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Cytotoxic effect of 13α-estrane derivatives on breast, endometrial and ovarian cancer cell lines

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ABSTRACT

Hormone-dependent cancers such as breast, uterine, and ovarian cancers account for more than 35% of all cancers in women. Worldwide, these cancers occur in more than 2.7 million women/year and account for 22% of cancer-related deaths/year. The generally accepted mechanism for the pathophysiology of estrogen-dependent cancers is estrogen receptor-mediated cell proliferation associated with an increased number of mutations. Therefore, drugs that can interfere with either local estrogen formation or estrogen action via estrogen receptors are needed. Estrane derivatives that have low or minimal estrogenic activity can affect both pathways. In this study, we investigated the effect of 36 different estrane derivatives on the proliferation of eight breast, endometrial, and ovarian cancer cell lines and the corresponding three control cell lines. Estrane derivatives **3** and **4_2Cl** showed a stronger effect on the endometrial cancer cell lines KLE and Ishikawa, respectively, compared with the control cell line HIEEC, with IC50 values of 32.6 microM and 17.9 microM, respectively. Estrane derivative **4_2Cl** was most active in the ovarian cancer cell line COV362 compared to the control cell line HIO80 with an IC50 value of 3.6 microM. In addition, estrane derivative **2_4I** showed a strong antiproliferative effect on endometrial and ovarian cancer cell lines, while the effect on the control cell line was slight or absent. The addition of halogen at carbon 2 and/or 4 in estrane derivatives **1** and **2** increased the selectivity for endometrial cancer cells. Overall, these results suggest that single estrane derivatives are efficient cytotoxic agents for endometrial and ovarian cancer cell lines, and thus potential lead compounds for drug development.

1. Introduction

Hormone-dependent cancers include breast cancer (BC), endometrial cancer (EC), and ovarian cancer (OC), which account for more than 35% of all cancers in women [1]. BC is the most common cancer worldwide with 2.3 million new cases and 684,996 deaths in 2020. EC and OC are the most frequent gynecological cancer and the deadliest hormone-dependent cancer, with 417,367 and 313,959 new cases in 2020, respectively [2]. BC and EC together account for 37% of all new cases, while together with OC account for 24% of cancer deaths [3]. The enormous number of patients and deaths attributable to these hormone-dependent diseases highlights the importance of new therapeutic strategies. The generally accepted mechanism for the pathophysiology of estrogen-dependent cancers, including BC and EC, is estrogen receptor-mediated cell proliferation associated with an increased number of mutations. Drugs currently used to treat estrogen receptor positive breast cancer interfere with local estrogen formation and estrogen action. Aromatase inhibitors have also been used in EC, but with less effect [4]. The presence of estrogen biosynthesis enzymes and estrogen receptors has been confirmed in ovarian cancer [5], demonstrating the involvement of estrogens in pathophysiology and justifying the use of estrogen deprivation therapy [6]. Current estrogen deprivation therapies, which include aromatase inhibitors and selective estrogen receptor modulators, are not optimal and lead to estrogen refractory

Abbreviations: BC, breast cancer; EC, endometrial cancer; OC, ovarian cancer; HGSOC, high grade serous ovarian cancer; IC50, half maximal inhibitory concentration; STS, steroid sulfatase; 17β-HSD1, 17-beta-hydroxysteroid dehydrogenase type 1; OATP2B1, solute carrier organic anion transporter family member 2B1; GPER, G protein-coupled estrogen receptor; AKR, aldo-keto reductase.

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disease. Estrone derivatives are potential inhibitors of estrogen biosynthesis and uptake and therefore may exhibit antiproliferative effects due to their low estrogenic activity. The lower estrogenic activity of estrone compared with that of its 17β-hydroxy counterpart could be further reduced by the introduction of one or two halogen atoms at positions C2 and/or C4 [7–9]. Other structural changes in the estrane skeleton could also lead to a reduction or complete loss of estrogenic activity. These include opening of the D-ring or configuration reversal at C13 [10–13]. We recently presented the results of biochemical studies of A-ring halogenated 13β- and 13α-estrogen derivatives with the D-ring intact or 17-deoxy [14–18]. These derivatives were previously tested for their inhibition of various enzymes involved in the estrogen pathway, including STS, 17β-HSD type 1 (HSD17B1), and AKR1C enzymes and the OATP2B1 transporter, and some of the compounds showed remarkable results [14–20]. Potent enzyme inhibitors with submicromolar or low micromolar IC_{50} values were identified, exhibiting selective or multiple inhibitory activities. Important structure-activity results were obtained that could make a valuable contribution to the development of selectively acting estrogen-based anticancer agents. In this research, we investigated a series of 36 estrane derivatives previously characterized for their inhibitory activity as potential agents against the proliferation of BC and EC and HGSOC, the most common subtype of OC.

2. Methods

2.1. Synthesis and characterization of estrane derivatives

A total of 36 compounds were tested in this study. The compounds were prepared from the starting compounds (1−4) shown in Fig. 1:

The epimeric 13β- or 13α-estrane derivatives investigated in this study (Supplementary Table 1) were synthesized and characterized as described previously (13−17).

2.2. Cell lines

In this study we used model cell lines from BC (MCF7), EC (Ishikawa, Hec-1-A, KLE, RL95–2), and high grade serous ovarian cancer (HGSOC; Kuramochi, COV362, OVSAHO) and the corresponding control cell lines (human breast epithelial cell line MCF10A, control endometrial cell line HIEEC, ovarian surface epithelial cell line HIO80). Cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C. All cell lines were cultured in media without antibiotics and were negative for mycoplasma infection, which was regularly tested using the MycoAlertTM mycoplasma detection kit (Lonza, Basel, Switzerland).

The control cell line **MCF10A** (CVCL_0598) was originally derived from the fibrocystic mammary gland of a 36-year-old parous premenopausal Caucasian woman. The cell line was acquired from LGC Standards GmbH, Wesel, Germany on 4th April, 2019 [21]. MCF10A cells were grown in DMEM:F12 medium (D0547) containing 5% FBS (F9665), 10 μg/mL insulin (19278), 20 ng/mL EGF (AF-100–15; stock solution 100 μg/mL), 0.5 μg/mL hydrocortisone (H0888–1 G, all from Sigma-Aldrich, St. Louis, MI, USA). Cells in passage + 8 to + 14 were used in this study and seeded at concentration of 1×10^4 cells/mL. Authentication by STR profiling was performed by ATCC in 2019.

