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Simultaneous Targeting of NPC1 and VDAC1 by Itraconazole Leads to Synergistic Inhibition of mTOR Signaling and Angiogenesis

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Abstract

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Supporting Information

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Supporting Figures S1–S7 and Materials and Methods (PDF)

Movie S1 (MPG)

Movie S2 (MPG)

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S.A.H., J.S.S., S.Y.H., and J.O.L. designed research; S.A.H., E.J.Y., B.A.N., and R-J.L. performed biological experiments; W.Q.S. and K.K.P. synthesized key chemical reagents; S.A.H. performed docking studies; S.A.H., E.J.Y., B.A.N., R-J.L., J.S.S., and J.O.L. analyzed data; and S.A.H. and J.O.L. wrote the manuscript. All authors edited, commented on, and approved the manuscript.

Notes

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The antifungal drug itraconazole was recently found to exhibit potent antiangiogenic activity and has since been repurposed as an investigational anticancer agent. Itraconazole has been shown to exert its antiangiogenic activity through inhibition of the mTOR signaling pathway, but the molecular mechanism of action was unknown. We recently identified the mitochondrial protein VDAC1 as a target of itraconazole and a mediator of its activation of AMPK, an upstream regulator of mTOR. However, VDAC1 could not account for the previously reported inhibition of cholesterol trafficking by itraconazole, which was also demonstrated to lead to mTOR inhibition. In this study, we demonstrate that cholesterol trafficking inhibition by itraconazole is due to direct inhibition of the lysosomal protein NPC1. We further map the binding site of itraconazole to the sterol-sensing domain of NPC1 using mutagenesis, competition with U18666A, and molecular docking. Finally, we demonstrate that simultaneous AMPK activation and cholesterol trafficking inhibition leads to synergistic inhibition of mTOR, endothelial cell proliferation, and angiogenesis.

Graphical abstract

The mechanistic Target of Rapamycin (mTOR) signaling pathway is a critical regulator of cell growth and proliferation, and as such it has been implicated in diseases such as cancer where growth and proliferation are dysregulated.¹ One of the mechanisms by which mTOR activity controls cancer progression is through regulation of angiogenesis, or new blood vessel growth from the preexisting vasculature.² Once primary tumors reach a certain size, they depend on the in-growth of new blood vessels to provide them with sufficient nutrients for continued rapid growth and, eventually, to enter the circulation and metastasize throughout the body.³ Tumors promote angiogenesis by secreting proangiogenic growth factors, which stimulate the endothelial cells lining blood vessels to proliferate and migrate toward the source of these factors. Inhibition of mTOR signaling blocks the transduction of these proangiogenic signals by preventing endothelial cell proliferation, and accordingly, mTOR inhibitors have been shown to be effective at inhibiting angiogenesis and cancer growth.^{4,5}

The clinically used antifungal drug itraconazole was recently found to inhibit mTOR signaling and angiogenesis through a mechanism unrelated to its antifungal target, 14- α demethylase (14DM), and unique to itraconazole over other azole antifungals.^{6,7} Itraconazole inhibited the proliferation of primary Human Umbilical Vein Endothelial Cells (HUVEC) and inhibited phosphorylation of mTORC1 substrates p70 S6K and 4EBP1 with an IC_{50} of about 200 nM, well below its plasma C_{max} of $>2 \mu M$ (Sporanox [package insert],

2014). It was also shown to selectively inhibit endothelial cells over other cell types and effectively block angiogenesis and tumor growth *in vivo*.^{6,8,9} In light of these results, itraconazole has entered several phase 2 trials as an investigational anticancer agent, and early results have shown promising efficacy against various types of cancer.^{10–12}

In a study aimed at unraveling the molecular mechanism of itraconazole's antiangiogenic activity, we reported that itraconazole indirectly inhibits mTOR signaling through another upstream regulatory kinase, the 5' AMP-dependent protein kinase (AMPK).¹³ AMPK acts as a homeostatic regulator of cellular energy levels by becoming activated upon increased AMP/ATP ratio, leading to stimulation of energy production and inhibition of energy consuming processes such as proliferation.¹⁴ Once activated, AMPK inhibits mTOR through direct phosphorylation of two mTOR-regulatory proteins, Raptor and TSC2, leading to the disassembly and inactivation of mTOR complex 1.¹⁵ The activation of AMPK by itraconazole was shown to be a result of direct binding to and inhibition of the mitochondrial Voltage-Dependent Anion Channel 1 (VDAC1), a critical regulator of mitochondrial metabolism, resulting in a drop in cellular energy levels.¹⁶ Thus, VDAC1 was shown to be a novel target for modulation of the AMPK/mTOR pathway in endothelial cells.¹³

