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Inhibition of 11 β -hydroxysteroid dehydrogenase 2 by the fungicides itraconazole and posaconazole

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Abstract

Impaired 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2)-dependent cortisol inactivation can lead to electrolyte dysbalance, hypertension and cardiometabolic disease. Furthermore, placental 11 β -HSD2 essentially protects the fetus from high maternal glucocorticoid levels, and its impaired function has been associated with altered fetal growth and a higher risk for cardio-metabolic diseases in later life. Despite its important role, 11 β -HSD2 is not included in current off-target screening approaches. To identify potential 11 β -HSD inhibitors amongst approved drugs, a pharmacophore model was used for virtual screening, followed by biological assessment of selected hits. This led to the identification of several azole fungicides as 11 β -HSD inhibitors, showing a significant structure-activity relationship between azole scaffold size, 11 β -HSD enzyme selectivity and inhibitory potency. A hydrophobic linker connecting the azole ring to the other,

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Conflicts of Interest

The authors declare no conflict of interest.

Chemical compounds studied in this article

Albendazole (PubChem CID: 2082); Butaconazole (PubChem CID: 47472); Climbazole (PubChem CID: 37907); Itraconazole (PubChem CID: 55283); Hydroxyitraconazole (PubChem CID: 108222); Ketoconazole (PubChem CID: 47576); Posaconazole (PubChem CID: 468595); Sertaconazole (PubChem CID: 65863); Terconazole (PubChem CID: 441383); Tioconazole (PubChem CID: 5482)

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more polar end of the molecule was observed to be favorable for 11 β -HSD2 inhibition and selectivity over 11 β -HSD1. The most potent 11 β -HSD2 inhibition, using cell lysates expressing recombinant human 11 β -HSD2, was obtained for itraconazole (IC₅₀ 139 \pm 14 nM), its active metabolite hydroxyitraconazole (IC₅₀ 223 \pm 31 nM) and posaconazole (IC₅₀ 460 \pm 98 nM). Interestingly, experiments with mouse and rat kidney homogenates showed considerably lower inhibitory activity of these compounds toward 11 β -HSD2, indicating important species-specific differences. Thus, 11 β -HSD2 inhibition by these compounds are likely to be overlooked in preclinical rodent studies. Inhibition of placental 11 β -HSD2 by these compounds, in addition to the known inhibition of cytochrome P450 enzymes and P-glycoprotein efflux transport, might contribute to elevated local cortisol levels, thereby affecting fetal programming.

Keywords

11 β -hydroxysteroid dehydrogenase; virtual screening; reproductive toxicity; itraconazole; posaconazole; glucocorticoid

1 Introduction

11 β -hydroxysteroid dehydrogenase 2 (11 β -HSD2) converts potent 11 β -hydroxyglucocorticoids (cortisol, corticosterone) into their inactive 11-keto forms (cortisone, 11-dehydrocorticosterone), thereby controlling tissue-specific activities of mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) [1]. 11 β -HSD2 is essentially involved in the regulation of electrolyte balance, vascular function, and angiogenesis, as well as in the fetal-placental barrier to inactivate cortisol and protect the fetus from high maternal glucocorticoid levels [2, 3].

The consequences of impaired 11 β -HSD2 function are manifested in patients with genetic loss-of-function mutations suffering from apparent mineralocorticoid excess (AME) [2, 4, 5]. In these patients the excessive cortisol-dependent MR activation in the kidney and colon results in hypokalemia, hypernatremia and water retention, leading to severe hypertension characterized by low renin, low aldosterone and increased plasma and urinary cortisol to cortisone ratios. 11 β -HSD2 activity is essential since very early on in life, because birth weights of individuals homozygous/compound heterozygous for *HSD11B2* mutations were found to be significantly lower than those of their unaffected siblings [6]. Milder acquired forms of AME can be caused by inhibition of 11 β -HSD2, for instance upon consumption of considerable amounts of licorice, containing the potent 11 β -HSD inhibitor glycyrrhetic acid (GA) [7].

The exposure to 11 β -HSD2 inhibitors is especially critical during pregnancy. 11 β -HSD2 builds a placental barrier by protecting the fetus from the 5-10 times higher maternal glucocorticoid levels in the course of a normal pregnancy [8–11]. Nevertheless, this barrier is not entirely complete, as a minor proportion of maternal cortisol is able to cross the placenta [11]. Glucocorticoids are important mediators of fetal growth, development and organ maturation. Rising total plasma cortisol levels during pregnancy, peaking in the third trimester at three-fold non-pregnant levels [12], are in parallel with progressive maturation of fetal organs, most notably the stimulation of surfactant production by the lung. However,

glucocorticoid administration, especially during late gestation, has been associated with reduced birth weight, elevated blood pressure, higher insulin, increased distractibility and attention deficit later in life [13–17]. The correlation between low birth weight, albeit in normal ranges, and subsequent diseases in adulthood was found to be largely independent of confounding life style factors such as smoking or obesity [15]. It has been hypothesized that a reduced placental 11 β -HSD2 activity is responsible for the high glucocorticoid concentrations reaching the fetus and subsequent programming of disease susceptibility [18]. Importantly, low 11 β -HSD2 expression was found to be associated with intrauterine growth restriction pregnancies in humans and in rodent models [19–22]. Determination of osteocalcin concentration in human cord blood samples, which is a sensitive marker of glucocorticoid exposure in adult humans, revealed a direct correlation with placental 11 β -HSD2 activity [23].

Treatment of pregnant rats with dexamethasone, which cannot be inactivated by 11 β -HSD2 [24], led to lower birth weights and caused HPA axis hyperactivity, hypertension, hyperglycemia and increased anxiety behavior [20, 25]. Similarly, administration of the unselective 11 β -HSD inhibitor carbenoxolone to pregnant rats resulted in reduced birth weight, and adult offspring showed enhanced HPA activity with increased glucocorticoid and CRH levels as well as elevated blood pressure [26, 27]. Evidence for the importance of placental 11 β -HSD2 was contributed by studies with placentas from 11 β -HSD2-deficient mice showing increased amino acid and reduced glucose transport as well as lower expression levels of genes important for angiogenesis [28]. Furthermore, maternal stress and malnutrition in rats were reported to be able to down regulate placental 11 β -HSD2 and program for diseases in adult life [29–31]. Factors including sex steroids, nitric oxide, prostaglandins, proinflammatory cytokines, infections and environmental pollutants were shown to have the potential to reduce 11 β -HSD2 activity in studies using placental cell lines [32, 33].

Observational studies showed that pregnant Finnish women consuming large amounts of licorice (containing the unselective 11 β -HSD inhibitor glycyrrhetic acid) had shorter gestation times [34] and gave birth to children with behavioral disturbances and poorer cognitive functions coupled with increased HPA axis activity, in a dose-dependent manner [35, 36]. Compromised 11 β -HSD2 function during pregnancy has also been implicated in preeclampsia, a major cause of maternal and perinatal mortality; however, the etiology is poorly understood [37, 38].

