A Salicylic Acid-Based Analogue Discovered from Virtual Screening as a Potent Inhibitor of Human 20α-Hydroxysteroid Dehydrogenase

Urmi Dhagat[a], Vincenzo Carbone[a], Roland P.-T. Chung[a], Toshihiro Matsunaga[b], Satoshi Endo[b], Akira Hara[b] and Ossama El-Kabbani[a,*]

[a]*Department of Medicinal Chemistry, Victorian College of Pharmacy, Monash University (Parkville Campus), Parkville, Victoria 3052, Australia;* [b]*Laboratory of Biochemistry, Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502-8585, Japan*

Abstract: 20α-hydroxysteroid dehydrogenase (AKR1C1) plays a key role in the metabolism of progesterone and other steroid hormones, thereby regulating their action at the pre-receptor level. AKR1C1 is implicated in neurological and psychiatric conditions such as catamenial epilepsy and depressive disorders. Increased activity of AKR1C1 is associated with termination of pregnancy and the development of breast cancer, endometriosis and endometrial cancer. Inhibition of the undesired activity of AKR1C1 will help reduce risks of premature birth, neurological disorders and the development of cancer. In order to identify potential leads for new inhibitors of AKR1C1 we adopted a virtual screening-based approach using the automated DOCK program. Approximately 250,000 compounds from the NCI database were screened for potential ligands based on their chemical complementarity and steric fit within the active site of AKR1C1. Kinetic analysis revealed 3,5-diiodosalicylic acid, an analogue of salicylic acid, as a potent competitive inhibitor with respect to the substrate 5β-pregnane-3α,20α-diol with a K_i of 9 nM. Aspirin, which is a well known salicylic acid-based drug, was also found to inhibit AKR1C1 activity. This is the first report to show aspirin (IC_{50}=21 μM) and its metabolite salicylic acid (IC_{50}=7.8 μM) as inhibitors of AKR1C1.

Key Words: Enzyme inhibitors, molecular docking, salicylic acid, 20α-hydroxysteroid dehydrogenase, aldo-keto reductase.

INTRODUCTION

Hydroxysteroid dehydrogenases (HSDs) regulate the availability of steroid hormones such as progesterone, estrogen, androgens, glucocorticoids and mineralocorticoids to their corresponding receptors. These enzymes act as molecular switches that regulate receptor occupancy and activation by catalysing the interconversion of potent steroid hormones to their inactive counterparts making them important drug targets [1-3]. HSDs belong to two distinct protein superfamilies, the short chain dehydrogenase/ reductases and the aldo-keto reductases (AKRs). There are four human 3α-HSD isoforms which belong to the AKR1C subfamily, namely, AKR1C1, AKR1C2, AKR1C3 and AKR1C4 [4, 5]. Although these isoforms share high sequence identity (>84%) they exhibit broad substrate specificity and are implicated in diverse physiological functions. Each of the four isoforms catalyse the reduction of 3-, 17-, 20-ketosteroids and also catalyse the oxidation of 3α-, 17β- and 20α-hydroxysteroids to varying extents. *In vivo*, these enzymes preferentially function as reductases with the oxidation reaction being limited by low concentrations of the cofactor NADPH [6].

AKR1C1, also known as 20α-HSD, exhibits significant 20α-hydroxysteroid activity and has a major role in progesterone metabolism [7]. Progesterone is essential for the maintenance of pregnancy and its conversion to an inactive progestin, 20-hydroxyprogesterone, by AKR1C1 is associ-

ated with premature birth leading to infant morbidity and mortality [8, 9]. Increased activity of AKR1C1 in the endometrium and in breast tissues leads to the formation of tumour promoting metabolites and to the development of endometriosis as well as endometrial cancer and breast cancer [10-12]. AKR1C1 also plays an important role in brain function where it modulates the occupancy of γ-aminobutyric acid type A (GABA_A) receptors through its 3α and 20α-HSD activity [13]. AKR1C1 reduces neuroactive steroids (3α,5α-tetrahydroprogesterone and 5α-tetrahydrodeoxycorticosterone) and their precursors (5α-dihydroprogesterone and progesterone) to inactive 20α-hydroxysteroids, thereby removing them from the synthetic pathway [14]. The elimination of the neuroactive steroids by AKR1C1 is implicated in symptoms of pre-menstrual syndrome (PMS) and other neurological disorders [15]. Therefore, it would be desirable to identify selective and potent inhibitors for AKR1C1 which may help prevent excess metabolism of progesterone in case of breast/endometrial cancer and maintain pregnancy or prevent miscarriage. In our efforts to discover new inhibitors of AKR1C1 using a virtual screening-based approach we identified 3,5-diiodosalicylic acid as a potent inhibitor of AKR1C1 with an IC_{50} (concentration for 50% inhibition) in the sub-micromolar range. We also report for the first time the potential role of aspirin (acetyl salicylic acid) and its metabolite salicylic acid in the counteraction of the adverse effects of AKR1C1 during pregnancy or PMS.