In a separate study, itraconazole was also shown to inhibit cholesterol trafficking in endothelial cells, leading to the accumulation of cholesterol in the late endosome/lysosome.⁷ This cholesterol localization defect is also observed in the cells of patients with a genetic disease called Niemann-Pick Type C (NPC), where a deficiency in one of two lysosomal cholesterol-binding proteins (NPC1 and NPC2) prevents the release of cholesterol from the lysosome to the rest of the cell (referred to as NPC phenotype).¹⁷ It was also demonstrated that inhibition of cholesterol trafficking could lead to mTOR inhibition, as either genetic knockdown of NPC1 or NPC2 or pharmacological inhibition of cholesterol trafficking with two other known NPC-inducing small molecules, U18666A and imipramine, could also inhibit mTOR signaling and proliferation in HUVEC (although at much higher concentrations than itraconazole, with IC₅₀ values of ~3 and 10 μ M, respectively).⁷ These results suggested that cholesterol trafficking is required for mTOR signaling in endothelial cells and might also play a role in itraconazole's mechanism of mTOR inhibition. However, the mechanism of NPC phenotype induction by itraconazole has remained unknown, leaving open the question of what molecular target of itraconazole might mediate this activity.

The existence of two different mechanisms by which itraconazole may exert its inhibitory effects on mTOR and endothelial cell proliferation raised a number of questions, including whether AMPK activation and NPC phenotype induction were interrelated or separate effects of itraconazole, and whether one or both of these effects was responsible for the downstream mTOR inhibition and antiproliferative effects. Herein, we demonstrate that NPC induction and AMPK activation are two parallel effects of itraconazole mediated by distinct molecular targets. Using an active photoaffinity probe of itraconazole, we show that itraconazole binds directly to NPC1, and map the binding site of itraconazole to the transmembrane sterol-sensing domain (SSD). Finally, we demonstrate that the combined effects of NPC induction and AMPK activation lead to synergistic inhibition of mTOR, suggesting that the simultaneous targeting of these two pathways can greatly enhance the potency of mTOR inhibition compared with targeting either pathway alone. This unique

dual-targeted mechanism of mTOR inhibition by itraconazole may underlie its increased potency for mTOR compared with other AMPK activators and NPC inducers alone and may allow itraconazole to overcome the weaknesses of other clinically used mTOR inhibitors such as feedback activation or acquired drug resistance.

RESULTS AND DISCUSSION

AMPK Activation and NPC Phenotype Induction Are Distinct, Parallel Effects of Itraconazole

AMPK is a well-established upstream regulator of mTOR signaling, and several pieces of evidence suggested that AMPK activation by itraconazole likely contributed to the observed mTOR inhibition in HUVEC.¹³ AMPK activation occurs rapidly after itraconazole treatment, as demonstrated by time-course Western blotting and live cell imaging with a FRET reporter of AMPK activity, and shortly before the onset of mTOR inhibition. Increased phosphorylation of raptor on the AMPK phosphorylation site demonstrated that a known direct AMPK-dependent mechanism of mTOR inhibition was also triggered. In addition, cell lines in which AMPK activation did not occur (A549, HeLa, and VDAC1^{-/-}MEFs) also displayed little to no mTOR inhibition by itraconazole. These results strongly suggested a causal relationship between AMPK activation and mTOR inhibition by itraconazole. However, the connection between AMPK activation and another previously observed effect of itraconazole, inhibition of cholesterol trafficking (aka NPC phenotype induction), was not clear. It remained a possibility that NPC induction by itraconazole somehow led to AMPK activation, or conversely that AMPK activation induced NPC phenotype by an unknown mechanism that then led to mTOR inhibition.