Due to its important physiological role and the adverse effects observed upon its inhibition, 11 β -HSD2 can be considered as an anti-target for drug development (with a few specific exceptions where these effects are wanted); however, it is not included in current off-target screening approaches. The present study addressed possible inhibitory effects of approved drugs toward 11 β -HSD2 by performing a virtual screening (VS) of the DrugBank database using an 11 β -HSD pharmacophore model. This was followed by a biological evaluation of selected hits, with a focus on the azole fungicides itraconazole and posaconazole.

2 Materials and methods

2.1 Chemicals and reagents

[1,2,6,7-³H]-cortisol, [2,4,6,7-³H]-estrone and [2,4,6,7-³H]-estradiol were purchased from PerkinElmer (Boston, MA, USA), [1,2-³H]-cortisone from American Radiolabeled Chemicals (St. Louis, MO), hydroxyitraconazole (OHI) from Carbosynth (Berkshire, UK) and all other chemicals from Sigma Aldrich (Buchs, Switzerland) of the highest grade available. Cell culture media were obtained from Sigma Aldrich (Buchs, Switzerland).

2.2 Pharmacophore modeling and virtual screening

The 11 β -HSD inhibitor pharmacophore model used for this study was previously reported and validated [39]. The model was initially based on three potent, structurally diverse 11 β -HSD inhibitors [40] and refined with recent literature and novel screening data (model 4new in reference [39], Fig. 1A).

To discover potential 11 β -HSD inhibitors amongst FDA-approved drugs and nutraceuticals, the DrugBank version 3.0 was downloaded as sd file (1543 approved drugs and 84 nutraceuticals) and transformed into a 3D-multiconformational database using the “build database” protocol of Discovery Studio 4.0 (Discovery Studio, Version 4.0, Biovia Inc., San Diego, CA, 2014). For each compound, a maximum of 255 conformers was calculated using fast settings. For the VS, the “search 3D database” protocol with BEST flexible search was used. The DrugBank database was screened with the 11 β -HSD inhibitors model using Discovery Studio 4.0.

2.3 Cell culture

Human Embryonic Kidney-293 cells (HEK-293) cells (used at passage number 15-30), human SW-620 colon carcinoma cells (passage number 11-15) and human MCF-7 breast cancer cells (passage number 19-27) were obtained from ATCC (Manassas, VA, USA) and were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/L glucose, 100U/mL penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, pH 7.4, and 10% MEM non-essential amino acid solution.

2.4 Determination of 11 β -HSD activity in cell lysates

Enzyme activities were determined as described earlier using lysates of HEK-293 cells stably expressing human 11 β -HSD1 and hexose-6-phosphate dehydrogenase (H6PDH; HHH7 clone) or 11 β -HSD2 (AT8 clone) [41]. 11 β -HSD1 reductase activity was measured by incubating the lysates for 10 min at 37°C in a total volume of 22 μ L containing 200 nM radiolabeled cortisone, 500 μ M NADPH and test substance or vehicle (DMSO at a maximal concentration of 0.1%). 11 β -HSD2-dependent oxidation was assessed in the presence of 200 nM radiolabeled cortisol and 500 μ M NAD⁺. The reactions were stopped by adding an excess amount of unlabeled cortisone and cortisol (1:1, 2 mM in methanol). Approximately 20-25% of the substrate were converted to the corresponding product. Steroids were separated by thin layer chromatography (TLC) using chloroform and methanol (9:1). Conversion of radiolabeled substrate was measured by scintillation counting. The substrate conversion was determined and compared to the enzyme activity in the control sample.

To exclude irreversible 11 β -HSD2 inhibition by the investigated compounds, HEK cell lysates were preincubated with the corresponding compounds for 0, 5 and 20 min at 4°C, followed by determination of enzyme activity. Alternatively, lysate were preincubated with the compounds for 10 min followed by subsequent dilution (1:2 or 1:4) and determination of enzyme activity [42]. Data (mean \pm SD) were normalized to vehicle control (DMSO) and obtained from at least three independent experiments.

2.5 Determination of 11 β -HSD2 activity in intact cells

11 β -HSD2 activity measurement in intact SW-620 and MCF-7 cells was determined as described earlier [43]. Briefly, 100 000 SW-620 and 50 000 MCF-7 cells per well were seeded in 96-well plates. The medium was replaced after 24 h by 40 μ L steroid- and serum-free DMEM (DMEMsf) containing either vehicle or inhibitor and 10 μ L medium containing 10 nCi radiolabeled cortisol and unlabeled cortisol to reach a final concentration of 50 nM. SW-620 cells were incubated for 4 h and MCF-7 cells for 5 h at 37°C, followed by analysis of steroid conversion by TLC and scintillation counting

2.6 Determination of 17 β -HSD1 and 17 β -HSD2 activity in cell lysates

Determination of 17 β -HSD1 and 17 β -HSD2 activity was conducted as described earlier [44]. Briefly, lysates of HEK-293 cells transiently expressing human 17 β -HSD1 and 17 β -HSD2 were incubated for 10 min at 37°C in a total volume of 22 μ L in the presence 200 nM estrone containing 50 nCi of [2,4,6,7-³H]-estrone and 500 μ M NADPH for 17 β -HSD1, and with 200 nM estradiol containing 50 nCi of [2,4,6,7-³H]-estradiol and 500 μ M NAD⁺ for 17 β -HSD2 activity measurements. Steroids were separated using TLC and substrate conversion determined by scintillation counting. Data (mean \pm SD) were normalized to vehicle control (DMSO) and obtained from at least three independent experiments.

2.7 Determination of 11 β -HSD activity in mouse and rat kidney homogenates

Mouse (C57BL6) and rat (Wistar) kidney tissue from adult males were snap frozen in liquid nitrogen and stored at -80°C until further use. For homogenate preparation, frozen tissue was sonicated in homogenization buffer (250 mM sucrose, 10 mM HEPES, pH 7.4; 900 μ L for 100 mg of kidney) and centrifuged at 2 000 \times g, 4°C for 10 min to remove cell debris. The total protein concentration was determined by BCA assay [45]. The homogenates were prediluted to a protein concentration of 3.75 mg of protein/mL (mouse) or 10 mg of protein/mL (rat) in homogenization buffer. Reactions were performed in a total volume of 22 μ L incubation buffer (300 mM NaCl, 20 mM Tris(hydroxymethyl)aminomethane-hydrochloride, 1 mM EDTA, 10% glycerol, pH 7.7) containing 0.075 mg/mL of mouse and 0.2 mg/mL of rat total kidney protein, respectively, as well as 10 μ M of test compound, 50 nM of corticosterone (containing 50 nCi [1,2,6,7-³H] corticosterone) and 500 μ M NAD⁺ at 37°C for 20 min. Approximately 20-25% of the substrate were converted. The steroids were separated by TLC and conversion of radiolabeled substrate was measured by scintillation counting.

