Divergence of Anti-angiogenic Activity and Hepatotoxicity of Different Stereoisomers of Itraconazole

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Translational Relevance

Itraconazole, a known antifungal drug, was found to possess anti-angiogenic and anti-hedgehog activity. However, its clinical development has been limited in part by its hepatotoxicity. We determined the antiangiogenic activity and hepatotoxicity of each of the four individual cis-stereoisomers of itraconazole. We found that one particular stereoisomer, the 2S4R2’S stereoisomer, designated IT-C, has more potent antiangiogenic and anticancer activity with less hepatotoxicity, both in vitro and in animal models, in comparison with the racemic mixture of itraconazole. These results revealed for the first time that stereochemistry of itraconazole has differential effects on its anti-angiogenic activity and hepatotoxicity, suggesting that IT-C is likely to be superior to itraconazole for future clinical development as an anticancer and anti-angiogenic drug.
Abstract

Purpose: Itraconazole is a triazole antifungal drug that has recently been found to inhibit angiogenesis. Itraconazole is a relatively well-tolerated drug but shows hepatotoxicity in a small subset of patients. Itraconazole contains three chiral centers and the commercial itraconazole is composed of four cis-stereoisomers (named IT-A, IT-B, IT-C, and IT-D). We sought to determine whether the stereoisomers of itraconazole might differ in their antiangiogenic activity and hepatotoxicity.

Experimental Design: We assessed in vitro antiangiogenic activity of itraconazole and each stereoisomer using human umbilical vein endothelial cell (HUVEC) proliferation and tube formation assays. We also determined their hepatotoxicity using primary human hepatocytes in vitro and a mouse model in vivo. Mouse Matrigel plug and tumor xenograft models were used to evaluate in vivo antiangiogenic and antitumor activities of the stereoisomers.

Results: Of the four stereoisomers contained in commercial itraconazole, we found that IT-A (2S,4R,2’R) and IT-C (2S,4R,2’S) were more potent for inhibition of angiogenesis than IT-B (2R,4S,2’R) and IT-D (2R,4S,2’S). Interestingly, IT-A and IT-B were more hepatotoxic than IT-C and IT-D. In mouse models, IT-C showed more potent antiangiogenic/antitumor activity with lower hepatotoxicity compared to itraconazole and IT-A.

Conclusions: These results demonstrate the segregation of influence of stereochemistry at different positions of itraconazole on its antiangiogenic activity and hepatotoxicity, with the 2 and 4 positions affecting the former and the 2’ position affecting the latter. They also suggest that IT-C may be superior to the racemic mixture of itraconazole as an anticancer drug candidate due to its lower hepatotoxicity and improved antiangiogenic activity.
Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, has been shown to play a critical role in both normal physiological and pathological processes. It is essential during development and wound healing, and is tightly regulated by endogenous pro- and anti-angiogenic factors. It has also been implicated in a number of diseases including cancer, rheumatoid arthritis and macular degeneration (1-3). Since the angiogenesis hypothesis was first proposed by Judah Folkman in 1971 (4), a number of angiogenesis inhibitors have been discovered and developed into anti-angiogenic drugs (5-7). A monoclonal antibody against vascular endothelial growth factor (VEGF), bevacizumab, was first approved by the US-Food and Drug Administration (FDA) in 2004 for the treatment of metastatic colon cancer in combination with standard chemotherapy (8). Subsequently, several small molecule angiogenesis inhibitors have been entered the clinic, including sorafenib (Nexavar®), sunitinib (Sutent®) and pazopanib (Votrient®). However, most of these small molecule antiangiogenic drugs are kinase inhibitors that lack specificity or lead to a high frequency of drug resistance (9-11), necessitating the development of small molecule angiogenesis inhibitors with novel mechanisms of action.

In an effort to accelerate drug discovery and development, we assembled the Johns Hopkins Drug Library over a decade ago and screened it for new anti-angiogenic activity among existing drugs using a proliferation assay with primary human umbilical vein endothelial cells. Among the most interesting hits was the antifungal drug itraconazole (12). Interestingly, itraconazole was also found to be an inhibitor of the hedgehog signaling pathway in a separate screen, rendering itraconazole a novel anticancer drug candidate capable of inhibiting the growth of both tumor vasculature and tumor cells themselves (13). A series of tests of itraconazole in preclinical angiogenesis and cancer models confirmed the anti-angiogenic and anticancer activity of itraconazole in vivo (12-14). Based on these promising preclinical results, itraconazole entered multiple Phase II human clinical studies for treating different types of cancer (http://www.clinicaltrials.gov/). Positive clinical results have been reported for advanced lung cancer, metastatic prostate cancer and basal cell carcinoma (15-17). Moreover, itraconazole was found to increase progression or overall survival among late-stage ovarian, triple-negative breast and metastatic pancreatic cancer patients upon retrospective analysis of previous clinical data.
Together, these encouraging results strongly suggest that itraconazole is a promising new anticancer drug lead.

Although the precise mechanism for the antiangiogenic and anti-hedgehog activities remains unknown, preliminary evidence suggests that the mode of action of itraconazole is distinct from any other known inhibitors of angiogenesis or the hedgehog signaling pathway. Early on, we ruled out the cholesterol biosynthetic enzyme, lanosterol 14α-demethylase, which mediates the antifungal activity of itraconazole, as the relevant target for its antiangiogenic activity (12). Subsequently, we serendipitously discovered that itraconazole blocks the traffic of cholesterol and likely lipids out of the lysosome, causing a phenotype similar to Niemann-Pick Disease Type C (NPC) in endothelial cells. We also found that itraconazole is a potent inhibitor of the mTOR pathway in endothelial cells (22). Importantly, the blockade of cholesterol trafficking through the endolysosome can only partially explain the inhibition of mTOR by itraconazole, suggesting that there exist unidentified molecular target(s) for itraconazole. Similarly, the mechanism underlying the inhibition of hedgehog signaling also remains unclear, even though it has been shown to block the translocation of Smoothened from intracellular vesicles into the primary cilium (13). Using a number of synthetic analogs of itraconazole, we explored the structure-activity relationship of itraconazole for its two distinct activities and found no correlation between the two, further suggesting that the antiangiogenic and anti-hedgehog activity of itraconazole are likely to be mediated by different molecular mechanisms (23).

