Itraconazole Inhibits Angiogenesis and Tumor Growth in Non–Small Cell Lung Cancer

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Abstract

The antiangiogenic agent bevacizumab has been approved for the treatment of non–small cell lung cancer (NSCLC), although the survival benefit associated with this agent is marginal, and toxicities and cost are substantial. A recent screen for selective inhibitors of endothelial cell proliferation identified the oral antifungal drug itraconazole as a novel agent with potential antiangiogenic activity. In this article, we define and characterize the antiangiogenic and anticancer activities of itraconazole in relevant preclinical models of angiogenesis and lung cancer. Itraconazole consistently showed potent, specific, and dose-dependent inhibition of endothelial cell proliferation, migration, and tube formation in response to both VEGF- and basic fibroblast growth factor–mediated angiogenic stimulation. In vivo, using primary xenograft models of human NSCLC, oral itraconazole showed single-agent growth-inhibitory activity associated with induction of tumor hypoxia-inducible factor 1 alpha expression and marked inhibition of tumor vascularity. Itraconazole significantly enhanced the antitumor efficacy of the chemotherapeutic agent cisplatin in the same model systems. Taken together, these data suggest that itraconazole has potent and selective inhibitory activity against multiple key aspects of tumor-associated angiogenesis in vitro and in vivo, and strongly support clinical translation of its use.

Based on these observations, we have initiated a randomized phase II study comparing the efficacy of standard cytotoxic therapy with or without daily oral itraconazole in patients with recurrent metastatic NSCLC.

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Introduction

Angiogenesis, the summation of multiple cellular and biologic processes culminating in the propagation of blood vessels, has been the subject of extensive examination in the context of tumor biology over the past 4 decades since it was first proposed by Judah Folkman in 1971 (1). Solid tumor growth and progression is dependent on tumor-associated angiogenesis. Tumor expression and circulating levels of angiogenic factors have been correlated with aggressive tumor growth, predilection for metastasis, and prognosis in a wide array of solid tumors, including lung cancer (2–4). Although many putative regulators of angiogenesis have been identified, 2 secreted factors, VEGF and basic fibroblast growth factor (bFGF), have been, in particular, strongly implicated in tumor-associated angiogenesis (5). VEGF and bFGF interact with distinct families of tyrosine kinase receptors (RTK) on the surface of endothelial cells and activate multiple downstream signaling pathways. Together, these pathways promote endothelial cell survival, proliferation, migration, invasion, and tube formation, resulting in the formation of new vascular networks (6, 7).

Lung cancer is the leading cause of cancer mortality in both men and women in the United States (8). The only antiangiogenic therapy currently approved for use in lung cancer is the α-VEGF monoclonal antibody bevacizumab. A landmark phase III clinical study, ECOG 4599, randomized patients with advanced non–small cell lung cancer (NSCLC) to a standard chemotherapy doublet with or without bevacizumab (9). This study showed a statistically significant improvement in both progression-free and overall survival rates in favor of the bevacizumab-containing arm. There are, however, major limitations to the clinical utility of bevacizumab. The absolute improvements in progression-free and overall survival rates in this study were modest (1.7 and 2 months, respectively). Due to episodes of fatal hemoptysis in a previous study (10), enrollment was restricted to patients with nonsquamous tumors, with no history of hemoptysis, with no brain metastases, with no indication for use of anticoagulants, and with good performance status. However, even in this carefully selected subpopulation, bevacizumab use was associated with increased treatment-related deaths (P = 0.001) including 5 episodes of fatal hemoptysis (vs. 0 in the control arm), and with significantly increased rates of hypertension, proteinuria, bleeding, neutropenia, febrile neutropenia, thrombocytopenia,
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hyponatremia, rash, and headache \((P < 0.05\) for each of these factors). Across disease types, bevacizumab use has been associated with increased treatment-related mortality (11). The financial cost of bevacizumab, given its limited efficacy and significant toxicity, was seen by many as excessive, at upwards of $500,000 per year of life gained (12, 13). A final concern comes from what was intended as a “confirmatory” trial, the Avastin in Lung (AVAIL) study, which randomized a similar patient population to a different standard chemotherapy doublet with or without bevacizumab (14). This study failed to show a statistically significant difference in survival. In summary, although evidence is strong that angiogenesis is critical to tumor growth and progression, there is a need for less toxic, less cost-prohibitive, and more effective therapies. These observations strongly support further development of novel antiangiogenic strategies for patients with lung cancer.

