Journal of Steroid Biochemistry & Molecular Biology 127 (2011) 255–261

Contents lists available at SciVerse ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Discovery of highly potent, nonsteroidal 17β-hydroxysteroid dehydrogenase type 1 inhibitors by virtual high-throughput screening

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ARTICLE INFO

Article history:
Received 12 April 2011
Received in revised form 15 July 2011
Accepted 14 August 2011

Keywords:
17β-HSD1
Nonsteroidal inhibitors
Virtual high-throughput screening
Estrogens
Breast cancer

ABSTRACT

17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) catalyzes the formation of the potent proliferation-stimulating hormone estradiol, and it is thus involved in the development of hormone-dependent breast cancer. Due to its high substrate specificity and the known relationships between its overexpression and disease incidence, 17β-HSD1 is considered an attractive target for drug development. Here, we have used structure-based virtual high-throughput screening to successfully identify potent nonsteroidal 17β-HSD1 inhibitors. Computational screening of a drug-like database containing 13 million compounds identified hits with a 2-benzylidenebenzofuran-3(2*H*)-one scaffold that we show to be highly potent 17β-HSD1 inhibitors. The most potent in the series, compound **1**, showed an IC_{50} of 45 nM in our 17β-HSD1 inhibition assay, and also showed good selectivity for 17β-HSD1 over 17β-HSD2.

1. Introduction

Estradiol (E2) is the most potent of the human estrogens and it binds to the estrogen receptors (ERs) in estrogen-responsive tissues and stimulates cell proliferation, thus contributing to development and progression of various hormone-dependent cancers [1]. Around 60% of all breast cancers and 75% of postmenopausal cases are hormone dependent, and they have ER-positive status [2]. After menopause, the great majority of estrogens are formed in the peripheral tissues, from adrenal precursors and inactive estrone-3-sulphate [3]. In these tissues, they have activities in the same cells where they are formed, so they act in an intracrine way [3]. The last step of E2 biosynthesis is the NADPH-dependent reduction of estrone (E1), which is catalyzed by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) [4]. The gene encoding the 17β-HSD1 sequence is located in the *q11-q12* region of chromosome 17, near to candidate genes that are responsible for hereditary breast-ovarian cancer syndrome [5,6]. Overexpression of 17β-HSD1 has been reported for several breast and endometrial cancer cell lines, and in breast cancer and endometriosis tissues, and this has also been characterized as a prognostic marker for disease progression [7–10].

17β-HSD1 is a cytoplasmic enzyme of the short-chain dehydrogenase/reductase superfamily that has high specificity and catalytic efficiency for E1 reduction [11,12]. Due to its pivotal role in specific E2 formation, 17β-HSD1 is an attractive clinical target for local and selective deprivation of E2 levels, and thus for treatment of hormone-dependent breast cancer and other estrogen-related diseases [4].

Important studies have been carried out recently in the field of discovery of potent and selective inhibitors of 17β-HSD1; however, intensive structure–activity relationship (SAR) studies and rational drug-design approaches have not yet provided any clinically useful drug candidates [13]. The development of steroidal 17β-HSD1 inhibitors has led to highly potent compounds; however, inhibitors with steroidal cores are not favorable, due to their potential low target specificity and undesirable pharmacokinetic profiles. Therefore, the design of nonsteroidal 17β-HSD1 inhibitors appears to be more attractive. In the early phases of the development of new 17β-HSD1 inhibitors, at least their selectivity relative to 17β-HSD2 must be evaluated, as 17β-HSD2 has a protective role through its inactivation of E2 to E1 [14,15]. Many crystal complexes of 17β-HSD1 with various steroidal ligands have been resolved, which offer the possibility for the structure-based design of new 17β-HSD1 inhibitors [16]. However, most of the drug discovery studies in this field are still based on optimizing the structure of the steroidal ligand through the experience and intuition of experts [17]. Although this approach has resulted in the discovery of very

Abbreviations: 17β-HSD1, 17β-hydroxysteroid dehydrogenase type 1; 17β-HSD2, 17β-hydroxysteroid dehydrogenase type 2; E1, estrone; E2, estradiol; ER, estrogen receptor; NAD^+, oxidized form of nicotinamide dinucleotide; NADPH, reduced form of nicotinamide dinucleotide phosphate; SARs, structure–activity relationships; VHTS, virtual high-throughput screening.

