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Identification of novel 11β-HSD1 inhibitors by combined ligand- and structure-based virtual screening

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ABSTRACT

11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) converts cortisone to cortisol in a NADPH dependent manner. Overexpression of 11β-HSD1 in key metabolic tissues is related to the development of type 2 diabetes, obesity, hypertension and metabolic syndrome. Using crystal structures of human 11β-HSD1 in complex with inhibitors as source of structural information, a combined ligand and structure-based virtual screening approach was implemented to identify novel 11β-HSD1 inhibitors. A selected group of compounds was identified in silico and further evaluated in cell-based assays for cytotoxicity and 11β-HSD1 mediated cortisol production inhibitory capacity. The expression of 11β-HSD1 and 11β-HSD2 in human LS14 adipocytes was assessed during differentiation. Biological evaluation of 39 compounds in adipocytes and steroids quantification by HPLC-MS/MS identify 4 compounds that exhibit 11β-HSD1 mediated cortisol production inhibitory activity with potencies in the micromolar range. Two compounds showed to be selective for the 11β-HSD1 reductase activity and over 11β-HSD2 isoform, and thus represent novel leads for the development of more active derivatives with higher efficacies targeting intracellular cortisol levels in type 2 diabetes and metabolic syndrome.

1. Introduction

The enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) has bidirectional activities, capable of carrying out both NADPH dependent 11-oxoreductase (cortisol to cortisone) and $NADP^+$ dependent dehydrogenase (cortisone to cortisol) reactions, with enzyme directionality varying according to the differentiation or developmental status of the particular cell type (Gathercole et al., 2013). 11β-HSD1 is widely distributed but is most abundantly expressed in brain, liver and adipose tissue. It is also expressed in the heart and vasculature, bone, eye, immune system, gastrointestinal tract, kidney, skin, pancreas, and placenta (Morris et al., 2003; Tomlinson et al., 2004). Its isozyme 11β-HSD2, is a unidirectional NAD^+ dependent dehydrogenase with distinct tissue-specific expression in classical mineralocorticoid target tissues, such as kidney, colon and salivary glands (Farman and Bocchi, 2000), where inactivates cortisol to cortisone, preventing its binding to the mineralocorticoid receptor (Stewart and Krozowski, 1999). Although both 11β-HSD1 and 11β-HSD2 catalyze the interconversion of glucocorticoids (Fig. 1A), they are functionally different and share only 21% identical amino acid sequence (Tomlinson and Stewart, 2001). Several studies have revealed that overexpression of 11β-HSD1 mRNA and enzyme activity are increased in subcutaneous abdominal adipose tissue of obese subjects (Morton, 2010), and related to the development of metabolic syndrome (MS) (Wake and Walker, 2004; Baudrand et al., 2010). Accordingly, 11β-HSD1 is today considered a therapeutic target to lower intracellular cortisol levels in type 2 diabetes, obesity, hypertension and metabolic syndrome (Tomlinson, 2005; Wamil and Seckl, 2007;

Abbreviations: 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; E, cortisol; F, cortisone; HPLC-MS/MS, high performance liquid chromatography coupled with tandem mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; MS, metabolic syndrome; BGA, 18beta-glycyrrhetinic acid.

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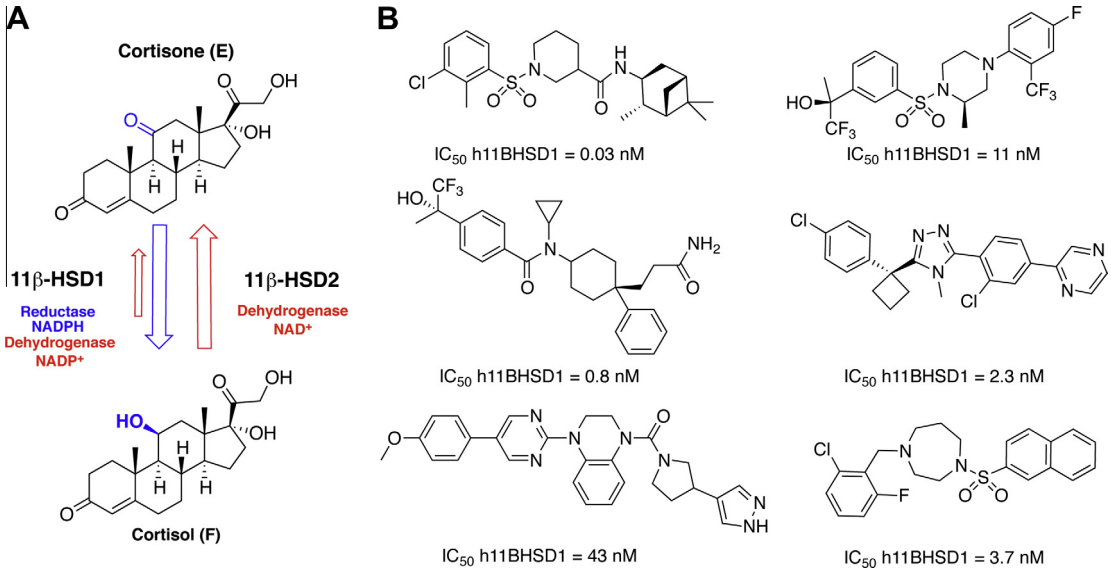


Fig. 1. (A) Mechanism of action of 11beta-hydroxysteroid dehydrogenases type 1 and 2. (B) Chemical structures of recently reported 11β-HSD1 inhibitors (IC_{50} are for reductase activity).

Schnackenberg, 2008; Gathercole and Stewart, 2010; Morgan and Tomlinson, 2010). Fig. 1B shows several reported examples of structural scaffolds with potent 11β-HSD1 inhibitory activities (Sun et al., 2011b,c; Venier et al., 2011; Wan et al., 2011; Xia et al., 2012).

Clinical trials with small-molecule inhibitors of 11β-HSD1 have shown inconclusive results (Hughes et al., 2012). For instance, a phase II study demonstrated that addition of INCB-13739 to metformin over 12 weeks resulted in statistically significant reduction in glycosylated hemoglobin A1, fasting plasma glucose, homeostasis model assessment-insulin resistance and total cholesterol in patients with type 2 diabetes inadequately controlled by metformin monotherapy (Rosenstock et al., 2010). On the other hand, a phase IIa study demonstrated that treatment with MK-0916 was well tolerated but not clinically effective in patients with type 2 diabetes and metabolic syndrome (Feig et al., 2011). Similarly, a 12-week study in overweight-to-obese patients with hypertension showed not statistical reduction in sitting diastolic blood pressure with MK-0736 and modest improvement in other BP endpoints (Shah et al., 2011).