DATABASE MINING AND MOLECULAR DOCKING

The National Cancer Institute (NCI) open database, comprising about 250,000 structures of known biologically-active

*Address correspondence to this author at the Department of Medicinal Chemistry, Victorian College of Pharmacy, Monash University (Parkville Campus), Parkville, Victoria 3052, Australia; Tel: +61 3 9903 9691; Fax: +61 3 9903 9582; E-mail: Ossama.el-kabbani@vcp.monash.edu.au

molecules [17], was used to screen for small molecules that might generate leads for potential inhibitors of AKR1C1. Structures of the 250,000 compounds were downloaded without altering their ionization states using the program Concord [18, 19]. Instead of doing a blind screen for potential inhibitors, a sub-library was created by adding physical and chemical filters to remove unfavorable compounds. Toxic compounds containing heavy metals such as Hg, Pt, Cr, Pu, Al, Au, Ag, Zn etc and compounds containing incorrectly-assigned rotable bonds that are associated with poor chemical stability were excluded from the search. The crystal structure of AKR1C1 complexed with 20α-hydroxyprogesterone and NADP$^+$ (PDB code:1MRQ) was first obtained from the RCSB protein database, after which the steroid ligand and water molecules were removed. A molecular surface of the active site was generated and the docking of ligands was restricted to this site. The structure-based screening program DOCK was used to screen for lead candidates and determine their binding energies based on their complementarity and steric fit within the active site pocket [20, 21].

INHIBITORS OF AKR1C1

The search using DOCK generated 29,250 compounds ranked according to their energy scores. Out of the top scoring compounds, only those that were commercially available were visually examined in more detail using the modeling program InsightII (Biosym Technologies, San Diego, CA, USA). Among these, compounds were selected based on the number and type of interactions with the active site residues and the coenzyme NADP$^+$. Out of these, compounds associated with poor solubility and permeability were excluded from further analysis [22]. Previous studies on the chemical characteristics of inhibitors of AKR1C1 have revealed that the presence of carboxyl or hydroxyl groups or halogen atoms along with a bulky hydrophobic ring system is necessary for potent inhibition [23, 24]. Based on these criteria, 16 candidate compounds were chosen for kinetic analysis.

Out of the 16 compounds that were predicted to inhibit AKR1C1, based on the number and type of interactions with the active site residues and the coenzyme NADP$^+$, only seven were active (Table **1**). The remaining 9 compounds were either insoluble or failed to inhibit AKR1C1 at 1 mM concentration and were therefore excluded from further analysis. Among the seven active compounds, anthraquinone-2-carboxylic acid, 3,5-diiodosalicylic acid, 3,5-dinitro-*p*-toluic acid and 3-phenyl-4,5-isoxazoledione-4-oxime showed potent inhibition with IC$_{50}$ values of 24 μM, 99 nM, 11 μM and 9.4 μM, respectively. The IC$_{50}$ values for 3,5-dinitro-4-hydroxyphenyl acetic acid and N-benzyl-2,2,2-trifluoroacetamide were not determined, because they exhibited 57% and 13% inhibition, respectively, at 1 mM concentration. Although our studies predicted 4-benzoylpyridine as a potential inhibitor of AKR1C1, it was later found to have been previously described as a substrate of the reduction reaction catalyzed by AKR1C1, and therefore its IC$_{50}$ value was not determined [25].