To address these possibilities, we tested whether two other commonly used NPC phenotype-inducing compounds that had previously been shown to inhibit mTOR, U18666A and imipramine, could also activate AMPK in HUVEC. Cells were treated with varying concentrations of each drug, and pathway effects were monitored by Western blotting with phospho-specific antibodies for both the AMPK substrate acetyl-CoA carboxylase (ACC; phospho-serine 79) and the mTOR substrate S6K (phospho-threonine 389). Whereas itraconazole caused both AMPK activation and mTOR inhibition, there was no detectable activation of AMPK by either U18666A or imipramine at concentrations where mTOR inhibition was clearly observed (Figure 1a). This result demonstrates that inhibition of cholesterol trafficking alone is not sufficient to activate AMPK, and therefore NPC phenotype induction is not upstream of AMPK activation by itraconazole.

We next tested whether other AMPK activators could induce the NPC phenotype in HUVEC. Two AMPK activating compounds commonly used in the literature are 2-deoxyglucose (2DG) and metformin. 2DG is an analog of glucose that cannot undergo glycolysis and thus causes a drop in cellular energy levels by acting as a competitive inhibitor of glycolysis.¹⁸ Metformin inhibits mitochondrial ATP production through a mechanism that is thought to involve inhibition of complex I of the respiratory chain.¹⁹ A third compound developed more recently, A769662, binds directly to and allosterically activates the heterotrimeric AMPK complex.^{20,21} HUVEC were treated with these three AMPK activating compounds alongside itraconazole, U18666A, and imipramine, and the

intracellular distribution of cholesterol was visualized by staining with the fluorescent cholesterol-binding dye filipin. As expected, itraconazole, U18666A, and imipramine all showed the characteristic redistribution of cholesterol (Figure 1b–e); however, none of the other three AMPK activating drugs caused any obvious change in cholesterol distribution compared with the control cells treated with vehicle alone (Figure 1f–h). Taken together, these results suggested that AMPK activation and NPC phenotype induction are separate, parallel effects of itraconazole.

Induction of NPC Phenotype Is Not Mediated by the Known Targets of Itraconazole

We previously demonstrated that knockdown of VDAC1 expression in HUVEC inhibited mTOR signaling and cell proliferation.¹³ To determine if VDAC1 knockdown could also induce the NPC phenotype, HUVEC were transduced with lentivirus expressing two different shRNA sequences targeting VDAC1. Cells expressing the shRNA were easily identifiable by the expression of GFP encoded by the same vector. There was no redistribution of cholesterol in cells expressing either sequence of VDAC1 shRNA compared with untransduced cells, demonstrating that the inhibition of mTOR resulting from VDAC1 knockdown is not due to NPC phenotype induction (Figure S1). Likewise, wild-type and VDAC1-knockout mouse embryonic fibroblasts (MEFs) did not display any difference in cholesterol localization (Figure S2a), and itraconazole induced the NPC phenotype equally in both cell lines despite the fact that VDAC1 knockout renders cells completely resistant to AMPK activation by itraconazole (Figure S2b), demonstrating that NPC induction by itraconazole occurs independently of VDAC1 status. In light of these results, we concluded that NPC phenotype induction by itraconazole is not mediated by VDAC1.

We also considered the possibility that inhibition of the antifungal target 14DM is responsible for NPC phenotype induction. However, HUVEC expressing shRNA targeting 14DM also showed no redistribution of cholesterol (Figure S3), and additionally, a triazole-deleted itraconazole analog (TD-itra) was clearly able to induce the NPC phenotype (Figure S4), demonstrating that the azole ring was not required for this activity. Because the azole ring mediates inhibition of 14DM by itraconazole and other azole antifungals, this result suggested that 14DM was unlikely to be the responsible target for NPC phenotype induction. Thus, we concluded that the NPC phenotype was likely mediated by another as yet unidentified target.