Itraconazole is an orally bioavailable, Food and Drug Administration Agency–approved agent belonging to the family of azole antifungal drugs that inhibit the enzyme lanosterol 14-demethylase, which is responsible for the conversions of lanosterol to ergosterol in fungi and lanosterol to cholesterol in humans, respectively. Initially reported by Chong and colleagues, itraconazole has been identified as a potent inhibitor of endothelial cell proliferation and Matrigel-stimulated angiogenesis, with inhibition of lanosterol 14-demethylase and sterol biosynthesis only partially explaining this novel antiproliferative activity (15). Further efforts to characterize the mechanism of inhibition of endothelial cell proliferation are ongoing, with recent reports suggesting perturbation of cholesterol-trafficking pathways imparted by itraconazole as a possible mechanism contributing to this activity (16). Notably, itraconazole has also recently been implicated as an antagonist of the hedgehog signaling pathway in models of hedgehog pathway deregulation (17).

Preclinical evaluation of the antiangiogenic capacity of itraconazole in relevant \textit{in vitro} models of angiogenesis and \textit{in vivo} models of cancer are clearly required to determine the viability of pursuing further clinical development of itraconazole as an antiangiogenic agent.

Tumor cell lines implanted into immunodeficient mice comprise the most commonly used platform for \textit{in vivo} preclinical cancer therapeutic testing. However, \textit{ex vivo} derivation of stable cell lines in tissue culture is associated with profound changes in cellular morphology, growth characteristics, chromosome structure, gene copy number, and gene expression (18–20), changes which are not reversed by reintroduction of cell lines into mice (21). In sharp contrast to the harsh biologic conditions in which tumors naturally arise, typical tissue culture conditions include relatively high oxygen tension, high glucose concentration, and low hydrostatic and oncotic pressures. These are precisely the conditions in which maintenance of angiogenic drive, in particular, is not relevant. To evaluate the \textit{in vivo} effects of itraconazole, in the present study we employed an alternative approach based on primary lung cancer xenografts. The primary xenograft model depends on immediate transfer of human cancers from patients into recipient mice, without intervening tissue culture or cell line derivation \textit{ex vivo}. We have previously reported that gene expression profiles of lung cancer primary xenografts more closely reflect those of the human cancers than do profiles of cell lines derived from the same parental tumor when reimplanted as standard (secondary) xenografts (21). These observations are supported by data from other investigators exploring primary xenografts (22, 23).

In this article, we describe the results of a series of \textit{in vitro} and \textit{in vivo} analyses evaluating the putative antiangiogenic activities of itraconazole. We employed several \textit{in vitro} assays using human umbilical vein endothelial cells (HUVEC) to separately probe specific hallmarks of endothelial cell function as they relate to angiogenic processes. These functional competencies include proliferative capacity, migration, chemotactic potential, and the ability to spontaneously form an extracellular matrix (ECM)-supported tube network. The capacity of itraconazole to modulate these functions was explored in the presence of multiple angiogenic stimuli including VEGF and bFGF. We further investigate the \textit{in vivo} activity of itraconazole as an inhibitor of tumor-associated angiogenesis and of tumor growth, both as a single agent and in combination with standard cytotoxic chemotherapy. These studies offer the first assessment of the efficacy of itraconazole as an antiangiogenic agent and as an anticancer therapeutic in a primary disease model.