Itraconazole has been widely used to treat fungal infections. Although itraconazole is known to be relatively well-tolerated, about 7% of patients who received the drug experience hepatotoxicity (24). In addition to lanosterol 14 alpha-demethylase (25), itraconazole is also known to be a potent inhibitor of human liver cytochrome P450 3A4 (CYP3A4), accounting for its extensive drug-drug interactions with other medications (26). It is believed that the effect of itraconazole on CYP3A4 is responsible for the human hepatotoxicity (27). The commercial itraconazole consists of four cis-stereoisomers of itraconazole, including IT-A (2S,4R,2’R), IT-B (2R,4S,2’R), IT-C (2S,4R,2’S), and IT-D (2R,4S,2’S). All four isomers are known to be ligands of CYP3A4 (28). However, only IT-B and IT-D among the four isomers were metabolized by CYP3A4 (28). To assess the antiangiogenic activity of the different stereoisomers, we previously synthesized each of cis-stereoisomers and determined their effects on both fungi and endothelial
cells (29). In this study, we conducted a large-scale purification of each stereoisomer from the racemic itraconazole mixture using the preparative supercritical fluid chromatography (SFC) and obtained gram quantities of each purified stereoisomer. Using the purified stereoisomers, we compared the antiangiogenic activity and hepatotoxicity of each stereoisomer of itraconazole and the racemic itraconazole both \textit{in vitro} and \textit{in vivo}. We found that IT-A and IT-C are more potent at inhibiting angiogenesis compared to the others, whereas IT-C and IT-D are less hepatotoxic compared to IT-A, IT-B and racemic itraconazole. Together, these results suggest that IT-C is the most potent antiangiogenic and anticancer agent with the least hepatotoxicity among the four stereoisomers of itraconazole.
Materials and Methods

Cells

Human umbilical vein endothelial cells (HUVEC) (Lonza, Allendale, NJ) were grown in endothelial cell growth medium-2 (EGM-2) using the EGM-2 bullet kit (Lonza) per manufacturer’s instructions. HUVEC phenotype was verified by morphological observation throughout serial passage by the manufacturer. Human pericytes (placental derived) were purchased from Zen-Bio (Research Triangle Park, NC) and were grown in the complete pericyte growth medium (Zen-Bio). HCC1954 human breast cancer and CWR22Rv1 human prostate cancer cells were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 1% antibiotic (penicillin and streptomycin) solution (Invitrogen). The cells were maintained in a humidified incubator at 37 °C adjusted to 5% CO₂.

Reagents

Racemic itraconazole and filipin were purchased from Sigma-Aldrich (St Louis, MO). Four individual cis-stereoisomers of itraconazole (IT-A, IT-B, IT-C and IT-D) were obtained using preparative supercritical fluid chromatography (SFC) separation of commercial itraconazole, which was performed by WuXi AppTec (Shanghai, China). Quality control of each purified stereoisomer was conducted by chiral HPLC and optical rotation using previously synthesized stereoisomer samples as standards (29). Low-density lipoprotein (LDL) from human plasma was purchased from Fisher Scientific (Pittsburgh, PA).

HUVEC proliferation assay

HUVEC (5000 cells per well) were seeded in 96-well plates containing 0.2 mL of EGM-2 medium and allowed to adhere at 37 °C for 24 hours. The cells were then treated with various concentrations of each compound for 24 hours. Cells were pulsed with 0.5 μCi [³H]-thymidine (PerkinElmer, Waltham, MA) for 16 hours, before the cells were treated with 1× trypsin-EDTA (Invitrogen, Carlsbad, CA). The detached cells were harvested onto FilterMat A glass fiber filters.
(Wallac, Turku, Finland) using a Harvester 96 cell harvester (Tomtec, Hamden, CT). $[^{3}H]$-thymidine counts were determined using a MicroBeta plate reader (Perkin Elmer). Each treatment was done in triplicate and the experiments were conducted four times independently. The IC$_{50}$ values of each compound and their 95% confidence intervals were calculated using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

**Western blot analysis**

Whole cell lysates were prepared by adding 2× Laemmli buffer to the cells (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl [pH 6.8]) and boiling for 5 minutes. Proteins were then separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). After blocking at room temperature for 1 hour, membranes were incubated at 4°C overnight with the primary antibodies including anti-VEGFR2 (Cell Signaling, Danvers, MA), anti-phospho-S6K (Cell Signaling), anti-S6K (Santa Cruz Biotechnologies, Santa Cruz, CA) and anti-GAPDH (Santa Cruz Biotechnologies) antibodies, followed by incubation with HRP-conjugated anti-mouse or anti-rabbit IgG at room temperature for 1 hour. Antibody-protein complexes were detected using enhanced chemiluminescence (ECL) immunoblotting detection reagent (GE Healthcare, Pittsburgh, PA).