To search for the molecular target responsible for mediating NPC phenotype induction by itraconazole, we returned to the live-cell photoaffinity labeling approach that was used in our previous study to identify VDAC1 as the target responsible for AMPK activation in endothelial cells.¹³ Live cell photoaffinity labeling enables the isolation and identification of small molecule-binding proteins through the use of a bioactive photoaffinity probe, with the advantage that binding and covalent labeling of the target protein occur within the native cellular environment, and thus the interaction is not disrupted upon cell lysis.²² The itraconazole probe contains a photo-activatable diazirine group for covalent cross-linking to the target protein upon irradiation with UV light, as well as a terminal alkyne for the attachment of an affinity handle via copper(I)-catalyzed azide–alkyne cycloaddition, or “click” reaction, allowing the subsequent isolation and identification of the target (Figure

S5a). This probe was previously shown to have similar activity in HUVEC to that of itraconazole in proliferation, mTOR inhibition, and AMPK activation assays, suggesting it likely binds to the same target protein(s).¹³

To determine whether the same photoaffinity probe could also be used to identify the target of itraconazole mediating its inhibition of cholesterol trafficking, we first tested whether the probe was also able to induce an NPC phenotype. We found that the probe was equally as good at inhibiting cholesterol trafficking as itraconazole itself (Figure S5b), suggesting both compounds likely act through the same mechanism and therefore that the probe should allow for the successful identification of the responsible target.

Itraconazole Binds Directly to NPC1

Niemann-Pick type C disease results from mutations in one of two lysosomal proteins, NPC1 and NPC2, with predicted molecular weights of 142 and 16.5 kDa, respectively. We decided to specifically test whether the itraconazole photoaffinity probe could bind to either or both of these proteins by expressing myc-tagged versions of the proteins in HEK293T cells and performing the live-cell photo-cross-linking experiment. Briefly, cells were incubated with 200 nM probe for 1h, with or without preincubation with 10 μ M itraconazole as a competitor, followed by UV irradiation, cell lysis, and protein denaturation. Click reaction of the lysates with biotin azide covalently attached the biotin group to the probe-cross-linked target proteins, which were purified on streptavidin beads and subsequently subjected to SDS-PAGE and Western blotting. myc-tagged NPC1 was clearly pulled down by the probe and was also competed away by excess itraconazole, indicating that itraconazole itself is able to bind NPC1 (Figure 2a). Myc-tagged NPC2, on the other hand, did not show any labeling by the probe. Endogenous NPC1, but not NPC2, could also be detected after pull-down from 293T cells (Figure S6a); however, the expression of NPC1 protein appeared to be fairly low in these cells, which may account for the lack of detection in our earlier study using the same photoaffinity probe. After testing NPC1 expression in several cell lines, we found that A549 expressed higher levels of NPC1 (Figure S6b) and also demonstrated NPC phenotype induction upon treatment with itraconazole (Figure S6c). Upon repeating the photolabeling experiment in A549, the pull-down of endogenous NPC1 could clearly be observed by Western blot (Figure S6d).

NPC1 is a 13-pass transmembrane protein, whose structure has recently been solved in two fragments by X-ray crystallography^{23,24} and in its entirety by electron cryomicroscopy.²⁵ Its major structural features include a soluble amino-terminal domain (NTD, aa 1–264) that faces into the lumen of the lysosome and functions to accept the handoff of cholesterol from the soluble protein NPC2; a second luminal loop (aa 371–615) thought to be involved in mediating the interaction with NPC2; and a sterol-sensing domain (SSD) comprising transmembrane segments 3–7 (aa 616–791).²⁶ To first determine whether itraconazole binds to the integral membrane or soluble portion of the protein, we expressed both the full-length protein and the NTD alone (aa 1–264) with a FLAG tag in 293T cells and repeated the photo-cross-linking and pull-down experiment. There was no observable photo-cross-linking to the NTD as compared with the full-length protein (Figure 2b), suggesting that the integral membrane portion of the protein is required for probe cross-linking.

The NPC-inducing small molecule U18666A was recently shown to bind directly to the SSD of NPC1 using a similar photoaffinity labeling approach.²⁶ To determine whether U18666A and itraconazole share the same binding site, we repeated the photo-cross-linking experiment using pretreatment with excess U18666A to test whether it could compete away binding of the itraconazole probe. Similarly to itraconazole, U18666A pretreatment blocked binding of the itraconazole probe to NPC1 (Figure 2c), demonstrating that the binding of these two molecules to NPC1 is mutually exclusive and indicating that they most likely share a common or overlapping binding site. To further confirm this, we introduced a point mutation in the SSD of NPC1 (P691S) that was shown to prevent binding of U18666A²⁶ and expressed this mutant in 293T cells. In contrast to the expressed wild-type protein, the P691S mutant could not be pulled down by the itraconazole probe (Figure 2d), indicating that the point mutation blocks itraconazole binding to NPC1 similar to U18666A. Taken together, these results provide strong evidence that itraconazole and U18666A both bind to the same site within the SSD of NPC1.