**Materials and Methods**

**Cell culture and reagents**

HUVECs (Lonza) were grown in endothelial cell growth medium-2 (EGM-2; Lonza) according to the manufacturer’s instructions. Cells were washed with PBS and serum-starved in endothelial cell basal medium-2 (basal media; Lonza) supplemented with 0.5% bovine serum albumin (RSA; Sigma) for 4 to 6 hours before assays. NSCLC cell lines, NCI-H358, NCI-H1838, NCI-H596, and NCI-H1975 were obtained from the American Type Cell Culture (ATCC) and cultured in RPMI-1640 supplemented with 10% FBS according to the ATCC’s recommendations. Cultures from LX-7 and LX-14 tumors were derived from preparations of single-cell suspensions grown in Media-2 plus 4.5 g/L glucose (RPMI-1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES buffer, and 1.5 g/L sodium bicarbonate). Cultures were incubated at 37°C with 95% air/5% CO\textsubscript{2} in a humidified incubator, unless otherwise stated. Itraconazole was obtained from Sigma and prepared as a solution in dimethyl sulfoxide (DMSO) for use in \textit{in vitro} experiments. Itraconazole oral solution (Sporanox; Ortho Biotech) and cisplatin (APP Pharmaceuticals) for \textit{in vivo} experiments were obtained from the pharmacy of The Sidney Kimmel Comprehensive Cancer Center and diluted as required with 40% hydroxypropyl-cyclodextrin, 2.5% propylene glycol, pH 4.5, in water and saline, respectively.

**Proliferation assays**

HUVECs were suspended in either EGM-2 or basal media containing 0.3% RSA and supplemented with 10 ng/mL VEGF-A or 12 ng/mL bFGF. NCI-H358, NCI-H1838, NCI-H596, NCI-H1975, LX-7, and LX-14 cells were suspended in respective

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RPMM-1640–based media. Cells were seeded at a density of 1 to 5 \times 10^5 cells per well and allowed to attach for 6 hours. Cells were then exposed to vehicle or drug treatment and incubated for 48 hours. Duplicate plates containing NCI-H1358, NCI-H1838, and NCI-H596 cells were also cultured under hypoxic conditions generated by flushing a modular incubator chamber with a 95% N2/5% CO2 preanalyzed air supply to generate a stable atmosphere of 1.5% O2. Relative cell numbers following treatment with itraconazole were quantified by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) as per the manufacturer’s recommendations using a SpectraMax M2e spectrophotometer and SoftMax Pro software (Molecular Devices).

**Phospho-RTK analysis**

HUVECs were cultured on 10-cm culture-treated dishes in EGM-2 medium and treated with vehicle or itraconazole for 24 hours. Cells were then harvested using a cell scraper and pelleted by centrifugation (300 \times g). Cells were then resuspended and lysed in modified radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 1 mmol/L ethylene diamine tetraacetic acid (EDTA), 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 1 mmol/L Na-orthovanadate, 1 mmol/L NaF, 1 \times Phosphatase Inhibitor Cocktails 1 and 2 (P2850 and P5726, respectively; Sigma), and 1 \times Protease Inhibitor Cocktail (P8340; Sigma), followed by centrifugation, yielding clarified lysates. Total protein content was quantified using Bradford assay. Lysates were analyzed using Proteome Profiler Human Phospho-RTK Array (R&D Systems) as per the manufacturer’s recommendations using 100 µg total protein.

**Migration assays**

**Transwell cell migration assay.** EGM-2, or basal media, containing 0.5% BSA supplemented with 10 ng/mL VEGF-A or 12 ng/mL bFGF was added to the lower wells of a CytoSelect Fluorometric 8 \mu mol/L Transwell Migration Assay plate (Cell Biolabs). Cells were suspended in basal media supplemented with 0.5% BSA and added to the upper wells of the plate and exposed to vehicle or drug treatment followed by incubation for 16 hours to allow for migration across the porous membrane. Cells remaining in the upper wells were then voided. Migratory cells were detached from the lower face of the Transwell membrane and lysed. Total DNA in the resulting lysates was stained using CyQuant GR dye (Millipore) and quantified by fluorometric analysis as per the manufacturer’s recommendations.