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doi:10.1016/j.jsbmb.2011.08.013

potent and highly selective 17β-HSD1 inhibitors, advanced drug-discovery efforts that cover wider chemical space with concomitant lower time and costs are more desirable [18,19].

Implementation of computer-assisted drug design for the discovery of new 17β-HSD1 inhibitors has been mostly used for interpretation of the inhibitory actions of the existing inhibitors and for further intuitive optimization of their potencies [20,21]. Fully computational, ligand-based drug-design approaches have been reported for the generation of three-dimensional (3D) quantitative SAR models by comparative molecular field analysis; however, these methods have only been used on small and structurally limited sets of compounds, which has resulted in their lower predictive value [13,22,23]. Structure-based drug-design approaches for the discovery of new 17β-HSD1 inhibitors have mostly been used for the generation of the pharmacophore models that have been derived from various co-crystal complexes of 17β-HSD1 [24–26]. These models have been validated by existing natural and synthetic 17β-HSD1 inhibitors, and they have served as pharmacophoric constraints for subsequent screening of virtual compound libraries. However, the high-ranked compounds that have emerged from these virtual screenings have at best been only modest inhibitors in *in vitro* 17β-HSD1 activity assays, with IC_{50} values in the μM range [24].

We performed virtual high-throughput screening (VHTS) based on the crystal structure of 17βHSD1 in complex with equilin and $NADP^+$ (PDB entry 1EQU) [27]. Implementation of simple pharmacophoric constraints resulted in the identification of highly potent (nanomolar) 17β-HSD1 inhibitors with a 2-benzylidenebenzofuran-3(2*H*)-one (aurone) scaffold.

2. Materials and methods

2.1. Chemicals

[2,4,6,7-^{3}H]-E1 and [2,4,6,7-^{3}H]-E2 were obtained from Perkin-Elmer (Boston, USA). E1 and E2 were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). NADPH and NAD^+ were purchased from Roth (Karlsruhe, Germany). The candidate inhibitory compounds were obtained from the National Cancer Institute, IBScreen, Asinex, ChemBridge, Enamine, Otava, Vitas-M and Life-Chemicals.

2.2. Initial virtual screening

All of the computational procedures were carried out on two workstations. The first workstation has four dual-core AMD Opteron 2.0 GHz processors, 16 GB of RAM, four 320 GB hard drives in a RAID10 array, and a Nvidia GeForce 7900 graphic card, and it runs 64-bit Fedora 7. The second workstation has two quad-core Intel Xeon 2.2 GHz processors, 8 GB of RAM, 320 GB and 1000 GB hard drives, and a Nvidia Quadro FX 4800 graphic card, and it runs the current version of 64-bit Arch Linux.

For initial virtual screening, the ZINC database that contains 13 million drug-like compounds was selected [28]. The database was first prepared with Omega2 software (OpenEye Scientific Software Inc.) to yield in average of 152 conformations per compound [29]. This database was screened using ROCS (OpenEye Scientific Software Inc.), to reduce it to compounds that fit well according to the size and shape of estrone, which was used as the query [30]. Only the 30,000 compounds with the highest shape similarities to estrone were retained and used in subsequent docking experiments.