Out of the seven active compounds listed above, 3,5-diiodosalicylic acid was the most potent competitive inhibitor of AKR1C1 (Fig. **1**) with respect to the steroidal substrate 5β-pregnane-3α,20α-diol with a K_i value of 9 ± 1.2
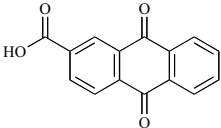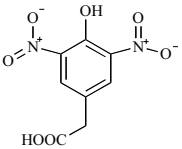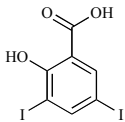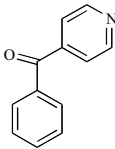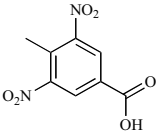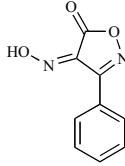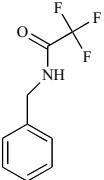
nM. The K_i value for 3,5-diiodosalicylic acid is much smaller than those for other steroid carboxylates and carboxyl-containing analogues of N-phenylanthranilic acid known to inhibit AKR1C isoenzymes [26], and comparable to those for benzbromarone and 3',3'',5',5''-tetrabromophenolphthalein, which are the most potent inhibitors of AKR1C1 known to date [24].

Previous crystallographic and molecular modeling studies of AKR1C1 in complex with substrates, inhibitors and coenzyme have helped identify residues within the active site that are involved in ligand binding [27, 28]. The catalytic tetrad, comprising residues Tyr55, His117, Asp50 and Lys84 together with the cofactor NADP$^+$ forms an oxyanion hole at the bottom of the steroid-binding pocket, and is involved in interactions with substrates and inhibitors. Tyr24, Leu54, Val128, Ile129, His222, and Trp227 are among the other residues that line the steroid-binding pocket and are also involved in hydrophobic or polar interactions with substrates and inhibitors [29]. A previous report on AKR1C1 inhibitors has revealed that carboxyl groups of the steroid carboxylates and N-phenylanthranilic acid derivatives are important requisites for inhibitor binding to the enzyme [26]. The acidic hydroxyl group and the dibromophenol part of benzbromarone and 3',3'',5',5''-tetrabromophenolphthalein are additional structural requisites for potent inhibition of AKR1C1 [24].

Docking simulations revealed that 3,5-diiodosalicylic acid binds to the active site of AKR1C1 with the carboxyl group pointing towards the oxyanion hole. The iodine atom at position 5 forms van der Waals interaction with the side chain of His117 (3.97Å) and the carboxylate forms hydrogen bonds with the hydroxyl group of Tyr55 (3.02Å) and the NE2 of His222 (2.92Å). The iodine atom at position 3 is involved in van der Waals interaction with the side chain of Trp227 (3.85Å). The proposed interactions between 3,5-diiodosalicylic acid and the active site residues are shown in Fig. (**2**).

Interactions between the remaining inhibitors and AKR1C1 were also analyzed and provided useful insights on the type of interactions between the different functional groups and the residues of the active site. Similar to the interactions described for other known inhibitors [26], the inhibitory activity of these compounds is due to their interactions with active site residues such as Tyr24, Tyr55, His117, His222 and Trp227. Most of these compounds either have a carboxyl or a nitro group or both and a benzene ring, characteristics that have been previously described as essential prerequisites for potent inhibition of AKR1C1 [24]. Additional interactions of these compounds include π stacking against the side chain of Trp227. 3,5-Dinitro-*p*-toluic acid forms hydrogen bonds with the NE2 of His117 (2.90 Å), NE1 of Trp227 (3.25 Å) and NE2 of His222 (2.98 Å), and van der Waals interaction with the side chain of Tyr55 (3.06 Å). Anthraquinone-2-carboxylic acid forms hydrogen bonds with the NE2 of His222 (3.35 Å) and is present within van der Waals contact with the side chain of Tyr24 (3.42 Å). The benzene ring of 3,5-dinitro-4-hydroxyphenyl acetic acid π stacks against the side chain of Trp227 (4.48 Å) and the nitro groups form hydrogen bonds with the NE1 of Trp227 (3.30 Å), NE2 of His117 (3.08 Å) and NE2 of His222 (3.34 Å), and van der Waals interaction

Table 1.   IC$_{50}$ Values, Energy Scores and Structures of Compounds Exhibiting Inhibitory Activity Against AKR1C1 Below 1 mM Concentration

