-azidobut-1-ynyl)-estrone with BODIPY alkyne via CuAAC furnished a **C-2 substituted fluorescent derivative** containing a C_4 linker unit [C1, C2].
Our newly established labeling strategy was extended to modification of an effective OATP2B1 inhibitor. A **BODIPY-13α-estrone organophosphorus conjugate** modified at C-3-*O* of the steroid, containing a four-carbon linker between the triazole moiety was synthesized [12].

2.3.2. Biochemical investigations

The **BODIPY-13α-estrone phosphonate** displayed efficient, **submicromolar OATP2B1 inhibitory potency** [12]. This result suggests that **the conjugation strategy** at the C-3-*O* moiety through a four-carbon-long linker seems to be **appropriate** concerning the binding to the transporter protein. Notably, to the best of our knowledge, this conjugate is **the first BODIPY-labeled estrone derivative with high affinity to the OATP2B1 transporter**. Nevertheless, the present labeling methodology allows the development of other fluorescently

labeled 13α-estrone-based OATP2B1 inhibitors with improved biological and/or optical properties.
The **BODIPY-labeled derivative of 15-*O*-propargyl estrone** displayed **potent 17β-HSD1 inhibitory action** without affecting the 17β-HSD2 function. The manuscript containing these results is under preparation. The synthetic work and the biochemical investigations have been finished, we are waiting for the results of computational simulations.

2.4. Modifications at ring D of estrone or 13α-estrone

2.4.1. Chemistry

Introduction of an endocyclic Δ^{15}-double bond into the ring D of 3-hydroxy, 3-*O*-methyl- or 3-*O*-benzyl-estrone or their 13α-estrone counterparts allowed the synthesis of various **15β-alkoxy** or **–azido** derivatives [13, 14]. Substituents differing in size and polarity were introduced onto C-15 with the aim of getting potential enzyme inhibitors. Special emphasis was placed to elaboration of **diastereoselective functionalizations at ring D**, since the biological activity of estrone derivatives greatly depends on the substitution pattern of this ring. Beside the syntheses of 15-substituted compounds, **derivatizations at C-16** were also performed. **Microwave-assisted phospha-Michael addition reactions** were carried out in the 13α-estrone series [15]. The exocyclic 16-methylene-17-ketones as α,β-unsaturated ketones were reacted with secondary phosphine oxides as nucleophilic partners. The addition reactions furnished the two tertiary phosphine oxide C-16 diastereomers in high yields. The main product was the 16α-isomer. The **Noyori-type** (1S,2S)- and (1R,2R)-*N*-(*para*-tosyl)-1,2-diphenylethylene-1,2-diamine **ligands complexed with ruthenium** have been found to be effective catalysts for the regiospecific transfer hydrogenation of 16-hydroxymethylidene-17-ketones to 16-hydroxymethylene-17-ketone diastereomers [16]. Further reduction of the isolated products with $NaBH_4$ in the presence of cerium(III) chloride (**Luche reduction** conditions) yielded the corresponding diols. In contrast to the previous preparation methods, this two-step simple hydrogenation/reduction protocol afforded all four possible isomers in almost equal amounts. With the aim of performing further stereoselective transformations on ring D of estrone, synthesis of **novel 16α,17α-oxazoline derivatives** was carried out [17]. The reaction of the sterically unhindered 16α-azido-17α-hydroxy-estrones with a range of benzaldehydes under the condition of the Schmidt rearrangement yielded 16α,17α-oxazoline derivatives.

2.4.2. Biochemical investigations

Concerning the biological activity of ring D substituted newly synthesized compounds, certain derivatives proved to be promising. Several **15-substituted derivatives** displayed substantial **17β-HSD1 inhibitory action** [14]. The **AKR1C1–3 inhibitory assays** reveal that the **15β-*O*-propargyl and the 15β-azido compound** inhibits only the **AKR1C2 enzyme**, thereby acting in a **selective** manner. The manuscript containing these results is under preparation. Concerning the **16-substitued** derivatives, the most potent compound, one **diphenylphosphine oxide derivative in the 3-*O*-methyl-13α-estrone series**, exerted **selective cell growth-inhibitory activity against UPCI-SCC-131 and T47D cell lines** with low micromolar IC_{50} values. Moreover, it displayed **good tumor selectivity property** determined against non-cancerous mouse fibroblast cells [15].

