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Molecular mechanisms of posaconazole- and itraconazole-induced pseudohyperaldosteronism and assessment of other systemically used azole antifungals

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ABSTRACT

Recent reports described cases of severe hypertension and hypokalemia accompanied by low renin and aldosterone levels during antifungal therapy with posaconazole and itraconazole. These conditions represent characteristics of secondary endocrine hypertension caused by mineralocorticoid excess. Different mechanisms can cause mineralocorticoid excess, including inhibition of the adrenal steroidogenic enzymes CYP17A1 and CYP11B1, inhibition of the peripheral cortisol oxidizing enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) or direct activation of the mineralocorticoid receptor (MR). Compared to previous experiments revealing a threefold more potent inhibition of 11β-HSD2 by itraconazole than with posaconazole, the current study found sevenfold stronger CYP11B1 inhibition by posaconazole over itraconazole. Both compounds most potently inhibited CYP11B2. The major pharmacologically active itraconazole metabolite hydroxyitraconazole (OHI) resembled the effects of itraconazole but was considerably less active. Molecular modeling calculations assessed the binding of posaconazole, itraconazole and OHI to 11β-HSD2 and the relevant CYP enzymes, and predicted important interactions not formed by the other systemically used azole antifungals, thus providing an initial explanation for the observed inhibitory activities. Together with available clinical observations, the presented data suggest that itraconazole primarily causes pseudohyperaldosteronism through cortisol-induced MR activation due to 11β-HSD2 inhibition, and posaconazole by CYP11B1 inhibition and accumulation of the mineralocorticoids 11-deoxycorticosterone and 11-deoxycortisol because of hypothalamus-pituitary-adrenal axis (HPA) feedback activation. Therapeutic drug monitoring and introduction of upper plasma target levels may help preventing the occurrence of drug-induced hypertension and hypokalemia. Furthermore, the systemically used azole antifungals voriconazole, isavuconazole and fluconazole did not affect any of the mineralocorticoid excess targets, offering alternative therapeutic options.

1. Introduction

Several recent reports documented cases of severe hypertension and hypokalemia accompanied by a suppression of renin and aldosterone levels during therapy with the azole antifungals posaconazole and itraconazole (reviewed in [1]). The affected patients displayed characteristics of secondary endocrine hypertension caused by mineralocorticoid excess [2]. Different molecular mechanisms can lead to mineralocorticoid excess; whereby posaconazole and itraconazole were proposed to exert their action through inhibition of either the adrenal steroid-metabolizing enzyme cytochrome P450 (CYP) 11B1 [3–5] or the peripheral cortisol oxidizing enzyme 11β-hydroxysteroid

Abbreviations: 11β-HSD, 11β-hydroxysteroid dehydrogenase; ACTH, Adrenocorticotropic hormone; AME, apparent mineralocorticoid excess; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle medium; DOC, 11-deoxycorticosterone; FBS, fetal bovine serum; HEK-293, Human Embryonic Kidney-293; HPA, Hypothalamus-pituitary-adrenal axis; MR, mineralocorticoid receptor; OHI, hydroxyitraconazole; TDM, therapeutic drug monitoring

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dehydrogenase type 2 (11β-HSD2) [5–7]. Nevertheless, the direct inhibition of CYP11B1 by posaconazole, itraconazole and other systemically used azole antifungals has not yet been demonstrated, and it remained unknown whether other targets are involved in the observed mineralocorticoid excess.

A reduced activity of CYP11B1 (also known as 11β-hydroxylase) results in a loss of the negative HPA feed-back with a compensatory increase of adrenocorticotropic hormone (ACTH) and an augmented steroidogenesis with an accumulation of the mineralocorticoid 11-deoxycorticosterone (DOC) and adrenal androgens, despite normal cortisol levels. Although DOC is a moderate mineralocorticoid receptor (MR) agonist, an excessive MR activation with volume expansion and hypertension can occur at supra-physiological DOC concentrations. Increased DOC levels can also be the result of deficient activity of CYP11B2 (also known as aldosterone synthase), which catalyzes the biosynthesis of aldosterone from DOC *via* the intermediates corticosterone and 18-hydroxycorticosterone. However, loss-of-function mutations in CYP11B2 result in the lack of aldosterone, with salt-wasting, hyperkalemia and hypotension [8]. Nevertheless, most compounds reported to cause adverse effects by inhibiting CYP11B enzymes are not selective, and inhibition of both enzymes result in the accumulation of DOC as a consequence of enhanced steroidogenesis in the attempt of the organism to generate sufficient cortisol to maintain body homeostasis. The relative inhibition of CYP11B1 and CYP11B2 by posaconazole, itraconazole and other systemically used azole antifungals has not yet been determined.

In contrast, 11β-HSD2 deficiency due to loss-of-function mutations or pharmacological inhibition leads to exaggerated cortisol-driven MR activation and can be detected by an increased (particularly urinary) cortisol-to-cortisone ratio. A previous *in vitro* study described the potent inhibition of 11β-HSD2 by itraconazole and more moderate inhibition by posaconazole, with IC_{50} values for both compounds in the nanomolar range [9]. Inhibition data on other systemically used azole antifungals were lacking. Regarding posaconazole-induced hypertension and hypokalemia, two case studies showed substantially increased 11-deoxycortisol and DOC serum levels [3,4], suggesting CYP11B1 inhibition. Both cases showed a concomitant increase of androstenedione and 17-hydroxyprogesterone concentrations, indicating stimulation of steroidogenesis. However, only one study assessed ACTH levels, serum cortisol-to-cortisone ratio and urinary steroids, showing elevated ACTH and cortisol-to-cortisone ratio [4]. The urinary steroid profile in this patient revealed unaffected 11β-hydroxylated androgen metabolites and cortisol-to-cortisone ratio, indicating a more pronounced inhibitory effect towards CYP11B1 than 11β-HSD2. In contrast, another case of posaconazole-induced hypertension showed normal serum DOC and androgen concentrations, along with an elevated serum cortisol-to-cortisone ratio and moderately enhanced 11-deoxycortisol and 17-hydroxyprogesterone levels [7], suggesting predominantly 11β-HSD2 inhibition. An increased serum cortisol-to-cortisone ratio and 11-deoxycortisol concentration was reported in an additional patient [10], but no information on adrenal/urinary steroid or ACTH levels were provided, impeding a mechanistic evaluation of the mineralocorticoid phenotype. In this regard, two recently described cases with posaconazole-induced hypertension revealed for one patient raised serum and urinary 11-deoxycortisol concentrations, pointing towards CYP11B1 inhibition, and for the second patient increased serum and urinary cortisol-to-cortisone ratios with only a mild increase in serum 11-deoxycortisol levels, indicating more pronounced 11β-HSD2 inhibition [5]. Notably, all published cases reported markedly increased serum posaconazole concentrations.

A similar phenotype of hypertension and hypokalemia with low renin and aldosterone concentrations was observed in two patients during antifungal therapy with itraconazole [6,11]. Both had high serum itraconazole concentrations. Of note, one of these patients displayed serum levels of hydroxyitraconazole (OHI), an active metabolite of intraconazole, above the therapeutic range [6]. Unfortunately, the assessed steroid profiles were incomplete. Nevertheless, both patients showed unremarkable cortisol, DOC, and ACTH levels, and in the patient where 11-deoxycortisol has been determined, it was only slightly elevated, suggesting more pronounced 11β-HSD2 than CYP11B1 inhibition.