2.8 Docking

Docking was performed using GOLD 5.2 (The Cambridge Crystallographic Data Centre, Cambridge, UK, [46]) based on a crystal structure of 11 β -HSD1 (co-crystallized with (2*R*)-4-[4-fluoro-2-(trifluoromethyl)phenyl]-2-methyl-1-([3-(1*H*-1,2,4-triazol-1-yl)phenyl]sulfonyl)piperazine, PDB code 3HFG [47]) and a homology model for human 11 β -HSD2 [48] and murine 11 β -HSD2 [49]. The PDB entry 3HFG was chosen for two reasons. First, it was the template for generating the homology model of human and murine 11 β -HSD2. Second, the co-crystallized inhibitor is of similar size as some of the investigated antifungals and contains a triazole moiety. It therefore probably constitutes a binding site conformation suitable for docking this class of 11 β -HSD1 inhibitors.

The ligand binding pocket was defined as a sphere with a 10Å radius around the coordinates X = -19.50 Y = 4.00, Z = 14.25. ChemPLP was used as scoring function. As workflow validation, redocking of the original ligand was performed using default settings and the best ranked pose deviates from the crystal structure with an RMSD of 0.856 for 11 β -HSD1. As both the homology models for human and murine 11 β -HSD2 were initially based on PDB entry 3HFG, the same coordinates were used to define the binding site for this docking. For human 11 β -HSD2, the docking was additionally repeated setting Arg212 as flexible amino acid. The binding poses were analyzed using LigandScout 4.1 [50] (Inte:Ligand GmbH, Vienna, Austria).

3 Results

3.1 Virtual screening of the DrugBank database using a 11 β -HSD pharmacophore model and virtual hit selection for biological evaluation

Despite its role in the regulation of electrolyte balance and cardiovascular function and its importance during pregnancy to control the *in utero* environment and therefore fetal growth and development, 11 β -HSD2 is not included in current drug off-target screenings. Thus, the present project aimed to evaluate some approved drugs for their ability to interfere with 11 β -HSD2 activity. For this purpose, the FDA-approved small molecule drug entries of the DrugBank database were subjected to VS using a previously developed 11 β -HSD pharmacophore model (Fig. 1A) [39]. This model is not expected to discriminate between 11 β -HSD1 and 11 β -HSD2 inhibitors because no crystal structure is available for 11 β -HSD2 and the model is, at least in part, built based on available 11 β -HSD1 ligands. Of the 1543 DrugBank entries, 101 approved drugs fitted into the model. Not surprisingly, several steroidal compounds (in total 18 hits) including mainly glucocorticoids, were among the hits. Besides, the virtual hit list contained several prostaglandin analogues as well as anti-infective agents, including antifungals, antibiotics and antiparasitic agents. Antifungals were especially represented by the class of azole fungicides, with sertaconazole, butoconazole and tioconazole as virtual hits (Fig. 1B). The majority of antibiotics comprised β -lactam antibiotics and lincosamides, with cloxacillin, flucloxacillin and nafcillin, as well as clindamycin and lincosamycin as representative structural classes. Further virtual hits included members of the classes of antihypertensives/antiarrhythmics, diuretics, lipid lowering drugs, antidiabetics, analgesics and antipsychotics. Several hits from the VS,

belonging to the different structural classes mentioned above and available through an in-house chemical repository, were selected for biological assessment.

3.2 Effect of selected virtual hits and further structurally related compounds on 11 β -HSD activity

The selected compounds were first tested for their potential to inhibit cortisol to cortisone conversion in lysates of HEK-293 cells stably expressing human 11 β -HSD2. The selectivity over the closely related 11 β -HSD1 was then determined by measuring the effect of the chemicals on the conversion of cortisone to cortisol. The selected steroids (nandrolone), antiarrhythmics (amiodarone), lipid lowering drugs (atorvastatin, simvastatin), diabetics (rosiglitazone), diuretics (ethacrynic acid), analgesics (indomethacine, nabilone), antipsychotics/sedativa (chlorpromazine), the uricosuric probenecid, antiparasitic agents (amodiaquine, chloroquine and pentamidine), β -lactam antibiotics (cloxacillin, flucloxacillin and nafcillin), lincosamides (clindamycin and lincomycin) and the steroid-like antibiotic fusidic acid showed no or weak inhibition (less than 40% inhibition) of 11 β -HSD1 and 11 β -HSD2 enzyme activity at a concentration of 20 μ M (data not shown). However, the azole fungicides sertaconazole, butoconazole and tioconazole showed moderate activity and inhibited 11 β -HSD2 at a concentration of 20 μ M, resulting in 61%, 44% and 50% residual activity, respectively. These azole fungicides were not selective, and they equally well or preferentially inhibited 11 β -HSD1, showing residual enzyme activities of 35%, 48% and 18%, respectively.

Due to their wide use and partial over-the-counter availability, and their previous association with 11 β -HSDs (triadimefon as a substrate of 11 β -HSD1 and ketoconazole as a weak inhibitor of 11 β -HSD1 and 11 β -HSD2 [39, 51]), additional, structurally related azole fungicides were selected for biological testing in order to provide proof of concept in applying pharmacophores as initial filter for the identification of hazardous compound classes [52]. The biological analyses using HEK-293 cell lysates expressing recombinant human enzymes revealed a clear structure-activity relationship between 11 β -HSD selectivity and structural size and shape of the azoles: the larger the structural size the more potent its inhibitory activity against 11 β -HSD2 and the higher the selectivity over 11 β -HSD1 (Fig. 2). One requirement of high activity was a hydrophobic central region of the azole scaffold linked to a more polar end. The compounds with a smaller scaffold preferentially inhibited 11 β -HSD1. In addition, the subdivision of azoles in imidazole and triazole derivatives revealed a further relationship. Imidazoles such as tioconazole, sertaconazole and butoconazole preferably inhibited 11 β -HSD1 over 11 β -HSD2, whereas triazoles such as terconazole, posaconazole and itraconazole more potently inhibited 11 β -HSD2. Albendazole, possessing a small azole scaffold containing a benzimidazol structure, was inactive against both enzymes. The most potent 11 β -HSD2 inhibition was found for itraconazole and posaconazole, with IC₅₀ values of 139 \pm 14 nM and 460 \pm 98 nM (Fig. 3), respectively, and selectivity over 11 β -HSD1 (Fig. 2). Furthermore, as itraconazole is mainly metabolized to the pharmacologically active OHI, this metabolite was also tested, yielding an IC₅₀ value of 223 \pm 31 nM against 11 β -HSD2. Irreversible 11 β -HSD2 inhibition by itraconazole, OHI and posaconazole was excluded by preincubation experiments; however,

preincubation did not alter the inhibitory effect, suggesting a competitive mode of inhibition in line with competition for substrate binding.