**Filipin staining**

HUVEC were seeded at $3 \times 10^4$ cells per well in a Nunc Lab-Tek II Chamber (8) Slide (Thermo Scientific, Rockford, IL) and allowed to adhere overnight. Following drug treatment for 24 hours, cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 minutes and then washed with PBS three times. The slides were incubated with PBS containing filipin (at a final concentration of 50 μg/mL) in the dark for 1 hour at room temperature. The cells were then washed with PBS three times, mounted with Immu-mount (Thermo Scientific), and stored in the dark prior to imaging with a Zeiss 510 Meta confocal microscope (Carl Zeiss, Thornwood, NY).
HUVEC tube formation assay

The tube formation assay, a model for assessment of angiogenesis in vitro, was conducted as previously described (30). Briefly, a 96-well plate was coated with Matrigel (BD Biosciences, San Jose, CA) by adding 50 μl of ice-cold Matrigel solution per well followed by incubation at 37°C for 1 hour. HUVEC were then seeded on the Matrigel-coated wells (2 × 10^4 cells per well). Cells were treated with compound alone or in combination with LDL and then incubated in a CO2 incubator for 16 hours. For the post-treatment experiments, compounds were added to the cells 6 hours after seeding on Matrigel and the incubation was continued for 18 hours. The cells were washed carefully with PBS once, and Calcein-AM (BD Biosciences) solution in PBS (2 μmol/L final concentration) was added. After incubation at 37°C for an additional 30 minutes, the cells were washed with PBS and the fluorescence-labeled HUVEC tubes were observed under the Nikon Eclipse TS100 fluorescence microscope (485-nm excitation and 520-nm emission) at magnification ×100. Each treatment was done in triplicate and the experiments were conducted three times independently. The total tube length, size and number of junctions from the fluorescence images were quantified using the AngioQuant v1.33 software (The MathWorks, Natick, MA).

HUVEC-pericyte co-culture tube formation

Growing pericytes in a 6-cm cell culture dish were labeled with Calcein-AM for 30 min prior to the tube formation experiments. Ibidi µ-Slide angiogenesis chamber (Ibidi, Planegg, Germany) was coated with Matrigel by adding 10 μl of ice-cold Matrigel solution per well followed by incubation at 37°C for 1 hour. HUVEC (1.5 × 10^4 cells per well) and pericytes (500 cells per well) were mixed at a ratio of 30:1 in complete EGM-2 media, seeded on the Matrigel-coated wells and then treated with compounds. After 20 hours of incubation, the co-culture tube formation was analyzed under a Zeiss AxioObserver Z1 fluorescence microscope with ApoTome apparatus (Carl Zeiss) at magnification ×50. The total tube lengths of the fluorescence-labeled pericytes were quantified using the AngioQuant v1.33 software.
Determination of hepatotoxicity in primary human hepatocytes

Fresh primary human hepatocytes (6-well plates) with matrigel overlay from three donors were obtained from XenoTech, LLC (Lenexa, KS). Ages and genders of donors were as follows: 22 year old male, 54 year old male and 63 year old male. Hepatocyte viability from each donor was at least 74%. Upon receipt of the hepatocytes, the shipping medium was replaced with Williams’ Medium E supplemented with 10% fetal bovine serum (Invitrogen), penicillin-streptomycin (Sigma-Aldrich) and L-glutamine (Invitrogen). The hepatocytes were then incubated overnight at 37°C in a 5% CO₂ humidified environment. Following the overnight incubation the medium was replaced with fresh William’s Medium E supplemented as described above and the hepatocytes were incubated with DMSO (vehicle control, 0.1% final volume), racemic itraconazole (2 μmol/L or 10 μmol/L) as well as stereoisomers denoted as IT-A, IT-B, IT-C and IT-D at a final concentration of either 2 μmol/L or 10 μmol/L for 72 hours. Following incubation, in order to monitor hepatocyte function, the medium was removed and alanine aminotransaminase (ALT) levels were measured using an alanine aminotransaminase activity assay kit purchased from BioVision Inc (Milpitas, CA). Cell viability following treatment with itraconazole and stereoisomers was determined using the Cell Death Detection ELISA kit according to the manufacturer’s instructions (Roche Diagnostics Corporation, Indianapolis, IN) as we have reported previously (31). Intracellular glutathione (GSH) was measured using HPLC by quantification of the 5-thio-2-nitrobenzoic acid formed following the addition of Ellman’s reagent to hepatocyte lysates as previously described (32).

Determination of hepatotoxicity in mice

Female mice (BALB/c, AnNCr) aged 6-8 weeks were purchased from the National Cancer Institute (Frederick, MD) and treated in accordance with Johns Hopkins Animal Care and Use Committee procedures. Mice (n = 5 per group) were treated intraperitoneally (i.p.) with vehicle (saline with 5% DMSO, 5% PEG500 and 5% tween-80), 60 mg/kg itraconazole, 60 mg/kg IT-A and 60 mg/kg IT-C once daily for 10 days. Mice were sacrificed and livers from each group were isolated, photographed and fixed in 10% neutral-buffered formalin (Sigma-Aldrich). Fixed liver...
tissues were embedded in paraffin, processed for histology sections and stained with hematoxylin and eosin (H&E).

**In vivo Matrigel plug angiogenesis assay**

Female mice (BALB/c, AnNCr) aged 6-8 weeks were used for the Matrigel plug angiogenesis assay. Mice (n = 5 per group) were pretreated i.p. with vehicle (saline with 5% DMSO, 5% PEG500 and 5% tween-80), itraconazole (20 and 50 mg/kg), IT-A (20 and 50 mg/kg) and IT-C (20 and 50 mg/kg) once daily for 2 days prior to Matrigel implantation. Matrigel (0.5 ml per injection) containing 200 ng/mL VEGF and 500 ng/mL basic fibroblast growth factor (bFGF) was implanted subcutaneously into mice. The drug treatment was continued once daily for an additional 8 days. Mice were sacrificed and whole Matrigel plugs with mouse skin were excised, fixed in 10% neutral-buffered formalin, embedded in paraffin. For staining infiltrated cells in the Matrigel, the fixed Matrigel plugs were processed for histochemical staining using Masson trichrome staining, which stains the Matrigel blue and the infiltrated cells and vessels red. For staining functional vasculatures, the fixed Matrigel plugs were stained with anti-CD31 (BD Pharmingen, San Jose, CA), a blood vessel marker, and anti-NG2 (Abcam, Cambridge, MA), a pericyte marker, to assess Matrigel plug angiogenesis. A cross-section of the Matrigel plug was photographed at ×100 magnification under a Nikon Eclipse TS100 microscope and CD31-positive blood vessels and NG2 stained cells were counted per field for quantification of angiogenesis.

