Benzimidazole Analogs as Potent Hypoxia Inducible Factor Inhibitors: Synthesis, Biological Evaluation, and Profiling Drug-like Properties

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Abstract. Aim: To develop potent HIF-1α inhibitors for potential treatment of cancer. Materials and Methods: Chemical synthesis, HIF-luciferase assay, cytotoxic assay, platelet aggregation assay, western blot analysis, quantitative real-time PCR, aqueous solubility, protein binding, metabolic stability, and metabolic pathways. Results: Thirteen novel benzimidazole analogs were synthesized. Compounds 3a and 3k showed the highest anti-HIF-1α activity. They are significantly more effective than YC-1 in the suppression of HIF-1α protein expression based on western blot assay. They showed comparable potency in inhibition of cancer cell migration. They are less potent in the inhibition of platelet aggregation. 3k had the most favorable drug-like properties, including long half-life in human liver microsomes, medium protein binding level and reasonable aqueous solubility. Conclusion: The potent anti-HIF-1α activity and favorable drug-like properties of compound 3k suggest that it may hold great potential as an adjuvant therapy for cancer treatment through repression of HIF-1α protein expression.

During hypoxic stress, tissues experience reduced oxygen levels due to insufficient supply of oxygen (1, 2). Tumor hypoxia is common in most solid tumors because the growth of tumors often outpaces the formation of new blood vessels. These new blood vessels are often leaky and non-functional, leaving portions of the tumor with significantly decreased oxygen tensions compared to normal tissues (1, 3-5). Hypoxic tumor cells execute several adaptive mechanisms to survive under hypoxic stress and thus are typically resistant to radiotherapy and chemotherapy (1, 4-7). The transcriptional response required for hypoxic adaptation is largely mediated through the hypoxia inducible factor (HIF) transcription factors, which regulate the expression of hundreds of downstream target genes that play critical roles in many cancer-related biological processes including angiogenesis, metabolism, stem cell activity, invasion, and metastasis.

HIF-1α was first identified and characterized by Semenza and colleagues (8) and is the most widely studied HIFα protein of the three HIFα subunits identified to date (HIF-1α, HIF-2α, and HIF-3α) (1, 3). HIF-1α is broadly expressed in many cancers and its over-expression has been strongly associated with tumor angiogenesis, progression, metastasis, and drug resistance (2, 7, 9-12). Many small-molecule HIF-1α inhibitors have been developed in the past decade (e.g., YC-1, E09 and PR-104, Figure 1A) and some are being tested in clinical trials (2). YC-1 (Figure 1A) is a HIF-α inhibitor widely used in pre-clinical studies as an
anticancer compound (13-19). However, the further development of YC-1 has been hampered by its potent ability to inhibit platelet aggregation, which may result in the deleterious side-effect of internal bleeding, as well as its limited efficacy compared to conventional therapies (20-22). To date, no drug that specifically targets HIF-α subunits has been approved by the US FDA for cancer treatment. The development of a highly efficacious HIF-1α inhibitor with reduced adverse effects could provide significant benefits to cancer patients at high risk of relapse due to resistance to currently available therapies.

In the present study, we have designed, synthesized and characterized the biological activities of a series of novel benzimidazole analogs as highly potent HIF-1α inhibitors based on the structure of YC-1. Two highly potent compounds, 3a and 3k, were identified from an in vitro high-throughput HIF-luciferase assay. We further conducted western blot and platelet aggregation assays to confirm the in vitro pharmacological activity and to evaluate the potential toxicity of 3a and 3k relative to that of YC-1. Both compounds, 3a and 3k, reduced HIF-1α expression more effectively than YC-1 and had less potency in inhibiting platelet aggregation. In addition, compound 3k demonstrated the most favorable in vitro pharmacokinetic properties, including satisfactory in vitro metabolic stability, reasonable water solubility, and medium protein binding. In summary, the present study provides the first evidence that these novel benzimidazole compounds we have generated are promising to be further developed into a new class of HIF-1α inhibitors.