As a result of the increasing search of 11β-HSD1 inhibitors, since the release of the first X-ray crystal structure of human 11β-HSD1 in 2004 (Hosfield et al., 2005), several crystal structures of 11β-HSD1 in complex with small molecule inhibitors have been deposited in the Protein Data Bank (PDB) database (Berman et al., 2000), thus providing a rich source of structural information for the development of novel and high affinity inhibitor compounds (Yan et al., 2010; Sun et al., 2011a,b; Thomas and Potter, 2011; Wu et al., 2011). 11β-HSD1 is NADPH-dependent enzyme that belongs to the short-chain dehydrogenase/reductase family and contains the structurally conserved nucleotide-binding Rossmann fold (Fig. 2A and B) (Rao and Rossmann, 1973), which is practically identical to that of the murine 11β-HSD1 structure, released in 2005 (Zhang et al., 2005). For the 11β-HSD2 isoform no crystal structure is available to date, but homology models have been developed (Carvajal et al., 2003; Manning et al., 2010).

In this work we report the use of a combination of computational and biological methods aimed to identify novel non-steroidal 11β-HSD1 inhibitors selective for the reductase activity and against 11β-HSD2 isoform in differentiated human adipose cell culture.

2. Methods

2.1. Protein–ligand complexes preparation and molecular modeling

Protein preparation and editing, atom typing (protein, ligand and cofactors), protein–ligand complexes structural alignment, and ligand clustering were performed using Accelrys Discovery Studio v2.1 (Accelrys Inc., San Diego, CA). The crystal structures of human 11β-HSD1 were retrieved from the Protein Data Bank database (Berman et al., 2000). After superimposition of monomers, the root-mean square deviation of alpha carbons (Cα-RMSD) for against the lower resolution structure was calculated for all residues and for residues within 6 Å from the center of mass of all co-crystallized ligands. Structural models for 11β-HSD1-cortisone and 11β-HSD2-cortisol complexes were developed and validated. Final full minimizations for all the systems were performed. The minimization protocol consisted in 20,000 minimization steps of the conjugate gradient algorithm with distance-dependent dielectric and the CHARMM22 force field (Brooks et al., 2009) until a gradient RMS of 0.001 kcal/mol Å was reached. Backbone atoms were restrained with an harmonic restraint of 15 kcal. See Supplementary information for full molecular modeling details.

2.2. Ligand-based shape query modeling

Once aligned in the same coordinate space, the co-crystallized ligand structures were clustered according to FPFC6 fingerprints, and the obtained 3D alignment for each cluster used to generate shape based query models using vROCS v3.1.2 (OpenEye Scientific Software). Each cluster hypothesis was validated according to the TanimotoComboScore metric using a library of decoys, and the obtained models retained and used as filters during docking procedure.

2.3. Virtual screening and compound selection

For docking purposes, the protein–ligand complex structure corresponding to each cluster center previously obtained was used (4), together with the 11β-HSD1-cortisone and 11β-HSD2-cortisol complexes. Receptors binding sites were automatically detected

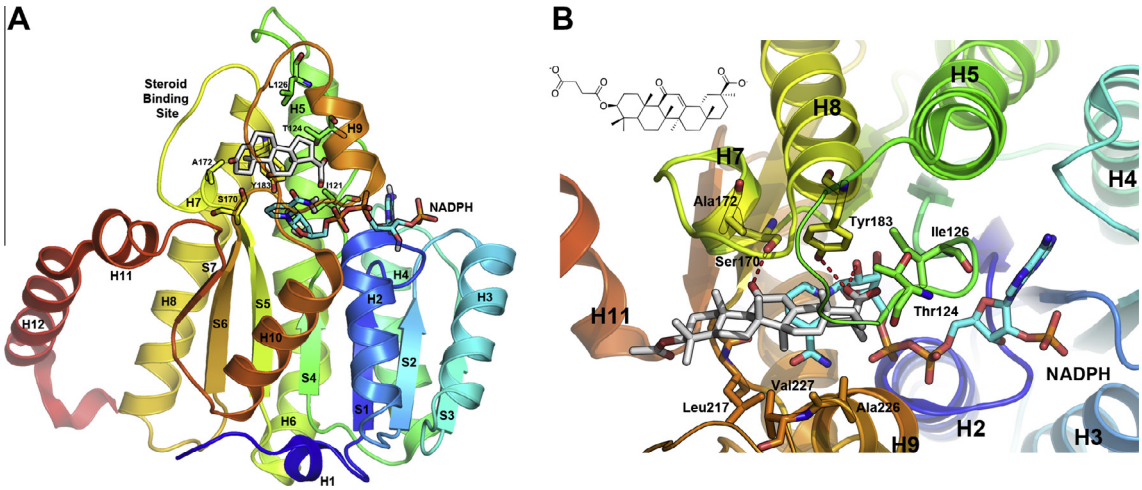


Fig. 2. (A) Schematic representation of murine 11β-HSD1-corticosterone complex (PDB id 1Y5R) secondary structure. 11β-HSD1 has a seven-stranded parallel β-sheets (S1–7) and 12 helices (H1–12) as seen also in the human 11β-HSD1 structures. (B) Carbenoxolone binding mode in the human 11β-HSD1 (PDB id 2BEL). Ligand binding site is located within the H7-H8, S4-H5 and S6-H9 connectors, while the nicotinamide head and the ribose ring of NADPH limits the bottom of the pocket.

using FRED receptor (OpenEye Scientific Software). The release 3 version of the OpenNCI database containing 260,071 structures was used for the screening. The library was retrieved from NCI, standardized and filtered by ADME/Tox constraints using FILTER v2.1, and multiple conformations for each compound in the database generated using OMEGA v2.4.3 (OpenEye Scientific Software) using default parameters (Hawkins et al., 2010; Hawkins and Nicholls, 2012). Virtual screening of the generated NCI conformer database was performed using FRED v2.2.5 program (OpenEye Scientific Software) with the ChemGauss3 scoring function (McGann et al., 2003). Top binding mode for the best 1000 scoring compounds on each protein binding site were selected according to a consensus score using the ChemGauss3, OEChemScore and PLP scoring functions (McGann, 2011). The top 100 scoring compounds on each docking run were visually analyzed, and overlap analysis of obtained solutions using the Tanimoto similarity metric performed with InstantJChem v5.9 (ChemAxon Inc.) to select 40 compounds that were requested along with beta-glycyrrhetinic acid (BGA) and obtained from to the Developmental Therapeutic Program at NCI-NIH (DTP/NIH, http://dtp.nci.nih.gov/).