**Oris cell migration assay.** HUVECs were plated at 95% confluency in Oris Cell Migration Assay plates ( Platypus Technologies) and incubated overnight to allow cell attachment. Wells were rinsed with PBS and incubated in basal media for 6 hours. Stopper inserts were then removed to reveal a migration zone that was 2 mm in diameter and cells were again rinsed in PBS and followed by exposure to EGM-2 media containing itraconazole (0–3 \mu mol/L), or basal media supplemented with 0.5% BSA. Assay plates were incubated for 24 hours to permit cell migration. Following migration, cells were labeled with 2 µg/mL calcein-AM for 30 minutes and visualized using an Olympus BX61 fluorescence microscope with images captured using an affixed CCD camera and Slidebook 5 software (Intelligent Imaging Innovations). Migration was quantified using ImageJ software (NIH) to measure the area of the migration zone remaining after migration and reported as the percentage decrease in migration zone area from the commencement of the migration period.

**Tube formation assay**

Wells of an assay plate were coated with growth factor–reduced basal membrane extract (Geltrex; Invitrogen) and were incubated for 30 minutes at 37°C. HUVECs were then suspended in either EGM-2 or basal media containing 0.5% BSA supplemented with 12 ng/mL VEGF or 10 ng/mL bFGF and seeded at 1.5 \times 10^5 cells per well. Cells were exposed to treatment followed by 16 hours incubation. Cells and resulting tube networks were visualized after 30 minutes of incubation with 2 µg/mL Calcein AM using an Olyamisu BX61 fluorescence microscope.

**NSCLC primary xenograft models**

The primary NSCLC xenograft models LX-14 and LX-7, representing squamous cell and adenocarcinoma histologies, respectively, were derived from primary tumor tissue obtained from treatment-naive patients at the Sidney Kimmel Comprehensive Cancer Center. Briefly, tumor cells isolated from bronchoscopic biopsy specimens were prepared into single-cell suspensions, mixed with Matrigel in equal volume, and injected as a subcutaneous bolus into nonobese diabetic (NOD)/severe combined immunodeficient mice (SCID) mice. Primary xenograft tumors were maintained solely in immunocompromised mice by serial passage. Tumor sections of primary xenografts were formalin-fixed, sectioned, and stained with hematoxylin and eosin at various passages to verify that they consistently maintained NSCLC histologic characteristics. Tissues were characterized for \textit{KRAS} and \textit{EGFR} mutations, EML4–ALK translocation, and cMET amplification by the Molecular Pathology Laboratory at Johns Hopkins University (Supplementary Table S1).

Freshly isolated primary xenograft tumors were mechanically dissociated into single-cell suspensions; 2.5 \times 10^6 viable cells were suspended in equal volumes of PBS and Matrigel and implanted subcutaneously in adult homozygous NOD/SCID mice (Charles River Laboratories). After tumors reached a size measuring \geq 150 mm^3, calculated as \(V = \frac{L \times W^2}{2}\), mice were treated with either vehicle control, itraconazole (75 mg/kg oral twice daily), cisplatin (4 mg/kg intraperitoneally every 7 days), or a combination of itraconazole and cisplatin. All treatments were administered in 10 mL/kg volumes with 12-hour separation between bi-daily treatments.