In the recently published crystal structure of near-full-length NPC1, U18666A and cholesterol were both modeled into a hydrophobic binding pocket created by transmembrane domains 3–5 within the SSD using molecular docking.²⁴ Given that U18666A was able to compete away binding of the itraconazole probe to NPC1, we hypothesized that the binding site of itraconazole when docked onto NPC1 would overlap with the binding site for U18666A. The highest affinity conformation calculated by the docking software AutoDock Vina places itraconazole into this same binding pocket, with the linear core of the molecule nestled into the hydrophobic channel and the isobutyl side chain facing toward the cytosolic end of this channel (Figure 3a, Movie S1). Notably, this orientation would allow for side-chain extensions, such as the bifunctional tail of the photoaffinity probe, to freely project outward along the side of the receptor, providing a structural basis for the high tolerability of modifications to the isobutyl side-chain we observed previously.²⁷ Interestingly, at the luminal end of the channel, a small space opens up within the protein that creates a secondary binding pocket, which is filled by the triazole and dichlorophenyl rings. Neither U18666A nor cholesterol appears to fill this space (Figure 3b, Movie S2), suggesting that the extended structure of itraconazole may allow for a bivalent interaction with the receptor by bridging both of these binding pockets. This observation raised the question of the relative affinity of itraconazole versus U18666A and cholesterol, as bivalent ligands often have higher affinity for their receptors owing to the forced proximity of two tethered pharmacophores.²⁸ Indeed, the binding affinity calculated by AutoDock Vina was -9.8 kcal/mol for itraconazole, while the affinities for cholesterol and U18666A were -8.0 and -7.1 kcal/mol, respectively. Collectively, these results are consistent with the hypothesis that itraconazole binds to the same binding site as cholesterol and U18666A within the SSD of NPC1.

The most recent model of NPC1 activity proposed by Li *et al.*²⁴ posits that initially free cholesterol molecules are transferred from NPC2 to the NTD of NPC1 through a “hydrophobic hand-off” mechanism, followed by a secondary transfer of cholesterol from the NTD to the SSD of NPC1, bringing cholesterol into contact with the lysosomal membrane from where it can diffuse away to join the general lipid pool within the luminal leaflet of the membrane. The results of our docking study suggest that itraconazole has a

higher affinity for the SSD than cholesterol itself. Therefore, itraconazole binding could theoretically block cholesterol transfer from the NTD to the SSD, causing a back-up of cholesterol in the late endosome and lysosome and leading to cholesterol accumulation. Alternatively, by binding to the SSD, itraconazole could prevent the proper sensing of cholesterol levels in the membrane, causing a false signal that prevents further trafficking activity. In either case, the fact that itraconazole binds to the SSD provides a plausible mechanistic explanation for the observed induction of NPC phenotype.

AMPK Activation and NPC Induction Lead to Synergistic Inhibition of mTOR, Proliferation, and Tube Formation

The fact that itraconazole binds directly to NPC1 as well as VDAC1 suggested that NPC phenotype induction and AMPK activation were two unrelated effects of itraconazole that both result in mTOR inhibition by different mechanisms. Inhibitors of cholesterol trafficking (U18666A, imipramine) and activators of AMPK (A769662, 2DG) have both been shown to inhibit mTOR and HUVEC proliferation individually, but significantly less potently than itraconazole.^{7,13,29} We therefore wondered if simultaneously targeting these two upstream processes would lead to synergistic inhibition of mTOR and endothelial cell proliferation. Synergy, or superadditivity, is observed when the effect of the combination of two or more compounds is greater than would be predicted by their individual potencies, allowing lower concentrations of each individual compound to be used to achieve the same overall effect.³⁰ To pharmacologically model the simultaneous activation of AMPK and inhibition of cholesterol trafficking by itraconazole, we therefore decided to test the direct AMPK activator A769662, which does not induce the NPC phenotype, in combination with the NPC inducer U18666A, which does not activate AMPK, to see if the two compounds would have a synergistic effect on mTOR and proliferation.