3. Novelties of the research

3.1. Elaboration of **new, microwave-assisted, transition metal–catalyzed cross-coupling or regioselective C–H activation methodologies** with special emphasis to green technologies.
3.2. Elaboration of **stereoselective synthetic methods** for modifications of ring D.
3.3. Development of **novel green, fluorescent labeling methodologies** of natural estrogens or estrone-based intracrine modulators.
3.4. Identification of **selective or multiple 17β-HSD1, STS, aromatase and/or OATP inhibitors** (on **hormonally inactive estrane core**) and verification of important structure-activity relationship data.
3.5. Identification of a **new compound group**, namely 13α-estrone derivatives, as **inhibitors of the AKR1C1–3 enzymes** or the **OATP transporters**.
3.6. Confirmation of **mechanism of action** of certain newly developed intracrine modulators.

4. Conclusions, dissemination and usability

New estrone derivatives, with substituents at C-2 and/or C-4, differing in size and polarity were synthesized *via* transition metal-catalyzed cross-coupling or C–H activation reactions. The cross-coupling reactions required prefunctionalization of the substrates, namely halogenation of ring A. However, the haloarenes proved to be especially promising concerning their biological activity. Utilization of **Pd- or Ni-catalysis** allowed the attachment of the phenyl group directly or indirectly (through a nitrogen or a phosphorus atom) to the aromatic ring of the steroid. The phenolic hydroxyl function of estrone derivatives was additionally modified. Besides ring A modifications, **diastereoselective transformations** were achieved on **ring D** of certain estrone derivatives. The knowledge gained in the directed modifications of steroidal compounds has been exploited in the **syntheses of fluorescent BODIPY dyes and their estrone conjugates. BODIPY–estrone (–estradiol) conjugates labeled at C-2, C-3-*O* or C-15** were synthesized. The measured fluorescence properties of the compounds suggest that they might be applicable for their observation in living cells.

The newly synthesized ring A and/or ring D modified estrone derivatives and BODIPY-conjugates have been subjected to **diverse biochemical investigations**. We expected that the targeted structure diversification might provide valuable structure-activity relationship data. **Our results confirm that structurally different enzymes with distinct catalytic mechanisms or transporters might be inhibited by the same inhibitor compounds**. Newly detected **multiple 17β-HSD1, STS, aromatase and/or OATP** inhibitors might be **superior to** compounds affecting the action of only a **single enzyme or transporter**. These multiple inhibitors may serve as good candidates for efficient suppression of local estrogen production in certain cancer tissues. A precise understanding of the substrates and modulators (inhibitors and stimulators) of OATPs would be of particular interest. However, this is not straightforward, since no crystal structures for OATPs are available. **Our comparative study provides novel insights into hepatic OATP-ligand interactions and selectivity.** Furthermore, **the integrative computational workflow** for structure-based modeling **can be leveraged for other pharmaceutical targets of interest**.

Considering that a labeled estrogen could allow determination of steroid uptake, transport and binding to certain proteins on a single-cell level, the understanding of the mechanism of action of such compounds would be of particular interest. **Our labeling strategy enables variations in the length of linkers, thereby providing a library of fluorescing conjugates.** The selection

of the site of conjugation as well as the nature of the substituents on the estrone moiety and the use of different linkers allow for the determination of the effect of structural modifications on the biological properties of the labeled compound. The methodologies developed should find extensive applications owing to the great importance of fluorescent labeled biomolecules. **The newly synthesized labeled estrone derivatives may serve as good candidates for the development of imaging probes for biological assays.** Enzymatic or receptorial assays are mainly based on radioisotope labeling. Nowadays, there is a need for the replacement of the harmful radioactive methods for greener and friendlier fluorescent ones. However, to the best of our knowledge, there is no reported general use of BODIPY-labeled estrone derivatives in fluorescent biological assays.

Concerning the **dissemination** and **usability** of project results, it should be underlined, that project results will be applied in **gradual and postgradual university education**. This project is **highly valuable in terms of scientific impact** on the science community. The outcomes of the project have **public health relevance**, too. **Our newly developed fluorescent labeled estrones might be used in estrone-based biochemical assays.**

5. Researcher employment – explanation

A full-time (40 hours per week) employment of one person in research assistant position at the Department of Organic Chemistry, University of Szeged was planned. However, a research assistant (Ildikó Bacsa) worked on this project only for some months. The remaining salary planned for this position was spent on the students working in this research. Students performed organic chemical synthesis, production, purification, structure determination of target compounds, and their preparation for biochemical tests, prepared laboratory reports, studied literature and monitored databases.

6. References

6.1. Scientific journal publications ΣIF = 65.927 (Web of Science)

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6.2. PhD dissertation

D1: Bacsa, I. Ösztrán vázas vegyületek A- és D-gyűrűben történő módosítása, PhD dissertation, University of Szeged, 2018.

6.3. Conferences

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C2 Szabó Vivien, Liliom Andrea, Mernyák Erzsébet: 17β-Ösztradiol fluoreszcens jelölése, MKE Vegyészkonferencia, Eger, 2019, 2019