3.3 Selectivity assessment of the azole fungicides over 17 β -HSD1 and 17 β -HSD2

Although 11 β -HSD2 and 11 β -HSD1 are responsible for the interconversion of the same substrate, i.e. glucocorticoids, they are distant homologs sharing only about 18% sequence identity [53]. In contrast, human 17 β -HSD2 is more closely related to 11 β -HSD2 with about 45% amino acid sequence identity. 17 β -HSD2 is predominantly involved in the metabolism of sex steroid hormones, inactivating estradiol to estrone, testosterone into 4-androstene-3,17-dione (androstenedione), 5 α -dihydrotestosterone (DHT) into 5 α -androstenedione or 5-androstene-3 β ,17 β -diol to dehydroepiandrosterone (DHEA). The reverse reaction of activating the weak estrogen estrone to the potent estradiol and to a minor extent DHEA to 5-androstene-3 β ,17 β -diol is catalyzed by 17 β -HSD1. Due to the high expression of 17 β -HSD1 and 17 β -HSD2 in placenta [54, 55], itraconazole, OHI and posaconazole were assessed for their potential to inhibit 17 β -HSD1 and 17 β -HSD2 enzyme activity in lysates of HEK-293 cells transiently expressing human 17 β -HSD1 and 17 β -HSD2. None of the compounds inhibited 17 β -HSD1 or 17 β -HSD2 activity (data not shown).

3.4 Species-specific 11 β -HSD2 inhibition by the selected azole fungicides

Earlier studies emphasized the importance to assess species-specific differences of 11 β -HSD1 and 11 β -HSD2 inhibitors, especially prior to conducting *in vivo* experiments [43, 51, 56]. Thus, prior to designing a rodent study to assess the effect of the selected azole fungicides on glucocorticoid inactivation, inhibition of 11 β -HSD2 by itraconazole, OHI and posaconazole was further analyzed in rat and mouse kidney homogenates incubated with 50 nM of the rodent substrate corticosterone in the absence or presence of 10 μ M test compound. In a qualitative comparison to the potent activity against recombinant human 11 β -HSD2, which was expressed in HEK-293 cells and measured upon incubation with 200 nM of the human substrate cortisol, considerably lower inhibitory activity was detected against the rat and mouse enzymes. The different concentrations of corticosterone and cortisol used for rodent and human 11 β -HSD2, respectively, reflect the approximately 5-10 fold affinity difference for these substrates [57, 58]. Itraconazole (Fig. 4A) and OHI (Fig. 4B) were at least 10-fold less potent toward rat and mouse 11 β -HSD2 compared to the human enzyme, while posaconazole (Fig. 4C) tended to be 2 times less active against rat 11 β -HSD2 and was about 4 times less active against mouse 11 β -HSD2. Although the activities of the human and rodent enzymes were measured under different conditions, considering their different physiological substrates, the data suggest a higher inhibitory activity of these azole fungicides toward human 11 β -HSD2 compared to the rodent enzymes.

3.5 Inhibition of 11 β -HSD2 in cell models with endogenous enzyme expression

To determine the inhibitory potential of itraconazole, OHI and posaconazole in intact cell systems, SW-620 and MCF-7 cells expressing relatively high endogenous 11 β -HSD2 levels [43] were applied. Itraconazole inhibited 11 β -HSD2 in a concentration-dependent manner with IC₅₀ values of 1.07 \pm 0.29 μ M in SW-620 and 1.19 \pm 0.24 μ M in MCF-7 cells. In contrast to the inhibitory potency ranking observed in lysates of stably transfected HEK-293

cells, OHI was slightly more potent than itraconazole with IC_{50} values of $0.82 \pm 0.18 \mu\text{M}$ in SW-620 and $0.68 \pm 0.20 \mu\text{M}$ in MCF-7 cells, respectively. Posaconazole did not substantially inhibit 11β -HSD2 activity, resulting in approximately 60% and 40% residual 11β -HSD2 activity in SW-620 and MCF-7 cells at a concentration of $5 \mu\text{M}$.

3.6 Predicted binding of selected fungicides to 11β -HSD1 and 11β -HSD2

Docking studies with 11β -HSD1 showed two important protein-ligand interactions for azole fungicides such as sertaconazole and tioconazole: a hydrophobic contact with Tyr183 and a hydrogen bond of the azole ring with the backbone nitrogen of Ala172 (Fig. 5A). This hydrogen bond interaction was also the only ligand-coordinating hydrogen bond observed in the 11β -HSD1 crystal structure co-crystallized with a sulfonyl-piperazine inhibitor and thus, presumably essential for the inhibitory activity. In contrast, terconazole does not form any hydrogen bonds with the binding pocket of 11β -HSD1. Furthermore, docking of itraconazole and posaconazole revealed steric clashes with Thr124, Thr222 and Ala226 of the 11β -HSD1 substrate binding pocket, providing an explanation for their inactivity towards 11β -HSD1. However, a major difference between 11β -HSD1 and 11β -HSD2 is the shape of the binding site entry. While Met233 flanks the binding site entry of 11β -HSD1, Arg212 replaces this amino acid at the analogous position in 11β -HSD2. The difference in size and electrostatic properties of these amino acids likely influences the inhibitory activity. Indeed, all 11β -HSD2 active compounds analyzed formed an interaction with Arg212. Furthermore, the extended azole scaffolds were found to form additional interactions in their docking poses with 11β -HSD2, pointing towards tighter binding to the protein. Interestingly, itraconazole and posaconazole did not fit entirely into the binding site of 11β -HSD2 but rather lined the surface next to the binding site entry with their azole part (Fig. 5B). Moreover, the additional hydroxyl group of posaconazole was able to form several hydrogen bonds with the phosphate moiety of NADPH.

To rationalize the relative selectivity of the three azole compounds to inhibit human 11β -HSD2, they were also docked into the murine homology model [49]. The murine 11β -HSD2 homology model revealed some crucial differences in the amino acid sequence compared to the human 11β -HSD2. Unlike the equivalent Arg212 on human 11β -HSD2, Arg279 on mouse 11β -HSD2 was not flanking the binding pocket entrance but oriented away (Fig. 5C). Furthermore, part of the binding cavity near the cofactor was occupied by Trp276, a residue located outside the binding pocket in human 11β -HSD2, and this residue caused itraconazole to adopt a different angle within the binding cavity in the docking simulation. Additionally, the docking poses for itraconazole predicted no hydrogen bonds with the protein (Fig. 5D). This missing structural anchoring provides an explanation for the weak activity of this fungicide towards murine 11β -HSD2. Interestingly, regarding murine 11β -HSD2, posaconazole and OHI showed greater inhibitory activity than itraconazole. Compared to itraconazole these two fungicides formed interactions with the cofactor via their hydroxyl groups (Figure 5E).

One important aspect of *in silico*-driven screening studies is the analysis of the predictions and a possible refinement of the model that was used for screening. The screening model performance was very powerful, as all three azole fungicides identified by the model

(tioconazole, sertaconazole and butoconazole) inhibited 11 β -HSD1 enzyme activity at a concentration of 20 μ M by at least 50%. However, although itraconazole and posaconazole were represented in the DrugBank, they were not found by the model as virtual hits. A previous refinement study on this model [39], addressed the shape restriction as one major restrictivity aspect. Shape deletion and subsequent VS of all tested compounds, including OHI, with this model retrieved all compounds except for climbazole as hits. For more thorough VS for potential 11 β -HSD inhibitors, the shapeless model version may therefore be more suitable.