2.4. LS14 cell line culture and differentiation

Our studies utilized the adipose cell line LS14, derived from a human metastatic liposarcoma (Hugo et al., 2006), which has been reported to be able to differentiate into lipid-laden adipocytes that express mature adipocyte genes (Hugo et al., 2006; Cifuentes et al., 2010). Preadipose LS14 cells were seeded on plastic culture dishes (Nunc, Rochester, NY) and grown in DMEM/Ham's F-12 (1:1) medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS, Hyclone) and antibiotics (penicillin–streptomycin). For adipogenic differentiation, cells were seeded at a density of 35,000 cells/cm^2, serum-starved overnight and cultured in the same medium (serum-free), supplemented with an adipogenic cocktail consisting of 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1.7 μM insulin (Eli Lilly & Co., Mexico) and 0.25 μM dexamethasone (Sigma). The medium was replaced every 2–3 days. Adipocyte differentiation was evidenced by Oil Red staining (Hausman, 1981). 11β-HSD1 and 11β-HSD2 mRNA expression was determined by RT-PCR.

Primer 11β-HSD1-forward 5′-AGGAAAGCTCATGGGAGGACTAG-3′ and 11β-HSD1-reverse 5′-ATGGTGAATATCATCATGAAAAAGATT-3′.

Western blot was performed under standard protocol conditions. Primary Rabbit polyclonal to 11β-HSD1 (ab83522, Abcam, Cambridge, UK); Rabbit polyclonal to 11β-HSD2 (AB115696, Abcam, Cambridge, UK); and secondary goat anti-rabbit IgG-HRP antibody (SC-2030, Santa Cruz Biotechnology, Santa Cruz, CA) were used.

2.5. Determination of 11β-HSD1 and 11β-HSD2 activities in differentiated LS14 cells

Dose response curves of cortisone were done to determine the lineal production of cortisol for 11β-HSD1 reductase enzymatic activity. LS-14 differentiated cells were incubated with DMEM/Ham's F-12 (1:1) and increasing substrate cortisone concentrations (37.5–4800 nM) for 24 h, NADPH was used as cofactor in excess (200 μM) and cortisol production was evaluated in supernatants by HPLC-MS/MS. 150nM was selected to perform the inhibition experiments because maximal enzyme activity and the lineal production of cortisol were found using this concentration. Determination of 11β-HSD1 and 11β-HSD2 dehydrogenase activity was determined in LS14 differentiated cells were incubated with DMEM/Ham's F-12 (1:1) and cortisol 600 nM concentrations for 24 h because maximal enzyme activity and an enough production of cortisone were found using this concentration, NADP+ or NAD+(200 μM) was used as cofactor, and cortisone or cortisol production was evaluated in supernatants by HPLC-MS/MS for 11β-HSD1 and 11β-HSD2 respectively.

2.6. 11β-HSD type 1 and type 2 activity inhibition assays

For the in vitro inhibition assays, all compounds from the NCI library and 18beta-glycyrrhetinic acid (BGA) were resuspended in dimethylsulfoxide (DMSO). The biological evaluation of the selected compounds was performed in LS14 differentiated cell culture. Differentiated LS14 cells were incubated with DMEM/Ham's F-12 (1:1) and antibiotics for 24 h with: (a) cortisone 150 nM or cortisol 600 nM (positive control and 100% of 11β-HSD1 reductase/dehydrogenase activity); (b) cortisone 150 nM or cortisol 600 nM plus increasing concentrations (between 0.03 and 40 μM) of each compound and (c) no cortisone/cortisol, considered as basal activity. NADPH, NADP+ or NAD+(200 μM) was used as cofactor in the corresponding assay. Each experiment contained a dose response curve of BGA between 0.015 and 10 μM as inhibition

control. The collected supernatants from LS14 cell cultures were purified in SPE columns and stored at −80 °C without preservatives until assayed. Cortisol and cortisone concentrations were measured in the incubation culture media by HPLC-MS/MS. Each solvent used was HPLC grade from Merck (Santiago, Chile) or Sigma (BGA). Cortisol (F), Cortisol-D4 (F-D4) and cortisone (E) were purchased from Steraloids, Inc. (Andover, MA, USA). The percentage of inhibition of each compound was obtained from the amount of cortisol/cortisone produced in each treatment relative to the positive control.

2.7. Triacylglycerol (TG) content

To assess adipocyte triacylglycerol content, LS14 cells were treated with 0.25% trypsin solution at 37 °C for 2 min or until cells were detached from the culture dish. The trypsin was then inactivated with culture medium containing FBS to a final concentration of 10%. Cells were sedimented by centrifugation at 800xg for 20 min and then incubated at room temperature for 5–10 min in a 1 μg/mL Nile Red (Sigma) solution in PBS. Fluorescence was evaluated using 485 nm excitation and 572 nm emission filters (Synergy 2 fluorimeter, BioTek Instruments). The results were normalized by total protein content, measured using the bicinchoninic acid method (Pierce).

2.8. Cell viability assays

The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) kit containing the tetrazolium compound MTS ([3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]) and an electron coupling reagent (phenazineethosulfate; PES) was used to monitor cell viability according to the manufacturer's protocols. Briefly, Human hepatocellular carcinoma (HepG2) cells (ATCC 1998, CRL-10741, Rockville, MD) were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), under standard cell culture conditions (37 °C, humidified, 5% CO_2). Cells were plated at a density of 1.5×10^4 cells/well in a 96-well plate and incubated in growth medium for 18 h. Cells were treated with 0, 0.02, 0.2, 2 and 20 μM of each compound in DMEM containing 10% FBS for 48 h. After the indicated time of incubation with the appropriate medium, 20 μL MTS/phenazinemethosulfate (PMS) (1:0.05) mixture was added per well, and cells were incubated for 1 h. MTS is reduced by viable cells to formazan, which then can be monitored on a ELX-800 universal plate reader (BioTek, Winooski, VT) at a 490-nm absorbance. Formazan production is time dependent and is proportional to the number of viable cells. The percentage of cell death was calculated by comparing absorbance values with untreated cells. Background absorbance from the control wells (same media, no cells) was subtracted. Six duplicate studies were performed for each experimental condition. Additional cytotoxicity assay using the MTS was performed for selected compounds using the LS-14 adipose cell line (see below), getting less than 10% of cell death for all of them.

3. Results

3.1. Protein–ligand complexes preparation and molecular modeling

Protein structure resolutions range from 1.55 to 3.10 Å. Fig. 3 depicts the structural alignment of the 18 structures of 11β-HSD1 in complex with small molecules revealed that all ligands bind to a common site located close to the nicotinamide ring in NADPH, surrounded by several of the residues that have been reported as essential for catalysis such as Ser170, Tyr183 and Ala172 among other (Sun et al., 2011a; Thomas and Potter, 2011). The alpha-carbon root-mean square deviation against the lower resolution 11β-HSD1 structure (PDB id 1XU9) range from 0.406 to 2.395 Å (average 1.68 Å), and the Cα-RMSD within 6 Å from the center of mass of all aligned ligands ranges from 0.399 to 2.349 Å (average 1.78 Å), showing that ligand binding does not induce significant conformational changes in the binding site.