Statistical analyses were carried out for each primary xenograft separately. Initial tumor volume measurements are summarized using means, SEM, and ranges. Growth patterns were summarized graphically by plotting the mean and SEM for each treatment group over time. Treatment groups were analyzed by 2-tailed paired Student \(t\) test, with a \(P < 0.05\) considered statistically significant.
Immunoblot analyses

Tumors were harvested 4 hours after the last dose following a 14-day treatment in tumor-bearing mice. Tumors were mechanically homogenized in 1:11 (w/v) modified RIPA buffer. Clarified lysates were then generated by centrifugation of crude lysates. The total protein content was quantified by Bradford assay and equally pooled by treatment group. Pooled lysates were mixed with lithium dodecyl sulfate sample buffer (Invitrogen), heated to 70°C for 5 minutes, and resolved by electrophoresis on a 4% to 12% Bis-Tris NuPage Gel (Invitrogen). Protein was then electrophoretically transferred to polyvinylidene fluoride membranes. Membranes were blocked using 5% condensed milk in Tris-buffered saline containing 0.2% Tween 20. Primary goat polyclonal antibodies to hypoxia-inducible factor 1 alpha (HIF1α; sc-8711) and β-actin (sc-1615; Santa Cruz Biotechnology) were used at a dilution of 1:1,000. Immunodetection was carried out using enhanced chemiluminescence (ECL), and autoradiographs were scanned using a GS-800 calibrated densitometer and analyzed using Quantity One software (BioRad).

Assessment of tumor vascular area

Nuclei of perivascular cells associated with perfusion-competent tumor vessels were detected by in vivo perfusion of Hoescht 33342 (HOE), as previously reported (24, 25). Briefly, animals were injected intravenously with 15 mg/kg HOE saline solution in a 10 mL/kg volume 4 hours after the last dose. Animals were euthanized by cervical dislocation 1.5 minutes after injection. Tumors were then harvested and frozen in Tissue Tek O.C.T. compound (Sakura Finetek). Tumors were serially sectioned with 6-μm thickness, mounted on Superfrost glass slides (Fisher) and allowed to air dry overnight. Tumor sections were then scanned and HOE staining of perivascular nuclei visualized under 10× objective on an Olympus BX61 fluorescence microscope affixed with an automated stage using Slidebook 5 software (Intelligent Imaging Innovations) to generate whole-section montage images. Tumor images were processed using ImageJ software by applying a threshold across all tumor images to generate binary representations of HOE-positive perivascular signal with hole filling used to approximate perfused vessel area (24). Perfused vessel area was then quantified as a percentage of total tumor area for each tumor sample, and mean vessel area ± SEM is reported as a percentage of total tumor area for each treatment group.

Results

Itraconazole inhibits HUVEC proliferation but has no direct antiproliferative effects on NSCLC cells

We initially sought to confirm the inhibitory activity of itraconazole on endothelial cell proliferation and to evaluate the specificity of this effect for distinct angiogenic stimuli. Itraconazole showed equipotent, dose-dependent inhibition of HUVECs in all growth factor-stimulated conditions tested, including stimulation with VEGF, bFGF, bFGF/VEGF, or EGM-2 media (containing these and multiple additional growth factors), with approximate IC50 [95% confidence interval (CI)] values of 588 (553–624), 689 (662–717), 691 (649–733), and 628 (585–672) nmol/L, respectively (Fig. 1A; the Dunn test not significant for differences in these values). Because this effect was observed both with VEGF and bFGF, we examined whether itraconazole exposure altered the activity state of the receptors primarily implicated in VEGF- and bFGF-mediated angiogenesis. Analysis of phospho-tyrosine levels on receptor tyrosine kinases (RTK) in HUVECs cultured in EGM-2 revealed dose-dependent decreases specifically in phospho-VEGFR2 and phospho-FGFR3 levels (Fig. 1B). None of the other RTKs present on the array were similarly affected. Together, these data show that itraconazole has inhibitory effects on multiple primary angiogenic pathways.

To assess the relative specificity of the inhibitory activity of itraconazole on endothelial cells, versus possible direct effects on tumor cell growth, the antiproliferative effects of itraconazole were examined in a panel of NSCLC cell lines representing a variety of genetically and phenotypically distinct lung cancer subtypes. In these NSCLC cell lines, itraconazole had no appreciable effect on proliferation below the maximum tested concentration of 100 μmol/L. To
mimic effects under varying oxygen tension, as might be seen in tumor cells in vivo, the assays were repeated under hypoxic culture conditions, again revealing no change in proliferation at concentrations less than 100 µmol/L of itraconazole (Supplementary Table S1).