Materials and Methods

Reagents and Materials. YC-1 was purchased from Cayman Chemicals (Ann Arbor, MI, USA). High Performance Liquid Chromatography (HPLC) or reagent grade solvents were purchased from either Sigma-Aldrich or Fisher Scientific and were used without further purification. Thin-layer chromatography (TLC) was performed on aluminum-backed Uniplates (Analtech, Newark, DE, USA). Melting points were measured using the Fisher-Johns melting point apparatus (uncorrected). Human, mouse, and rat liver microsomes were purchased from Xenotech, LLC (Lenexa, KS, USA). Plasma used for protein binding assays was purchased from Innovative (Novi, MI, USA).

Cell culture and cytotoxicity assay of MDA-MB-231. The metastatic breast cancer cell line MDA-MB-231 was originally obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and was authenticated prior to use in these experiments by DDC Medical (www.ddmedical.com). Cell culture supplies were purchased from Cellgro Mediatech (Herndon, VA, USA). MDA-MB-231 cells were maintained in DMEM/Hi high glucose media supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA), and 1x antibiotic/antimycotic (Sigma-Aldrich, St. Louis, MO). The cytotoxic activity of test compounds 3a-m was investigated in MDA-MB-231 cells using the sulforhodamine B (SRB) assay. Cultured cells were plated in 96-well plates (at 5,000 cells/well) and allowed to adhere overnight at normoxia, before pre-incubation with a titrated range of test compounds for 1h before culture at normoxia (21% O2; 5% CO2) or hypoxia (1% O2; 5% CO2). The cells were stained with Sulforhodamine B (SRB) after 96 h and the optical density of stained cells was measured at 540 nm on a Synergy HT multi-mode microplate reader (BioTek Instruments, Winooski, VT, USA) (23).

Wound-healing assay. Forty thousand MDA-MB-231 cells were seeded into each well of a 6-well plate and allowed to adhere overnight to form a monolayer. A 200 μL sterile pipette tip was used to “scratch” the center of the monolayer in a straight line following established protocols (24, 25). The culture medium was gently removed and wells were washed once with fresh medium. FBS-free DMEM/Hi high glucose medium containing the compound of interest or vehicle (DMSO) control was added per well in a volume of 1 mL (n=3 wells/dose/treatment) and plates were incubated at normoxia for the indicated period of time to allow cells to migrate into the scratched area. Images of cell monolayers were captured by an EVOS® FL Cell Imaging System (Thermo Fisher Scientific Inc., NY, USA) and the mean migration distance was calculated and expressed as mean±SEM in arbitrary units (n=3).

HIF-1α luciferase assay in MDA-MB-231-Luc cells. A stable luciferase-transfected breast cancer cell line, MDA-MB-231-Luc, was obtained from Dr. Robert J. Gillies at the Moffitt Cancer Center. MDA-MB-231-Luc cells were maintained in DMEM/Hi high glucose media supplemented with 10% FBS, and 1x antibiotic/antimycotic and 1 μg/ml puromycin for selection. The luciferase activity of cells cultured in the presence of each compound (3a-m) was quantified using the Luciferase Assay system (E1500, Promega, Madison, WI, USA) per manufacturer’s instructions. Cultured cells were plated in 96-well plates (at 5,000 cells/well) and allowed to adhere overnight at normoxia, then the cells were treated with test compounds and incubated at normoxia (21% O2; 5% CO2) for 24h, followed by culture at hypoxia (1% O2; 5% CO2) for another 24h. Cells were lysed and luciferase reagents were added through an automated injector. The luminescence signal was read by a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT, USA).

Western blot. MDA-MB-231 cells were seeded into P10 dishes so that the next day they were 70-80% confluent. Cells were treated with vehicle or compounds 3a or 3k (10 μM or 50 μM) for 24h at normoxia. At harvest, cells were placed on ice, scraped into cold PBS (Phosphate Buffer Solution), pelleted and flash frozen in liquid nitrogen. Cell pellets were lysed in a modified RIPA buffer (150 mM NaCl) to generate whole cell extracts (WCE), followed by re-extraction of insoluble material with a high-salt lysis buffer containing 400 mM NaCl, generating HS-WCE as previously described (26). Protein concentration was determined using the Qubit Quan-iT Assay kit (Invitrogen, Carlsbad, CA, USA). HS-WCE protein extracts were loaded on a precast 8% Precise polyacrylamide gels (Thermo Scientific, Rockford, IL) (1 μg HS-WCE/per lane) and subject to Protein blotting. Next, blots were probed with 1:10,000 antibodies, and incubation in anti-rabbit IgG-HRP (Bio-Rad, Hercules, CA) (1:5,000) and β-tubulin (cat# ab6046, AbCam, Boston, MA, USA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at RT (room temperature) for 1 hour and incubated at 4°C overnight with both anti-HIF-1α (cat# 100-479, Novus Biologicals, Littleton, CO, USA, 1:5,000) and β-tubulin (cat# ab6046, AbCam, Boston, MA, USA, 1:10,000) antibodies, and incubation in anti-rabbit IgG-HRP.
secondary antibody (1:40,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antigen-antibody complexes were visualized using the Immobilon ECL western detection kit (Millipore).