4 Discussion

Molecular modeling-based *in silico* approaches are important in drug development for the identification of bioactive molecules. Pharmacophore-based VS is a powerful strategy to enrich potentially active compounds among a large number of test compounds, thereby facilitating the identification of drug lead structures [59]. Such *in silico* tools are also applied in anti-target screenings; however, in contrast to lead compound identification this is more challenging since this approach aims to identify all potentially harmful substances. The present study applied a pharmacophore model based on 11 β -HSD1 crystal structures for VS of the DrugBank database to find approved drugs that might inhibit 11 β -HSD2, an enzyme not included in current off-target screenings during drug development. The goal was to identify, as a first step, structural compound classes inhibiting 11 β -HSD2, followed by a more detailed analysis of a selected compound class including an *in vitro* validation of selected virtual hits.

The pharmacophore model used in this study demonstrated already earlier high predictive power [60], and azole fungicides have been previously associated with 11 β -HSDs: The azole fungicide triadimefon was reported to be a substrate of 11 β -HSD1 (K_m 3.5 μ M), thereby acting as a weak competitive inhibitor (IC_{50} 15 μ M) [51]. Ketoconazole was found to have weak inhibitory effects toward 11 β -HSD1 and 11 β -HSD2 [39, 61]. Thus, this compound class was chosen for further investigations. Biological evaluation revealed several azole fungicides as 11 β -HSD inhibitors, showing a significant structure-activity relationship between azole scaffold size and 11 β -HSD enzyme selectivity and potency of inhibition. The large scaffolds of itraconazole and posaconazole, which were not initial VS hits, potently inhibited 11 β -HSD2 enzyme activity. Compared to terconazole, which was considerably less active on 11 β -HSD2, itraconazole and posaconazole contain an extended hydrophobic central region and an additional triazolone side chain, which may form additional stabilizing interactions with the binding pocket, including Arg212, thereby providing an explanation for the potent inhibition. Thus, the VS approach has proven useful to identify azole fungicides as 11 β -HSD2 inhibitors and to prioritize this compound class for further biological analyses, which were necessary to identify the most potent compounds itraconazole and posaconazole.

Itraconazole and posaconazole are clinically used for the prophylaxis and treatment of systemic mycotic infections and exert their mode of action by inhibiting the biosynthesis of ergosterol, an essential component of the fungal cell membrane [62, 63]. They can be applied for a prolonged period of time, up to years when used as prophylactic treatment, especially in immunosuppressed patients [64]. After oral exposure, itraconazole is

extensively metabolized in the liver by cytochrome P450 3A4 (CYP3A4) to its main metabolite OHI, which retains potent 11 β -HSD2 inhibitory activity. Thus, cortisol-dependent MR and GR activation due to prolonged 11 β -HSD2 inhibition may contribute to the observed adverse effects of these fungicides. In contrast to itraconazole, hepatic metabolism of posaconazole plays a minor role and mainly involves conjugation by UDP-glucuronyltransferase UGT1A4 [65]. Although posaconazole is not a substrate of CYP3A4, it acts like itraconazole and OHI as a potent CYP3A4 inhibitor and it is therefore prone to considerable pharmacokinetic interactions with CYP3A4 substrates, including glucocorticoids [66, 67]. Inhibition of CYP3A4 by a single dose of itraconazole was shown to significantly decrease the formation clearance (CL_f) of the metabolites 6 β -hydroxycortisol and 6 β -hydroxycortisone [68]. Plasma cortisol and cortisone concentrations were not altered, indicating negative feedback regulation by the HPA axis and suggesting that cortisol levels are locally increased in CYP3A4 expressing tissues, particularly in the liver. Itraconazole, OHI and posaconazole might further promote local glucocorticoid effects by inhibiting P-glycoprotein (P-gp) mediated cortisol efflux [69, 70]. Besides its important role in the liver, P-gp expression has been detected in the human placenta from the first trimester on towards full-term [71] and P-gp was shown *in vitro* to support the placental 11 β -HSD2 glucocorticoid barrier [72].

Inhibition of 11 β -HSD2 by itraconazole and posaconazole might impair glucocorticoid inactivation in the kidney, colon, vasculature and placenta. Indeed, Denolle et al. reported a case study of a patient on long-term itraconazole treatment developing hypokalemia, edema, hypertension, low plasma renin and aldosterone concentrations and normal serum cortisol, typical symptoms of AME as a result of cortisol-dependent MR activation [73]. Similar side effects including hypokalemia, edema, hypertension and mildly reduced aldosterone serum levels were described by Sharkey et al. for several patients during long-term itraconazole use [74]. Importantly, the drug safety sheet of Sporanox[®] (itraconazole) reports hypokalemia and edema as occasionally (1/1000, <1/100 patients) occurring, whereas the safety sheet for the *i.v.* solution of Noxafil[®] (posaconazole) notes hypokalemia as one of the most likely occurring adverse effect (22% of the reports) and a rise in blood pressure as frequent (1/100, <1/10) [75, 76].

Due to the high plasma protein binding capacity of itraconazole (99.8%), OHI (99.6%) and posaconazole (98%), predominately to albumin, the circulating fraction of unbound drug is low [75, 76]. However, in specific situations the plasma protein binding capacity can be reduced. Pregnancy leads to several metabolic and physiologic changes, including a decrease of serum albumin levels to about half of those in non-pregnant women. Considering this change, the unbound fraction of itraconazole/OHI and of posaconazole in plasma at steady state conditions during 200 mg b.d.i. itraconazole or 400 mg b.d.i posaconazole treatment [75, 77] can be roughly estimated to reach IC₂₅ values of itraconazole (78 nM) and posaconazole (237 nM) for 11 β -HSD2 as determined in HEK-293 cell lysates. Despite the high plasma protein binding ability, extensive extravascular distribution and accumulation in tissues are of considerable importance for these lipophilic compounds. Itraconazole was found to exceed plasma concentrations in fat tissue approximately 17-fold and in lung, liver, muscle and kidney about 2-3 fold [64]. Less data are available for posaconazole, although it was shown that posaconazole concentrations are 31-42 fold higher in pulmonary alveolar

cells compared with plasma [78]. Thus, the concentration of these triazole fungicides might also be elevated in placental tissue compared with plasma and therefore may reach relevant concentrations to inhibit 11 β -HSD2 enzyme activity.