Interestingly, four of the reported patients suffering from posaconazole- and itraconazole-induced pseudohyperaldosteronism received initially either voriconazole [7,12] or fluconazole [5,6] without exhibiting signs of mineralocorticoid excess. Moreover, substitution of posaconazole or itraconazole administration by fluconazole [12], voriconazole [6] or isavuconazole [5,13] led to the resolution of the pseudohyperaldosteronism-mediated hypertension and hypokalemia.

This study aimed to define how clinically used azole antifungals can induce pseudohyperaldosteronism-mediated hypertension and hypokalemia. Using bioassays and molecular modeling calculations, the present study compared the inhibitory effects of posaconazole, itraconazole, OHI, and other systemically used azole antifungals towards CYP11B1, CYP11B2, CYP17A1, and 11β-HSD2, and assessed their potential to activate the MR.

2. Materials and methods

2.1. Chemicals and reagents

[1,2,6,7-^{3}H]-cortisol was purchased from PerkinElmer (Boston, MA, USA), [1,2-^{3}H]-cortisone and [1,2-^{3}H]-11-deoxycorticosterone from American Radiolabeled Chemicals (St. Louis, MO), OHI from Carbosynth (Berkshire, UK), isavuconazole from MedChemExpress, Lucerna Chem (Luzern, Switzerland) and all other chemicals from Sigma Aldrich (Buchs, Switzerland) of the highest grade available. Cell culture media were obtained from Sigma Aldrich.

2.2. Cell culture

Hamster V79MZh cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4.5 g/L glucose, 100 U/mL penicillin/streptomycin, 4 mM L-glutamine and 1 mM sodium pyruvate. Human Embryonic Kidney-293 (HEK-293) cells were cultured in DMEM supplemented with 10% FBS, 4.5 g/L glucose, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, pH 7.4, and 1% MEM non-essential amino acid solution.

2.3. Determination of 11β-HSD activity in cell lysates

Lysates of HEK-293 cells stably expressing human 11β-HSD1 and hexose-6-phosphate dehydrogenase or 11β-HSD2 were used to measure enzymatic activities as described earlier [14]. 11β-HSD1-dependent oxoreduction was assessed by incubating the lysates for 10 min at 37 °C with 200 nM radiolabeled cortisone and 500 μM NADPH, in the absence or presence of different azole antifungals or vehicle. 11β-HSD2 activity was determined by adding 50 nM radiolabeled cortisol and 500 μM NAD^+, with or without antifungals or vehicle. The reactions were terminated by adding an excess amount of unlabeled cortisone and cortisol (2 mM each, in methanol). The steroids were then separated by thin layer chromatography using chloroform and methanol (9:1). The conversion of radiolabeled substrate was measured by scintillation counting. Data (mean ± SD) obtained from two independent experiments performed in triplicate were normalized to the vehicle control (0.1% DMSO). GraphPad Prism 5 software and non-linear regression for curve fitting was used to analyze inhibition (GraphPad Software, San Diego, USA).

2.4. Determination of CYP11B1 and CYP11B2 activity

CYP11B enzyme activities were determined as described earlier with minor modifications [15]. Briefly, V79MZ cells stably expressing

human CYP11B1 or CYP11B2 [16] were grown in 24-well plates until confluence in DMEM supplemented with 5% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine and 1 mM sodium pyruvate at 37 °C and 5% CO_2. DMEM was removed and 450 µL of fresh DMEM containing 5.6 µL of the azole antifungal solutions in ethanol were added to each well. After preincubation for 1 h at 37 °C, the reaction was started by adding 50 µL of DMEM containing 100 nM DOC plus 0.15 µCi [1,2-^{3}H]-11-deoxycorticosterone. Final inhibitor concentrations ranged from 0.32 nM - 1 µM for OHI, 0.8 nM - 2.5 µM for itraconazole and posaconazole and 0.32 nM - 10 µM for fluconazole, voriconazole, isavuconazole and ketoconazole. Final ethanol concentration in the incubation medium was 1%. The cells were incubated for 20 min and 1 h for CYP11B1 and CYP11B2 expressing cells, respectively. To determine the conversion of DOC to the products corticosterone, 18-hydroxycorticosterone and aldosterone (CYP11B2) as well as DOC to the product corticosterone (CYP11B1), steroids were extracted from the incubation medium using 500 µL ethyl acetate. The organic phase was transferred into fresh vials, and following evaporation of the solvent, the residue was dissolved in 40 µL of methanol-water (65/35; v/v) and analyzed by radio-HPLC (Agilent 1200 HPLC system, Santa Clara, CA, USA) with a Nucleosil-gel 5C 18 column (Macherey-Nagel, Düren, Germany). Enzymatic conversion of radiolabeled substrate into product (s) was determined by evaluating the peak areas of the substrate and product(s). Inhibition was analyzed by GraphPad Prism 5 using non-linear regression for curve fitting. Data (mean ± SD) were normalized to the vehicle control and obtained from two independent experiments.

2.5. Determination of CYP17A1 activity

Protein preparation of human CYP17A1 from recombinant E. coli pJL17/OR co-expressing human CYP17A1 and rat NADPH-P450-reductase and enzyme activity measurements were conducted as described earlier [17,18]. Briefly, 17α-hydroxylase activity was determined by measuring the enzymatic conversion of the substrate progesterone to the product 17α-hydroxyprogesterone and the by-product 16-hydroxyprogesterone. In a first step, 140 µL of phosphate buffer (0.05 M, pH 7.4, 1 mM Mg Cl_2, 0.1 mM EDTA and 0.1 mM dithiothreitol) containing 6.25 nmol progesterone, 5 µL of the corresponding azole antifungal compound and 50 µL NADPH regenerating system (10 mM $NADP^+$, 100 mM glucose-6-phosphate, 2.5 U glucose-6-phosphate dehydrogenase) were pre-incubated at 37 °C for 5 min, before adding 50 µL of protein suspension (0.8–1 mg protein per mL). The final substrate concentration was 25 µM, azole antifungal concentrations were 400 nM, 2 µM or 10 µM, except for ketoconazole 8 nM - 10 µM. After incubation for 30 min at 37 °C the reaction was stopped by adding 1 N HCl. Steroids were extracted with ethyl acetate and analyzed by UV spectroscopy. GraphPad Prism 5 with non-linear regression for curve fitting was used to analyze inhibition. Data (mean ± SD) were normalized to vehicle control, obtained from two independent experiments.