**Quantitative real-time PCR.** 3.5×10^5 MDA-MB-231 cells were seeded into each well of a six-well plate overnight before being treated with 3k or YC-1 solution in culture medium (10 μM) for 8 h in hypoxia (1% oxygen) or normoxia (n=3). Cells under the same condition but treated with culture medium containing 5% DMSO for 8 h were used as control groups. Total RNA was isolated using RNA-Bee™ reagent (TEL-TEST INC., TX, USA) then cDNA was created using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) as per manufacturer’s instructions. Real-time quantitative PCR (qPCR) was performed using 40ng cDNA for each well (384 well plates) with the Light Cycler 480 SYBR Green1 master mix on a LightCycler® 480 system (Roche Applied Science, IN, USA). Results for gene HIF1A, PHD3 and VEGF were normalized to those for Cyclophilin A level (n=3).

**Platelet aggregation.** Blood was obtained by venipuncture into acid-citrate-dextrose (ACD) anti-coagulant from normal adults who denied ingestion of medications that alter platelet function. Platelet-rich citrate-dextrose (ACD) anti-coagulant from normal adults who denied ingestion of medications that alter platelet function. Platelet aggregation.

**Aqueous solubility.** The aqueous solubility of YC-1, 3a, and 3k was estimated following a reported method (23, 27). 1.0 mg of each of the aforementioned YC-1, 3a, and 3k compound was added to either 1 mL water or pH 7.0 phosphorous buffer and placed in a room temperature shaker and shaken for 24 h at 450 rpm. The resultant suspension was centrifuged at 10,000 RPM for 15 min, and the supernatant was injected into an API-3000 triple-quadrupole LC-MS/MS (AB Sciex, Foster City, CA, USA) equipped with an electrospray ionization source (ESI) to determine the concentration of the dissolved compound. High performance liquid chromatography (HPLC) was conducted using a Kinetex FFP column (100x4.6 mm i.d., 3.0 μm particle size, Phenomenex, Torrance, CA, USA) with a pre-column filter. HPLC mobile phase A: 0.1% Formic acid; B: MeOH. 0-0.5 min: 2% B; 0.5-0.6 min: 2-98% B; 0.6-9 min: 98% B; 9-9.1 min: 98-2% B; 9.1-12 min: 2% B. The flow rate was set to 0.4 mL/min.

**In Vitro metabolic stability.** Metabolic stability study was conducted following a reported procedure.(28) Briefly, 0.5 μM test compound was incubated with liver microsomes (1 mg/mL) in a 37°C incubator shaking at 200 RPM. The NADPH regenerating solution A and B was purchased from Xenotech, LLC (Lenexa, KS, USA). 100 μL of the reaction solution was sampled at 5, 10, 20, 30, 60, 90 min, and 120 min. 200 μL cold acetonitrile spiked with internal standard (200 nM) were added to 100 μL reaction solution to extract the test compound. The resultant mixture was centrifuged at 10,000 RPM for 15 min, and the supernatant was injected into LC-MS/MS for analysis. For metabolite identification, 50 μM of each of the test compound were incubated with 1mg/mL liver microsomes for 2 h under the abovementioned conditions.