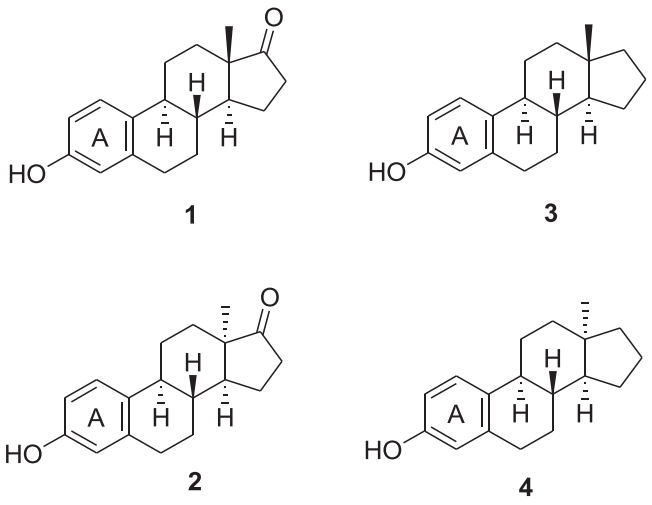


Fig. 1. Starting compounds for the synthesis of estrane derivatives.

The human breast adenocarcinoma cell line **MCF-7** (CVCL-0031) was originally derived from a 69-year-old patient [22], and it was acquired on 12th January 2016 from Sigma-Aldrich (86012803; lot number 14/018; Sigma-Aldrich, St. Louis, MI, USA). MCF-7 cells were grown in Minimum Essential Medium Eagle (M5650; Sigma–Aldrich GmbH) containing 10% FBS (F9665) and 0.01 mg/mL insulin (I9278) (all from Sigma-Aldrich, St. Louis, MI, USA). Cells in passage + 15 to + 26 were used in this study and seeded at concentration of 4.5×10^4 cells/mL. Authentication by STR profiling was performed by ECACC on 5th October 2016.

The endometrial control cell line **HIEEC** was obtained from Michael A. Fortier (Laval University, Quebec, Canada) on 4th April 2014 as p14. It was originally prepared from a primary culture obtained from an endometrial biopsy taken from a 37-year-old woman with confirmed absence of neoplasia and endometriosis [23], on day 12 of her menstrual cycle. HIEEC cells were grown in RPMI-1640 medium (R5886) supplemented with 2 mM L-glutamine (G7153) and 10% fetal bovine serum (F9665, all from Sigma-Aldrich, St. Louis, MI, USA). HIEEC cells in passage + 12 to + 19 were used in this study and seeded at concentration 5×10^4 cells/mL. Cells in passage p + 8 were authenticated by STR profiling performed by ATCC on 8th March 2018.

The **Ishikawa** cell line (CVCL_2529) was originally derived from an endometrial adenocarcinoma from a 39-year-old Asian woman [24], and was acquired on 18th December 2012 from Sigma–Aldrich (ECACC99040201; Sigma-Aldrich Chemie GmbH; Deisenhofen, Germany) as p + 3. Cells were cultured in Eagle's Minimum Essential Medium (M5650) supplemented with 2 mM $NaHCO_3$ (S3817) and 5% FBS (F9665, all from Sigma-Aldrich Chemie GmbH; Deisenhofen, Germany). Cells in passage + 12 to + 16 were used in this study and seeded at concentration of 4×10^4 cells/mL. Cells p + 13 were authenticated by STR profiling by ATCC on 22nd February 2018.

The **HEC-1-A cell line** (CVCL_0293) was originally derived from an endometrial adenocarcinoma from a 71-year-old patient [25], and it was acquired from the American Type Culture Collection (ATCC_HTB-112TM) on 31st May 2012 as p125. The growth medium for HEC-1-A cells was McCoy's 5 A medium (M4892; Sigma-Aldrich, St. Louis, MI, USA), containing10% FBS (F9665). HEC-1-A cells in passage + 10 to + 17 were used in this study and seeded at concentration of 5×10^4 cells/mL. Cells in passage p + 15 were authenticated by STR profiling performed by ATCC on 22nd February 2018.

The **KLE** cell line (CVCL_1329) was originally derived from a poorly differentiated endometrial carcinoma from a 68-year-old patient [26], and was acquired from the American Type Culture Collection (ATCC_CRL-1622™, lot 70001143) on 18th October 2017 as p + 12. The growth medium for KLE cells was DMEM:F12 (D6421; Sigma–Aldrich GmbH) supplemented with 10% FBS (F9665) and 2.5 mM L-glutamine (G7153). KLE cells in passages + 18 and + 27 were used in this study and seeded at concentration of 8×10^4 cells/mL. Authentication by STR profiling was performed by ATCC in 2017.

The **RL95–2** (CVCL_0505) cell line was originally established from a grade 2 adenosquamous endometrial carcinoma from a 65-year-old patient [27], and was acquired from the American Type Culture Collection (ATCC_ CRL-1671™, lot 62130010) on 18th October 2017 as p125. Growth medium for RL95–2 cells was DMEM/F12 (D6421; Sigma–Aldrich GmbH), containing 10% FBS (F9665), 2.5 mM L-glutamine (G7153) and 5 μg/mL insulin (I9278). For this study, RL95–2 cells were used in passages + 12 and + 15 and seeded at a concentration of

3.5×10^4 cells/mL. Authentication by STR profiling was performed by ATCC in 2017.

The control ovarian cell line **HIO80** (CVCL_E274) was obtained from Andrew K. Godwin (University of Kansas Medical Center, Kansas, USA) on 20th October 2017 as p + 72. It was originally derived from ovarian surface epithelium [28]. HIO80 cells were grown in 1:1 mixture of medium 199 (M5017; Sigma-Aldrich GmbH) and MCDB105 medium (M6395) supplemented with 4% FBS (F9665) and 7.5 μg/mL insulin (I9278). HIO80 cells in passage + 10 to + 12 were used in this study and seeded at concentration 2×10^4 cells/mL. HIO80 in passage p + 10 were authenticated by STR profiling performed by ATCC on 22nd Februar 2019.