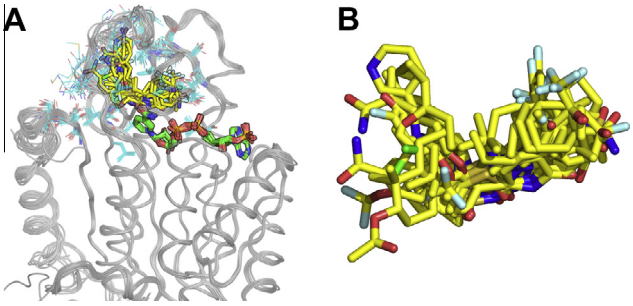


Fig. 3. (A) Structural alignment of 18 crystal structures of complexes of human 11β-HSD1 with small molecules. Secondary structures are shown as gray ribbons, NADPH cofactor and ligands are shown with carbon atoms in green and yellow respectively. (B) Structure-based ligand alignment used for clustering and shape-based query generation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

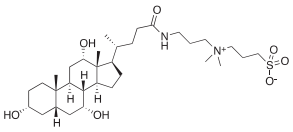
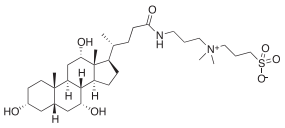
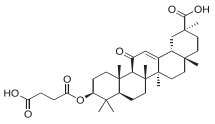
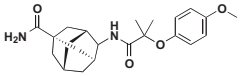
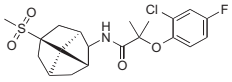
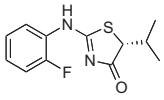
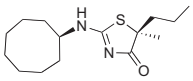
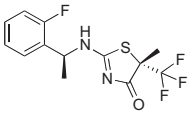
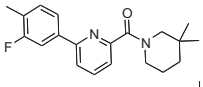
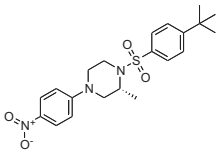
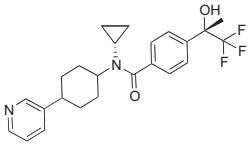
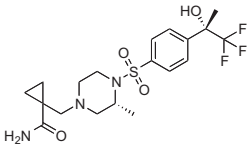
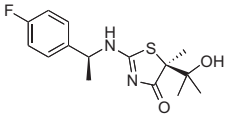
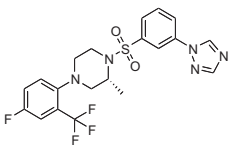
3.2. Ligand-based shape-query modeling

Similarity values were calculated using the Tanimoto distance for the molecular function class fingerprints of maximum diameter 6 (FCFP_6) available within the cluster ligand protocols in Discovery Studio v2.1. After protein structure superposition, the procedure identifies 4 main clusters according to the FCFP_6 fingerprints, and within each cluster ligands are very similar as estimated by the Tanimoto coefficients (Table 1). Cluster 1 includes only carbenoxolone (CBX), as steroidal compounds were purposely excluded from the analysis in order to avoid similarity with this type of scaffolds within the virtual screening results. The obtained 3D alignment for each cluster was then used to generate shape based query models. Each cluster hypothesis was validated according to the TanimotoComboScore metric using a library of decoys. The enrichment curve plots the number of active compounds recovered versus the proportion of the database screened. The AUC (area under the curve of an ROC plot) is simply the probability that a randomly chosen active has a higher score than a randomly chosen inactive. As seen in Table 2, the area under the curve (AUC) of the probability obtained for the hypotheses from clusters 2, 3 and 4 are higher that 73% at ±95% confidence (ROC curves in supplementary figure X), suggesting the hypotheses queries can be considered moderately selective as most actives rank higher than most of the decoy molecules.

3.3. Modeling of 11β-HSD1-cortisone and 11β-HSD2-cortisol complexes

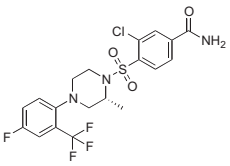
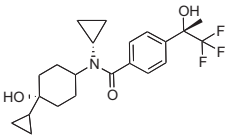
Models for 11β-HSD1 in complex with cortisone and 11β-HSD2 in complex with cortisol were generated by two ways. In the first case we used the position of corticosterone in the murine 11β-HSD1-corticosterone complex to obtain a putative position for the steroidal scaffold of cortisone within the protein, and then we applied molecular modeling techniques to create a human 11β-HSD1-cortisone complex. In the second case, we used comparative modeling to generate a 3D structure of 11β-HSD2-cortisol complex using several structures of 17β-HSD1 as templates. The overall sequence identity and sequence similarity were 23.5% and

Table 1
Summary of crystal structures of 11β-HSD1-ligand complexes used in this study.

PDB ID (Ref.)	Resolution (Å)	Ligand structure	RMSD 1XU9 (Å)	RMSD BS (Å)[a]	Cluster ID	Tanimoto distance[b]
1XU9 (Hosfield et al., 2005)	1.55		–	–	N.A	N.A
1XU7 (Hosfield et al., 2005)	1.80		0.406	0.399	N.A	N.A
2BEL (*tbp*)	2.11		1.492	1.796	1	0.000
2IRW (Patel et al., 2007)	3.10		1.799	1.956	4	0.000
2ILT (Sorensen et al., 2007)	2.30		1.770	1.874	4	0.448
2RBE (Yuan et al., 2007)	1.90		2.027	2.049	3	0.712
3BYZ (Johansson et al., 2008)	2.69		1.754	1.749	3	0.667
3BZU (Hale et al., 2008)	2.25		2.019	2.146	3	0.000
3CH6 (Wang et al., 2008)	2.35		1.628	1.713	2	0.733
3CZR (Sun et al., 2008)	2.35		1.741	1.720	2	0.605
3D3E (Julian et al., 2008)	2.60		0.573	1.093	4	0.739
3D4N (Julian et al., 2008)	2.50		1.803	1.849	2	0.730
3EY4 (tpb)	3.00		2.395	2.349	3	0.417
3HFG (Wan et al., 2009)	2.30		1.849	1.827	2	0.000

(*continued on next page*)

Table 1 (*continued*)

PDB ID (Ref.)	Resolution (Å)	Ligand structure	RMSD 1XU9 (Å)	RMSD BS (Å)[a]	Cluster ID	Tanimoto distance[b]
3H6K (Wan et al., 2009)	2.19		1.903	2.006	2	0.406
3FCO (McMinn et al., 2009)	2.65		2.041	2.196	4	0.723

tbp: to be published.
[a] Residues within 6 Å from the center of mass of ligand alignment.
[b] Tanimoto score.

Table 2
Summary of receptor characteristics and area under the curve (AUC) for shape-query models validation.