2.6. MR reporter gene assay

V79MZ cells (150,000 per well) were seeded in 24-well plates and cultured for 6 h. Then, the cells were transiently transfected with plasmids for MR (400 ng/well), truncated MMTV promoter luciferase reporter (pHHluc, 50 ng/well) and pCMV-lacZ β-galactosidase (5 ng/well) as transfection control using polyethyleneimine. At 4 h post-transfection, the medium was replaced with fresh culture medium and the cells were incubated for another 16 h. The medium was again replaced by serum- and phenol red-free DMEM (SF DMEM) for 1 h, before the treatment solutions were added to the cells. Fresh SF DMEM containing either 50 nM cortisol or 1 µM of the respective azole antifungal were added to the cells and incubated for 6 h. The cells were then lysed with Tropix lysis solution (Applied Biosystems, Foster City, CA) containing 0.5 µM dithiothreitol and placed at −80 °C for at least 20 min. Luciferase activity was determined by adding D-luciferin-firefly substrate solution (0.47 mM D-luciferin, 53 mM ATP, 0.27 mM coenzyme A, 0.13 mM EDTA, 33.3 mM dithiothreitol, 8 mM $MgSO_4$, 20 mM tricine, pH 7.8) and β-galactosidase activity by using the Tropix kit. Data (mean ± SD) were normalized to the vehicle control (0.1% DMSO) and obtained from three independent experiments performed in triplicates.

2.7. Docking calculations

Docking calculations were performed using GOLD 5.2 (The Cambridge Crystallographic Data Centre, Cambridge, UK, [19]) based on a homology model for human 11β-HSD2 [20] and on crystal structures for human CYP11B2 (PDB code 4DVQ) [21], CYP11B1 (PDB code 6M7X) [22] and CYP17A1 (PDB code 3RUK) [23]. The ligand binding pockets were defined as a sphere with a 10 Å radius around the coordinates X = -27.46, Y = 27.98, Z = -16.91 for CYP11B2, X = 48.54, Y = -42.29, Z = -1.57 for CYP11B1 and X = 28.53, Y = -8.76, Z = 36.51 for CYP17A1. As a scoring function ChemPLP was used. In order to validate the workflow, re-docking of the original ligands (DOC for CYP11B2, fadrozol for CYP11B1 and abiraterone for CYP17A1) was performed using the default settings. The best-ranked pose of DOC deviated from the co-crystallized ligand with an RMSD value of 0.390, for fadrozole with an RMSD value of 0.370, and for abiraterone with and RMSD value of 0.522.

The settings used for the 11β-HSD2 homology model were defined as described previously [9]. Briefly, a 10 Å radius around the coordinates X = -19.50 Y = 4.00, Z = 14.25 were used to define the binding site. In addition, Arg212 was set as flexible amino acid. The binding poses were analyzed using LigandScout 4.2 (Inte:Ligand GmbH, Vienna, Austria) [24].

3. Results

3.1. Inhibition of CYP11B1 and CYP11B2 enzyme activities by systemically administered azole antifungals

In order to assess the underlying mechanisms of the posaconazole- and itraconazole-dependent mineralocorticoid effects, the most commonly prescribed azole antifungals for systemic applications were tested for their potential to inhibit the enzymatic activities of CYP11B1 (assessed as conversion of 11-DOC to corticosterone) and CYP11B2 (conversion of 11-DOC to corticosterone, 18-hydroxycorticosterone and aldosterone) in stably transfected V79MZh cells (Fig. 1, Table 1). Besides ketoconazole, a known inhibitor of CYP11B1 and CYP11B2 (reviewed in [1]), posaconazole exhibited the most potent inhibitory capacity towards CYP11B1 and CYP11B2 with IC_{50} values of 59 ± 4 nM and 9 ± 3 nM, respectively. The IC_{50} values for itraconazole were found also in the nanomolar range, *i.e.* for CYP11B1 439 ± 43 nM and CYP11B2 23 ± 8 nM, with a strong preference to inhibit CYP11B2. OHI, the major pharmacologically active metabolite of itraconazole, was found to be about two times less active towards CYP11B1 (IC_{50} value of 948 ± 167 nM) and approximately 13 times less active towards CYP11B2 (IC_{50} value of 298 ± 62 nM) compared to its parent compound. Isavuconazole, voriconazole and fluconazole showed only very weak inhibitory capacity towards CYP11B1 and CYP11B2 with IC_{50} values above 1 µM.

To rationalize the results obtained in the enzyme activity evaluations, the compounds were docked into the substrate binding pockets of CYP11B2 and CYP11B1. The docking calculations provided a possible explanation for the potent inhibition of posaconazole and ketoconazole towards CYP11B2 (Fig. 2): Hydrogen bond interactions stabilized both, the inner part of the molecule as well as that oriented towards the outside of the binding pocket, *i.e.* one close to the catalytic center and one at the protein surface. Arg120, Arg51 and Phe239 were the residues predicted to interact with posaconazole by hydrogen bonds, whereas Phe381 and Arg51 were proposed to form hydrogen bond interactions

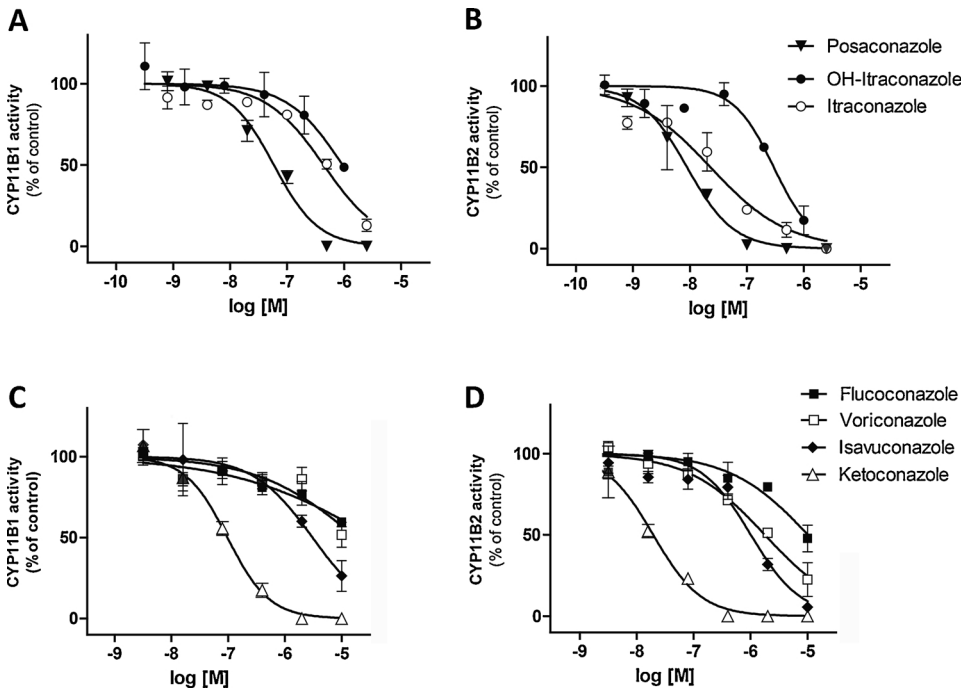

Fig. 1. Inhibition of CYP11B1 and CYP11B2 enzyme activities by azole antifungals. The conversion of 11-DOC to corticosterone and 11-DOC to corticosterone, 18-hydroxycorticosterone and aldosterone was determined in V79MZh cells expressing human CYP11B1 (A, C) and CYP11B2 (B, D), respectively, in the presence of various concentrations of inhibitor. (A, B) Concentration-dependent inhibition of CYP11B1 and CYP11B2 activities by posaconazole, itraconazole and hydroxyitraconazole (OHI). (C, D) Concentration-dependent inhibition of CYP11B1 and CYP11B2 activities by fluconazole, voriconazole, isavuconazole, and ketoconazole. Enzyme activities were normalized to vehicle control samples. Inhibition curves were fitted and analyzed by non-linear regression. Data represent mean ± SD of two independent experiments.