The **Kuramochi** cell line (CVCL_1345) was originally derived from high grade ovarian serous adenocarcinoma from a metastatic site in the ascites [29] and was acquired from JCRB (JCRB0098 lot 06302015) on 23rd October 2017 as p17. Growth medium for Kuramochi cells was RPMI (R5886; Sigma–Aldrich GmbH), containing 10% FBS (F9665) and 2 mM L-glutamine (G7153). In this study Kuramochi cells in passages 125 + 7–125 + 12 were used and seeded at concentration of 7.5×10^4 cells/mL. Authentication by STR profiling was performed by JCRB on 30th October 2017.

The **COV362** (CVCL_2420) cell line was originally established from a high grade ovarian serous adenocarcinoma from metastatic site in pleural effusion [30] and it was acquired from ECACC (ECACC 07071910) on 13th October 2017 as p37. Growth medium for COV362 cells was DMEM (D5546; Sigma–Aldrich GmbH), containig 10% FBS (F9665) and 2 mM L-glutamine (G7153). In this study COV362 cells in passages 37 + 12–37 + 22 were used and seeded at concentration of 3.5×10^4 cells/mL. Authentication by STR profiling was performed by ECACC in 2017.

The cell line **OVSAHO** (CVCL_3144) was originally established from a serous papillary adenocarcinoma from a metastatic site in the abdomen [31] from a 56-year-old woman and was acquired from JCRB (JCRB1046 lot 04062015) on 4th June 2018 as p44. Growth medium for OVSAHO cells was RPMI (R5886; Sigma–Aldrich GmbH), containig 10% FBS (F9665) and 2 mM L-glutamine (G7153). In this study OVSAHO cells in passages 125 + 8 and 125 + 12 were used and seeded at concentration of 35×10^4 cells/mL. The cells were authenticated by JCRB in 2018.

2.3. Proliferation assay

Cells were seeded in a 96-well plate in 100 μL medium and allowed to adhere and grow for 24 h. Estrane derivatives were dissolved in DMSO to a final 0.01 M stock solution. After the initial incubation, cells were treated with either 100 μM of compound or increasing concentrations of compounds (0.001 μM - 100 μM) at final volume of 180 μL to determine the IC_{50}. Because DMSO affects cultured cells, its concentration was kept below 1%. For control, DMSO only was added to the cells, in the same final volume as the test compound. After 48 h, 20 μL AlamarBlue reagent (A50100, Thermo Fisher Scientific Chemie GmbH; MA, USA) was added per well and the plate was incubated at 37 °C and 5% CO_2 for 4 h The absorbance was measured using the BioTek microplate spectrophotometer at 570 nm, with the reference wavelength set at 600 nm. All the experiments were performed with three technical replicates and at least two biological replicates. The survival rate was determined as a ratio between the test compound and the control (DMSO only). The half-maximal concentration (IC_{50}) was determined for estrane derivatives with high cell growth-inhibitory effects by generating a dose-response curve (Graph Pad Prism, Version 8.0). Statistical analysis was performed using the Kruskal-Wallis test.

3. Results and discussion

We evaluated the anti-proliferative effect of synthesized estrane derivatives on cancer cell lines of three hormone-dependent cancers (BC, EC and HGSOC). A total of 36 different compounds were tested at a final concentration of 100 μM. Cell viability in the presence of estrane derivatives was determined by measuring metabolic activity using the AlamarBlue assay [32]. The assay is based on the reduction of resazurin to resorufin, which occurs only in viable cells, by electron transfer within the respiratory chain. The advantage of this assay is the formation of a water-soluble fluorescent product, sensitivity and low costs, while the limited linear range and further reduction in some cells to dihydroresorufin, which is non fluorescent and highly toxic, are the disadvantages of this assay.

3.1. Estrane derivatives with high antiproliferative effects on cancer cells

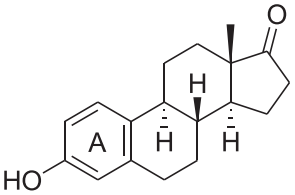
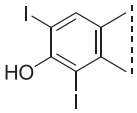
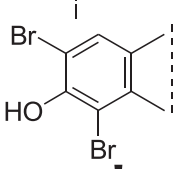
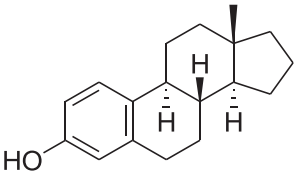
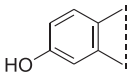
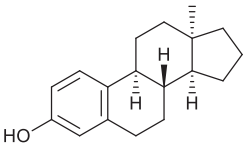
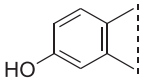
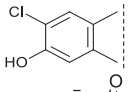
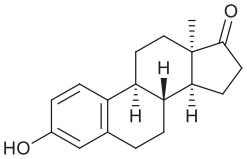
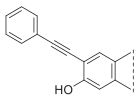
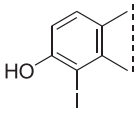
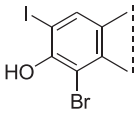
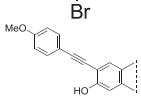
Based on the results of this screening (Supplementary table 1), we selected compounds that reduced cancer cells growth by more than 80%. The IC_{50} was determined for the most potent antiproliferative compounds. The compounds that had strong growth inhibitory effects in the BC cell line MCF7 were compounds **3**, **4**, **4_2Cl**, **2_2HPh**, **4_2Br**,**4Br**, **4_2I**,**4I** (Table 1). All of the compounds also had a strong antiproliferative effect on the breast control cell line MCF10A, as the survival rate in the presence of these compounds was less than 20%. The compounds that were strongly antiproliferative for at least one EC cell line were compounds **1_2I**,**4I**, **1_2Br**,**4Br**, **3**, **4**, **4_2Cl**, **2_2HPh**, **2_4I**, **2_2I**, **4Br**, **2_2MeOPh**, **2_2Br**, **4Cl**, **2_2Br**, **4I**, **2_2I**, **4Cl**, **4_2Br**, **4Br**, **4_2I**,**4I**. However, seven of the fourteen compounds decreased survival of the control endometrial cell line HIEEC by more than 80%, while six other compounds also had strong antiproliferative effect and decreased survival by more than 48%. Only one of the compounds tested, **2_4I**, had little or no antiproliferative effect on the control HIEEC cell line. The compounds that had strong antiproliferative effects on at least one HGSOC cell line were compounds **4_2Cl**, **4Cl**, **3**, **4**, **2_2Cl**, **4_2Br**, **4Br**, **4_2I**, **4I**. All of these compounds were also strongly antiproliferative for the ovarian control cell line HIO80 with survival rate less than 20% in presence of the compounds.