The docking was performed on the crystal structure of 17β-HSD1 co-crystallized with equilin and $NADP^+$ (PDB entry 1EQU), using FlexX 3.1.2 (BiosolveIT GmbH) [27,31]. The active site was defined as the area of 17β-HSD1 within 6.5 Å of the co-crystallized equilin. The cofactor $NADP^+$ was left in the model. The docking experiments were set up with FlexX-implemented pharmacophore constraints, which were selected to keep only the compounds that could form interactions with crucial polar amino-acid residues at the opposite sides of the substrate-binding domain (H-bonds with either the catalytic Ser142 or Tyr155 at one side of the active site, and with His221 or Glu282 at the other side). Method was validated by docking of known inhibitors into the active site of 17β-HSD1. Published potent non-steroidal inhibitors like diosmetin [32], coumarines [19] and bis(hydroxyphenyl)thiophenes [20] got high FlexX scores ranging between −24 and −26.5 kcal/mol thereby confirming the predicting ability of this software. All of these 30,000 compounds from the ROCS search were then docked to the active site. Only 2805 of these compounds matched the pharmacophore constraints and were successfully docked and ranked according to their predicted scores. Visual inspection of 51 the highest ranking compounds was then performed, and all of the compounds with potential estrogenic effects (steroids, flavonoids and other phytoestrogens) were eliminated. From the remaining set of highest ranking compounds, 18 of those available were ordered from various commercial suppliers according to current availability, and used in the 17β-HSD1 *in vitro* inhibition assay.

2.3. Two-dimensional similarity search and second virtual screening

To obtain initial SAR data on compounds with the 2-benzylidenebenzofuran-3(2*H*)-one scaffold that came from this initial virtual screening, simple 2D similarity searches were carried out using the ZINC built-in search engine. Two different queries were used: compound **1** and compound **2** (Table 1). Only compounds with similarities greater than 90% to these queries were considered. This yielded 329 new compounds with a 2-benzylidenebenzofuran-3(2*H*)-one scaffold. To facilitate the selection of compounds to be assayed *in vitro*, molecular docking was performed. This docking was set up in the same way as stated in Section 2.2, and the compounds were ranked according to their predicted scores. Nineteen compounds with the highest ranking scores were ordered from the different suppliers and used in the 17β-HSD1 *in vitro* inhibition assay (Tables 2 and 3).

2.4. Preparation of 17β-HSD1 and 17β-HSD2 enzymes for activity assays [32]

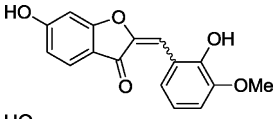
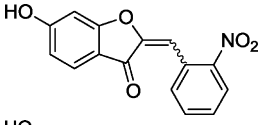
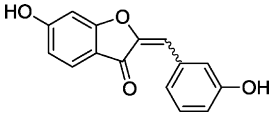
Recombinant His-tagged human 17β-HSD1 was overexpressed in the BL21-CodonPlus (DE3)-RIL strain of *Escherichia coli* using the pQE30-17β-HSD1 construct (prepared at the Institute of Experimental Genetics, Neuherberg, Germany). The bacteria were resuspended in PBS and sonicated; the resultant cell homogenate was used as the source of the recombinant 17β-HSD1, and it was stored frozen until the determination of enzymatic activity.

Recombinant N-29 truncated 17β-HSD2 was overexpressed in the JM-107 *E. coli* strain using the pGEXII-N-29 17β-HSD2 construct (prepared at the Institute of Experimental Genetics, Neuherberg, Germany). The bacteria were resuspended in phosphate-buffered saline and sonicated; the resultant cell homogenate was used as the source of the recombinant 17β-HSD2, and it was stored frozen until the determination of enzymatic activity.

2.5. In vitro 17β-HSD1 inhibition assay

All of the commercially obtained candidate inhibitory substances were tested for inhibition of 17β-HSD1, in terms of their percentages of inhibition of E1 reduction, using the *E. coli* cell homogenates (see Section 2.4). The bacterial homogenate was incubated in 100 mM sodium phosphate buffer, pH 6.5, with E1 and

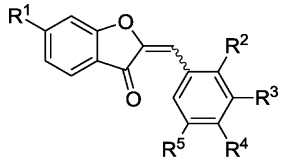
Table 1
Initial hits identified in the *in vitro* 17β-HSD1 activity assay.