Thus, HUVEC were first treated with moderate doses of both compounds alone or in combination, and the effect on S6K phosphorylation was assessed by Western blot (Figure 4a). At the chosen concentrations (15 μ M U18666A and 75 μ M A769662), the two compounds had little to no effect on mTOR activity individually. However, when the compounds were combined, S6K phosphorylation was completely inhibited, to a similar extent as with 2 μ M itraconazole treatment. This result supported the hypothesis that the combined effect of cholesterol trafficking inhibition and AMPK activation could synergistically inhibit mTOR.

We next determined whether the synergy observed at the level of mTOR would translate to cell proliferation. Proliferation was monitored using [³H]-thymidine incorporation as a readout, and full dose–response curves were obtained for each compound in the absence or presence of varying concentrations of the other to determine whether the IC₅₀ of the individual compound was decreased by addition of the other compound. A decrease in the IC₅₀, which would be illustrated graphically by a leftward shift of the dose–response curve, was taken to demonstrate synergy, as it indicates that lower concentrations of the compound are required to achieve the same antiproliferative effect. Thus, HUVEC were treated with the two compounds individually or in combination before being pulsed with [³H]-thymidine and harvested for scintillation counting. A769662 alone had an IC₅₀ of ~77 μ M, consistent with

previously published results.¹³ However, upon addition of increasing concentrations of U18666A (12.5, 25, 50 μ M), the IC₅₀ of A769662 dose-dependently decreased to approximately 45 μ M, 30 μ M, and 16 μ M, respectively (Figure 4b). Likewise, the IC₅₀ of U18666A alone was ~50 μ M, but when cotreated with A769662 (20, 40, 80 μ M) the IC₅₀ of U18666A correspondingly decreased (approximately 33 μ M, 17 μ M, and 6 μ M, respectively; Figure 4c). These results demonstrate a synergistic effect of the combination at the level of cell proliferation, consistent with the effects observed on mTOR signaling. In contrast, itraconazole itself did not demonstrate synergy with either U18666A or A769662 (Figure S7), likely because itraconazole treatment already affects both pathways, and therefore combining it with another compound that affects one or the other pathway would not be expected to have a significant additional effect.

Finally, we tested the same combination of drugs for synergy in the tube formation assay, a widely used *in vitro* model of angiogenesis. In this assay, endothelial cells are plated on an extracellular matrix, and their ability to differentiate into capillary-like structures is quantified. Thus, fluorescence-labeled HUVEC were mixed with compounds as indicated and then seeded onto a Matrigel-coated chamber. After 6 h, the tube networks were visualized by fluorescence microscopy and quantified using specialized software. Compared with DMSO-treated cells, treatment with 5 μ M itraconazole abolished the tube network formation (Figure 5a,b). Whereas U18666A (Figure 5c) and A769662 (Figure 5d) had no significant effect on tube lengths individually, when combined the two compounds drastically decreased tube lengths (Figure 5e,f). Morphologically, the tube networks formed by cells in the combination treatment group were also most similar to the itraconazole treated cells, with shortened tube lengths, increased number of junctions and broken tube networks (Figure 5b vs e). In sum, these results indicate that simultaneous inhibition of cholesterol trafficking and activation of AMPK using two specific inhibitors leads to a significantly enhanced inhibitory effect on mTOR signaling, proliferation, and angiogenesis, closely modeling the effects of dual-pathway targeting by itraconazole in endothelial cells.

The concept of polypharmacology, or a drug acting on more than one target, is not new but has been recently gaining a renewed appreciation.³¹ Until recently, most people considered so-called “dirty drugs” to be undesirable, as a high specificity and potency for a single target would presumably have powerful activity against the target with minimal side effects. However, this idea of a “magic bullet” to cure disease has often not been borne out clinically; in many situations, cells can compensate for inhibition of a single target either by developing resistance mutations or otherwise decreasing their dependence on that target. In cancer in particular, due to the high rate of genetic mutation, treatment with single targeted drugs often leads to selection of drug-resistant cancer cell populations resulting in treatment failure over time. Furthermore, drugs with multiple targets can have utility in treating multiple different diseases, as in the case of itraconazole, which in addition to its antifungal, antiangiogenic, and anticancer properties was recently shown to possess antiviral activity mediated by inhibition of OSBP.³² Therefore, there is an increasing appreciation that simultaneous inhibition of multiple targets may be a beneficial property in drug development, particularly in drug repurposing.