The results show that compounds **3**, **4**, **4_2Br**, **4Br** and **4_2Cl** decreased survival by more than 80% in all cell lines, regardless of cancer type and cell type. Two of them, 3 and 4, are nonhalogenated estrane derivatives, whereas **4_2Cl** and **4_2Br, 4Br** are their halogenated counterparts (Table 1). IC_{50} values were determined for the most active compounds (Table 2). None of the compounds had a lower IC_{50} for the BC cell line MCF-7 compared with the control cell line MCF10A. Compound 3 had a lower IC_{50} for the EC cell line KLE and compound **4_2Cl** had a lower IC_{50} for the EC cell line Ishikawa compared with the control cell line HIEEC (Table 2). **4_2Cl** also showed a higher antiproliferative effects on the HGSOC cell line COV362 with a 4-fold lower IC_{50} compared with the control cell line HIO80 (Table 2). The effects on the HGSOC cell line Kuramochi were much weaker. The antiproliferative effects of **4_2Cl** could be explained by the inhibition of STS and 17β-HSD1 enzymes, as this compound acts as a potent inhibitor of these two estrogen biosynthetic enzymes [15]. Moreover, COV362 exhibits higher expression of *STS* and lower expression of *HSD17B1* compared with Kuramochi [33,34], suggesting that the preferred mechanism of antiproliferative action may be via inhibition of STS. In addition, 2-chloro substituted compounds have a lower estrogenic effect than C4 substituted compounds and also the parent compounds [7,8,35]. Therefore, the results suggest that **4_2Cl** might have a lower effect via the estrogen receptor compared with compound **4**. Structure-activity relationship analysis suggests that the absence of the keto-group at C17 (starting compounds 3 and 4) increases nonspecific cytotoxic activity compared with starting compounds **1** and **2**.

3.2. Estrane derivatives with potent antiproliferative effect on cancer cells and little on control cells

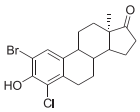
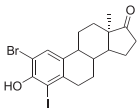
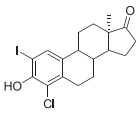
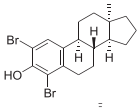
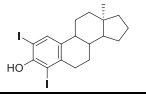
Based on the results of the previous section, we found that most compounds that had strong antiproliferative effects on cancer cell lines

Table 1
Cell viability of breast (BC), endometrial (EC) and high grade serous ovarian cancer (HGSOC) cell lines, and corresponding control cell lines in the presence of 100 μM estrane derivatives with high cytotoxic activity. Indicated is the mean percentage of cell growth + /- SD from two to three independent experiments.

Comp. number	Structure	control	BC	control	EC				control	HGSOC		
		MCF10A	MCF7	HIEEC	KLE	HEC-1-A	Ishikawa	RL95-2	HIO80	COV362	Kuramochi	OVSAHO
												
1_2I,4I		2.1 ± 15.6	118.5 ± 115.4	15.8 ± 15.1	45.6 ± 20.9	33.1 ± 35.6	1.1 ± 3.7	30.5 ± 8.6	8.1 ± 5.6	29.3 ± 28.4	126.3 ± 32.0.	< 20% act.
1_2Br,4Br		5.6 ± 1.6	> 80%	25.4 ± 11.5	50.9 ± 10.6	17.5 ± 3.0	8.2 ± 2.3	> 80%	0.7 ± 2.3	78.7 ± 13.8	155.6 ± 14.3	> 80%
												
3		13.9 ± 16.0	-4.9 ± 8.0	-0.4 ± 2.3	-6.7 ± 12.5	2.9 ± 3.9	12.9 ± 4.8	-7.2 ± 8.8	0.6 ± 2.1	-10.1 ± 31.7	12.4 ± 27.8	-9.7 ± 11.7
												
4		-6.0 ± 4.8	-2.0 ± 4.6	-1.0 ± 4.0	-3.4 ± 0.4	-7.4 ± 4.0	-3.6 ± 2.1	-3.6 ± 3.2	-0.2 ± 5.3	-0.6 ± 26.0	-11.9 ± 16.4	-0.0 ± 0.4
4_2Cl		5.3 ± 3.6	-2.7 ± 11.7	0.6 ± 2.0	20.2 ± 27.3	3.7 ± 3.1	-0.3 ± 0.4	-3.3 ± 3.4	1.7 ± 2.5	3.9 ± 18.5	-8.8 ± 5.8	-1.4 ± 1.5
												
2_2CCPh		9.6 ± 9.4	-6.4 ± 9.1	34.2 ± 17	59.3 ± 6.6	13.0 ± 11.5	23.1 ± 9.4	27.6 ± 38.6	43.6 ± 4.4	21.7 ± 9.5	68.2 ± 20.1	57.4 ± 6.8
2_4I		> 80%	< 20% act.	> 80%	22.2 ± 18.7	27.9 ± 10.4	-5.7 ± 6	141.2 ± 20.6	> 80%	45.1 ± 29.9	< 20% act.	< 20% act.
2_2I,4Br		4.5 ± 7.4	20.7 ± 18.1	5.5 ± 0.4	> 80%	59.5 ± 7.4	28.7 ± 16.7	0.6 ± 21.8	12.8 ± 9.7	45.4 ± 74.2	< 20% act.	48. ± 6.8
2_2MeOPh		40.5 ± 6.0	60.8 ± 35.7	51.8 ± 20.0	68.6 ± 4.8	> 80%	12.5 ± 7.2	51.7 ± 0.7	39.9 ± 1.9	62.3 ± 38.1	130.4 ± 33.0	147.1 ± 31.0