Compound	Structure	17β-HSD1 inhibition[a] (%)[b]	
		0.6 μM[c]	6 μM[c]
1		91	100
2		83	100
3		72	93

[a] Data are mean values of two determinations, with RSD less than 5%;
[b] Recombinant human enzyme overexpressed in *E. coli*, [^{3}H]E1 + E1 [67 nM] and NADPH [100 μM].
[c] Concentration of inhibitor.

the tracer [2,4,6,7-^{3}H]-E1 (final concentration, 67 nM), at 37 °C, in the absence and presence of the potential inhibitors at concentrations of 0.6 μM and 6 μM. For the most potent 17β-HSD1 inhibitors, the IC_{50} values were also determined, with the compounds used at 12 nM to 12 μM. The concentration of the cofactor NADPH was 100 μM. Inhibitor stock solutions were prepared in DMSO and diluted with acetonitrile prior to use, with the final concentration of acetonitrile in the reactions adjusted to 1%. The reactions were stopped after 10 min by the addition of ethyl acetate (the time needed to convert ca. 25% of the substrate in a control assay, without any inhibitors). The samples were extracted with ethyl acetate, then this solvent was evaporated, and the steroids were dissolved in acetonitrile for separation on a reverse-phase HPLC ODS Hypersil (Thermo Scientific Inc., Waltham, MA, USA)

Table 2
[a]FlexX scores for compounds **1**–**19** compared to their *in vitro* 17β-HSD1 and 17β-HSD2 inhibition potencies.

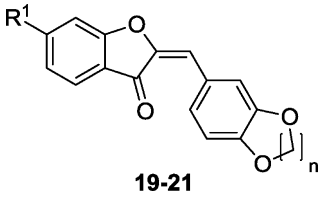


1-18

Cpd.	R^1	R^2	R^3	R^4	R^5	FlexX score[b] (kcal/mol)/configuration	Inhibition of 17β-HSD1[c]			Inhibition of 17β-HSD2[f] (%)[d]	
							% Inh.[d]		IC_{50}[e] (nM)	6 μM	60 μM
							0.6 μM[g]	6 μM			
1	–OH	–OH	–OMe	–H	–H	−26.82/*E*	91	100	45 ± 1	33	91
2	–OH	$-NO_2$	–H	–H	–H	−33.68/*E*	83	100	74 ± 1	26	75
3	–OH	–H	–OH	–H	–H	−23.50/*Z*	72	93	100 ± 1	11	56
4	–OH	–OH	–H	–H	–H	−21.06/*E*	74	96	105 ± 1	41	87
5	–OH	–H	–H	–OEt	–H	−19.10/*E*	73	89	168 ± 2	27	46
6	–OH	–H	–OMe	–H	–H	−22.01/*Z*	71	92	180 ± 1	29	57
7	–OH	–H	–OMe	–OEt	–H	−18.21/*Z*	71	89	183 ± 1	32	79
8	–OH	–H	–H	$-OCH_2{=}CH_2$	–H	−18.35/*E*	65	87	n.d.	n.d.	n.d.
9	–OH	$-NO_2$	–H	–OH	–OMe	−30.22/*Z*	65	90	n.d.	n.d.	n.d.
10	–OH	–H	–H	–OMe	–H	−19.15/*E*	63	88	n.d.	n.d.	n.d.
11	–OAc	–H	–OMe	–OMe	–H	−23.86/*Z*	33	61	n.d.	n.d.	n.d.
12	$-OCH_2CONH_2$	–H	–OMe	–OMe	–H	−23.11/*E*	23	82	n.d.	n.d.	n.d.
13	–OMe	–H	–H	–OH	–H	−23.88/*Z*	23	75	n.d.	n.d.	n.d.
14	$-OCH_2COOH$	–OMe	–OMe	–H	–H	−24.40/*Z*	n.i.	61	n.d.	n.d.	n.d.
15	$-OCH_2COOH$	–H	–OMe	–H	–H	−26.49/*Z*	n.i.	18	n.d.	n.d.	n.d.
16	–OMe	–H	–OH	–H	–H	−23.94/*Z*	n.i.	9	n.d.	n.d.	n.d.
17	$-OCH_2COOH$	–H	–OMe	–OMe	–H	−26.15/*Z*	n.i.	n.i.	n.d.	n.d.	n.d.
18	$-OCH_2CO$(4-MeOPh)	–H	–H	–H	–H	−23.80/*Z*	n.i.	n.i.	n.d.	n.d.	n.d.