**Plasma protein binding.** Plasma protein binding assay of compound 3k was performed by a reported ultra-filtration method (29). 1 mL of human, rat, and mouse plasma samples was spiked with 5 μL of 100 μM test compound and incubated for 30 min at 37°C followed by ultra-filtration. 400 μL of the reaction mixture were sampled and placed in the Amicon centrifugal filter units with 10KDa cutoff (Millipore Corp, Billerica, MA, USA) and centrifuged at 10,000 RPM for 20 min. 150 μL of acetonitrile spiked with internal standard were mixed with 50 μL of the ultra-filter for LC-MS analysis. For protein binding, 0.5 μM of 3k were incubated with 1 mg/mL liver microsomal proteins without NADPH. The protein binding was calculated using the following equation: PB %=(1-CF×CSD/(CPD×CSF))×100%, where CP is the peak area ratio of analyte to internal standard in the filtrate from the plasma donor sample, and CSF is the peak area ratio in the plasma donor sample. CSD is the peak area ratio in the filtrate from the PBS donor sample, and CPD is the peak area ratio in the PBS donor sample before centrifugation.

**Prediction of in vivo clearance (Cl) of 3k in human, mouse, and rat.** In vivo clearance was calculated by using the half life obtained from metabolic stability, and the protein binding data from the plasma and liver microsomes. The intrinsic hepatic clearance (Cl_in,vitro) of 3k was calculated as follows:

\[
\text{Cl}_\text{in,vitro} = \frac{0.693}{(t_{1/2} \text{ in minutes} \times \text{protein concentrations in mg/mL})}
\]

The scaled Cl_in,vitro was used to calculate the in vivo clearance in the liver. Scaling factors [(mg of protein per gram of liver weight)×(gram of liver weight per kg body weight)] are 1980 and 2400 for human and rat, respectively (28-31). Cl_h (In vivo intrinsic hepatic clearance in the liver, mL/min/kg body weight) was calculated by multiplying scaling factors by Cl_in,vitro. The Cl_h (In vivo hepatic clearance) was predicted by combining Cl_h, f_u (Fraction of Unbound) and Q_h in the venous equation:

\[
\text{Cl}_\text{h} = \frac{Q_h \times f_u}{\text{f}_\text{u}(\text{Cl}_\text{h} \times f_u \times \text{f}_\text{u})}
\]

Whereas Q_h is hepatic blood flow, f_u is fraction of unbound in plasma, and f_u,m is fraction bound in liver microsomes, respectively. Hepatic extraction ratio was calculated by dividing hepatic clearance (Cl_h) by hepatic blood flow (Q_h).

**Analytical method.** LC-MS/MS analysis was conducted on an API-3000 triple-quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) with a Turbo Ion Spray source. The parameters were set as follows: spraying needle voltage: 5.5 kV. Curtain gas: 8 psi, nebulizing gas: 8 psi. Collision Activated Dissociation (CAD) gas: 7 psi. Probe heater temperature: 400°C. Data was processed by Analyst 1.4.2 software (AB Sciex, Foster City, CA, USA). 10 μL of the sample was injected into a Shimadzu (Canby, OR) HPLC system with Shimadzu LC-10ADVP pump. Compound 3g was used as internal standard for the analysis of 3a. Compound 3e was used as internal standard for the analysis of 3k. Test compounds were extracted from 100 μL of liver microsomal reaction mixture or plasma (protein binding assay) with 200 μL of cold acetonitrile spiked with 200 nM internal standard. The samples were processed for LC-MS/MS.
analysis. Multiple-reaction-monitoring (MRM) was applied to determine metabolic stability, protein binding, and aqueous solubility for 3a and 3k. The optimized MRM parameters for analytes and internal standards are as follows: 342.1911 (3a), 362.0911 (3g), internal standard for 3a), 342.1911 (3k), 387.1911 (3e, internal standard for 3a), 305.1911 (YC-1). Two different solvent gradients were used. For metabolic stability study for 3a and 3k, the following chromatography condition was used: mobile phase A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile (ACN). 0-0.5 min: 2%-B; 0.5-0.6 min: 2-98% B; 0.6-9 min: 98%-B; 9-9.1 min: 98%-2% B; 9.1-12 min: 2%-B. The flow rate was set to 0.4 mL/min. For metabolite identification of 3k, the following condition was applied: mobile phase A: 0.1% formic acid in water; B: 0.1% formic acid in ACN. 0-3.5 min: 2%-B; 3.5-9 min: 98%-B; 9.1-9.1 min: 98%-2% B; 9.1-12 min: 2%-B. The flow rate was set to 0.4 mL/min. High resolution mass spectrometry (HRMS) data were acquired on a Waters Xevo G2-S QTOF system equipped with an Acquity I-class UPLC (Ultra-high Pressure Liquid Chromatography) system. Nominal mass spectra of synthesized compounds were acquired on a Bruker ESQUIRE-LC mass spectrometer with ion trap source. Nuclear magnetic resonance spectra were acquired on a Varian Inova-500 NMR spectrometer (Agilent Technologies, Santa Clara, CA, USA) and a Bruker ARX 300 (Bruker Biospin Corporation, Billerica, MA, USA) spectrometer. Chemical shifts are reported as parts per million (ppm) relative to TMS (Tetramethylsilane) in CDCl3.