(*continued on next page*)

Table 1 (*continued*)

Comp. number	Structure	control	BC	control	EC				control	HGSOC		
		MCF10A	MCF7	HIEEC	KLE	HEC-1-A	Ishikawa	RL95-2	HIO80	COV362	Kuramochi	OVSAHO
2_2Br, 4Cl		-2.1 ± 17.1	70.4 ± 4.5	46.9 ± 5.6	66.6 ± 5.0	42.7 ± 0.4	7.8 ± 6.0	52.9 ± 23.0	8 ± 17.1	> 80%	131.8 ± 4.3	73.7 ± 12.2
2_2Br,4I		12.8 ± 6.5	64.0 ± 4.7	48.5 ± 12.1	> 80%	59.9 ± 24.6	11.5 ± 26.3	84.3 ± 13.6	16.8 ± 16.6	> 80%	134.3 ± 15.6	> 80%
2_2I,4Cl		5.7 ± 3.3	20.7 ± 12.2	38.7 ± 32.4	34.5 ± 33.4	41 ± 2.5	15.4 ± 19	38.3 ± 9.2	23.4 ± 25.1	> 80%	< 20% act.	58.0 ± 1.7
4_2Br,4Br		7.8 ± 30.6	-2.9 ± 0.7	-0.6 ± 1.0	3.9 ± 7.4	0.5 ± 0.1	-0.8 ± 2.5	-3.7 ± 4.8	-1.7 ± 0	-6.9 ± 31.4	14.5 ± 19.2	-4.0 ± 4.9
4_2I,4I		-3.7 ± 5.5	7.2 ± 0.8	2.2 ± 2.3	-1.0 ± 1.0	12.1 ± 11.9	3.6 ± 2.5	-1.2 ± 3.9	-2.4 ± 1.1	14.9 ± 6.2	43.6 ± 9	21.4 ± 2.9

BC – breast cancer, EC – endometrial cancer, HGSOC – high-grade serous ovarian carcinoma

Table 2

IC_{50} values (μM) for estrane derivatives with antiproliferative effects on cancer and control cell lines. The following cell lines were included: control breast cell line MCF10A and BC cell line MCF-7, endometrial control cell line HIEEC and four EC cell lines KLE, HEC-1-A, and Ishikawa, ovarian control cell line HIO80 and two HGSOC cell lines COV362 and Kuramochi. Estrane derivatives with lower IC_{50} for cancer cells compared with control are indicated in bold. IC_{50} is given in μM.

Compound number	Breast control	BC	Endometrial control	EC			Ovarian control	HGSOC	
	MCF10A	MCF7	HIEEC	KLE	HEC-1-A	Ishikawa	HIO80	COV362	Kuramochi
3	18.9	35.7	**~ 45.3**	**32.7**			14.4	40.4	
4	14.2	59.0	25.8	28.3			11.9	~ 25.7	
4_2Cl			**25.3**			**17.9**	**15.1**	**3.6**	32.2
2_4I						37.2		73.5	

BC – breast cancer, EC – endometrial cancer, HGSOC – high-grade serous ovarian carcinoma.

(less than 20% survival) were also cytotoxic to control cell lines. Therefore, in this part, we weakened the criterion and selected the compounds that reduced the survival of cancer cell lines by more than 50%, whereas they did not reduce the survival of control cell lines by more than 20%. The results show that none of the estrane derivatives met this criterion in breast cancer cells.

3.2.1. Anti-proliferative effects on model cell lines of endometrial and ovarian cancer

Several estrane derivatives showed antiproliferative effect on EC cell

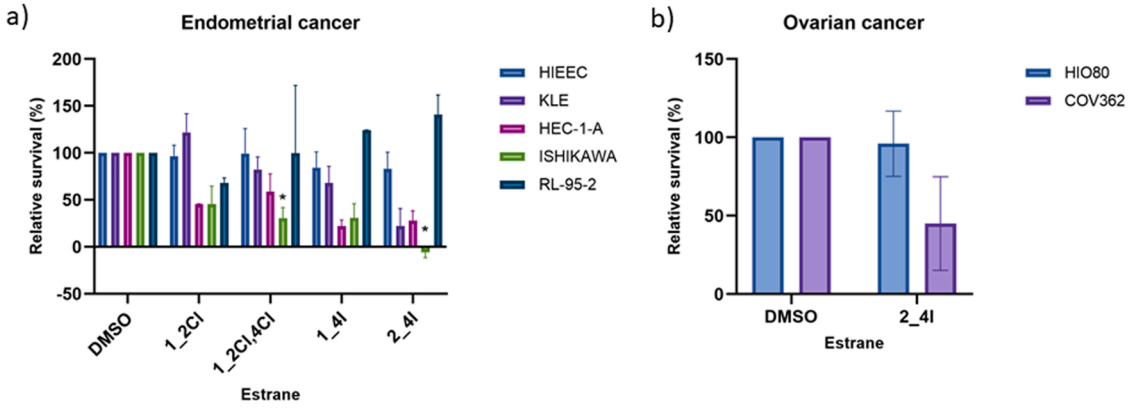


Fig. 2. Cell viability of EC and HGSOC cell lines in the presence of estrane derivatives with cytotoxic effect on cancer cell lines and little or no cytotoxicity on control cells. Cytotoxicity was determined using the AlamarBlue assay. The compounds were tested at a final 100 μM concentration on the endometrial control cell line HIEEC and on four EC cell lines KLE, HEC-1-A, Ishikawa and RL95–2 (a). The compounds were also tested also at 100 μM concentration on ovarian control cell line HIO80 and OC cell lines COV362 (b). The column shows the mean survival + /- SD. Kruskal Wallis statistical test was performed between treatment and control (DMSO). * $p < 0.05$.

lines with little or no effect on the control HIEEC. The strongest effect was observed with compound **2_4I** (more than 70% decreased proliferation in all cell lines except RL95–2) (Fig. 2a) and with IC_{50} of 37.2 μM in Ishikawa cells. Previous studies suggest that **2_4I** may affect estrogen pathway at multiple levels. It may act as an inhibitor of AKR1C3 (85.7% enzyme inhibition at 100 μM), which can activate estrone to estradiol [17] and as an inhibitor of the OATP2B1 transporter [20], which is also involved in the up-take of E1S and DHEAS [20]. However, Laczko-Rigo et al. showed in epidermoid carcinoma that the antiproliferative effect of this compound was not related to OATP2B1 [19]. The mode of action of this compound in endometrial cancer cells thus remains to be determined. This compound is also a potent inhibitor of STS and 17β-HSD type 1 [15], indicating its multiple functions.