Receptor	Cluster ID	AUC[a]	Site box volume	Outer contour volume	Inner contour volume	Interaction constrain acceptor	Interaction constrain donor
2BEL	1	N.D.	5459	1322	152	SER170, TYR183, WAT5	None
3HFG	2	0.737	5222	1186	268	ALA172	None
3BZU	3	0.869	3755	844	167	ALA172, TYR183	None
2IRW	4	0.779	5440	1031	197	SER170, TYR83	None
11β-HSD1-E	N.D	N.D	4296	1222	460	SER170, TYR183	O3 from NADPH
11β-HSD2-F	N.D.	N.D.	3938	875	206	TYR232, ASN171	None

[a] Using the TanimotoComboScore.

43.6% respectively (Supplementary Fig. 2). Although 11β-HSD1 and 11β-HSD2 proteins have only ~18% sequence identity they share a similar binding site architecture as shown in Fig. 4. The overall quality of the obtained 11β-HSD2 model was assessed by several methods. A Cα-RMSD < 1 Å was obtained when superimposing the 11β-HSD2 model with the template structures (Supplementary Fig. 3A). Here, more than 98% of the residues fall in the allowed regions of the Ramachandran plot and less than 2% of the residues were outliers, which are located in loop regions that are away from the steroids binding site. The PROSA Z-score of −4.79 indicates that the model is of similar quality to that of experimentally determined X-ray and NMR structures of the same length. Similar results were obtained using the ProQ server that scores the model with an LGscore of 3.131, which is indicative of a very good model (LGscore > 2.5). Thus, the obtained model has a reasonable fold and can be used with confidence for further docking procedures. Table 2 shows the details of the receptors used in the virtual screening protocol.

3.4. Virtual screening and compound selection

Physicochemical profiling (ADME/Tox) of the 260,071 compounds of the OpenNCI library yields a filtered library of 159,317 compounds and a library of 11,238,156 conformers. The conformer library was screened and the top 1000 ranked compounds resulting from the docking procedure on each cluster center protein were filtered based on docked ligand poses that satisfy essential binding features according to interaction constrains. In-house

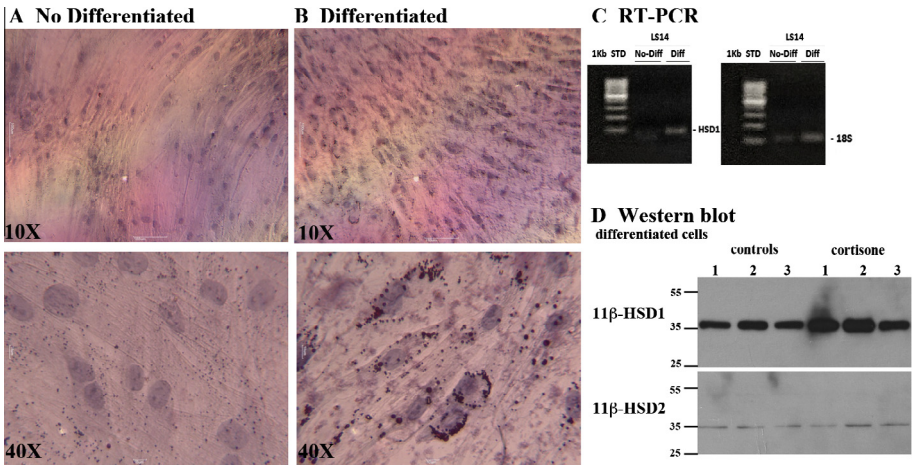


Fig. 4. Differentiation of LS-14 cell. (A) Two representative images from non-differentiated LS-14 cells (column A) and LS-14 differentiated cell (column B) Oil red O staining in two magnifications (10X and 40X). (B) Agarose gel ethidium bromide, staining showing RT-PCR product for 11β-HSD1 (upper panel) or the control gene 18s (lower panel). In non-differentiated (No-Diff) or differentiated (Diff)LS-14 cells.

developed scripts allow us to additionally identify the top scoring compounds that are able to bind to more than one 11β-HSD1 binding site. Visual inspection and compound availability determined the selection of the 39 compounds that were requested and obtained from the Developmental Therapeutics Program at the NCI/NIH. During the selection of the docked molecules, we wanted to select compounds able to bind to 11β-HSD1 and not to 11β-HSD2. None of the compounds was able to bind to all binding sites, and the top 39 compounds were selected according to (I) their ability to replace the substrate by forming hydrogen bonds with the active site residues (number of Hbonds > 1) and (II) the calculated ChemGauss3 score (<−60.00). The remaining docking results were analyzed visually and molecules were selected based on (I) the number and type of interactions established, (II) which residues they interacted with, and (III) comparison of the structures of the active sites of 11β-HSD1. Molecules that were predicted to interact with 11β-HSD1 and not 11β-HSD2 active site were preferentially selected.

As shown in Table 3, BGA (non selective 11β-HSD1 inhibitor) scored similar in 11β-HSD1 sites and 11β-HSD2, while the compounds NSC83182 and NSC111660 are predicted to be selective for 11β-HSD1. NSC131747 has a selectivity ratio (score 11β-HSD1/score 11β-HSD2) of 1.27 compared to that of NSC158971 (0.92) and BGA (1.03), suggesting that these compounds are not selective for 11β-HSD1.

Analysis of the binding mode for the most potent compounds show that the dihydrochalcone derivative NSC83182 (Fig. 7) binds in a V-shape conformation to 11β-HSD1. This molecule docked well in four out of five 11β-HSD1 binding sites. Hydrogen bonds are formed between the carboxylate group of the ligand and the OH groups of the Ser170 and Tyr183 side chains (2BEL and 2IRW). Alternative binding modes where no polar contacts are made (3HFG) or a binding mode where the carboxy group is stabilized with the main-chain amide nitrogen atoms from residues Leu171 and Ala172 (3BZU) were also obtained for this compound. In the case of the isoquinolinone derivative NSC131747, no common binding mode was observed, despite the fact that several hydrogen-bond interactions can be formed between the ligand nitrogen and carbonyl oxygen atoms and the side chains of Tyr183, Ser170, Ala172 or Tyr177 (data not shown).

3.5. LS14 cell line culture and differentiation

The LS14 is a liposarcoma derived adipose cell line that exhibits a gene expression pattern similar to that in primary human preadipocytes, are capable of terminal differentiation, and accurately represent the full spectrum of hormonal and metabolic characteristics of human adipocytes (Hugo et al., 2006). As shown in Fig. 6, only differentiated LS14 cells showed Oil Red O staining (Fig. 6B) and 11β-HSD1 mRNA expression, as determined by real-time PCR (Fig. 6C). The protein abundance was studied in differentiated cells by Western blot with specific antibodies (Abcam). Basal expression was detected in control cells (no cortisone) but when cells were incubated the presence of cortisone the immunoreactivity to 11β-HSD1 increased. 11β-HSD2 expression was less abundant than 11β-HSD1 (Fig. 4D). As shown in Fig. 5, the activity of 11β-HSD1 in LS14 differentiated cell is predominantly reductase (Fig. 5A), and low 11β-HSD1 as well as 11β-HSD2 dehydrogenase activities detected (Fig. 5B and C). Maximal substrate conversion under assay condition for 11β-HSD1 reductase activity was 49.3%, whereas for 11β-HSD1 and 11β-HSD2 dehydrogenase activity were 4.18% and 1.32%, respectively (Fig. 5D, Supplementary Table 1).