Chemistry. 3a and 3k were synthesized using methods outlined in Figure 1B. Synthesis of the benzimidazole analogs was carried out following a reported procedure (33) with minor modifications. To a solution of appropriate substituted benzoic acids (compound 2, 1.0 mmol) and triethylamine (TEA, 1.2 mmol) in dimethyl formamide (DMF) (6 mL) was added N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) (1.1 mmol) and 1-Hydroxybenztriazole (HOBt) (1.1 mmol). The mixture was stirred for 10 min at room temperature. 2-Amino-1-benzyl-benzimidazole (compound 1, 1.0 mmol) was added to the reaction mixture and stirred overnight. After the reaction (monitored by TLC) was completed, the mixture was quenched by water followed by extraction with ethyl acetate. The organic layer was concentrated. The product was purified by column chromatography (eluent: hexane: ethyl acetate 5:1). 3a: yield: 80%. 1H NMR (Nuclear Magnetic Resonance) (500MHz, CDCl3) δ 8.08-8.11 (m, 2 H), 7.35-7.38 (m, 2 H), 7.30-7.43 (m, 1 H), 5.86 (s, 2 H), 4.17 (s, 2 H). ESI-MS: calculated for C22H19N3O, 341.2, found 364.1 [M+Na]+. HRMS-ESI (m/z) (M+H)+ calculated for C22H20N3O: 342.1606, found: 342.1605. 3k: yield: 79%. 1H NMR (300MHz, CDCl3) δ 7.47 (d, J=6.9 Hz, 2 H), 7.30-7.43 (m, 12 H), 5.86 (s, 2 H), 4.17 (s, 2 H). ESI-MS: calculated for C22H20N3O, 341.2, found 364.1 [M+Na]+. HRMS-ESI (m/z) (M+H)+ calculated for C22H20N3O: 342.1606, found: 342.1682. Chromatographic data (NMR, routine low resolution Mass spectrometry, and HRMS) for compounds 3b-m are provided in supplementary material.

Results

Identification of compounds 3a and 3k as potent HIF inhibitors through HIF-luciferase assay on the MDA-MB-231-Luc cells. The current work was based on the structure of YC-1 (Figure 1A), a well-established HIF-1α inhibitor (14). We performed molecular modeling studies to identify similar molecular scaffolds with the scaffold of YC-1 using the University of Cincinnati Drug Discovery Center compound library, which contains over 360,000 diversity-selected, high-quality, drug-like molecules. We first conducted a virtual screening based on the structure of YC-1 using the Accelrys Pipeline Pilot program, took the highest ranked hits, and ordered the compounds for in vitro assays. This approach provided several lead compounds that showed potent inhibitory effect on HIF-1α expression, with compound G3 (Figure 1A) being more potent than YC-1. Using G3 as a new template, we designed and synthesized 13 new analogs. Using a novel high-throughput HRE reporter-based luciferase assay, 3a and 3k were identified to have the best potency with half maximal inhibitory concentration (IC50) values in the nanomolar range (0.6 and 2.2 μM for 3a and 3k respectively, Table I). Because 3a and 3k demonstrated the highest inhibitory effect on HIF, they were selected for further biological studies, as detailed below.