Animal studies revealed embryotoxicity and teratogenicity with craniofacial and skeletal anomalies at concentrations exceeding the maximum recommended human dose by 5-20 fold during itraconazole treatment but at lower than therapeutic doses during posaconazole treatment [75, 76]. Therefore, it is recommended to use these drugs in pregnancy only if the benefit outweighs the potential risk. Extrapolation of findings from animal studies to human is often difficult due to species-specific differences. The present study provides evidence for a considerably weaker inhibition of mouse and rat 11 β -HSD2 by itraconazole and OHI, and lower activity of posaconazole towards the rodent enzymes. Thus, 11 β -HSD2-related effects on electrolyte balance, cardiovascular system and placental barrier function would be detected in rodent models only at much higher concentrations than in humans. Furthermore, regarding the latter, animal studies might be unreliable due to the highly variable 11 β -HSD2 expression levels during gestation between different species. Mouse placental 11 β -HSD2 mRNA drops towards late gestation, while rat placental 11 β -HSD2 reduction occurs later and less pronounced. In contrast, human placental 11 β -HSD2 levels are rising during gestation (reviewed by [13]).

In a prospective cohort study, de Santis *et al.* evaluated first trimester exposure to itraconazole in 206 pregnant women compared with 207 unexposed controls [79]. The mean duration of the therapy was 6.9 ± 6.4 days with a daily dose of 182 ± 63 mg. No statistical difference between the exposed and the control group was found in terms of major congenital anomalies, premature birth or birth weight, but rates of live births and spontaneous abortion were higher in the exposed group. Bar-Oz *et al.* reported in a retrospective cohort study four times higher congenital malformation rates after first trimester exposure to itraconazole; however, strongly suggesting reporting bias due to retrospective data analysis [80]. The same authors found no difference in the rate of malformations compared to the control in a prospective cohort study with 229 women exposed to 50-800 mg itraconazole daily throughout 8.5 ± 12.4 days during the first trimester of pregnancy [81]. However, the rate live birth and the mean birth weight were lower in the exposed group compared to the control. No human data for prenatal posaconazole exposure have been published so far.

Developmental programming implies that an environmental factor affects fetal development during a sensitive time window to predispose the fetus towards diseases permanently throughout life [15]. A critical parameter includes the duration of the exposure to an environmental factor. The mean prenatal itraconazole exposure reported by de Santis *et al.* and Bar-Oz *et al.* was 6.9-8.5 days, thus shorter than the 15 days of treatment needed to reach plasma steady-state levels of itraconazole. This is important regarding 11 β -HSD2 inhibition and thus prenatal programming through glucocorticoids. Several investigations found a reduction in birth weight in infants exposed to multiple courses of antenatal glucocorticoid therapy when adjusting for gestational age [82–84]. In addition, only first trimester itraconazole exposures were examined in the above described studies, but the effects of dexamethasone exposure on birth weight in rats was reported to be more

pronounced when administered during later stages of pregnancy [85]. Chronic glucocorticoid treatment in mice was observed to impair the development of the cerebellum through inhibition of Sonic hedgehog (Shh) induced proliferation, a pathway important during embryonic growth and postembryonic tissue homeostasis. By upregulation of 11 β -HSD2 expression Shh signaling partly antagonized the glucocorticoid-dependent effects, thus representing a feedback mechanism [86]. Interestingly, itraconazole, OHI and posaconazole were found to inhibit Shh signaling [87–89], which may result in downregulation of 11 β -HSD2 expression, thereby adding to the direct inhibitory effect of these azole fungicides.

Elevated glucocorticoid levels are known to inhibit angiogenesis, a crucial process during placental development that was shown to be inhibited by itraconazole [88, 90, 91]. Insufficient placental vascularization has been associated with intrauterine growth restriction, preeclampsia and fetal death, both in human and animal studies (reviewed by [92]).

In conclusion, itraconazole, OHI and posaconazole were identified as novel potent inhibitors of human 11 β -HSD2 by VS of the DrugBank database using an 11 β -HSD pharmacophore model. Inhibition of placental 11 β -HSD2 by these azole fungicides might contribute to an elevated local increase of cortisol levels, in addition to the known inhibition of cytochrome P450 enzymes and P-gp, thereby affecting fetal programming. Due to the observed species-specific differences, the consequences of placental 11 β -HSD2 inhibition may have been overlooked in preclinical studies using rodents, although similar biological responses might be detected in rodents, albeit at higher concentrations.

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Abbreviations

11β-HSD2	11 β -hydroxysteroid dehydrogenase 2
AME	apparent mineralocorticoid excess
androstenedione	4-androstene-3,17-dione
CRH	corticotrophin releasing hormone
DHEA	dehydroepiandrosterone
DHT	5 α -dihydrotestosterone
DMEM	Dulbecco's modified Eagle medium
GR	glucocorticoid receptor

H6PDH	hexose-6-phosphate dehydrogenase
HEK-293	Human Embryonic Kidney-293 cells
HPA	hypothalamic-pituitary-adrenal
MR	mineralocorticoid receptor
OHI	hydroxyitraconazole
P-gp	P-glycoprotein
TLC	thin layer chromatography
VS	virtual screening

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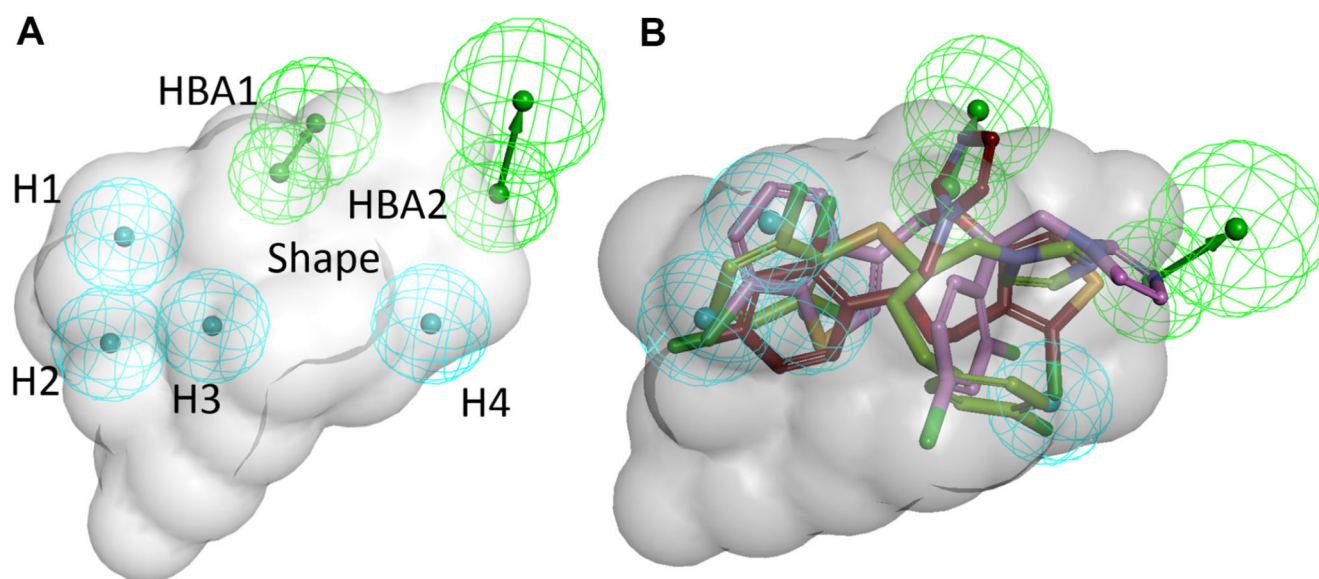