Itraconazole potently inhibits HUVEC migration and chemotaxis

Endothelial cell migration is an essential process in angiogenesis and is functionally distinct from proliferation (26, 27). Migration is regulated by multiple chemotactic stimuli, resulting in the activation of signaling pathways that mediate cytoskeletal remodeling. We explored the effects of itraconazole on endothelial cell migration across stimulus gradients using a Boyden chamber assay. HUVEC migration was also assessed in a modified wound-healing assay.

The Boyden chamber assay was employed to assess chemotactic potential of HUVECs across gradients of angiogenic factors. Itraconazole inhibited VEGF-stimulated, bFGF-stimulated, and EGM-2-stimulated HUVEC migration in a dose-dependent manner, with similar potencies to those shown in the endothelial proliferation assays described above (Fig. 2A). In addition, itraconazole markedly inhibited migration of HUVECs under non-gradient stimulation with EGM-2, as assessed in the modified wound-healing assay (Fig. 2B and C).

Itraconazole potently inhibits HUVEC tube formation

Beyond simple endothelial cell proliferation and chemotactic migration, neovascularization is dependent on angiogenic stimuli driving formation and organization of tubular networks, that is, a capillary bed, requiring breakdown and restructuring of extracellular connective tissue. This capacity for formation of invasive and complex capillary networks can be modeled ex vivo with the provision of ECM components as a growth substrate, promoting spontaneous formation of a highly cross-linked network of HUVEC-lined tubes (28). We utilized this model to further define dose-dependent effects of itraconazole in response to VEGF, bFGF, and EGM-2 stimuli. In this assay, itraconazole inhibited tube network formation in a dose-dependent manner across all stimulating culture conditions tested and exhibited similar degree of potency for inhibition as shown in HUVEC proliferation and migration assays (Fig. 3).

Itraconazole inhibits growth of NSCLC primary xenografts as a single agent and in combination with cisplatin therapy

The effects of itraconazole on NSCLC tumor growth were examined in the LX-14 and LX-7 primary xenograft models, representing a squamous cell carcinoma and adenocarcinoma, respectively. NOD/SCID mice harboring established progressive tumors treated with 75 mg/kg itraconazole twice
Itraconazole treatment increases tumor HIF1α and decreases tumor vascular area in NSCLC xenografts

Markers of hypoxia and vascularity were assessed in LX14 and LX-7 xenograft tissue obtained from treated tumor-bearing mice. Probing of tumor lysates by immunoblot indicated elevated levels of HIF1α protein in tumors from animals treated with itraconazole, whereas tumors from animals receiving cisplatin remained largely unchanged relative to vehicle treatment (Fig. 4C and D). HIF1α levels associated with itraconazole monotherapy and in combination with cisplatin were 1.7- and 2.3-fold higher, respectively in LX-14 tumors, and 3.2- and 4-fold higher, respectively in LX-7 tumors, compared with vehicle treatment. In contrast, tumor lysates from mice receiving cisplatin monotherapy showed HIF1α expression levels equivalent to 0.8- and 0.9-fold that seen in vehicle-treated LX-14 and LX-7 tumors, respectively.

To further interrogate the antiangiogenic effects of itraconazole on lung cancer tumors in vivo, we directly analyzed tumor vascular perfusion by intravenous pulse administration of HOE dye immediately before euthanasia and tumor resection. This procedure allows for assessment of functional vasculature based on fluorescent dye delivery to and concentration in perivascular nuclei. Analysis of perfused tumor sections by fluorescence microscopy showed significant reduction of tumor microvessel density associated with itraconazole therapy in both LX-14 and LX-7 primary xenografts (Fig. 5). Vehicle-treated tumors showed 14.9% and 21.9% mean tumor vascular area for LX-14 and LX-7 xenografts, respectively, whereas itraconazole monotherapy resulted in reduction of mean tumor vascular area to 5.8% (P < 0.001) and 9.7% (P < 0.001) in LX-14 and LX-7 tumors, respectively. Addition of itraconazole to a cisplatin regimen resulted in a similarly significant reduction in tumor vasculature, with LX-14 showing a decrease in mean tumor vascular area from 11.2% to 6.1% (P < 0.001) and LX-7 showing a decrease from 20.8% to 10.3% (P < 0.001) tumor vascular area.