**In vivo tumor xenograft assay**

Female athymic nude mice (BALB/c, nu/nu-NCr) aged 6-8 weeks were purchased from the National Cancer Institute (NCI)-Frederick and treated in accordance with Johns Hopkins Animal Care and Use Committee procedures. HCC1954 human breast cancer cells (2 × 10⁶) were implanted subcutaneously into the mice. After tumors became palpable, the mice (n = 7 per group) were treated i.p. with vehicle (saline with 5% DMSO, 5% PEG500 and 5% tween-80), itraconazole (25 and 50 mg/kg) and IT-C (25 and 50 mg/kg) once daily for 30 days. For
CWR22Rv1 human prostate cancer xenografts, male athymic nude mice (BALB/c, nu/nu-NCr) aged 6-8 weeks (NCI-Frederick) were used for tumor cell implantation (2 × 10^6 cells per injection). The tumor bearing mice (n = 6 per group) were treated i.p. with vehicle, itraconazole (20 and 60 mg/kg) and IT-C (20 and 60 mg/kg) once daily for 24 days. The tumor volume was measured periodically using a vernier caliper and calculated according to the modified ellipsoid formula: tumor volume (mm³) = (width)^2 × (length) × π/6. After treatment, the mice were sacrificed and whole tumor tissues from the HCC1954 xenograft were excised. Tumor tissues were then fixed in 10% neutral-buffered formalin, embedded in paraffin and sliced to obtain 5-μm sections. The blood vessels in the tumor tissue sections were stained with an anti-CD31 antibody (BD Pharmingen, San Jose, CA), a blood vessel marker, to assess tumor angiogenesis. A cross-section of the tumor tissue was photographed at magnification ×100 under the Nikon Eclipse TS100 microscope and blood vessels stained with anti-CD31 antibody were counted per field for the quantification of tumor angiogenesis. The lumen sizes of the blood vessels were also quantified using the Zen image analysis tool (Carl Zeiss).

**Statistical analysis**

Statistical analysis of the data was performed using GraphPad Prism version 5.0. Statistical significance, determined by two-tailed Student’s t-test (between two groups) and two-tailed Single Sample t-test (test group vs hypothetical control value), is indicated throughout by the following: *, P ≤ 0.05 and **, P ≤ 0.01.
Results

Racemic itraconazole and its stereoisomers inhibited HUVEC proliferation with different potencies.

Racemic itraconazole and each of the four purified cis-stereoisomers were tested for HUVEC proliferation by measuring the ability of the cells to incorporate [3H]-thymidine into DNA. Itraconazole dose-dependently inhibited the thymidine incorporation of HUVEC with an IC₅₀ value of 137 nmol/L (Table 1). Under the same assay conditions, the 2S4R stereoisomers including IT-A and IT-C showed more potent inhibition against HUVEC proliferation compared to itraconazole with IC₅₀ values of 103 and 98 nmol/L, respectively. However, the 2R4S stereoisomers including IT-B and IT-D were less potent than itraconazole with IC₅₀ values of 223 and 199 nmol/L, respectively. Collectively, 2S4R stereoisomers were roughly two times more potent than 2R4S stereoisomers in HUVEC proliferation. A one-to-one combination of IT-A and IT-C or IT-B and IT-D showed no synergy in HUVEC proliferation, suggesting that they might share the same mechanism of action to inhibit HUVEC proliferation (Table 1).

Itraconazole stereoisomers inhibited VEGFR2 glycosylation, mTOR activity and cholesterol trafficking in HUVEC.

We next determined if all stereoisomers still share a common mechanism of action to inhibit endothelial cell proliferation by assessing three previously identified activities of itraconazole, i.e., inhibition of VEGFR2 glycosylation, mTOR and cholesterol trafficking (NPC phenotype). Itraconazole inhibited VEGFR2 glycosylation in HUVEC as evidenced by the dose-dependent shift of high-molecular weight, hyper-glycosylated form of VEGFR2 to the low molecular weight, hypo-glycosylated form. All four stereoisomers inhibited VEGFR2 glycosylation with slight different potencies. All four stereoisomers as well as racemic itraconazole dose-dependently inhibited mTOR activity as measured by S6 kinase (S6K) phosphorylation, a downstream target of mTOR. It was apparent that IT-A and IT-C (2S4R series) were more potent than IT-B and IT-D (2R4S series) for the inhibition of VEGFR2 glycosylation and mTOR activity (Fig. 1A). Next, we determined the effects of itraconazole and the stereoisomers on cholesterol trafficking in HUVEC. Cellular cholesterol was fluorescently labeled by filipin and...
the cholesterol distribution was observed under a confocal microscope. HUVEC were treated with either vehicle (DMSO) or 50 nmol/L of itraconazole or stereoisomers. The concentration of 50 nmol/L that we used here was the lowest concentration of itraconazole that is sufficient to induce an NPC phenotype. At this concentration, IT-A and IT-C as well as itraconazole showed an NPC phenotype as judged by the accumulation of cholesterol fluorescence in the perinuclear region of HUVEC. However, IT-B and IT-D failed to show NPC phenotype at this concentration (Fig. 1B). At concentrations over 100 nmol/L, IT-B and IT-D were able to induce an NPC phenotype in HUVEC (data not shown). Thus, for inhibition of cholesterol trafficking, mTOR activity and VEGFR2 glycosylation, 2S4R series were more potent than 2R4S series, which correlated with the relative potency of the stereoisomers against HUVEC proliferation.

Inhibition of angiogenesis by itraconazole stereoisomers is dependent on cholesterol.