Table 1
IC_{50} values of selected azole antifungals for CYP11B1, CYP11B2 and CYP17A1. CYP11B1 and CYP11B2 activities were measured by determining the conversion of 11-DOC to corticosterone and 11-DOC to corticosterone, 18-hydroxycorticosterone and aldosterone, respectively, in intact stably transfected V79MZh cells. CYP17A1 activity was determined as conversion of progesterone to 17α-hydroxyprogesterone in a cell-free assay. Inhibition curves were fitted and analyzed by non-linear regression. Data were normalized to vehicle control and represent mean ± SD of two independent experiments.

Compound	CY11B1 IC_{50} [nM]	CYP11B2 IC_{50} [nM]	CYP17A1 IC_{50} [nM]
Posaconazole	59 ± 4	9 ± 3	n.d.*
Itraconazole	439 ± 43	23 ± 8	n.d.
OHI	948 ± 167	298 ± 62	n.d.
Ketoconazole	98 ± 11	20 ± 6	1,500 ± 300
Isavuconazole	3,079 ± 569	1,016 ± 170	n.d.
Voriconazole	> 10,000	1,788 ± 487	n.d.
Fluconazole	> 10,000	> 10,000	n.d.

*n.d., not determined.

with ketoconazole. Regarding itraconazole and OHI, solely hydrophobic interactions were predicted, not explaining the potent activity of itraconazole. Comparison of the binding poses of itraconazole and OHI revealed an unfavorable positioning of the hydroxyl group of OHI in a hydrophobic region of the binding pocket. It needs to be noted that the applied docking approach is limited by its rigidity and cannot detect interactions formed upon an induced fit. Thus, molecular dynamic simulations could be performed to provide further information. The smaller scaffolds isavuconazole, voriconazole and fluconazole showed several hydrophobic contacts as well as hydrogen bond interactions; however, these interactions may be insufficient to allow potent inhibition.

The docking calculations for CYP11B1 showed two important hydrogen bond interactions for posaconazole and ketoconazole, *i.e.* Tyr485 and Glu114 as well as Phe381 and Arg404, respectively (Fig. 3). Itraconazole and OHI both formed only one hydrogen bond, with Phe381, in addition to several hydrophobic interactions, providing a possible explanation for the weaker activity compared to posaconazole. As with CYP11B2, the hydroxyl group of OHI is in a hydrophobic region of the CYP11B1 binding pocket, likely explaining the weaker activity compared to itraconazole. Also in analogy with CYP11B2, isavuconazole, fluconazole and voriconazole may exert insufficient interactions with residues of the CYP11B1 ligand-binding pocket for a potent activity because of their smaller size.

3.2. Assessment of possible cross-reactivity of the selected azole antifungals towards CYP17A1

Since ketoconazole has been reported earlier to inhibit CYP17A1 activity [25], the systemically used azole antifungals were further tested for their ability to abolish 17α-hydroxylase activity (Fig. 4). For this purpose, lysates of recombinant E. coli pJL17/OR co-expressing human CYP17A1 and rat NADPH-P450-reductase were used to determine the enzymatic conversion of progesterone to 17α-hydroxyprogesterone. Neither itraconazole nor OHI, isavuconazole, voriconazole or fluconazole were able to inhibit CYP17A1 17α-hydroxylase activity at concentrations up to 10 μM. Due to interference with the UV spectroscopy analysis of the steroids, the highest concentration of posaconazole applied was 400 nM; however, as for other systemically used azole antifungals no interference with CYP17A1 17α-hydroxylase activity could be observed.

Docking calculations of azole antifungals to CYP17A1 predicted only for ketoconazole a comparable binding pose to that of the known CYP17A1 inhibitor abiraterone (Fig. 5A and B). Whereas abiraterone displayed an iron binding interaction with the heme and a hydrogen bond to Asn202, ketoconazole showed a hydrogen bond to the closely located Tyr201. Additionally, both showed stabilizing hydrophobic interactions in the catalytic center close to the heme. In contrast, the docking predicted only several hydrophobic contacts for posaconazole, itraconazole and OHI but no other stabilizing interactions.

3.3. Effects of selected azole antifungals on 11β-HSD2 activity

Itraconazole, OHI and posaconazole have been described previously as potent inhibitors of the 11β-HSD2-dependent conversion of cortisol to cortisone [9], thus, potentially increasing the amount of cortisol in mineralocorticoid target tissues. However, no information about the potential inhibitory activities of voriconazole, fluconazole and isavuconazole towards 11β-HSD2 was available. Therefore, lysates of HEK-