Though the precise binding modes of itraconazole with VDAC1 and NPC1 remain to be determined, a comparison of the structures of the two targets of itraconazole reveals little obvious sequence or structural similarity. Interestingly, however, VDAC1 is also known to contain binding sites for cholesterol,^{33–35} and the fact that itraconazole binds to NPC1 in a cholesterol binding site (the SSD) raises the possibility that it may bind in an analogous fashion to VDAC1. It is also possible that the key interactions between itraconazole and each of the two target proteins rely on different portions of itraconazole. Thus, the identification of the dual mechanism of action necessitates that future efforts to improve the structure of itraconazole will have to take into consideration binding to both VDAC1 and NPC1.

The clinical development of mTOR inhibitors as anticancer drugs has been hindered by problems with acquired insensitivity. The canonical inhibitor for which the mTOR pathway was named, rapamycin, and its analogs have been demonstrated to have antiangiogenic and antitumor activity; however, in practice their efficacy in clinical trials has been limited, thought to be due to insensitivity resulting from a signaling feedback loop that leads to activation of AKT upon prolonged treatment.^{36,37} Other direct mTOR inhibitors have been designed which are mostly active site ATP-competitive kinase inhibitors, but like other drugs of this class such inhibitors often have off-target effects on other kinases and can lead to the development of resistance conferring mutations.⁵ Thus, mTOR inhibitors with novel mechanisms may be useful in overcoming these limitations. By simultaneously targeting two pathways that regulate mTOR activity—i.e., AMPK signaling and cholesterol trafficking—itraconazole not only affects mTOR activity through a novel mechanism, it also generates a synergistic effect at the levels of mTOR, endothelial cell proliferation, and tube formation, providing strong evidence that this synergy is physiologically relevant for its antiangiogenic activity.

In summary, this work has unraveled a novel antiangiogenic mechanism of action of itraconazole, whereby direct inhibition of both VDAC1 and NPC1 lead to mTOR inhibition *via* different upstream mechanisms, i.e., AMPK activation and cholesterol trafficking inhibition, that when combined synergize to result in an enhanced antiproliferative and antiangiogenic effect. Due to this unique dual-targeted mechanism, itraconazole could potentially have several clinical advantages over other currently used mTOR inhibitors. First, unlike rapamycin, itraconazole is nonimmunosuppressive.³⁸ Second, having two distinct targets decreases the likelihood of developing drug resistance, as the chance of simultaneously developing resistance to two targets is low. Third, specifically targeting endothelial cells rather than cancer cells further decreases the occurrence of resistance-causing mutations, as endothelial cells are genetically stable while cancer cells mutate rapidly. Finally, the synergistic effect of dual pathway inhibition means lower doses of drug can be used to achieve the same effect and thus minimize the risk of side effects. The relative efficacy of itraconazole and other anti-mTOR agents as anticancer drugs remains to be determined; however, because itraconazole is well-tolerated in most patients and has demonstrated efficacy in numerous types of cancer, there is a strong rationale for further clinical trials using itraconazole as an anticancer agent, particularly in cancers refractory to existing treatments.

METHODS

Cell Culture

Primary HUVEC pooled from four donors (Lonza) were cultured in complete EGM-2 (Lonza) and subcultured every 2 days at a density of 1:4, or 3 days at 1:8, and discarded after passage 8. HEK 293T, HeLa, and A549 were cultured in low glucose DMEM (Gibco; Gaithersburg, MD) supplemented with 10% filtered FBS (Gibco) and 1% penicillin/streptomycin (Gibco). VDAC1 wild-type and knockout MEFs were generated as previously reported³⁹ and cultured in high glucose DMEM supplemented with 10% filtered FBS and 1% penicillin/streptomycin. All cells were cultured at 37 °C with 5% CO₂.