We next employed an in vitro tube formation assay to assess the activity of the stereoisomers. Upon seeding on top of solidified Matrigel, individual endothelial cells migrate and elongate to form tubule-like networks, reminiscent of new blood vessel formation (Fig. 1C). Treatment of HUVEC with 3 µmol/L itraconazole, IT-A and IT-C strongly inhibited HUVEC tube formation (Supplementary Fig. S1A and B). However, treatment with IT-B and IT-D showed little or no inhibition of HUVEC tube formation at the same concentration (Supplementary Fig. S1A and B), again demonstrating that IT-A and IT-C are more potent than the other two stereoisomers. To see if the compounds are able to inhibit pre-formed tubes of HUVEC, itraconazole and stereoisomers were added 6 hours after cell seeding on Matrigel and the incubation was continued for an additional 18 hours. At a higher concentration (6 µmol/L), itraconazole and the two potent stereoisomers (IT-A and IT-C) significantly inhibited the pre-formed tubes of HUVEC, again verifying that IT-A and IT-C are more potent anti-angiogenic agents than IT-B and IT-D (Supplementary Fig. S2A and B). As the antiproliferative activity of itraconazole was shown to be dependent on cholesterol trafficking (22), we conducted a rescue experiment of angiogenesis inhibition by itraconazole and stereoisomers with low-density lipoprotein (LDL). Treatment of HUVEC with LDL slightly enhanced the tube formation of HUVEC. When HUVEC was treated with itraconazole or stereoisomers together with LDL, the inhibition of tube formation was completely reversed to the control level (Fig. 1C and D). Furthermore, inhibition of VEGFR2
glycosylation by itraconazole or stereoisomers was also completely reversed by LDL (Fig. 1E and F). These results indicated that, similar to itraconazole, the antiangiogenic activity of the different stereoisomers is dependent on their ability to inhibit cholesterol trafficking.

**Racemic itraconazole and its stereoisomers inhibited HUVEC-pericyte co-culture tube formation.**

Pericytes are vascular supporting cells that interact with endothelial cells and play an important role in initiation of angiogenesis by guiding the endothelial cell sprouting and stabilizing the vessels (33). Therefore, pericytes, together with endothelial cells, have been recognized as a putative target in cancer treatment (34). To examine the effects of racemic itraconazole and its stereoisomers on pericyte angiogenesis, we performed pericyte alone and pericyte-HUVEC co-culture tube formation experiments. Under our experimental conditions, and as shown in other reports, pericytes alone could not form tubular networks on Matrigel (data not shown) (35). We therefore used HUVEC-pericyte co-culture tube formation at a ratio of 30:1 of HUVEC and pericytes, respectively. At a concentration of 3 μmol/L, racemic itraconazole, IT-A and IT-C significantly inhibited pericyte tube networks as well as overall co-culture tube formation on Matrigel (Fig. 2A and B). In contrast, IT-B and IT-D showed negligible effects on co-culture tube formation. These results indicated that racemic itraconazole and the two stereoisomers IT-A and IT-C inhibited angiogenesis in the presence of pericytes.

**Itraconazole stereoisomers possess distinct hepatotoxicity profiles.**

Having shown that the 2S4R pair of itraconazole stereoisomers are more potent than 2R4S pair in all *in vitro* angiogenesis-related assays, we wondered whether the stereochemistry had a similar influence on the relative hepatotoxicity of the different stereoisomers. Using freshly prepared human primary hepatocytes, we determined three representative markers of hepatotoxicity, including hepatocyte death, intracellular GSH depletion and alanine transaminase (ALT) release. As expected, incubation with 2 and 10 μmol/L itraconazole increased primary human hepatocyte death 1.6- and 1.8-fold ($P \leq 0.01$), respectively, as compared to vehicle
control (Fig. 3A). Similarly, itraconazole effects on GSH depletion (76%; \( P \leq 0.01 \)) and ALT levels in the cell culture medium (92.5 IU/L; \( P \leq 0.01 \)) were consistent with the hepatotoxicity (Fig. 3B and C). Interestingly, IT-A and IT-B (both have a 2’R stereochemistry) appeared to have slightly higher hepatotoxicity than racemic itraconazole as judged by induction of cell death by 2.2- (\( P \leq 0.01 \)) and 2.0-fold (\( P \leq 0.01 \)), respectively, at a final concentration of 10 \( \mu \)mol/L as well as increases in GSH depletion (70% (\( P \leq 0.01 \)) and 67% (\( P \leq 0.01 \)), respectively) and extracellular ALT levels (117 IU/L (\( P \leq 0.01 \)) and 128 IU/L (\( P \leq 0.01 \)), respectively). In contrast, IT-C and IT-D (both have a 2’S stereochemistry) did not show appreciable hepatotoxicity, as judged by cell death, hepatic GSH levels or the concentration of ALT in the culture medium (Fig. 3A-C). These results were quite unexpected as the hepatotoxicity profile is totally different from the antiangiogenic profile of stereoisomers. Thus, the stereochemistry at the C2 and C4 chiral centers of itraconazole appeared to be important for the antiangiogenic activity while that of the C2’ chiral center influences hepatotoxicity (the chemical structure of itraconazole is shown in Table 1).

We next assessed the hepatotoxicity of the stereoisomers in mice. Female mice were administered with vehicle or itraconazole stereoisomers (60 mg/kg) i.p. once daily for 10 days. After drug treatment, we first determined the ALT levels in the blood samples of the mice, but found no significant change in blood ALT levels from the mice treated with stereoisomers (data not shown). Next, livers from each mouse were isolated and processed for H&E staining. The livers from vehicle or IT-C treated mice appeared normal, while those from IT-A treated mice showed abnormalities, i.e., white colored and quite sticky to surrounding tissues. In comparison, livers in mice treated with itraconazole looked partially abnormal (data not shown). From the H&E staining of the liver sections, we found that IT-A caused severe fatty liver in the mice (Fig. 3D). Mice treated with racemic itraconazole had partial fatty liver, while livers from those treated with vehicle or IT-C were normal (Fig. 3D). Once again, IT-C showed less hepatotoxicity in mice than either IT-A or the racemic itraconazole, consistent with the results from human hepatocytes.