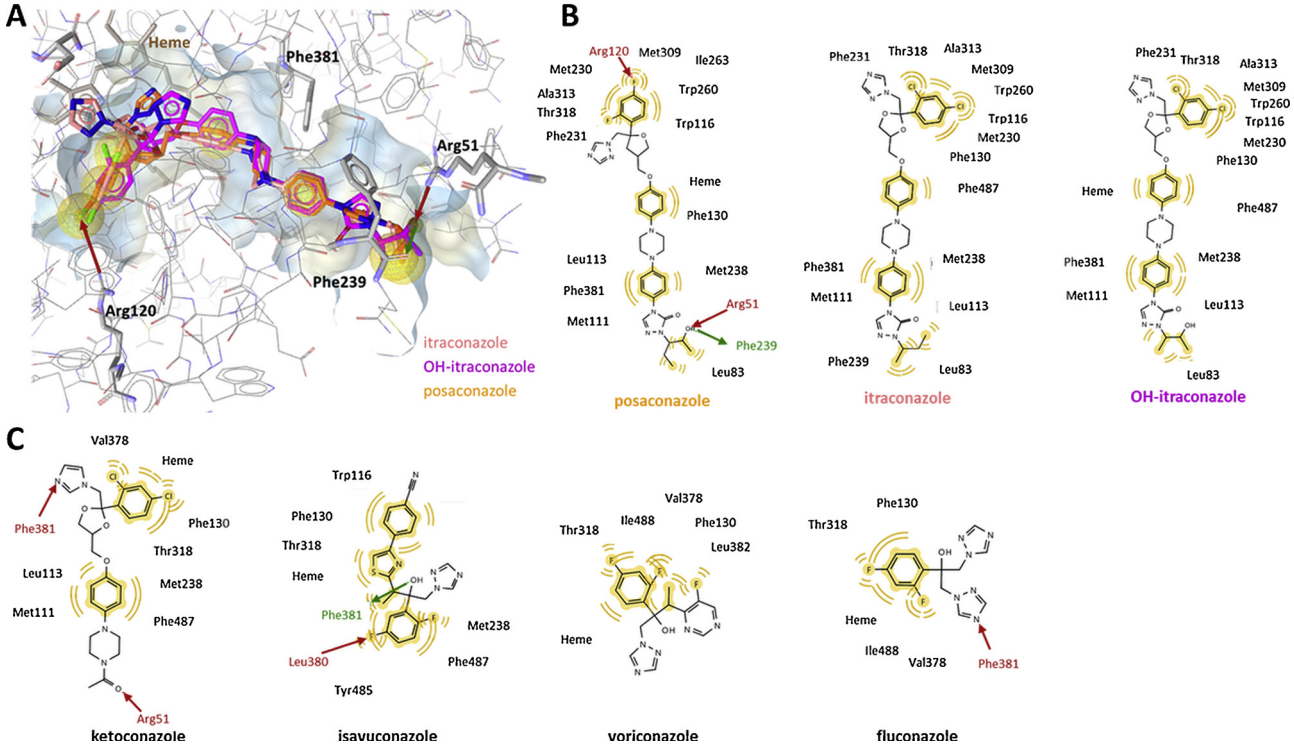

Fig. 2. Predicted binding calculations of selected azoles to the substrate binding pocket of CYP11B2. (A) Representative binding poses of itraconazole, OHI and posaconazole in the binding pocket of CYP11B2. Important interactions for protein-ligand binding and the cofactor are shown in ball-and-stick style. Hydrophobic contacts between the ligand and the binding site are represented as yellow spheres, hydrogen bond acceptors as red and hydrogen bond donors by a green arrow. Two-dimensional representation of the binding interactions of (B) posaconazole, itraconazole, and OHI and (C) ketoconazole, isavuconazole, voriconazole and fluconazole.

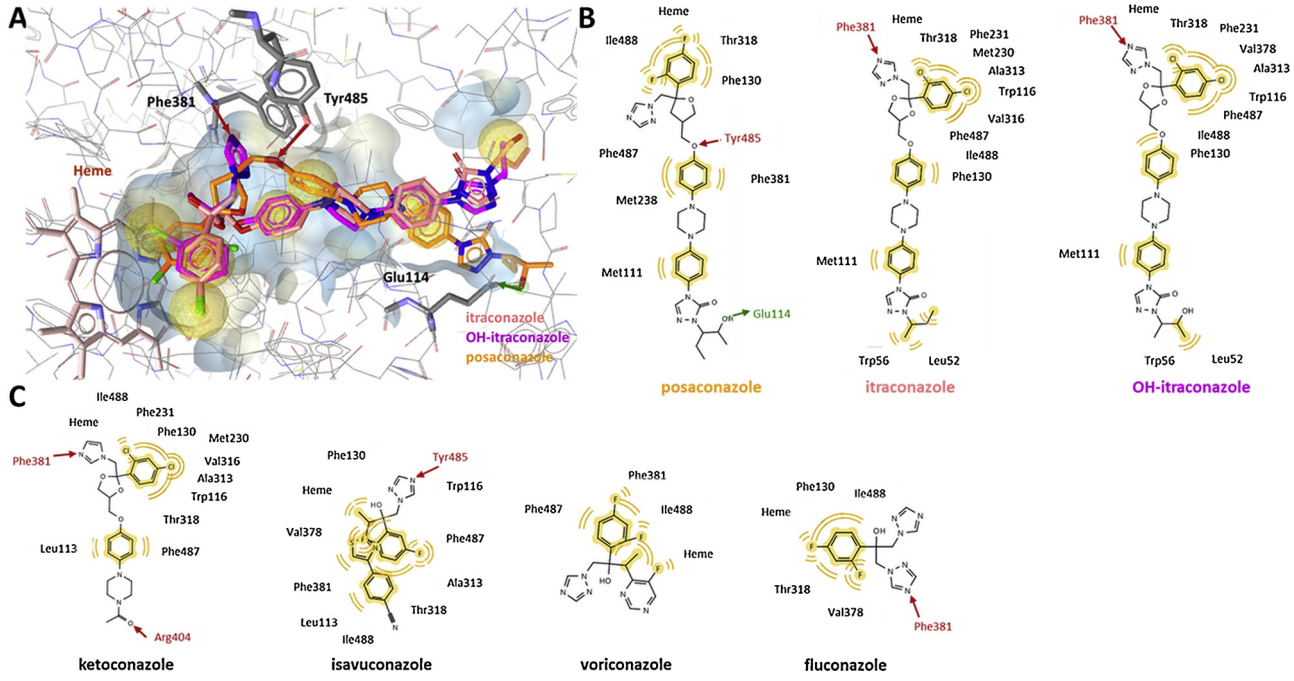

Fig. 3. Predicted binding calculations of selected antifungals to the substrate binding pocket of CYP11B1. (A) Representative binding poses of itraconazole, OHI and posaconazole in the binding pocket of CYP11B1. Important interactions for protein-ligand binding and the cofactor are shown in ball-and-stick style. Hydrophobic contacts between the ligand and the binding site are represented as yellow spheres, hydrogen bond acceptors as red and hydrogen bond donors by a green arrow. Two-dimensional representation of the binding interactions of (B) itraconazole, OHI and posaconazole and (C) ketoconazole, isavuconazole, voriconazole and fluconazole.

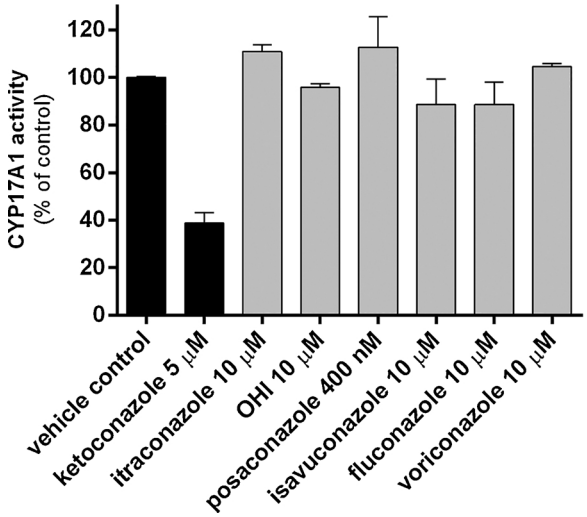

Fig. 4. Inhibition of CYP17A1 17α-hydroxylase activity by the selected azole antifungals. The conversion of progesterone to 17α-hydroxyprogesterone in bacterial lysates expressing human CYP17A1 was determined in the presence of various concentrations of inhibitor. The enzyme activity was normalized to that in the vehicle control. Data represent mean ± SD from two independent experiments.

293 cells stably expressing 11β-HSD2 were incubated with 50 nM cortisol as substrate and 10 μM or 1 μM of the corresponding test compounds (Fig. 6A). Neither voriconazole nor fluconazole were able to inhibit 11β-HSD2 at a concentration up to 10 μM. Isavuconazole showed weak inhibitory activity towards 11β-HSD2 with a residual enzyme activity of 44% at a concentration of 10 μM. Furthermore, an evaluation for possible cross-inhibitory activity of these compounds towards the related 11β-HSD1 was performed but none of the compounds was able to inhibit the cortisone to cortisol conversion at a concentration of 10 μM (Fig. 6B).