Table 3
Comparative docking scores of selected compounds.

Compound	2BEL	2IRW	3HFG	3BZU	Average	11β-HSD2
NSC83182	−67.06	−100.58	−80.51	−88.52	−84.17	ND
NSC111660	−58.29	−62.27	−77.63	−72.79	−67.75	ND
NSC131747	−69.26	−96.86	−89.85	−81.05	−84.26	−66.38
NSC158971	−65.57	−93.72	−85.21	−76.96	−80.37	−87.62
BGA	−86.73	−44.12	−92.25	ND	−74.37	−75.32

All scores calculated with the ChemGauss3 scoring function for the top scored binding mode obtained for each compound.
ND: not docked.

3.6. Inhibition of 11β-HSD1 reductase activity

The inhibitory rate of the compounds at 5 μM was in the range from 1.29% to 57.2% (Table 3). Of the 39 compounds, two showed greater than 50% inhibition of 11β-HSD1 mediated conversion of cortisone to cortisol (Table 4). Compound NSC18294 did not dissolve in DMSO, and therefore it was not tested (*ND*). The active compounds showed a dose-dependent inhibition of human 11β-HSD1 with IC_{50} values ranging from 5.31 to 16.34 μM (Fig. 6A). Although IC_{50} for BGA has been reported to be in the range of 8.6–300 nM (Su et al., 2007; Zhang et al., 2009; Xia et al., 2011), in our in vitro model BGA shows a calculated IC_{50} of 0.32 μM for 11β-HSD1 reductase activity.

3.7. Inhibition of 11β-HSD1 and 11β-HSD2 dehydrogenase activity

The most potent compounds were also evaluated in their capacity to inhibit 11β-HSD1 and 11β-HSD2 dehydrogenase activity. Neither NSC83182 nor NSC158971 inhibits dehydrogenase activity. As shown in Fig. 6B, compound NSC83182 instead increases dehydrogenase activity of both 11β-HSD1 and 11β-HSD2 by 4–10-fold against control respectively. On the other hand, compound NSC158971 does not seem to produce significant changes compared to control dehydrogenase activity.

3.8. Cell viability assays

Cell viability assay was performed primarily in HepG2 cells using the MTS method. The compounds were assayed under increasing concentrations from 0.002 to 20 μM. The greater part of the tested compounds did not show signs of toxicity (defined as the value obtained after subtracting 2 standard deviations from the average value of control wells treated only with vehicle) even at 20 μM (Supplementary Fig. 5). NSC83182 (20 μM) reduced HepG2 mean cell viability to near 80% compared to control. However NSC111660; NSC131747 and NSC158971 at 20 μM did not affect HepG2 cell viability. NSC169996 showed a greater toxic effect in HepG2, reducing cell viability to 60% of control. When assayed in the LS14 cell line, both NSC83182 and NSC158971 did not reduce cell viability compared to control (Fig. 6C).

3.9. LS14 adipocyte triacylglycerol content

To evaluate a physiological endpoint after the exposure of LS14 cells to the 11β-HSD1 inhibitors, we assessed the TG content, which represents the most important physiological function of adipocytes. Exposure to cortisone in 11β-HSD1-positive cells such as LS14, and the consequent elevation in cortisol levels, is expected to decrease adipocyte lipid content (Morris et al., 2003). Our observations show this trend (Fig. 6D). Exposure to cortisone together with the 11β-HSD1 inhibitor NSC83182 (one of the most potent in our inhibition studies) reverted this trend, with TG content significantly greater than cortisone-exposed cells and not different from cells not exposed to cortisone. This result suggests that NSC83182 modulate adipocyte lipid handling, a main

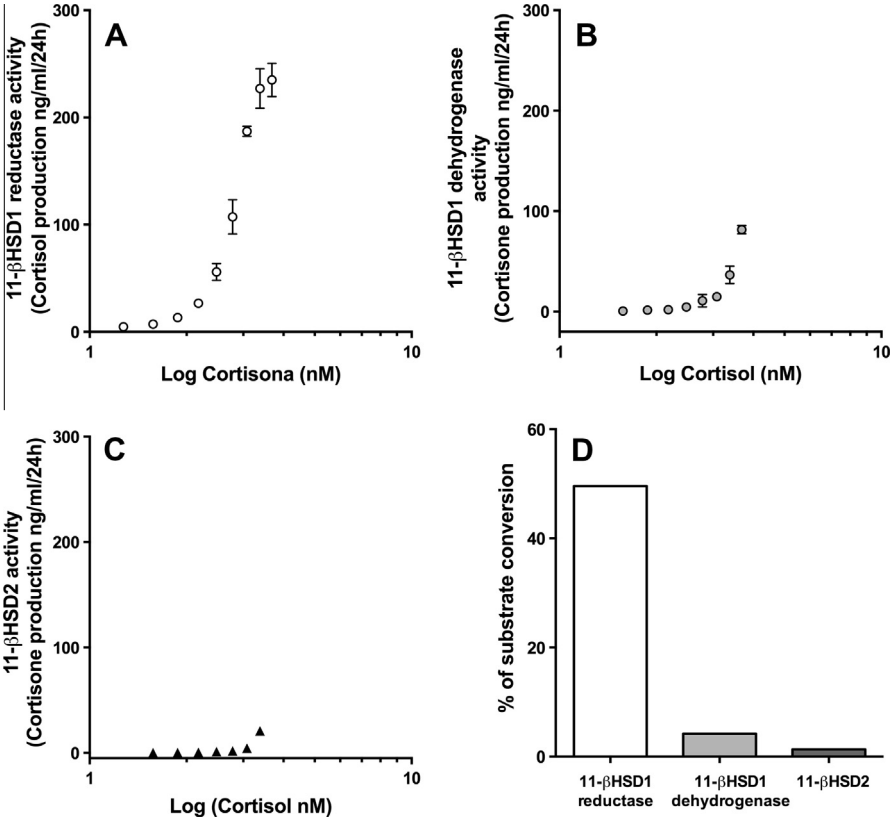


Fig. 5. Determination of 11β-HSD1 and 11β-HSD2 activities in LS-14 cell line. (A) 11β-HSD1 reductase activity, (B) 11β-HSD1 dehydrogenase activity, (C) 11β-HSD2 dehydrogenase activity and, (D) percentaje of substrate conversion by 11β-HSD enzymes.