**IT-C inhibited angiogenesis in vivo.**
We next determined and compared the *in vivo* antiangiogenic activity of IT-A and IT-C along with racemic itraconazole as a control. We excluded the 2R4S series (IT-B and IT-D) from these experiments since they had lower antiangiogenic activity than 2S4R series. Female mice were pre-treated with IT-A, IT-C or itraconazole at the doses of 20 and 50 mg/kg for each compound for 2 days. The mice were then implanted subcutaneously with Matrigel plugs containing angiogenic factors including bFGF and VEGF. Treatment with vehicle or compounds was continued once daily for additional 8 days. Mice were sacrificed and Matrigel plugs were isolated from the mice for the immuno-histological analysis of blood vessel infiltration into the Matrigel using anti-CD31 (endothelial cell marker) and anti-NG2 (pericyte marker) antibodies. In addition to the antibody staining, entire population of cells infiltrated into the Matrigel plugs was analyzed using Masson’s trichrome staining (Supplementary Fig. S3A and B). Compared to the vehicle control, itraconazole inhibited the number of CD31-positive blood vessels infiltrated into the Matrigel by 19% and 46% at the dosage of 20 mg/kg and 50 mg/kg, respectively (Fig. 4A and B). IT-A also inhibited the CD31-positive blood vessels by 19% and 48%. IT-C showed more significant inhibition on CD31-positive Matrigel angiogenesis, with 41% and 60% inhibition at 20 mg/kg and 50 mg/kg, respectively (Fig. 4A and B). Similar results were observed in NG2-positive pericyte staining. IT-C exhibited the strongest inhibition on the infiltration and vessel formation by NG2-positive pericytes, with 47% and 70% inhibition at 20 mg/kg and 50 mg/kg, respectively (Fig. 4C and D). These results suggested that IT-C is more active than IT-A for the antiangiogenic activity.

**IT-C inhibited tumor growth and tumor-induced angiogenesis in mice.**

Taking into consideration both the antiangiogenic activity and hepatotoxicity, IT-C was deemed superior to all other stereoisomers due to a combination of its higher antiangiogenic potency and lack of hepatotoxicity in both human cells and mice. We thus assessed the antitumor activity of IT-C in two tumor xenograft models (breast cancer and prostate cancer) in parallel with racemic itraconazole. We also took the opportunity to determine the effect of IT-C on tumor-induced angiogenesis. For the breast cancer xenograft model, female athymic nude mice bearing HCC1954 cells were treated i.p. with vehicle, itraconazole or IT-C at 25 and 50 mg/kg for each compound, once daily, for 30 days. Racemic itraconazole at 50 mg/kg significantly inhibited the
growth of HCC1954 xenografts compared to the vehicle alone (Fig. 5A). Tumor volume was reduced by 40% at Day 28 of treatment with 50 mg/kg itraconazole compared to the vehicle control. Importantly, IT-C showed more pronounced inhibition of the tumor xenograft growth than itraconazole at both 25 and 50 mg/kg (Fig. 5B). It inhibited the tumor volume by 53% at Day 28 at 50 mg/kg. There were no apparent toxicities associated with itraconazole or IT-C based on assessment of mouse body weight (Supplementary Fig. S4). Similar results were observed in the CWR22Rv1 prostate cancer xenograft model. Treatment of male athymic nude mice with either itraconazole (60 mg/kg) or IT-C (60 mg/kg) significantly inhibited the tumor growth (Fig. 5C and D). To assess tumor-associated angiogenesis in the breast cancer xenografts, the tumor tissues were harvested from the mice and processed for immunohistochemical analysis of CD31, a blood vessel marker. Compared to the vehicle, both racemic itraconazole and IT-C significantly inhibited angiogenesis in the HCC1954 xenografts (Fig. 5E and F), with IT-C being more potent than itraconazole.
Discussion

Although itraconazole has been widely used to treat fungal infections, several adverse effects have been reported. The most frequent side effects associated with itraconazole are gastrointestinal complaints including nausea, vomiting, diarrhea, flatulence and constipation (36). Fortunately, these adverse effects are mostly transient and mild, and thus are tolerable to patients (37). However, rare but severe hepatic toxicity associated with itraconazole has also been reported. Up to 7% of patients receiving itraconazole have elevated ALT levels (24). Moreover, fatalities due to hepatic failure have been reported in patients receiving itraconazole (38). Major efforts have been made to overcome the adverse effects of itraconazole on the liver. A pulse therapy of itraconazole (typically, 400 mg itraconazole b.i.d. for 1 week followed by a three-weeks holiday) was introduced in superficial fungal infections based on the pharmacokinetic (PK) properties of itraconazole (39). Upon cessation of drug intake, itraconazole is rapidly cleared from systemic circulation (7-10 days), but persists for 3-4 weeks in the stratum corneum and for up to 6-12 months in the nail (40). This PK property of itraconazole makes pulse therapy for onychomycosis and dermatomycoses possible, which could significantly reduce the hepatotoxicity-related adverse effects while showing therapeutic efficacies similar to continuous therapy (41). However, this strategy may not be applicable for the cancer therapy, which requires a high dose of itraconazole in systemic circulation. Thus, the hepatotoxicity associated with itraconazole still poses a significant challenge for the long-term, high-dose treatment of cancer patients with itraconazole.