Docking calculations using a homology model of 11β-HSD2 revealed close alignment of itraconazole, OHI and posaconazole, fitting not entirely into the substrate binding site of the enzyme but blocking the surface of the binding site entry with their azole groups (Fig. 7A), as it has been observed earlier [9]. Moreover, two protein-ligand interactions providing an explanation for the potent inhibitory activities of itraconazole, OHI and posaconazole towards 11β-HSD2 were observed. First, the nitrogen of the triazole ring of itraconazole and OHI, not belonging to the azole part, served as hydrogen bond acceptor for an interaction with the catalytic Tyr232, whereas the hydroxyl group of posaconazole formed a hydrogen bond with Tyr232 (Fig. 7B); second, itraconazole and OHI formed an additional aromatic interaction with Arg279, which might support the more pronounced inhibitory activity of itraconazole and OHI towards 11β-HSD2 compared to posaconzole. Ketoconazole was found earlier to exhibit moderate inhibitory activity towards 11β-HSD2 [26]. Docking calculations revealed a hydrogen bond with Tyr232, as seen for the larger scaffolds, as well as a second hydrogen bond with Arg279, proposed to stabilize ligand binding. The binding of isavuconazole was also found to be stabilized by hydrogen bonds, *i.e.* with Arg279 and Ser269. Voriconazole and fluconazole, which did not inhibit the 11β-HSD2 enzyme activity, only showed a single hydrogen bond to Arg279 and few stabilizing hydrophobic interactions.

3.4. Effects of selected azole antifungals on MR activity

Besides an inhibition of adrenal steroidogenesis and peripheral glucocorticoid metabolism by the systemically applied azole antifungals, a possible direct activating effect of these compounds on the MR was assessed by a reporter gene assay. V79MZh cells expressing the MR and a MR-dependent luciferase reporter gene were treated with either 50 nM cortisol in the presence or absence of spironolactone as positive control or 1 μM of the respective azole antifungals (Fig. 8). None of the tested azole antifungals altered the activity of the MR under these conditions.

4. Discussion

Several recent reports described cases of posaconazole- and itraconazole-induced hypertension and hyperkalemia (reviewed in [1]). Two different mechanisms underlying the observed pseudohyperaldosteronism were proposed, namely the inhibition of CYP11B1, resulting in 11-DOC-mediated MR activation, and 11β-HSD2, leading to cortisol-dependent MR activation. Whilst the inhibition of 11β-HSD2 by posaconazole and itraconazole has been biochemically confirmed using recombinant enzyme, the evidence for CYP11B1 inhibition was derived from clinical analyses, revealing low serum aldosterone but elevated 11-DOC and 11-deoxycortisol levels; however, a direct inhibition of CYP11B1 was not assessed so far. To extend the existing knowledge, the current study elucidated the inhibitory effects of these two drugs and additional systemically applied azole antifungals on the activities of the most important targets of mineralocorticoid excess, namely CYP11B1 and CYP11B2, CYP17A1, 11β-HSD2 and the MR.

Posaconazole was found to be the most potent CYP11B1 inhibitor, whereas itraconazole and OHI were more moderate inhibitors. Interestingly, both posaconazole and itraconazole showed more pronounced inhibition of CYP11B2, which likely contributes to the observed low serum aldosterone levels in the treated patients. However, an exclusively abolished activity of CYP11B2, as observed in patients with rare genetic loss-of-function mutations [8], is expected to lower blood pressure and increase serum potassium levels, and therefore the azole antifungals-induced hypertension and hypokalemia is a result of pseudohyperaldosteronism caused by CYP11B1 and/or 11β-HSD2 inhibition. A comparison of the inhibitory activities revealed posaconazole as a potent inhibitor of CYP11B1 and a moderate one of 11β-HSD2, whereas itraconazole exhibited the opposite pattern by potently inhibiting 11β-HSD2 but moderately CYP11B1. Although the inhibitory potencies towards CYP11B1 and 11β-HSD2 by a given compound cannot be directly compared because of the different assay protocols used, the results suggest that the occurrence of pseudohyperaldosteronism during posaconazole therapy is mainly caused through inhibition of CYP11B1 and that by itraconazole *via* 11β-HSD2 blockade. Importantly, neither isavuconazole, nor voriconazole or fluconazole showed relevant inhibitory activities towards CYP11B enzymes or 11β-HSD2.

Furthermore, none of the systemically used azole antifungals inhibited CYP17A1 or stimulated the MR activity under the conditions applied. Thus, using biochemical assays combined with computational modeling calculations, the present study provides an explanation for the molecular mechanisms underlying the mineralocorticoid symptoms observed under posaconazole and itraconazole therapy but not upon administration of isavuconazole, fluconazole or voriconazole.

Effects of itraconazole and posaconazole related with adrenal toxicity and inhibition of steroid hormone synthesis have been described in preclinical repeated-dose toxicity studies in rats and dogs at exposure levels greater than or even equal to therapeutic concentrations in humans [27,28]. Nonetheless, the first case reports on hypertension and hypokalemia due to pseudohyperaldosteronism during itraconazole and posaconazole therapy appeared almost a decade after their market launch. Importantly, the plasma concentrations reached are dependent on drug formulation. For example, significant interindividual and intraindividual pharmacokinetic variabilities limited the use of the oral posaconazole suspension particularly due to saturable absorption and a highly variable bioavailability, depending on the concomitant food intake, gastric pH and gut motility [29,30]. Moreover, several potential drug-drug interactions can further contribute to the variations in