A potent antiproliferative action on HEC-1-A and Ishikawa, was also observed for the estrane derivative **1_4I**, which decreased proliferation by 87% and 69%, respectively. This effect may be mediated by inhibition of STS and 17β-HSD type 1, which are inhibited by submicromolar concentrations of this compound [15].

The strongest antiproliferative effect on the RL95–2 cell line was observed for **1_2Cl**, with a decrease in proliferation of only 24%. **1_2Cl** inhibits aromatase, STS and 17β-HSD type 1 with low micromolar IC_{50} [15]. This compound also inhibits AKR1C1 (91.6% at 100 μM), AKR1C2 (88% at 100 μM), and AKR1C3 (86.8% inhibition at 100 μM) [17]. Interestingly, RL95–2 exhibits higher gene expression of *STS* and *HSD17B1* compared with HEC-1-A and KLE, which may explain their susceptibility to the antiproliferative effects of this compound [33,34]. Nevertheless, RL95–2 is a cellular model of grade 2 EC, which is generally well-manageable [36].

For HGSOC, three cancer cell lines were included in the study, COV362, Kuramochi and OVSAHO. None of the estrane derivatives tested selectively affected proliferation of Kuramochi or OVSAHO cell lines (Supplementary table 1), whereas one of the estrane derivatives, **2_4I**, impaired proliferation of COV362, decreasing proliferation by 48% (Fig. 2**b**) and showed IC50 of 73.5 μM. The expression of *AKR1C3* was higher in COV362 than in Kuramochi and OVSAHO, whereas the expression of *OATP2B1* in COV362 was comparable to Kuramochi and higher than in OVSAHO. The high expression of *AKR1C3* and *OATP2B1* in COV362 may explain the potential pathway of **2_4I** -mediated antiproliferative effects [33,34]. **2_4I** was also selective for EC cells compared with the control endometrial cell line HIEEC.

The structure-activity relationship suggests that the presence of a keto-group at C17 increases the selectivity of the compounds for cancer cells, whereas it has little or no effect on control cells. Starting compounds **1** and **2** with iodine at C4 have a cytotoxic effect on KLE, while the other compounds have no effect, indicating a specific effect on the KLE cell line. The presence of iodine at C4 in compound **2** compared with **1** also had a higher effect on Ishikawa as well as significantly decreased survival of HGSOC cell line COV362. In general, the addition of halogen to C2 and/or C4 on starting compounds **1** and **2** increased selectivity for cancer cells compared with control, as starting compounds **1** and **2** are cytotoxic to the endometrial control cell line HIEEC and the ovarian control cell line HIO80.

Halogenated estranes generally have little or no estrogenic effect. However, this effect needs to be verified because it may lead to undesirable proliferation at low concentrations. We checked the the effects of **2_4I** and **4_2Cl** (results of IC_{50} determination) at nanomolar concentrations. We found that the results varied between cell lines, as the proliferative effect of **2_4I** was observed in Ishikawa whereas it was absent in COV362. For **4_2Cl**, no proliferative effect was observed in Ishikawa and Kuramochi, whereas the proliferative effect was observed in COV362. The stimulation of proliferation by derivatives **2_4I** and **4_2Cl** can be explained by their binding to one of the estrogen receptors ERα (*ESR1*), ERβ (*ESR2*) and G protein-coupled estrogen receptor (*GPER*) [37]. Both Ishikawa and COV362 express *ESR1*, *ESR2* and *GPER* [38] [39] and it seems that these derivatives may act through different receptors.

4. Conclusion

Overall, our results indicate that **4_2Cl** and **2_4I** are lead compounds for drug development as they act on EC and OC cell lines. These compounds are inhibitors of several enzymes, including AKR1C enzymes, STS, 17β-HSD1 and also OATP2B1 uptake transporter, thus they may prevent local estrogen formation and action and may thus act as selective intracrine modulators. It is also possible, that these compounds target other enzymes, receptors or transporters and their precise mechanism of action should be further investigated. To better assess the clinical potential of the selected compounds, they could be tested in disease models, that better reflect the original tumor, such as patient-derived organoids [40–42].

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Conflict of interest

Authors declare no conflict of interest.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2023.106350.