DOCK energy score (kcal)	Compound	Molecular formula	Inhibition (%)	IC$_{50}$ (µM)	Structure
-32.34	Anthraquinone-2-carboxylic acid	$C_{15}H_8O_4$	80*	24 ± 0.6	
-29.96	3,5-Dinitro-4-hydroxyphenyl acetic acid	$C_8H_6N_2O_7$	57	n.d	
-29.94	3,5-Diiodosalicylic acid	$C_7H_4I_2O_3$	100	0.099 ± 0.006	
-28.12	4-Benzoylpyridine	$C_{12}H_9NO$	97	n.d	
-27.78	3,5-Dinitro-*p*-toluic acid	$C_8H_6N_2O_6$	100	11 ± 0.5	
-27.20	3-Phenyl-4,5-isoxazoledione-4-oxime	$C_9H_6N_2O_3$	100	9.4 ± 0.2	
-26.35	N-Benzyl-2,2,2-trifluoroacetamide	$C_9H_8F_3NO$	13	n.d	

IC$_{50}$ values were not determined (n.d.) for two compounds with approximately 50% or less inhibition at 1 mM concentration. Percentage inhibition at 100 µM concentration (*) was determined for compounds that precipitated out at 1 mM concentration.

with the side chain of Tyr55 (3.64 Å). The benzene ring of N-benzyl-2,2,2-trifluoroacetamide π stacks against the side chain of Trp227 (4.30 Å) and is also involved in a hydrogen bond with the hydroxyl group of Tyr24 (3.54 Å). The fluorine atoms are within van der Waals contact with the side chains of His222 (3.69 Å) and Tyr55 (3.42 Å). 3-Phenyl-4, 5-isoxazoledione-4-oxime forms hydrogen bonds with the NE2 of His222 (3.29 Å) and the NE2 of His117 (3.05 Å),

and the benzene ring π stacks against the side chain of Trp227 (4.32 Å).

A NEW USE FOR AN OLD DRUG

Since 3,5-diiodosalicylic acid was the most potent inhibitor identified in our study, its analogues salicylic acid and acetylsalicylic acid (aspirin) were also tested for activity
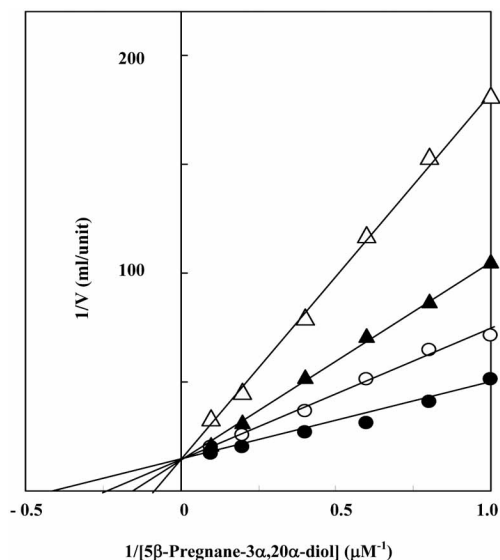
Fig. (1). Inhibition pattern for AKR1C1 by 3,5-diiodosalicylic acid. The activity was determined using the six concentrations of 5β-pregnane-3α,20α-diol as the substrate in the absence (●) or presence of the inhibitor. The inhibitor concentrations were 10 nM (○), 20 nM (▲) and 40 nM (△).

against AKR1C1. Both salicylic acid and acetyl salicylic acid were inhibitory below 1 mM concentration, which could be attributed to the presence of the carboxyl group that forms two hydrogen bonds within the active site and the bulky benzene ring, which might be necessary for hydrophobic interactions
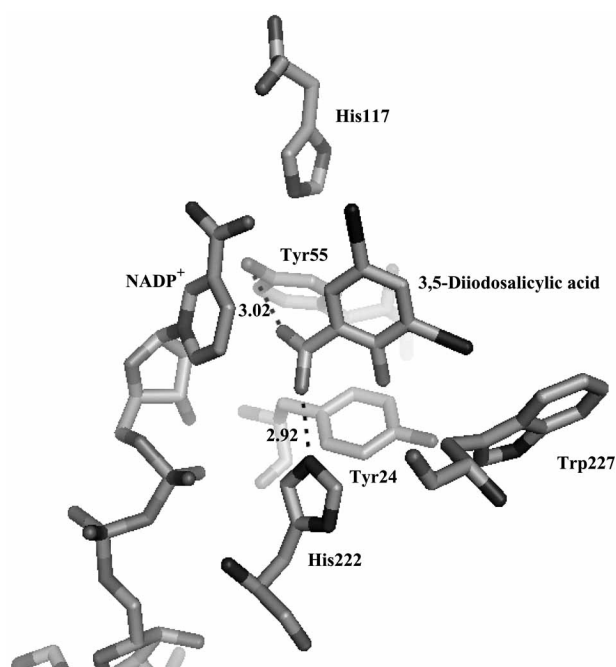