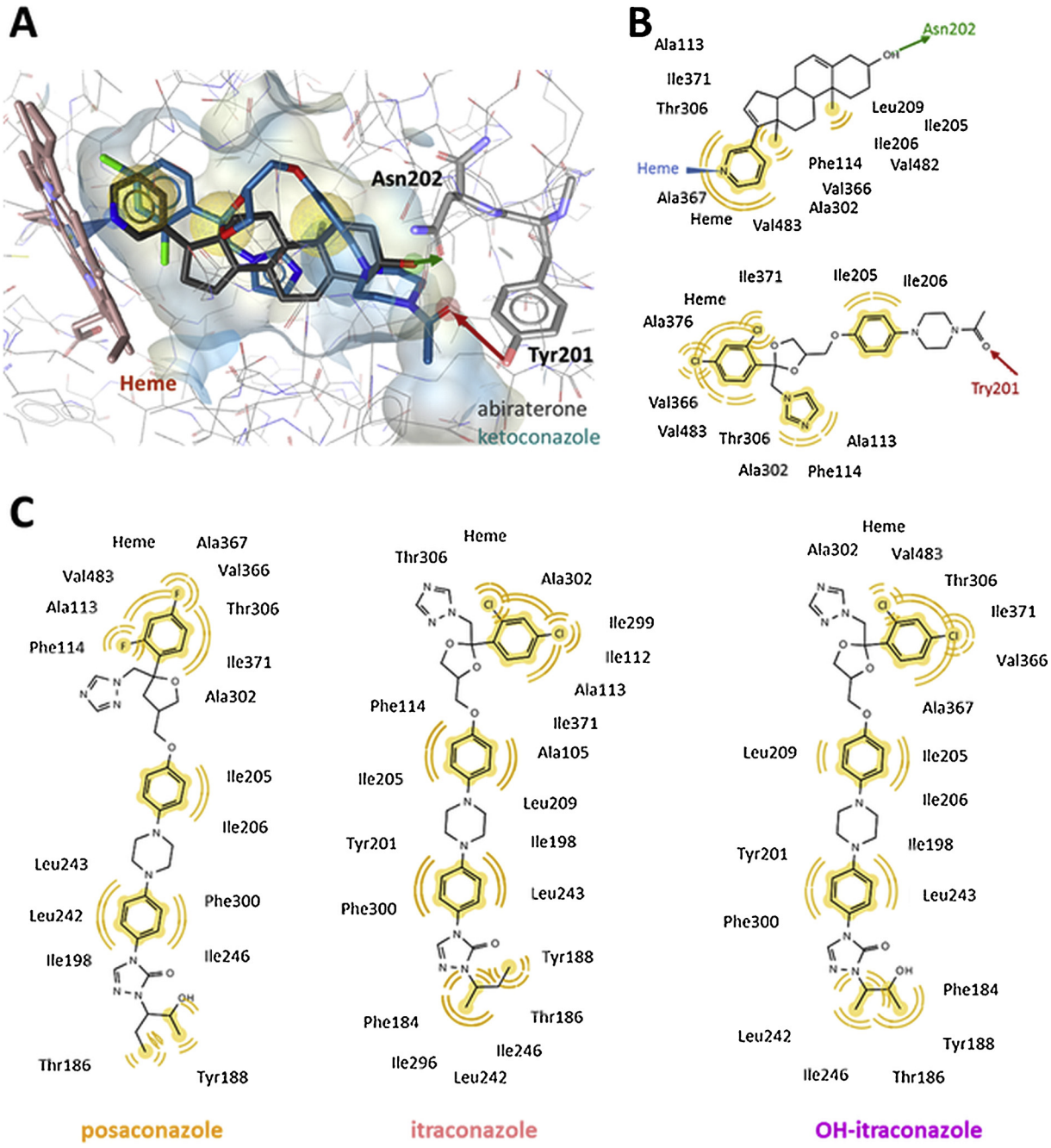

Fig. 5. Predicted binding calculations of selected azoles to the substrate binding pocket of CYP17A1. (A) Representative binding poses of abiraterone and ketoconazole in the binding pocket of CYP17A1. Important interactions for protein-ligand binding and the cofactor are shown in ball-and-stick style. Hydrophobic contacts between the ligand and the binding site are represented as yellow spheres, hydrogen bond acceptors as red arrow, hydrogen bond donors green arrow, and iron binding locations as blue cone. Two-dimensional representation of the binding interactions of (B) ketoconazole and abiraterone, and (C) itraconazole, OHI and posaconazole.

itraconazole and posaconazole exposure levels [28,31–34]. Thus, regarding patients receiving posaconazole suspension, therapeutic drug monitoring (TDM) has been recommended mainly to ensure an adequate drug exposure and thereby efficacy [35].

The newer delayed-release posaconazole tablets are considerably less prone to gastrointestinal absorption variabilities, leading to higher and more consistent posaconazole plasma levels [36,37], and limited evidence supports routine TDM to evaluate patient drug exposure [35–40]. However, a recent single-center retrospective observational study in outpatients newly starting posaconazole therapy assessed the occurrence of posaconazole-induced pseudohyderaldosteronism and found positive correlations between serum posaconazole levels and systolic blood pressure as well as 11-deoxycortisol levels [41]. Patients with posaconazole-induced pseudohyperaldosteroism had significantly higher serum posaconazole levels (3 μg/mL; 4.3 μM) than those without (1.2 μg/mL; 1.7 μM). Further support that the azole antifungal-induced pseudohyperaldosteronism is dose-dependent is provided by earlier studies on patients treated with different itraconazole doses [42] and a case where lowering posaconazole dose ameliorated the hypertension and hypokalemia [5]. Moreover, all cases of itraconazole- and posaconazole-induced pseudohyperaldosteronism reported so far had elevated serum drug concentrations [1].

Regarding the clinical phenotype and relative distribution of CYP11B1 and 11β-HSD2 inhibition, the concentrations reached at the

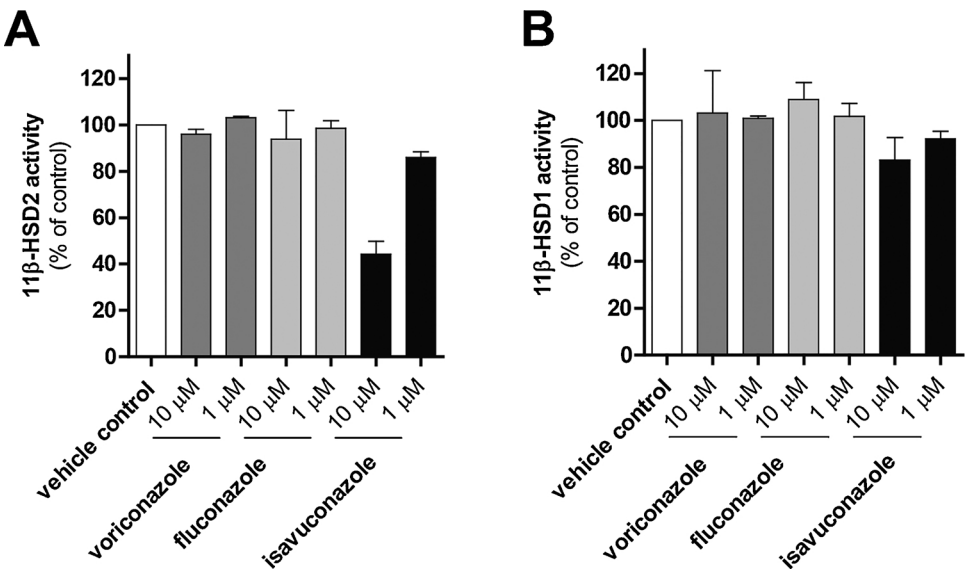

Fig. 6. Effects of selected azole antifungals on 11β-HSD2 and 11β-HS1 activity determined in HEK-293 cell lysates. The conversion of cortisol to cortisone and the reverse reaction were determined in lysates of HEK-293 cells stably expressing 11β-HSD2 and 11β-HSD1, respectively. The activity in the presence of inhibitor was normalized to that of the vehicle control. Data represent mean ± SD from three independent experiments.

site of the respective enzyme, *i.e.* in the adrenals and in kidney/colon, are crucial, but to date, intra-tissue concentrations are not available. Both posaconazole and itraconazole are highly protein bound in plasma (> 98% and > 99%, respectively) [28,43]. Regarding the plasma concentrations determined in the recent clinical study [41], the estimated free posaconazole concentration in the group with pseudohyperaldosteronism (85 nM when considering 2% unbound drug) is at or below IC_{50} values for CYP11B1 and 11β-HSD2 obtained in this study. However, posaconazole and itraconazole are lipophilic compounds, with a larger distribution volume and a tendency to penetrate preferentially into tissues with high lipid content [44]. Thus, the tissue/plasma concentration ratios for these compounds may be considerably higher than 1. A recent study comparing posaconazole trough concentrations of the delayed-release tablet formulation and the oral suspension showed that despite the known increase in posaconazole exposure levels with the tablet formulation, the variability of the posaconazole trough levels was not significantly lower than that of the suspension [45]. Thus, the dose-dependent occurrence of posaconazole- and itraconazole-induced hypertension and hypokalemia suggests that TDM could be beneficial to prevent overexposure. However, there are currently no studies reported that define an upper plasma target level for posaconazole (and the less frequently used itraconazole) that is associated with toxicity, and pharmacokinetic studies supporting the registration of the tablet formulation by the European Medicines