Fig. 1. Pharmacophore model used for virtual screening of the DrugBank database. (A) The model consists of four hydrophobic (H) features (cyan), two hydrogen bond acceptors (HBA, green) and a sterical shape restriction (grey) [39]. (B) Virtual hits from the DrugBank screening fitted into the model. Compounds are color-coded: red – tioconazole; green – butoconazole; violet – sertaconazole.

compound	structure	residual enzyme activity [% of control] (20 μ M)		IC ₅₀ values [μ M]	
		11 β -HSD1	11 β -HSD2	11 β -HSD1	11 β -HSD2
Albendazole		105 \pm 5	100 \pm 15	n.d.	n.d.
Climbazole		57 \pm 10	86 \pm 12	n.d.	n.d.
Tioconazole		18 \pm 3	44 \pm 5	4.97 \pm 0.64	n.d.
Sertaconazole		35 \pm 3	61 \pm 5	12.73 \pm 1.69	n.d.
Butoconazole		48 \pm 6	50 \pm 6	n.d.	n.d.
Ketoconazole		67 \pm 4 ^a	26 \pm 2 ^a	n.d.	n.d.
Terconazole		97 \pm 6	62 \pm 5	n.d.	n.d.
Posaconazole		88 \pm 11	8 \pm 5	n.d.	0.460 \pm 0.098
Itraconazole		89 \pm 6	4 \pm 3	n.d.	0.139 \pm 0.014

Fig. 2. Structure-activity relationship of azole fungicides inhibiting 11 β -HSDs.

11 β -HSD1-dependent reduction of cortisone (200 nM) to cortisol and 11 β -HSD2-dependent oxidation of cortisol (200 nM) to cortisone were measured using HEK-293 cell lysates in the presence of 500 μ M NADPH or NAD⁺, respectively. Residual enzyme activity upon exposure to 20 μ M test substance (mean \pm SD) and IC₅₀ values (mean \pm SD) were obtained from three independent experiments. For compounds with residual enzyme activities (% of control) >40%, at a compound concentration of 20 μ M, IC₅₀ values were not determined (n.d.). ^a, values reported earlier by Vuorinen et al. [39].

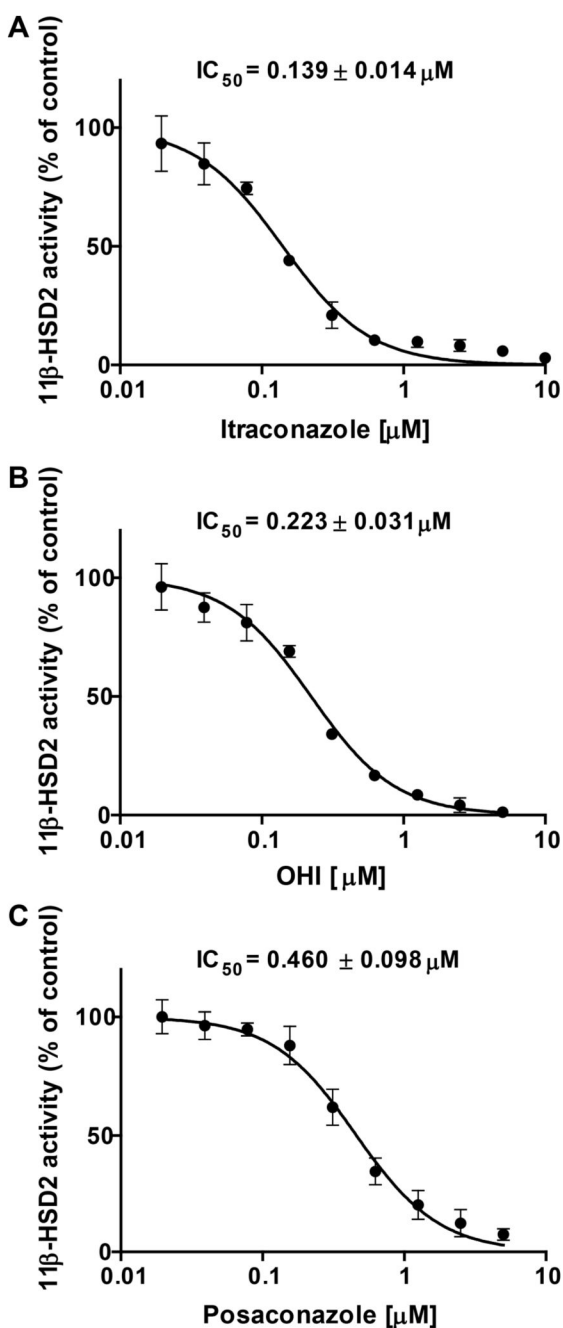


Fig. 3. Inhibition of 11 β -HSD2 enzyme activity determined in HEK-293 cell lysates.

Lysates of HEK-293 cells stably expressing recombinant human 11 β -HSD2 were incubated for 10 min at 37°C with 200 nM radiolabeled cortisol, 500 μM NAD⁺ and increasing concentrations of itraconazole (A), hydroxyitraconazole (OHI) (B) and posaconazole (C). The substrate conversion was determined and compared to the enzyme activity in the control samples (0.1% DMSO). Data represent mean \pm SD from three independent experiments.