[a] The compounds are ordered according to their *in vitro* 17β-HSD1 inhibitory potencies.
[b] Predicted interaction energies according to FlexX score; the configuration of the isomers is noted.
[c] Recombinant human enzyme overexpressed in *E. coli*, [^{3}H]E1 + E1 [67 nM] and NADPH [100 μM].
[d] Data are means of two determinations, with RSDs less than 10%.
[e] Data are means of three determinations, ±SD.
[f] Recombinant human enzyme overexpressed in *E. coli*, [^{3}H]E2 + E2 [300 nM] and NAD^+ [100 μM].
[g] Concentration of inhibitor.

Table 3
[a]FlexX scores for compounds **19–21** compared to their *in vitro* 17β-HSD1 and 17β-HSD2 inhibition potencies.



19-21

Cpd.	R^1	*n*	FlexX score[b] (kcal/mol)/configuration	Inhibition of 17β-HSD1[c]			Inhibition of 17β-HSD2[d] (%)	
				% Inh.[e]		IC_{50}[f] (nM)	6 μM	60 μM
				0.6 μM	6 μM			
19	–OH	2	−20.08/*E*	79	91	67 ± 1	n.i.	n.i.
20	–OH	1	−23.12/*Z*	77	87	79 ± 1	n.i.	n.i.
21	–OMe	2	−23.05/*Z*	n.i.	n.i.	n.d.	n.d.	n.d.

[a] Compounds are ordered according to their *in vitro* 17β-HSD1 inhibitory potencies.
[b] Predicted interaction energies according to FlexX scores; the configuration of the isomer is noted.
[c] Recombinant human enzyme overexpressed in *E. coli*, [^{3}H]E1 + E1 [67 nM] and NADPH [100 μM].
[d] Recombinant human enzyme overexpressed in *E. coli*, [^{3}H]E2 + E2 [300 nM] and NAD^+ [100 μM].
[e] Data are means of two determinations, with RSDs less than 5%.
[f] Data are means of three determinations, ±SD.

C18 column. Isocratic HPLC runs were performed at 25 °C and a flow rate of 1 mL/min of acetonitrile:water (45:55) as mobile phase. Detection and quantification of the radioactive steroids were performed using Quickszint Flow 302 (Zinsser Analytic, Frankfurt, Germany) as scintillation fluid, and a Ramona 2000 radioflow detector (Raytest). The assays were performed in duplicates for the determination of the percentages of inhibition, and in triplicates for determination of the IC_{50} values, with the standard deviations (SDs) also reported. The conversions were calculated according to the following formula: %conversion = (%E2/(%E2 + %E1)) × 100, and the inhibition was calculated according to: %inhibition = [(%conversion of control − %conversion of sample)/%conversion of control] × 100. Equilin was used as positive control and had % of inhibition 81 and 100 at 0.6 and 6 μM concentration, respectively.

2.6. In vitro 17β-HSD2 inhibition assay

The most potent of the 17β-HSD1 inhibitors were also evaluated for their *in vitro* 17β-HSD2 inhibition. The assay was performed as described for the 17β-HSD1 inhibition assay in terms of the percentages of inhibition, but with the following modifications: sodium phosphate buffer, pH 7.5, with E2 and the tracer [2,4,6,7-^{3}H]-E2 (final concentration, 300 nM), with the compounds again at concentrations of 6 μM and 60 μM, with 100 μM cofactor NAD^+ used; the reactions were stopped after 5 min (time needed to convert ca. 20% of the substrate in a control assay without any inhibitor).

2.7. Compound characterization

For compounds **1–21**, their purity was determined using reversed-phase high-performance liquid chromatography (HPLC) analyses performed on an Agilent 1100 system (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump and a multiple-wavelength detector, using an Agilent Eclipse Plus C18, 5 μm (150 mm × 4.6 mm) column. The compounds were dissolved in 40% acetonitrile/water at 0.16 mg/mL final concentration, and 10 μL was injected onto the column. Acetonitrile was used as an organic modifier and 0.1% trifluoroacetic acid in water as an aqueous buffer. The elution was performed with a 1.0-mL/min flow rate using a linear gradient from 30% to 70% acetonitrile over 15 min, followed by 2.5 min at 70% acetonitrile, then back down to 30% acetonitrile over 30 s, and followed by 7 min equilibration between samples. Detection was at 220 nm.

Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer.

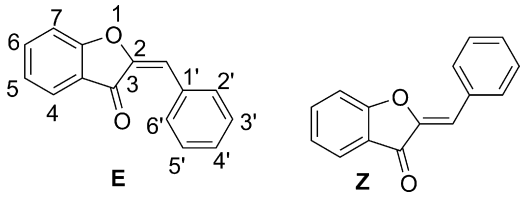


Fig. 1. *E* and *Z* forms of the 2-benzylidenebenzofuran-3(2*H*)-one structure. The numbering of the system is shown.

3. Results and discussion

3.1. Of 51 virtual hits, only those with the 2-benzylidenebenzofuran-3(2H)-one scaffold showed potent inhibition of 17β-HSD1

In the initial virtual screening, we identified 51 virtual hits of various structural classes. Compounds with scaffolds with potential estrogenic effect (steroids, flavonoids and other phytoestrogens) were eliminated so only 18 available compounds without potential estrogenic effects were obtained and evaluated *in vitro*; however, only compounds **1–3**, with a 2-benzylidenebenzofuran-3(2*H*)-one structure, showed potent *in vitro* 17β-HSD1 inhibition; they were defined as hits following more than 70% inhibition of 17β-HSD1 at 0.6 μM (Table 1).

2-Benzylidenebenzofuran-3(2*H*)-one is the central scaffold of the aurones (Fig. 1), which are natural compounds that are known to have anticancer and antiprotozoal properties [33].

However, compound **1**, which was the most potent inhibitor discovered in our study, and compound **2** have not yet been reported in terms of their biological activities. Only compound **3** was already described as potent 17β-HSD1 inhibitor [34]. This shows that this computational approach that used simple pharmacophoric constraints resulted in the discovery of a favorable structure for 17β-HSD1 inhibition.

The predicted binding conformation of the *E* isomer of compound **1** in the active site of 17β-HSD1 explains its highly potent

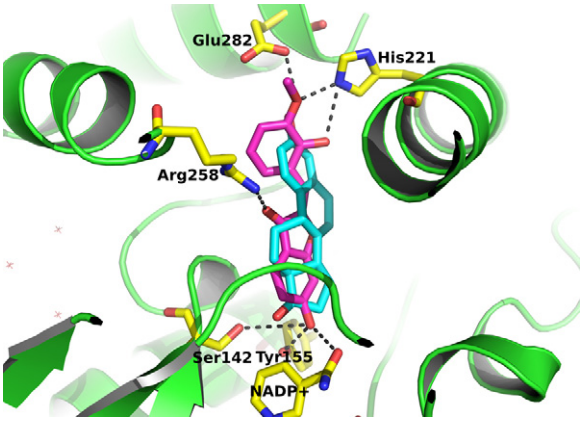


Fig. 2. Compound **1** (magenta) docked to the active site of 17β-HSD1 (PDB entry 1EQU). Comparison of the binding modes between compound **1** and equilin (blue) is shown. The amino-acid residues and the cofactor moiety (yellow sticks) can form H-bonds (gray dashes) with compound **1**. The figure was prepared using PyMOL [35]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

inhibitory activity (Fig. 2). Compound **1** occupies a similar region to the co-crystallized equilin, where its benzofuran-3-one core overlaps well with the C and D rings of equilin. The 6-OH group of compound **1** can form three potential H-bonds, with Ser142 and Tyr155, and with the $NADP^+$ carboxamide oxygen. At the opposite side of the substrate-binding region, the 3′-methoxy group of compound **1** can form potential H-bonds with the substrate-recognition residues His221 and Glu282; an additional H-bond interaction is provided between its 2′-OH group and His221. Similar to the steroid ligand, the aurone lipophilic core of compound **1** forms van der Waals interactions with the central hydrophobic part of the substrate-binding region.