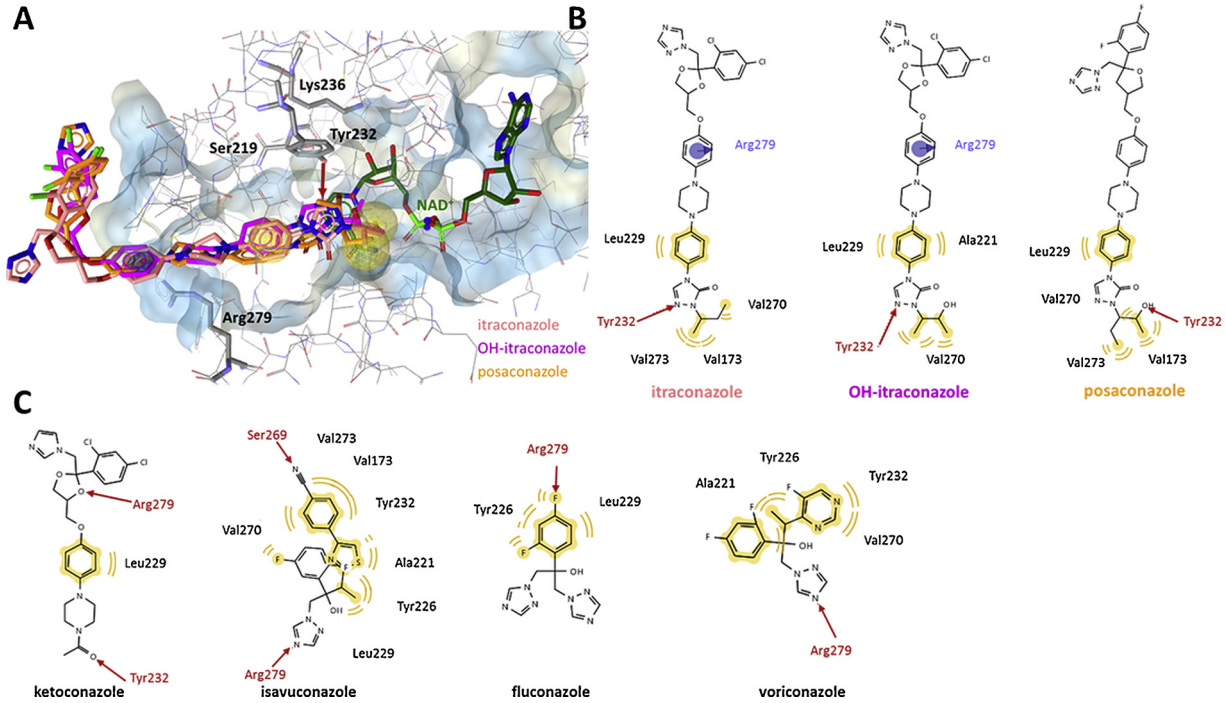

Fig. 7. Predicted binding calculations of the selected azoles using a homology model of 11β-HSD2. (A) Representative binding poses of itraconazole, OHI and posaconazole in the binding pocket of 11β-HSD2. Important interactions for protein-ligand binding and the cofactor are shown in ball-and-stick style. Hydrophobic contacts between the ligand and the binding site are represented as yellow spheres, hydrogen bond acceptors as red arrow and the aromatic stacking as blue circle. Two-dimensional representation of the binding interactions of (B) itraconazole, OHI and posaconazole, and (C) ketoconazole, isavuconazole, fluconazole and voriconazole.

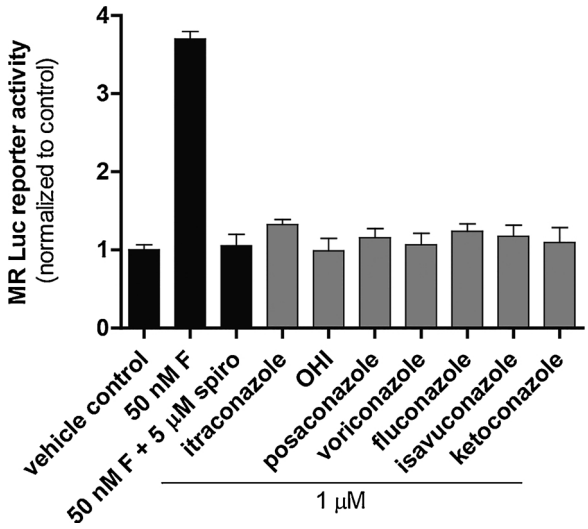

Fig. 8. Effects of selected azole antifungals on the MR-mediated activation of a luciferase reporter. The effect of systemically applied azole antifungals on the transcriptional activity of the MR was measured in V79MZ cells expressing an MR-responsive luciferase reporter. Spironolactone served as positive and DMSO as negative controls. Data of a representative experiment performed in triplicates were normalized to the vehicle control and are shown as mean ± SD from triplicates.

Agency (EMA) used a provisional cutoff of 3.75 µg/mL [37,46]. These studies described that 3% of all patients had steady-state trough levels of ≥3.75 µg/mL and 10% between ≥2.5 µg/mL and ≤3.75 µg/mL [46]. To compare these values with trough levels for TDM of patients on oral posaconazole suspension, the following levels are recommended during steady-state conditions (after 7 days of treatment): > 0.7 µg/mL for prophylactic purposes and > 1-1.25 µg/mL during therapy of a suspected or documented invasive aspergillosis [29,35,37]. Given the mineralocorticoid excess symptoms observed at serum posaconazole concentration of 3 µg/mL [1,41], a TDM strategy with an upper plasma target level, defined by pharmacokinetic studies, seems appropriate during therapy with posaconazole delayed-release tablets although this exact upper limit remains undefined pending the results of ongoing studies.

In conclusion, biochemical experiments and molecular modeling revealed that posaconazole and itraconazole are potent inhibitors of CYP11B1 and CYP11B2, with a preference for CYP11B2. The results provide an explanation for the observed pseudohyperaldosteronism caused by posaconazole and itraconazole, with posaconazole being a potent inhibitor of CYP11B1 but a moderate one of 11β-HSD2, whilst itraconazole potently inhibits 11β-HSD2 but moderately CYP11B1. The other systemically applied azole antifungals, voriconazole, fluconazole and isavuconazole, did neither inhibit CYP11B1/2 nor 11β-HSD2. None of the tested azole antifungals inhibited CYP17A1 or activated MR. Evidence for interindividual pharmacokinetic variabilities with posaconazole delayed-release tablets suggests a TDM strategy to avoid pseudohyperaldosteronism. Moreover, introduction of upper plasma target levels may help to prevent the occurrence of drug-induced hypertension and hypokalemia.

CRediT authorship contribution statement

Katharina R. Beck: Conceptualization, Investigation, Data curation, Validation, Formal analysis, Visualization, Writing - review & editing. **Lucija Telisman:** Investigation, Visualization. **Chris J. van Koppen:** Investigation, Validation, Resources, Writing - review & editing. **George R. Thompson:** Conceptualization, Writing - review & editing. **Alex Odermatt:** Conceptualization, Validation, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

Chris J. van Koppen was an employee of Elexopharm, Germany. The other authors declare no conflict of interest.

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