Discussion

Cancer-associated angiogenesis is a critical component of solid tumor establishment, growth, and spread, and remains a primary target of anticancer drug development (29). Antiangiogenic therapies to date have primarily focused on 2 approaches: (i) monoclonal antibodies or antibody derivatives that bind and sequester soluble endothelial growth factors or that inhibit ligand interaction with specific endothelial receptors; and (ii) small-molecule RTKs with specificity for endothelial receptors including VEGFR2 and FGFR3 (30). These strategies typically have a narrow focus, specifically targeting one of the most critical defined pathways of angiogenic stimulation. These novel drugs exemplify a broader ascendency of rationally designed targeted therapeutic drug development as the predominant focus of therapeutic cancer research over the past 2 decades.

Narrowly targeted therapeutic strategies, the so-called "smart bombs" for cancer, are conceptually attractive in terms of selectively targeting tumor growth and survival pathways while limiting off-target toxicities. It is becoming clear that, for complex biologic processes such as cancer cell growth and angiogenic drive, focused inhibition of a critical node in a single signaling axis, although it is the predominant signaling axis, invites emergence of resistance pathways. In lung cancer, most notably, targeting the driver mutation in EGFR-mutant NSCLC can lead to dramatic initial responses in advanced disease, but is essentially never curative (31). Secondary mutations of EGFR itself (32), upregulation of alternative RTKs...
such as c-MET (33, 34), constitutive activation of downstream pathways such as phosphoinositide 3-kinase (PI3K) and Akt (35, 36), as well as a large-scale shift in gene expression and morphology known as epithelial–mesenchymal transition (37, 38) have all been implicated as mechanisms of acquired resistance. These and similar observations have led to an ongoing debate about whether highly selective inhibitors or multi-targeted inhibitors will ultimately be more effective, and more durably effective, drugs.

Itraconazole as an antiangiogenic agent appears to fall into the latter category, that is, an inhibitor that coordinately affects multiple angiogenic stimulatory pathways. In this study, we evaluated the influence of itraconazole on multiple aspects of endothelial cell function that contribute to angiogenic potential. In assays of HUVEC proliferation, itraconazole appears reasonably cell-type specific, because high selective inhibitors or multi-targeted inhibitors will ultimately be more effective, and more durably effective, drugs.

Itraconazole inhibits growth of NSCLC primary xenografts, with itraconazole-treated tumors showing increased expression of HIF1α. A, mice bearing established LX-7 tumors were treated with vehicle (n = 9), oral itraconazole 75 mg/kg twice daily (ITRA; n = 9), cisplatin 4 mg/kg intraperitoneally every 7 days (CDDP; n = 9), or a combination of itraconazole and cisplatin (ITRA + CDDP; n = 8). Mean ± SEM tumor volumes are reported for each treatment group. B, mice bearing established LX-14 tumors were treated with vehicle (n = 6), oral itraconazole 75 mg/kg twice daily (ITRA, n = 7), cisplatin 4 mg/kg intraperitoneally every 7 days (CDDP, n = 6), or a combination of itraconazole and cisplatin (ITRA + CDDP, n = 7). Mean ± SEM tumor volumes are reported for each treatment group. C, tumor lysates from LX-7 xenografts were generated from mice treated for 14 days with vehicle (n = 4), oral itraconazole (ITRA, n = 4), intraperitoneal cisplatin (CDDP, n = 4), or a combination of itraconazole and cisplatin (ITRA + CDDP, n = 4). Pooled lysates were probed for HIF1α. D, HIF1α immunoblots on pooled tumor lysates from LX-14 xenografts treated for 14 days with vehicle (n = 4), oral itraconazole (ITRA, n = 4), intraperitoneal cisplatin (CDDP, n = 4), or a combination of itraconazole and cisplatin (ITRA + CDDP, n = 3).