The PDB 1EQU crystal structure is the only 17β-HSD1 crystal structure where Arg258 protrudes into the substrate-binding cavity, a feature that is not seen with the other 17β-HSD1 crystal structures [21]. Our docking study shows that the 3-keto oxygen of compound **1** can form potential H-bond with this strong proton-donor residue (Fig. 2), which results in strong stabilization of binding. This shows that the residues in the 17β-HSD1 active site have some mobility, and the selection of the correct crystal structure can influence the results of virtual screening.

3.2. No further potent inhibitors were identified by two-dimensional similarity searches with compounds *1* and *2* as queries

The compounds that emerged from the virtual 2D-similarity search that was performed with compounds **1** and **2** as queries were all 2-benzylidenebenzofuran-3(2*H*)-ones, and some of these have already been reported as potent 17β-HSD1 inhibitors (compounds **4**–**21**; Tables 1 and 2) [34]. Although the docking using FlexX predicted larger interaction energies (FlexX scores) for several of these than for compound **1**, none of these was shown to be more potent as *in vitro* 17β-HSD1 inhibitors. However, the correlation between the FlexX scores for interaction energies and the *in vitro* 17β-HSD1 inhibitory potencies of these 2-benzylidenebenzofuranones is known not to be optimal. Relatively active, and even inactive, compounds might give higher FlexX scores than derivatives that have been shown to be potent 17β-HSD1 inhibitors *in vitro*.

The benzylidenebenzofuran-3(2*H*)-ones are generally prepared by aldol condensation between benzofuran-3(2*H*)-ones and various benzaldehydes [33]. The products are frequently obtained in the form of *E*/*Z* isomeric mixtures; however, in theory, the *Z* isomer is more stable than the *E* isomer [36]. The configurations of the aurones are difficult to establish with certainty by relying simply on data collected by basic analytical methods [37]. Therefore, we have not performed these for the commercially obtained compounds. The docking program predicted different interaction energies for each isomer. The most potent 17β-HSD1 inhibitor in the series was predicted to be the *E* isomer of compound **1** (Fig. 2), but other potent 17β-HSD1 inhibitors (**3**, **6**, **7**, **9** and **21**) were calculated to be more active in their *Z* isomeric states (Tables 2 and 3). The aurones from the VHTS were thus selected according to their scores and independently of whether they were predicted to be either in their *E* or *Z* isomeric states, since we were unable to establish their conformations.

3.3. The presence of the 6-OH group is essential for potent 17β-HSD1 inhibition by the 2-benzylidenebenzofuran-3(2H)-ones

The results of the *in vitro* 17β-HSD1 inhibition assays allowed us to establish some important SAR data for these 2-benzylidenebenzofuran-3(2*H*)-one inhibitors of 17β-HSD1. The presence of the phenolic 6-OH group is essential for potent 17β-HSD1 inhibition by the 2-benzylidenebenzofuran-3(2*H*)-ones, as its replacement resulted in dramatic losses in the inhibitory activities (Tables 2 and 3). Compounds **1**–**10**, **19** and **20**, all of which have the free 6-OH group, showed at least 60% inhibition at 600 nM. These inhibitors most probably occupy an ideal orientation in the substrate-binding site, and therefore the strong proton donor phenolic 6-OH group can form triple H-bonds with catalytic residues Ser142 and Tyr155, and also with the proton-acceptor oxygen of the cofactor carboxamide, as suggested by the docking (Fig. 2). In this series of 6-OH compounds, there were no clear relationships between the various benzylidene substitutions, and 17β-HSD1 inhibitory potencies could be seen, although few general conclusions can be derived. The most potent 17β-HSD1 inhibitor, compound **1**, has an IC_{50} of 45 nM and has the 2′-OH and 3′-OMe groups, which can both form H-bonds with the substrate-recognition residues His221 and Glu282 (Fig. 1). The presence of either the 2′-OH or the 3′-OH groups reduces the 17β-HSD1 inhibitory potencies, as seen with compounds **4** and **3**, respectively. This implies that the 2′,3′-disubstitution pattern with polar substituents achieves the most optimal interactions with the active site. However, the presence of the –OH group on the benzylidene ring is not obligatory, and the introduction of the sole electron-withdrawing 2′-NO_2 in compound **2** shows preservation of good inhibitory activity, with an IC_{50} of 74 nM. The reduced inhibitory activity of compound **9**, when compared to compound **2**, shows that the additional the 4′-OH and 5′-OMe groups counteract the influence of the 2′-NO_2 substituent and decrease the interactions with 17β-HSD1. Compounds that have only alkoxy substituents on the benzylidene ring (**5**–**8**, **10**) retain high potencies; however, they are less active than compounds **1**, **3** and **4**, which have the –OH group on the benzylidene ring. The position, number and size of these alkoxy substituents clearly might not be related to the inhibitory potency.