Filipin Staining

HUVEC were plated in an eight-well slide chamber at a density of 1000/well in 1 mL of media and allowed to settle overnight. Cells were treated with drugs for 24 h as indicated. The medium was removed, and cells were fixed with 4% paraformaldehyde for 15 min at RT. Cells were then washed twice with PBS before being incubated with 500 μ L of filipin solution (diluted from 5 mg mL⁻¹ DMSO stock solution to 50 μ g/mL in PBS) for 1 h in the dark. The cells were then carefully washed twice more with PBS, mounted, and covered with a coverslip and observed using a 710NLO-Meta multiphoton microscope (Carl Zeiss, Thornwood, NY) using DAPI wavelengths (360/460 nm) at the Johns Hopkins Microscope Facility. Cells transduced with lentivirus were stained with filipin 3 days post-transduction, and visualization of GFP was used to monitor expression of the shRNA-containing plasmid. More detailed materials and methods can be found in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

AMPK activation and NPC phenotype induction as parallel effects of itraconazole. (a) HUVEC were treated with drugs as indicated for 1 h, and AMPK/mTOR activity was monitored by phosphorylation of their respective substrates. Unlike itraconazole, the NPC phenotype-inducing compounds U18666A and imipramine inhibited mTOR without affecting AMPK activity. (b–h) HUVEC were treated with (b) DMSO, (c) itraconazole (1 μ M), (d) U18666A (5 μ M), (e) imipramine (10 μ M), (f) A769662 (75 μ M), (g) metformin (5 mM), and (h) 2-deoxyglucose (2 mM) for 24 h before filipin staining to visualize intracellular cholesterol. Compared to the NPC inducers U18666A and imipramine, none of the AMPK activators (A769662, metformin, 2DG) induced an NPC phenotype in HUVEC.

Figure 2.

Itraconazole binding directly to NPC1. (a) Photoaffinity labeling (PAL) with the itraconazole probe was performed in 293T cells expressing both myc-tagged NPC1 and NPC2, followed by biotin pull-down and Western blotting with an antimyc antibody. Pretreatment with 10 μ M itraconazole was used to demonstrate competition, indicating specific binding of the probe. (b) 293T cells were transfected with either full-length, myc-tagged NPC1 or FLAG-tagged NPC1(NTD), followed by PAL and Western blotting. (c) PAL with the itraconazole probe was performed in A549 cells using either 10 μ M itraconazole or 20 μ M U18666A as competitors. (d) PAL was performed in 293T cells transfected with either wild-type (WT) or mutant (P691S) myc-tagged NPC1.

Figure 3.

Crystal structure of near full-length NPC1 with itraconazole docked into the sterol-sensing domain. (a) Surface representation of NPC1 with itraconazole (cyan) docked using AutoDock Vina.⁴⁰ Transmembrane domains 3–7 of the sterol-sensing domain (SSD) are colored purple. (b) Close-up of the SSD binding pocket with docked itraconazole (cyan), cholesterol (yellow), and U18666A (magenta) demonstrating the extended binding area of itraconazole in comparison with cholesterol and U18666A.

Figure 4.

Combination treatment with AMPK activator (A769662) and NPC inducer (U18666A) synergistically inhibiting mTOR and proliferation. (a) HUVEC were treated with drugs as indicated for 30 min before harvesting for Western blot. Neither A769662 nor U18666A alone significantly inhibits mTOR, while the combined treatment shows dramatically enhanced inhibition. (b) Addition of U18666A causes a dose-dependent decrease in the IC₅₀ of A769662 in the ³H-thymidine incorporation assay, demonstrating synergistic inhibition of HUVEC proliferation. (c) Addition of A769662 causes a dose-dependent decrease in IC₅₀ of U18666A in ³H-thymidine incorporation assay, demonstrating synergistic inhibition of HUVEC proliferation.

Figure 5. Synergistic inhibition of endothelial tube formation by A769662 and U18666A. Fluorescent-labeled HUVEC were treated with drugs as indicated for 6 h, and the effect on tube formation was quantified. Whereas neither A769662 nor U18666A alone had a significant effect on tube formation, the combination treatment significantly inhibited tube formation, demonstrating a synergistic effect of the combination on angiogenesis *in vitro*.