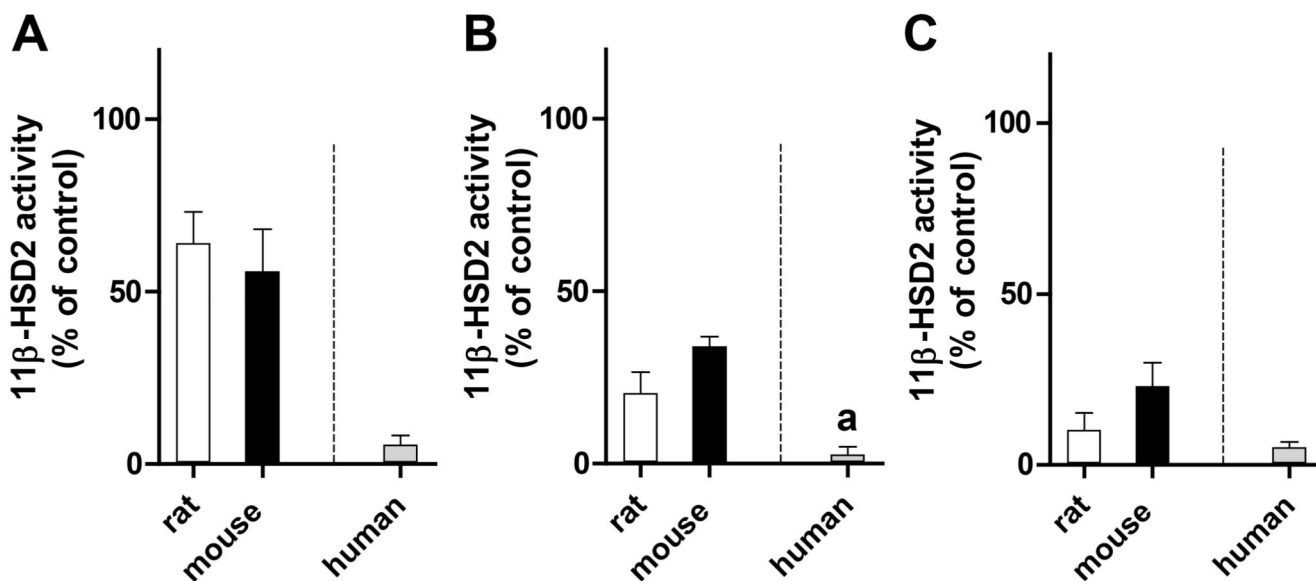


Fig. 4. Inhibition of rat and mouse 11β-HSD2 activity measured in kidney homogenates, compared to human 11β-HSD2 in HEK-293 cell lysates.

Rat and mouse kidney homogenates were incubated for 20 min at 37°C with 50 nM radiolabeled corticosterone in the absence or presence of 10 μM itraconazole (A), 10 μM hydroxyitraconazole (OHI) (B) or 10 μM posaconazole (C). Human 11β-HSD2 activity was measured in HEK-293 cells stably expressing recombinant human 11β-HSD2 upon incubation for 10 min at 37°C with 200 nM radiolabeled cortisol, 500 μM NAD⁺ and the corresponding concentration of azole fungicide (10 μM itraconazole, 10 μM posaconazole or 5 μM OHI). The substrate conversion was calculated (in nmoles/min) and normalized to the enzyme activity in the control sample (0.1% DMSO). Data represent mean ± SD from at least three independent experiments. **a**, 5 μM was used (due to complete inhibition higher concentrations were not analyzed).

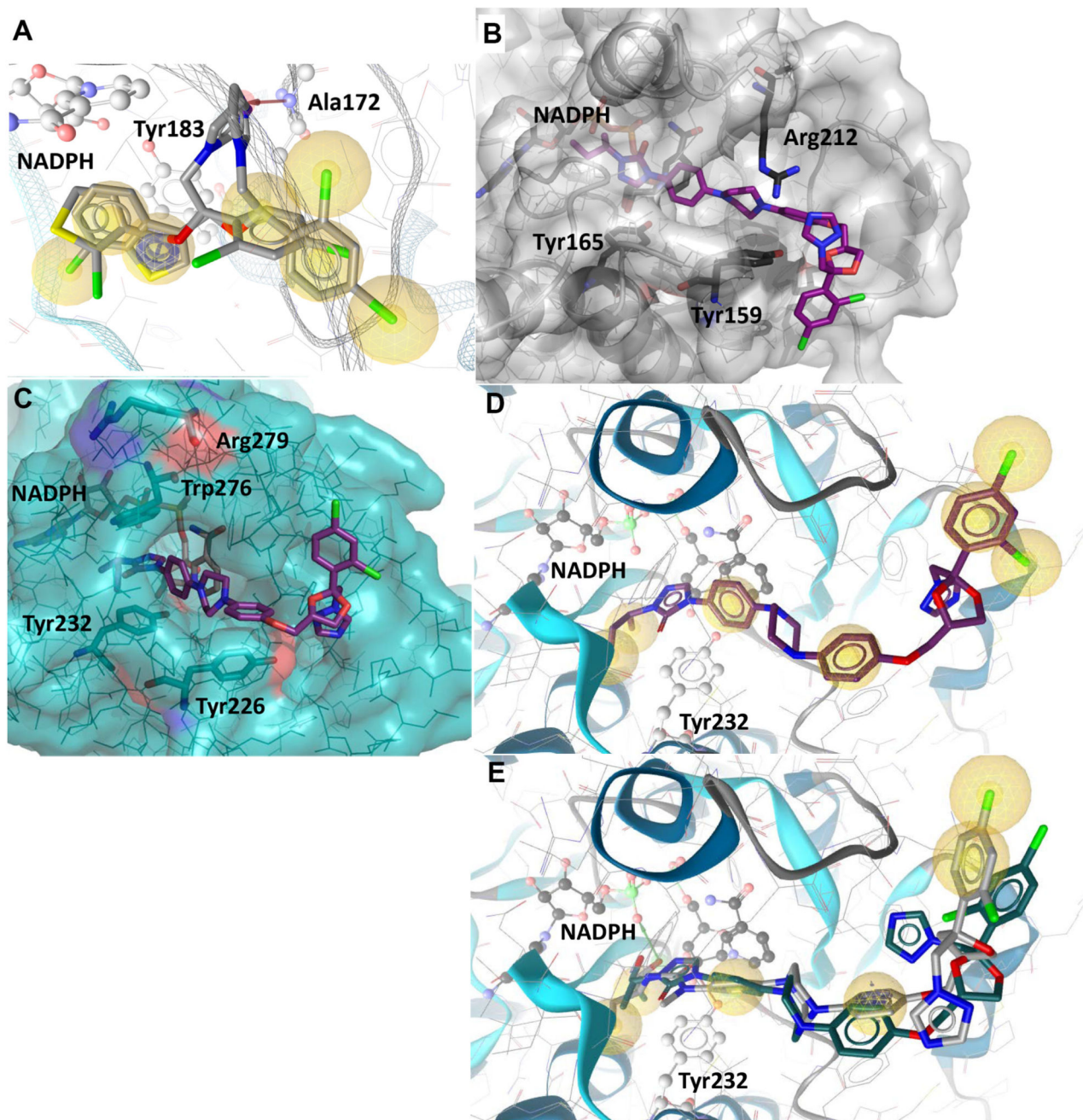


Fig. 5. Predicted binding of selected fungicides to 11β-HSD1 and 11β-HSD2.

(A) Representative binding poses of inhibitors in human 11β-HSD1 exemplified by tioconazole and sertaconazole. Important interactions for protein-ligand binding and the cofactor are shown in ball-and-stick style. Hydrophobic contacts between the ligand and the binding site are represented as yellow spheres, the HBA to Ala172 as red arrow and the aromatic stacking with Tyr183 as blue circle. (B) Binding mode of itraconazole docked into the homology model of human 11β-HSD2. (C) Itraconazole docked into the homology model of murine 11β-HSD2. Arg279 (equivalent to Arg212 in human 11β-HSD2) is

pointing away from the binding pocket, while Trp276 blocks the binding pocket next to the cofactor NADPH. (D) Itraconazole docked into the homology model of murine 11 β -HSD2. The ligand is not anchored via hydrogen bonds or charged interactions, only hydrophobic contacts (yellow spheres) are formed. (E) Posaconazole (grey) and OHI (green) docked into murine 11 β -HSD2. Their hydroxyl groups form hydrogen bonds (green arrow) with the cofactor, which provides an explanation for their higher activity compared to itraconazole.