Compounds **11**–**18** do not have the free 6-OH group, and they all show significantly lower 17β-HSD1 inhibitory activity than the compounds with the free 6-OH group. Compound **11**, where the 6-OH group was esterified with acetic acid, retained some inhibitory activity, probably because the 6-acetyl substituent is still a good proton-acceptor. Compounds **12**–**18** that have ether groups at position 6 were mostly inactive at 0.6 μM. Here, compounds **12** and **13** still showed significant inhibition at 6 μM, possibly because the 6-carboxamidomethyleneoxy functionality in compound **12** and the 4′-OH group in compound **13** can form polar interactions with the active site.

Compounds **19** and **20**, with 3′,4′-ethylenedioxy and 3′,4′-methylenedioxy groups on the benzylidene ring, respectively, have

free 6-OH groups and show very potent 17β-HSD1 inhibition at 0.6 μM, although they do not have polar substituents on the benzylidene ring (Table 3). Compound **19** is the second most potent inhibitor in this series of aurones, with an IC_{50} of 67 nM. Their very potent 17β-HSD1 inhibitory activities can be attributed to additional hydrophobic interactions of the rigid methylene and ethylene groups with the active site. Compound **21** is inactive, probably due to the 6-methoxy substituent.

3.4. Correct modifications of the benzylidene ring to larger bicyclic systems can lose 17β-HSD2 inhibition

The most potent of these 17β-HSD1 inhibitors were also evaluated for their 17β-HSD2 inhibition (Table 2). The compounds with polar groups and smaller alkoxy substituents (**1**–**7**) inhibited 17β-HSD2 at 60 μM. Conversely, compounds **20** and **21**, which have the larger 3′,4′-ethylenedioxy and 3′,4′-methylenedioxy groups, respectively, did not inhibit 17β-HSD2 (Table 3). This implies that by the correct modification of the benzylidene ring to a larger bicyclic system, 17β-HSD2 inhibition can be avoided, with the concomitant retention of very potent selective 17β-HSD1 inhibition. This should be considered when designing new inhibitors with the 2-benzylidenebenzofuran-3(2*H*)-one scaffold.

4. Conclusions

Our VHTS approach with implementation of simple pharmacophoric constraints resulted in the identification of new aurone derivatives as potent nonsteroidal 17β-HSD1 inhibitors. Although, aurones were previously reported for their 17β-HSD1 inhibition, their SAR for inhibition of this target enzyme has not been clarified yet. By introduction of specific computational programs for 2D-similarity search using *in vitro* validated virtual hits as queries and further biochemical evaluation, we successfully elucidated the key structural elements of aurones that are crucial for their potent 17β-HSD1 inhibition. Moreover, we have shown that properly substituted aurones could have good selectivity for 17β-HSD1 over 17β-HSD2 and thus could be considered as promising starting points for the development of clinically useful drugs for local deprivation of E2 levels in severe hormone-dependent diseases.

Acknowledgments

We thank OpenEye Scientific Software Inc. for free academic licenses of their software, Dr. Jerzy Adamski (Institute of Experimental Genetics, Genome Analysis Center, Neuherberg, Germany) for providing the *E. coli* BL21-CodonPlus (DE3)-RIL and JM-107 cells with pQE30-17β-HSD type 1 and pGEXII-N-29 17β-HSD type 2 constructs, respectively, and Government of the Republic of Slovenia, Ministry of Higher Education, Science and Technology of the Republic of Slovenia for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.08.013.

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