References

[1] J. Ferlay, E. Steliarova-Foucher, J. Lortet-Tieulent, S. Rosso, J.W. Coebergh, H. Comber, D. Forman, F. Bray, Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012, Eur. J. Cancer 49 (6) (2013) 1374–1403.
[2] J. Ferlay, M. Colombet, I. Soerjomataram, T. Dyba, G. Randi, M. Bettio, A. Gavin, O. Visser, F. Bray, Cancer incidence and mortality patterns in Europe: estimates for 40 countries and 25 major cancers in 2018, Eur. J. Cancer 103 (2018) 356–387.
[3] R.L. Siegel, K.D. Miller, H.E. Fuchs, A. Jemal, Cancer statistics, 2021, CA Cancer J. Clin. 71 (1) (2021) 7–33.
[4] C. Gao, Y. Wang, W. Tian, Y. Zhu, F. Xue, The therapeutic significance of aromatase inhibitors in endometrial carcinoma, Gynecol. Oncol. 134 (1) (2014) 190–195.
[5] J.C. Chura, C.H. Blomquist, H.S. Ryu, P.A. Argenta, Estrone sulfatase activity in patients with advanced ovarian cancer, Gynecol. Oncol. 112 (1) (2009) 205–209.
[6] J.W. Mueller, L.C. Gilligan, J. Idkowiak, W. Arlt, P.A. Foster, The regulation of steroid action by sulfation and desulfation, Endocr. Rev. 36 (5) (2015) 526–563.
[7] R. Kuruto-Niwa, T. Ito, H. Goto, H. Nakamura, R. Nozawa, Y. Terao, Estrogenic activity of the chlorinated derivatives of estrogens and flavonoids using a GFP expression system, Environ. Toxicol. Pharm. 23 (1) (2007) 121–128.
[8] H. Nakamura, T. Shiozawa, Y. Terao, F. Shiraishi, H. Fukazawa, By-products produced by the reaction of estrogens with hypochlorous acid and their estrogen activities, J. Health Sci. 52 (2) (2006) 124–131.
[9] G.M. Anstead, K.E. Carlson, J.A. Katzenellenbogen, The estradiol pharmacophore: ligand structure-estrogen receptor binding affinity relationships and a model for the receptor binding site, Steroids 62 (3) (1997) 268–303.
[10] B. Schonecker, C. Lange, M. Kotteritzsch, W. Gunther, J. Weston, E. Anders, H. Gorls, Conformational design for 13alpha-steroids, J. Org. Chem. 65 (18) (2000) 5487–5497.
[11] D. Ayan, J. Roy, R. Maltais, D. Poirier, Impact of estradiol structural modifications (18-methyl and/or 17-hydroxy inversion of configuration) on the in vitro and in vivo estrogenic activity, J. Steroid Biochem Mol. Biol. 127 (3–5) (2011) 324–330.
[12] K.M. Penov Gasi, D.A. Miljkovic, L.D. Medic Mijacevic, E.A. Djurendic, S. Z. Stojanovic, M.N. Sakac, M. Djurendic, S.M. Stankovic, D. Lazar, S. Andric, R. Kovacevic, Synthesis, X-ray crystal structures and biological activity of 16-