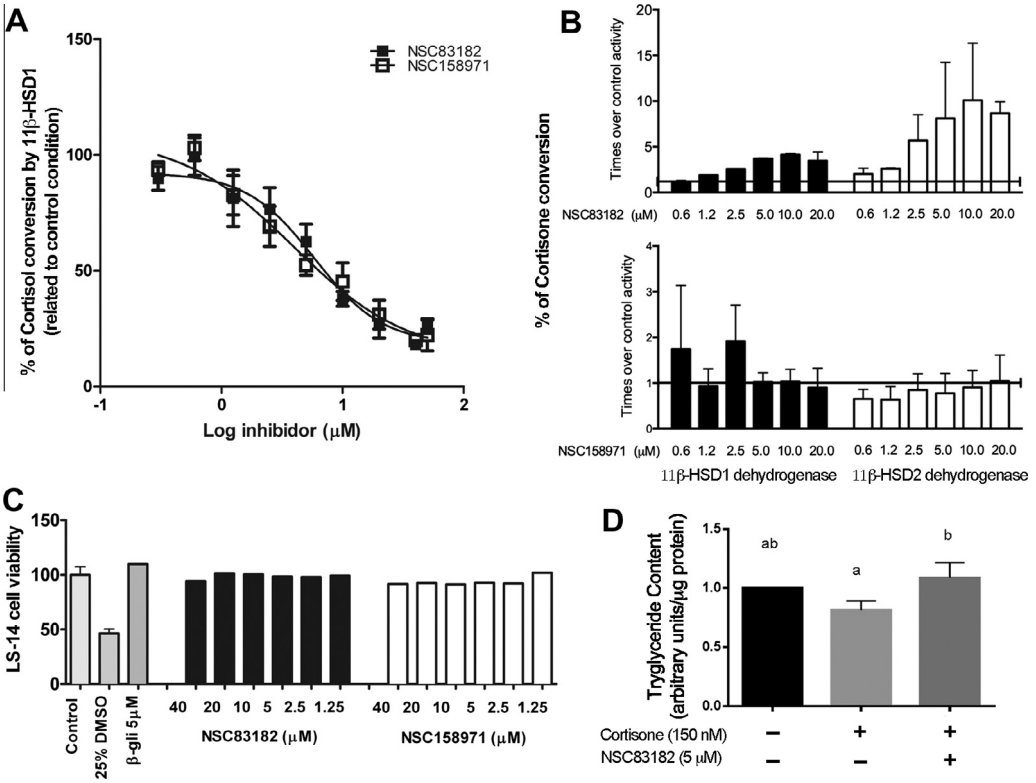


Fig. 6. In vitro inhibition assay of novel identified 11β-HSD1 inhibitors in LS-14 cell line. (A) Dose response curve for the most active compounds in the 11β-HSD1 reductase assay, (B) inhibition of 11β-HSD1 and 11β-HSD12 dehydrogenase activities, (C) Effect of the compounds on LS-14 cell viability and, (D) triacylglycerol content in differentiated LS14 adipocytes exposed to the specified conditions for 72 h. Bars represent mean ± SEM; values have been normalized to the negative control for visual purposes only. Bars with different letters differ, $p < 0.05$, Friedman and Dunn's multiple comparison test (raw data were used for the analysis), $n = 4$ independent experiments.

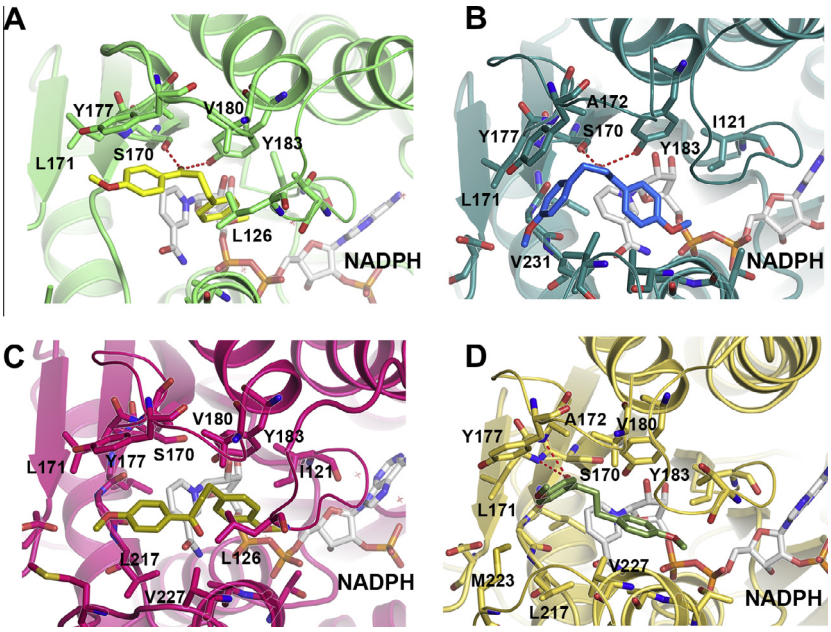


Fig. 7. Binding mode obtained for active compound NSC83182 within various 11β-HSD1 binding sites: (A) 2BEL, (B) 2IRW, (C) 3HFG and (D) 3BZU.

Table 4
Inhibition of 11β-HSD1 mediated cortisol production in LS14 cell lysates and average docking scores for tested compounds.

Compound identifier	ChemGauss3 score	% inhibition[a]	IC_{50} (μM)[b]	Compound identifier	ChemGauss3 score	% inhibition[a]	IC_{50} (μM)[b]
NSC13440	−65.90	4.83		NSC127489	−86.30	2.86	
NSC18294	−70.69	ND		**NSC131747**	**−84.26**	**43.73**	**16.34**
NSC23834	−93.91	4.96		NSC132823	−72.70	11.83	
NSC27040	−98.32	2.04		NSC141992	−87.39	6.69	
NSC32979	−61.81	2.33		NSC147861	−95.36	3.70	
NSC37908	−95.83	2.31		NSC157865	−96.66	6.59	
NSC69353	−94.95	17.34		**NSC158971**	**−80.37**	**55.75**	**5.31**
NSC78561	−69.72	6.06		NSC159878	−90.21	5.95	
NSC83182	**−84.17**	**57.2**	**5.85**	NSC169996	−87.04	3.06	
NSC83495	−93.22	13.16		NSC173318	−93.83	1.29	
NSC83503	−88.12	4.09		NSC196538	−86.41	3.05	
NSC89589	−96.18	4.29		NSC201553	−75.56	1.90	
NSC91351	−92.21	3.83		NSC205438	−81.03	2.80	
NSC91362	−95.74	5.81		NSC277468	−93.89	24.16	
NSC93300	−90.65	2.76		NSC277469	−97.41	12.71	
NSC101558	−94.91	19.27		NSC302561	−101.99	17.85	
NSC111575	−95.21	5.37		NSC308795	−93.98	2.48	
NSC111660	**−67.75**	**44.40**	**9.87**	NSC312899	−93.67	4.55	
NSC115811	−96.18	4.73		NSC356219	−95.56	5.46	
NSC116373	−97.33	4.25		BGA	−74.37	100	0.32

The most active compounds are highlighted in bold.
[a] Percentage of inhibition 11β-HSD1 reductase activity was measured at 5 μM in LS14 adipocytes, results are reported as a mean of at least three independent determinations with a standard deviation of typically ±5%.
[b] IC_{50} values (μM) were obtained with six inhibitor concentrations.

pathophysiological player in the metabolic syndrome, by inhibiting 11β-HSD1 activity.