alteration of VEGFR2 and FGFR3 phosphorylation states does not appear to be directly related to the previously noted effects of itraconazole on cholesterol trafficking and mTOR pathway inhibition (16). The mechanism(s) responsible for this targeted receptor inhibition has not been fully defined, and is the subject of ongoing analyses in our laboratories. These effects on multiple key drivers of angiogenesis may be important to the consistent inhibitory effects on multiple downstream angiogenic functions.

Beyond proliferation, endothelial cell migration, directional chemotaxis, and complex tube formation are all critical, and distinct, functional components of tumor-associated angiogenesis. Itraconazole potently inhibited each of these functional competencies as indicated by MTS, wound-healing, Boyden chamber, and tube formation assays. Extending these analyses in vivo, itraconazole showed marked tumor growth inhibition in our primary xenograft models of squamous cell and adenocarcinoid NSCLC. When administered in combination with cytotoxic chemotherapy, itraconazole contributed to a durable cytostatic tumor growth response. These in vivo effects appeared to be consistent with a potent antiangiogenic effect, associated with significant inhibition of angiogenic biomarkers, most notably intratumoral induction of the hypoxia-responsive gene, HIF1α, and depletion of perfusion-competent tumor vascularity. Taken together, these in vitro and in vivo analyses support the hypothesis that itraconazole inhibits angiogenic potential across all models tested and shows intriguing efficacy in the first evaluation of this agent alone.
and in combination with cytotoxic chemotherapy in a preclinical primary cancer model.

Angiogenesis is an essential contributor to the growth and spread of solid tumors. Few antiangiogenic agents have shown improved outcomes in randomized phase III trials, including only 1 such agent in lung cancer patients studied to date. The benefits provided by bevacizumab in lung cancer represent an important proof of principle; however, these benefits are typically modest, improving survival by a few weeks in patients treated with first-line chemotherapy. The lack of antiangiogenic therapeutic options and limitations associated with bevacizumab therapy contribute to the need for development and evaluation of additional angiogenesis targeting agents, including agents with mechanisms of action distinct from the several monoclonal antibodies and RTKs currently competing in this space. Itraconazole is an orally bioavailable, well-tolerated, widely available off-patent medication that has a well-characterized safety profile in thousands of patients. The initial findings described here have important clinical implications for potential use of itraconazole as a novel antiangiogenic agent and strongly support both further investigation of the molecular mechanisms of action of this drug in endothelial cells, and clinical investigation in patients with lung cancer and other solid tumors. Based on these several considerations, we have recently initiated a randomized phase II clinical trial randomizing patients with advanced recurrent NSCLC to standard chemotherapy with or without oral itraconazole.

Disclosure of Potential Conflicts of Interest

The intellectual property covering itraconazole has been licensed from Johns Hopkins University to Accelas Holdings, Inc., in which J.O. Liu owns equity. No potential conflicts of interest were disclosed by the other authors.

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References


7. Cross MJ, Claesson-Welsh L, FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibi-
11. Ranpura V, Hapani S, Wu S. Treatment-related mortality with bev-
26. Zeng H, Dvorak HF, Mukhopadhyay D. Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) receptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell prolifera-
tion, but not migration, through phosphatidylinositol 3-kinase-depen-
37. Thomson S, Buck E, Pett F, Griffin G, Brown E, Ramnrahn N, et al. Epithelial to mesenchymal transition is a determinant of sensitivity of non–small-cell lung carcinoma cell lines and xenografts to epi-
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