Hepatotoxicity associated with itraconazole has long been recognized, but the mechanism underlying the liver toxicity of itraconazole has remained obscure. Cytochrome P450, specifically CYP3A4, is a main liver enzyme responsible for the metabolism of itraconazole (42). Itraconazole also inhibits the enzyme activity of CYP3A4 (26). When rats were pretreated with phenobarbital, an inducer of cytochrome P450, hepatotoxicity induced by itraconazole was significantly reduced (27, 37). On the other hand, pretreatment of rats with SKF 525A, an inhibitor of cytochrome P450, significantly enhanced itraconazole-induced hepatotoxicity (27). These results suggest that inhibition of cytochrome P450, probably CYP3A4, by itraconazole is, at least in part, responsible for itraconazole-induced hepatotoxicity. Commercial itraconazole currently used in the clinic is a racemic mixture of four cis-stereoisomers. It is interesting to note
that CYP3A4 metabolizes itraconazole in a stereoselective manner. Only IT-B and IT-D are metabolized by CYP3A4 among the four stereoisomers. Although all four stereoisomers are strong binders and inhibitors of CYP3A4, IT-B and IT-D have 2- to 4-fold stronger binding affinity for CYP3A4 and 2- to 4-fold lower IC₅₀ values for inhibition of the enzymatic activity of CYP3A4 in comparison to IT-A and IT-C (28). In the present study, we confirmed that IT-A and IT-C were more potent inhibitors of angiogenesis than IT-B and IT-D. In contrast, IT-A and IT-B were much more hepatotoxic than IT-C and IT-D both in mice and in human hepatocytes. These results suggest that inhibition of CYP3A4 by itraconazole or metabolism of itraconazole by CYP3A4 may not be solely responsible for its hepatotoxicity. One interesting possibility is that a cytochrome P450 enzyme with specificity for the C2’ chiral center of itraconazole could be the main enzyme mediating the hepatotoxicity of itraconazole. In addition, the segregation of antiangiogenic activity and hepatotoxicity of the four stereoisomers of itraconazole suggests that the cellular target protein(s) responsible for angiogenesis inhibition and hepatotoxicity might be different. Answers to these questions may become clear upon identification of the molecular targets mediating the different effects of itraconazole.

Although itraconazole was shown to elevate serum ALT levels in rats, it did not increase the levels in mice in this study. Instead, itraconazole and IT-A induced moderate to severe fatty liver in mice. Rats and mice have shown different hepatotoxicity responses to certain compounds, such as coumarin (43). Inter-strain differences of hepatotoxicity responses have also been observed in mice treated with cocaine and phenobarbital (44). This is thought to be due to the different liver metabolic enzymes present in different strains and animal species. Itraconazole has shown similar hepatotoxicity profiles in humans and rats, suggesting that they respond similarly to this compound. In mice, the compound did not show ALT elevation, but induced fatty liver, suggesting that racemic itraconazole and IT-A still have negative impact on mice liver but in a way different from rats or humans. However, IT-C at doses up to 60 mg/kg did not cause apparent adverse effects on the mouse liver. In the experiments using human primary hepatocytes, IT-C showed no apparent signs of hepatotoxicity at concentrations up to 10 µmol/L for all three hepatotoxicity markers. In contrast, IT-A was strongly hepatotoxic and racemic itraconazole showed a medium degree of hepatotoxicity. Together, these results points to IT-C as the most promising candidate for future clinical development with improved potency and decreased hepatotoxicity in comparison to racemic itraconazole. That the C2’ stereochemistry
has a major influence on the hepatotoxicity of itraconazole should also facilitate the development of next generations of itraconazole analogs as new leads of antiangiogenic and anticancer drugs.

Several anti-angiogenic agents, such as sunitinib and sorafenib, have successfully entered into the clinic as targeted cancer therapies. Although these agents have dramatically changed the management of cancer in patients these days, many clinical challenges still remain, including the huge financial burden to patients and the emergence of drug resistance that occurs with most of the kinase inhibitor drugs. Development of new anti-angiogenic and anticancer agents from existing non-cancer drugs (the drug repositioning strategy) offers the possibility of reducing the financial burden to patients (45). In addition, this approach can also diversify molecular targets as exemplified with itraconazole, which is not a kinase inhibitor. Development of agents with diverse molecular targets provides better opportunities to find drug combinations with standard chemotherapy or current targeted drugs to reduce the emergence of cancer drug resistance. As evidenced from recent clinical investigations, itraconazole has shown positive clinical outcome in patients with non-small cell lung cancer, metastatic prostate cancer and basal cell carcinoma. It also showed prolonged survival in patients with late-stage ovarian, triple-negative breast and metastatic pancreatic cancer. IT-C, as an equipotent isomer of itraconazole with reduced hepatotoxicity, will have a wider range of therapeutic window than racemic itraconazole, and thus is a promising lead for future development as an antiangiogenic and anticancer drug.
References


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Figure legend

Figure 1. Itraconazole and stereoisomers inhibit VEGFR2 glycosylation, mTOR activity, cholesterol trafficking and tube formation in endothelial cells. A, HUVEC were treated with itraconazole (ITRA) and its stereoisomers at various concentrations (0, 0.125, 0.25, 0.5 and 1 µmol/L) for 24 hours. Whole cell lysates were subjected to Western blot analysis using antibodies specific for VEGFR2, phospho-S6K (pS6K) and total S6K. In the VEGFR2 western blots, the upper and the lower bands correspond to hyperglycosylated and hypoglycosylated forms, respectively. B, HUVEC were treated with 50 nmol/L of itraconazole and stereoisomers for 24 hours. The cells were stained with filipin and the fluorescent images were captured under a confocal microscope. C, HUVEC were seeded on Matrigel and treated with itraconazole and stereoisomers at the concentration of 3 µmol/L and LDL (50 µg/mL) for 16 hours. Cells were then stained with Calcein-AM and the tube formation was observed under a fluorescent microscope. D, total tube lengths, sizes and number of junctions from the fluorescence images were quantified using the AngioQuant software and plotted using Graphpad Prism. Data represent the mean ± SE of three independent experiments. **, P ≤ 0.01 for drug alone versus drug + LDL. E and F, HUVEC were treated with indicated concentrations of itraconazole and stereoisomers with or without LDL (50 µg/mL) for 24 hours. Western blot analysis was done using antibodies specific for VEGFR2 and GAPDH as a loading control.