Fig. (2). Proposed interactions between AKR1C1 residues and the inhibitor 3,5-diiodosalicylic acid. Hydrogen bonds are shown as dashed lines and distances are given in Å.

Although the carboxyl group necessary for interaction with the catalytic Tyr55 is common to all three compounds, aspirin and salicylic acid are less potent inhibitors of AKR1C1 than 3,5-diiodosalicylic acid (Table **2**). The loss of the iodine atoms at positions 3 and 5 in salicylic acid results in a 78-fold loss in potency and in case of aspirin the acetylation of the hydroxyl group of salicyclic acid further decreases the inhibitory potency. These results emphasize the importance of the van der Waals interactions between the iodine atoms at positions 3 and 5 and the side chains of residues His117 and Trp227 in AKR1C1.

Table2.   **Chemical Structures and IC_{50} Values for Salicylic Acid and Acetylsalicylic Acid**

Compound	Molecular formula	IC_{50} (μM)	Structure
Salicylic acid	$C_7H_6O_3$	7.8 ± 0.3	
Acetyl salicylic acid (aspirin)	$C_9H_8O_4$	21 ± 2	

CONCLUSION

The use of virtual screening has identified a varied group of inhibitors of the dehydrogenase activity of AKR1C1. We identified 3,5-diiodosalicylic acid as a potent inhibitor of AKR1C1 with an IC_{50} value in the sub-micromolar range. A crystal structure of 3,5-diiodosalicylic acid bound in the active site of AKR1C1 would enhance our understanding of the mode of interaction and help identify any changes in the enzyme's tertiary structure that may be associated with inhibitor binding. Although 3,5-diiodosalicylic acid was the most potent inhibitor of AKR1C1, the inhibitory effects of aspirin cannot be disregarded due to its wide spread use as a therapeutic agent for a host of conditions varying from headaches to prevention of cardiovascular disease and cancer [30]. Despite the fact that aspirin is less potent compared to other known inhibitors of AKR1C1, it is inexpensive, relatively safe and easily available over the counter. Inhibitors identified in this study may aid the development of new drugs for the treatment of cancer, maintain pregnancy or prevent miscarriage.

ENZYME ASSAY

The assays were conducted at 25 °C in 0.1 M potassium phosphate buffer, pH 7.5 in a 96-well plate with a final reaction volume of 200 μl. The substrate S-tetralol and inhibitor were dissolved in methanol with the final concentration of methanol not exceeding 2% of the total volume of the reaction mixture. Initially, all 16 compounds were tested to determine their inhibitory effect at 1 mM concentration and IC_{50} values were determined only for compounds that exhibited more than 50% inhibition at 1 mM concentration. The final concentrations of $NADP^+$, S-tetralol and inhibitor in the reaction mixture were 2.5 mM, 2.5 mM and 1 mM, respectively. The reaction was commenced upon addition of appro-

priately diluted AKR1C1 and its dehydrogenase activity was determined by measuring the rate of change of NADPH fluorescence at 455 nm with an excitation wavelength of 340 nm [31]. The inhibitor constant K_i was determined from a Lineweaver-Burke plot with 5β-pregnane-3α,20α-diol as the substrate. One unit of enzyme activity was defined as the amount that catalyses the formation of 1 μmol of NADPH per minute at 25 °C. All reported values are the means or means ± S.D. from three determinations.

ABBREVIATIONS

AKR = Aldo-keto reductase

IC_{50} = Concentration required for 50% inhibition

HSD = Hydroxysteroid dehydrogenase

$NADP^+$ = Nicotinamide adenine dinucleotide phosphate

$GABA_A$ receptor = γ-Aminobutyric acid type A receptor

PMS = Pre-menstrual syndrome

NCI = National Cancer Institute

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