amino-17-substituted-D-homo steroid derivatives, Steroids 68 (7–8) (2003) 667–676.
[13] S. Jovanovic-Santa, J. Petrovic, S. Andric, R. Kovacevic, E. Durendic, M. Sakac, D. Lazar, S. Stankovic, Synthesis, structure, and screening of estrogenic and antiestrogenic activity of new 3,17-substituted-16,17-seco-estratriene derivatives, Bioorg. Chem. 31 (6) (2003) 475–484.
[14] I. Bacsa, R. Jojart, G. Schneider, J. Wolfling, P. Maroti, B.E. Herman, M. Szecsi, E. Mernyak, Synthesis of A-ring halogenated 13alpha-estrone derivatives as potential 17beta-HSD1 inhibitors, Steroids 104 (2015) 230–236.
[15] I. Bacsa, B.E. Herman, R. Jojart, K.S. Herman, J. Wolfling, G. Schneider, M. Varga, C. Tomboly, T.L. Rizner, M. Szecsi, E. Mernyak, Synthesis and structure-activity relationships of 2- and/or 4-halogenated 13beta- and 13alpha-estrone derivatives as enzyme inhibitors of estrogen biosynthesis, J. Enzym. Inhib. Med Chem. 33 (1) (2018) 1271–1282.
[16] J. Szabo, Z. Pataki, J. Wolfling, G. Schneider, N. Bozsity, R. Minorics, I. Zupko, E. Mernyak, Synthesis and biological evaluation of 13alpha-estrone derivatives as potential antiproliferative agents, Steroids 113 (2016) 14–21.
[17] M. Sinreih, R. Jojart, Z. Kele, T. Budefeld, G. Paragi, E. Mernyak, T.L. Rizner, Synthesis and evaluation of AKR1C inhibitory properties of A-ring halogenated oestrone derivatives, J. Enzym. Inhib. Med Chem. 36 (1) (2021) 1500–1508.
[18] I. Bacsa, R. Jojart, J. Wolfling, G. Schneider, B.E. Herman, M. Szecsi, E. Mernyak, Synthesis of novel 13alpha-estrone derivatives by Sonogashira coupling as potential 17beta-HSD1 inhibitors, Beilstein J. Org. Chem. 13 (2017) 1303–1309.
[19] R. Laczko-Rigo, E. Bakos, R. Jojart, C. Tomboly, E. Mernyak, C. Ozvegy-Laczka, Selective antiproliferative effect of C-2 halogenated 13alpha-estrones on cells expressing Organic anion-transporting polypeptide 2B1 (OATP2B1), Toxicol. Appl. Pharm. 429 (2021), 115704.
[20] R. Laczko-Rigo, R. Jojart, E. Mernyak, E. Bakos, A. Tuerkova, B. Zdrazil, C. Ozvegy-Laczka, Structural dissection of 13-epiestrones based on the interaction with human Organic anion-transporting polypeptide, OATP2B1, J. Steroid Biochem Mol. Biol. 200 (2020), 105652.
[21] H.D. Soule, T.M. Maloney, S.R. Wolman, W.D. Peterson Jr., R. Brenz, C. M. McGrath, J. Russo, R.J. Pauley, R.F. Jones, S.C. Brooks, Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10, Cancer Res 50 (18) (1990) 6075–6086.
[22] H.D. Soule, J. Vazquez, A. Long, S. Albert, M. Brennan, A human cell line from a pleural effusion derived from a breast carcinoma2, JNCI: J. Natl. Cancer Inst. 51 (5) (1973) 1409–1416.
[23] P. Chapdelaine, J. Kang, S. Boucher-Kovalik, N. Caron, J.P. Tremblay, M.A. Fortier, Decidualization and maintenance of a functional prostaglandin system in human endometrial cell lines following transformation with SV40 large T antigen, Mol. Hum. Reprod. 12 (5) (2006) 309–319.
[24] M. Nishida, K. Kasahara, M. Kaneko, H. Iwasaki, K. Hayashi, [Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors], Nihon Sanka Fujinka Gakkai Zasshi 37 (7) (1985) 1103–1111.
[25] H. Kuramoto, Studies of the growth and cytogenetic properties of human endometrial adenocarcinoma in culture and its development into an established line, Acta Obstet. Gynaecol. Jpn 19 (1) (1972) 47–58.
[26] G.S. Richardson, G.R. Dickersin, L. Atkins, D.T. MacLaughlin, S. Raam, L.P. Merk, F.M. Bradley, KLE: a cell line with defective estrogen receptor derived from undifferentiated endometrial cancer, Gynecol. Oncol. 17 (2) (1984) 213–230.
[27] D.L. Way, D.S. Grosso, J.R. Davis, E.A. Surwit, C.D. Christian, Characterization of a new human endometrial carcinoma (RL95-2) established in tissue culture, Vitro 19 (3 Pt 1) (1983) 147–158.
[28] W.L. Yang, A.K. Godwin, X.X. Xu, Tumor necrosis factor-alpha-induced matrix proteolytic enzyme production and basement membrane remodeling by human ovarian surface epithelial cells: molecular basis linking ovulation and cancer risk, Cancer Res 64 (4) (2004) 1534–1540.
[29] T. Motoyama, [Biological characterization including sensitivity to mitomycin C of cultured human ovarian cancers (author's transl)], Nihon Sanka Fujinka Gakkai Zasshi 33 (8) (1981) 1197–1204.
[30] C.A.M. van den Berg-Bakker, A. Hagemeijer, E.M. Franken-Postma, V.T.H.B. M. Smit, P.J.K. Kuppen, H.H.V.R. Claasen, C.J. Cornelisse, P.I. Schrier, Establishment and characterization of 7 ovarian carcinoma cell lines and one granulosa tumor cell line: growth features and cytogenetics, Int. J. Cancer 53 (4) (1993) 613–620.
[31] T. Yanagibashi, I. Gorai, T. Nakazawa, E. Miyagi, F. Hirahara, H. Kitamura, H. Minaguchi, Complexity of expression of the intermediate filaments of six new human ovarian carcinoma cell lines: new expression of cytokeratin 20, Brit J. Cancer 76 (7) (1997) 829–835.
[32] K. Prabst, H. Engelhardt, S. Ringgeler, H. Hubner, Basic colorimetric proliferation assays: MTT, WST, and resazurin, Methods Mol. Biol. 1601 (2017) 1–17.
[33] E. Cerami, J. Gao, U. Dogrusoz, B.E. Gross, S.O. Sumer, B.A. Aksoy, A. Jacobsen, C. J. Byrne, M.L. Heuer, E. Larsson, Y. Antipin, B. Reva, A.P. Goldberg, C. Sander, N. Schultz, The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data, Cancer Discov. 2 (5) (2012) 401–404.
[34] J. Gao, B.A. Aksoy, U. Dogrusoz, G. Dresdner, B. Gross, S.O. Sumer, Y. Sun, A. Jacobsen, R. Sinha, E. Larsson, E. Cerami, C. Sander, N. Schultz, Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal, Sci. Signal 6 (269) (2013) pl1.
[35] B.T. Zhu, G.Z. Han, J.Y. Shim, Y. Wen, X.R. Jiang, Quantitative structure-activity relationship of various endogenous estrogen metabolites for human estrogen receptor alpha and beta subtypes: Insights into the structural determinants favoring a differential subtype binding, Endocrinology 147 (9) (2006) 4132–4150.
[36] R. Pavlic, M. Gjorgoska, E. Hafner, M. Sinreih, K. Gajser, S. Poschner, W. Jager, T. L. Rizner, In the model cell lines of moderately and poorly differentiated endometrial carcinoma, estrogens can be formed via the sulfatase pathway, Front Mol. Biosci. 8 (2021), 743403.
[37] N. Fuentes, P. Silveyra, Estrogen receptor signaling mechanisms, Adv. Protein Chem. Struct. Biol. 116 (2019) 135–170.
[38] N. Hevir-Kene, T.L. Rizner, The endometrial cancer cell lines Ishikawa and HEC-1A, and the control cell line HIEEC, differ in expression of estrogen biosynthetic and metabolic genes, and in androstenedione and estrone-sulfate metabolism, Chem. Biol. Inter. 234 (2015) 309–319.
[39] R. Pavlic, M. Gjorgoska, T.L. Rizner, Model cell lines and tissues of different HGSOC subtypes differ in local estrogen biosynthesis, Cancers 14 (11) (2022).
[40] C.J. de Witte, J. Espejo Valle-Inclan, N. Hami, K. Lohmussaar, O. Kopper, C.P. H. Vreuls, G.N. Jonges, P. van Diest, L. Nguyen, H. Clevers, W.P. Kloosterman, E. Cuppen, H.J.G. Snippert, R.P. Zweemer, P.O. Witteveen, E. Stelloo, Patient-derived ovarian cancer organoids mimic clinical response and exhibit heterogeneous inter- and intrapatient drug responses, Cell Rep. 31 (11) (2020), 107762.
[41] J.T. Neal, X. Li, J. Zhu, V. Giangarra, C.L. Grzeskowiak, J. Ju, I.H. Liu, S.H. Chiou, A.A. Salahudeen, A.R. Smith, B.C. Deutsch, L. Liao, A.J. Zemek, F. Zhao, K. Karlsson, L.M. Schultz, T.J. Metzner, L.D. Nadauld, Y.Y. Tseng, S. Alkhairy, C. Oh, P. Keskula, D. Mendoza-Villanueva, F.M. De La Vega, P.L. Kunz, J.C. Liao, J. T. Leppert, J.B. Sunwoo, C. Sabatti, J.S. Boehm, W.C. Hahn, G.X.Y. Zheng, M. M. Davis, C.J. Kuo, Organoid modeling of the tumor immune microenvironment, Cell 175 (7) (2018) 1972–1988, e1916.
[42] M. Boretto, N. Maenhoudt, X. Luo, A. Hennes, B. Boeckx, B. Bui, R. Heremans, L. Perneel, H. Kobayashi, I. Van Zundert, H. Brems, B. Cox, M. Ferrante, I.H. Uji, K. P. Koh, T. D'Hooghe, A. Vanhie, I. Vergote, C. Meuleman, C. Tomassetti, D. Lambrechts, J. Vriens, D. Timmerman, H. Vankelecom, Patient-derived organoids from endometrial disease capture clinical heterogeneity and are amenable to drug screening, Nat. Cell Biol. 21 (8) (2019) 1041–1051.