Figure 2. Itraconazole and stereoisomers inhibit the tube formation of HUVEC-pericyte co-culture. A, pericytes were labeled with Calcein-AM for 30 min before being harvested for tube formation. HUVEC and pericyte mixture (30:1 ratio) were treated with compounds at 3 µmol/L, seeded on Matrigel and allowed to form tube networks for 20 hours. Total cells (HUVEC and pericytes) are shown in the brightfield images and pericytes are shown in the green fluorescent images. Scale bar = 200 µm. B, total tube length from the green fluorescence images were quantified as the pericyte tube formation using the AngioQuant software and plotted using Graphpad Prism. Data represent the mean ± SE of three independent experiments. *, P ≤ 0.05; **, P ≤ 0.01 for control versus drug.
Figure 3. Itraconazole and each stereoisomer have distinct hepatotoxicity profiles. A, B and C, freshly prepared human primary hepatocytes were incubated with itraconazole and stereoisomers at the indicated concentrations for 72 hours. Cell viability of the hepatocytes (A), intracellular glutathione levels from the hepatocyte lysates (B) and the alanine aminotransaminase (ALT) levels from the hepatocyte culture media (C) were measured as described in the Materials and Methods. *, $P \leq 0.05$; **, $P \leq 0.01$ for 10 µmol/L versus 0 µmol/L (for A and B, two-tailed Single Sample $t$-test). **, $P \leq 0.01$ for drug versus DMSO control (for C, two-tailed Student’s $t$-test). D, female BALB/cAnNCr mice were treated i.p. with itraconazole and stereoisomers at the dosage of 60 mg/kg for 10 days. Livers from each mouse were isolated and processed for H&E staining. Representative images from five mice per group are shown. Scale bar = 100 µm.

Figure 4. Itraconazole and the 2S4R series of stereoisomers inhibit Matrigel angiogenesis in vivo. A and C, female BALB/cAnNCr mice with Matrigel implantation were treated i.p. with itraconazole, IT-A and IT-C at dosages of 20 and 50 mg/kg for total of 10 days (2-day pre-treatment and 8-day post-treatment of Matrigel implantation). Matrigel plugs with mouse inner skin were isolated and processed for immunohistochemical (IHC) staining of CD31 (endothelial cell marker) and NG2 (pericyte marker). Representative IHC images of CD31 staining (A) and NG2 staining (C) are shown. B and D, the number of CD31-positive vessels (B) and NG2-positive cells (D) were counted from the IHC samples and plotted using Graphpad Prism. Data represent mean ± SE of three independent IHC samples per group. *, $P \leq 0.05$; **, $P \leq 0.01$ for each drug versus vehicle control. Scale bar = 1.79 mm.

Figure 5. Itraconazole and IT-C inhibit the tumor growth and angiogenesis in vivo. A and B, female athymic nude mice (BALB/c, nu/nu-NCr) bearing HCC1954 human breast cancer cells were treated with itraconazole (ITRA, A) or IT-C (B) for 30 days. C and D, male athymic nude mice (BALB/c, nu/nu-NCr) bearing CWR22Rv1 human prostate cancer cells were treated with itraconazole (ITRA, C) or IT-C (D) for 24 days. Tumor volume was measured periodically by a vernier caliper and plotted using Graphpad prism. Data represent mean ± SE of tumor volumes in each group (n = 6-7 mice). *, $P \leq 0.05$; **, $P \leq 0.01$ for the drug versus vehicle control at each
time point. E, HCC1954 tumor xenograft tissues were processed for immunohistochemical (IHC) staining of a blood vessel marker, CD31. Representative images of CD31 staining of tumor sections from each group are shown. Scale bar = 100 µm. F, the number of CD31-positive blood vessels were counted from photos taken under a bright-field microscope and plotted using Graphpad Prism. The lumen size of blood vessels were measured using the Zen image analysis tool. Data represent mean ± SE of the number and the lumen size of blood vessels from the IHC images (n = 5 per group). **, P ≤ 0.01 for each drug versus vehicle control.
Table 1. Chemical structures of itraconazole *cis*-stereoisomers and their antiproliferative activities against HUVEC

![Chemical structure of itraconazole](image)

<table>
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<tr>
<th>Compound</th>
<th>Stereochemistry</th>
<th>IC$_{50}$ (nmol/L)</th>
<th>95% CI (nmol/L)</th>
<th>Inhibitory index*</th>
<th>P #</th>
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<td>Itraconazole</td>
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<td>IT-A</td>
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<td>103.6</td>
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<td>199.4 to 249.6</td>
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<tr>
<td>IT-C</td>
<td>2S4R2’S</td>
<td>98.3</td>
<td>86.8 to 111.4</td>
<td>0.71</td>
<td>0.003</td>
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<tr>
<td>IT-D</td>
<td>2R4S2’S</td>
<td>199.5</td>
<td>182.6 to 218.0</td>
<td>1.45</td>
<td>0.005</td>
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<td>IT-A (1:1) + IT-C (1:1)</td>
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<td>101.2</td>
<td>91.4 to 112.0</td>
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<td>0.013</td>
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<tr>
<td>IT-B (1:1) + IT-D (1:1)</td>
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<td>211.0</td>
<td>187.4 to 237.5</td>
<td>1.53</td>
<td>0.009</td>
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Mean IC$_{50}$ values and their 95% confidence intervals (CI) from 4 independent experiments are shown. *Inhibitory index represents the ratio of IC$_{50}$ of stereoisomers or mixtures to racemic itraconazole. #P values of IC$_{50}$s for stereoisomers vs racemic itraconazole.
Fig. 2

A

<table>
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<th></th>
<th>DMSO</th>
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B

Pericyte

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* * 

* *
Fig. 5
Clinical Cancer Research

Divergence of Anti-angiogenic Activity and Hepatotoxicity of Different Stereoisomers of Itraconazole

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