4. Discussion

11 beta-hydroxysteroid dehydrogenase type 1 (11β-HSD1) overexpression in key metabolic tissues are related to the development of type 2 diabetes, obesity, hypertension and metabolic syndrome. Accordingly, several chemical families have been developed as potential 11β-HSD1 inhibitors discussed in comprehensive reviews (Boyle and Kowalski, 2009; Ge et al., 2010; Singh and Tice, 2010; Sun et al., 2011a).

Virtual screening has been successful in prioritizing large chemical libraries to identify experimentally active compounds, serving as a practical and effective alternative to high-throughput screening (Polgar and Keseru, 2011; Tang and Marshall, 2011). Previous publications from several groups have reported the use of either ligand or structure-based methods for the identification of 11β-HSD1 inhibitors (Schuster et al., 2006; Yang et al., 2008, 2009; Singh and Tice, 2010; Tice et al., 2010; Paderes et al., 2011; Xia et al., 2011; Xu et al., 2011). Given the rich source of structural information available for 11β-HSD1 we designed a virtual screening protocol which combines both methods by including the obtained shape-based query hypotheses as a filter during our virtual screening procedure, with the aim of identifying non-steroidal compound selective for the reductase state of the enzyme and selective over its isoform 11β-HSD2.

To date, 33 crystal structures of 11β-HSD1 in complex with small molecule inhibitors are available at the PDB Database

(http://www.pdb.org), of which 26 correspond to human 11β-HSD1. When we started this work, only 18 structures had been released (See Table 1). The alpha-carbon root-mean square deviation against the lower resolution 11β-HSD1, and the Cα-RMSD within 6 Å from the center of mass of all aligned, shows that ligand binding does not induce significant conformational changes in the binding site. Therefore, in order to reduce the structural complexity, we used a clustering approach based on molecular fingerprints, which are representations of chemical structures originally designed to assist in chemical database searching, but later used for analysis tasks such as similarity searching, clustering or recursive partitioning (Hassan et al., 2006). Based on this clustering approach, a set of representative binding sites of 11β-HSD1 in complex with small molecules was obtained. Although several crystal structures are available for 11β-HSD1, currently there is no reported crystal structure for either of 11β-HSD1 in complex with cortisone or for 11β-HSD2 in complex with cortisol. Thus, we constructed a model for each of these complexes. To obtain a 11β-HSD2-cortisol complex we used several structures of related 17β-HSD1 as templates. The template selection was based on the BLAST searches and considering previous phylogenetic studies (Baker, 2010). The virtual screening protocol allows us to identify a set of putative 11β-HSD1 inhibitors able to bind to several of the representative 11β-HSD1 binding sites, which have low affinity for the 11β-HSD2 isoform.

The adipose tissue, which is a target for 11β-HSD1 inhibitors and also an integral component within the endocrine system (Liu et al., 2011), has serious pathophysiological consequences when misregulated. In adipose tissue, 11β-HSD1 predominantly functions as an oxoreductase, facilitating glucocorticoid receptor-mediated hormone action (Bujalska et al., 2002, 2007). 11β-HSD1 inhibitors have been historically evaluated in cell lines that are further differentiated into adipose cells, and therefore, a cell model of adipose tissue to study the effect of 11β-HSD1 inhibition that better mimics the physiological conditions should provide an improved tissue-specific compound selection tool. In our work, we used the LS-14 cell line, a liposarcoma derived adipose cell line that exhibits a gene expression pattern similar to that in primary human preadipocytes. Our results showed that 11β-HSD2 expression was less abundant than 11β-HSD1 in this cell line, in agreement with previous reports of up to 22-fold lower 11β-HSD2 versus 11β-HSD1 expression in adipose tissue (Uckaya et al., 2008; Mericq et al., 2009; Munoz et al., 2009; Svendsen et al., 2009; Veilleux et al., 2009; Baudrand et al., 2010; Veilleux et al., 2010; Mlinar et al., 2011; Sledzinski et al., 2011; Wamil et al., 2011). We have shown that LS-14 cells capable of terminal differentiation, might be considered as model for the evaluation of the inhibitory capacity of compounds targeting 11β-HSD1.

All the novel identified active compounds have chemical scaffolds that have not been previously reported as 11β-HSD1inhibitors. A PubChem (http://www.pubchem.ncbi.nlm.nih.gov) search shows that NSC83182, the most potent inhibitor identified, has been reported to induce beta-cell replication by 5.33%, and to inhibit/block calcium-activated chloride channels. NSC131747 and NSC111660 have been found to be active in various yeast anticancer drug screens, and NSC158971 does not present any record of bioassay activity. Interestingly, selective inhibition of reductase activity of 11β-HSD1 by similar flavanone compounds has been reported by the Oddermatt group (Schweizer et al., 2003). In all binding modes, an important π-stacking interaction between Tyr177 and one of the 4-methoxyphenyl rings is established. Tyr177 represents the largest difference between the human and the rodent enzymes (Zhang et al., 2005), and might represent a critical binding partner for NSC83182. For this compound it may be plausible that its mechanism over cell replication may explain the increase in 11β-HSDs dehydrogenase activities, although further experiments are needed to address this issue.

Considering that the disappointing efficacy of 11β-HSD1 inhibitors in clinical studies has been explained by lack of selectivity for 11β-reductase activity (Hughes et al., 2012), our method provides a novel approach for the development of selective agents that inhibits preferentially the reductase activity of 11β-HSD1 and selectively over the 11β-HSD2 isoform.

5. Conclusions

We have successfully identified novel non-steroidal 11β-HSD1 inhibitory compounds, which show activity in the micromolar range in cell-based assay, by means of a combined ligand and structure-based virtual screening approach. Furthermore, we have developed a method for the screening of potential 11β-HSD1 inhibitors, which combines a cell-based assay using human derived adipose cells and enzymatic product determination by HPLC-MS/MS. Forthcoming work will be focused on the activity optimization of these identified lead compounds.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2014.01.011.

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