3a and 3k are significantly more potent in reducing HIF-1α protein levels than YC-1 in MDA-MB-231 cells. In order to confirm that these compounds reduced expression of HIF-1α protein levels, we assessed the inhibitory effect of 3a and 3k on the expression of HIF-1α by western blotting. As indicated in Figure 2, compounds 3a and 3k inhibited the expression of HIF-1α in a dose-dependent manner under normoxic conditions. At 50 μM, HIF-1α expression was completely suppressed by both 3a and 3k (Figure 2A). At a lower concentration of 10 μM, the HIF-1α expression level is very low for both compounds 3a and 3k (68% and 36% relative to vehicle control, respectively), compared to non-treated control (vehicle-only) and YC-1-treated cells (Figure 2B). These results clearly indicate that both 3a and 3k can strongly reduce HIF-1α protein levels, and that both compounds are more potent than YC-1 in the inhibition of HIF-1α protein expression in vitro. When we repeated this experiment under hypoxic conditions (1% oxygen), none of the three compounds (YC-1, 3a and 3k) showed detectable reduction of HIF-1α protein levels, indicating the HIF-1α protein accumulation under hypoxic stress in MDA-MB-231 cells is not inhibited by these compounds. It should be noted that our method of protein extraction is more efficient in extracting HIF-1α from the nuclear compartment than conventional RIPA buffer, which may be a reason why other groups have reported a YC-1-dependent reduction of HIF-1α protein after hypoxic culture (34, 35).

3k may promote HIF-1α degradation by enhancing prolyl hydroxylase 3 (PHD3) expression. Reduced HIF-1α protein levels detected using western blots could result from either reduced protein synthesis or enhanced protein degradation. To determine whether HIF-1 transcriptional activity is inhibited by the most potent compound 3k, in comparison to YC-1, we performed qRT-PCR experiments to compare the mRNA levels of HIF-1α, prolyl hydroxylase 3 (PHD3), which is itself a direct HIF-1 target gene and is one of the
proteins responsible for HIF-1α protein degradation (36), and vascular endothelial growth factor (VEGF), a well-characterized HIF-dependent target gene. As shown in Figure 3A, the most striking effect of compound treatment is the enhanced expression of \( \text{PHD3} \). Compound 3k was significantly more potent to induce \( \text{PHD3} \) mRNA levels compared to YC-1, which is consistent with the results from western blot in which we observed less HIF-1α protein in response to 3k treatment than with YC-1. Expression levels of both \( \text{HIF1A} \) and \( \text{VEGF} \) were also significantly reduced by treatment, but the relative reduction (YC-1 >3k) was not consistent with the western blot (3k >YC-1).

When this experiment was repeated under hypoxic condition (1% oxygen, Figure 3B), the enhancement of \( \text{PHD3} \) mRNA levels by YC-1 or 3k that was observed under normoxia became insignificant, whereas expression of both \( \text{HIF1A} \) and \( \text{VEGF} \) remained repressed by either compound. Interestingly, YC-1 showed stronger inhibition for all three genes compared to effects with 3k under these conditions. Collectively, these results suggested that even though 3k retains certain structural similarities to YC-1, 3k likely interacts with the HIF-1 pathway differently. It is possible that YC-1 may function to inhibit HIF-1α protein synthesis, whereas 3k may rely more on enhancing HIF-1α protein degradation. Understanding the differential mechanisms of action between YC-1 and its derivatives requires more extensive study and will be presented in a future report.

3a and 3k are less potent in inhibition of platelet aggregation than YC-1 and may cause less risk of bleeding in patients. One major potential side-effect for the use of YC-1 is its high potency in inhibition of platelet aggregation to collagen and α-thrombin, which could result in an increased risk of bleeding in cancer patients. To determine whether compounds 3a and 3k that are significantly more potent than YC-1 for HIF-1α inhibition, would still have such potential side-effects, we assessed platelet reactivity in the presence of all three compounds using washed human platelets and light transmission aggregometry vs. the DMSO vehicle control (Figure 4). In the absence of compounds but in the presence of the DMSO vehicle control, the mean aggregation response (n=3) to collagen was 73±5% and to α-thrombin was 71±3%. These responses were in the expected normal range for healthy, human adult donors (37), indicating that the DMSO control (0.5% v/v) did not alter the platelet aggregation test results. Addition of the three compounds at 50 and 100 μM did inhibit collagen-induced aggregation compared to vehicle control (Figure 4A and 4B). 3k had significantly lower inhibition than 3a at the 50 μM concentration (\( p=0.01 \)). There was no significant difference in inhibition of collagen-induced aggregation between 3a and 3k vs. YC-1; however, the inhibition levels had a definite lower trend for 3a and 3k vs. YC-1 for both concentrations. In contrast, when tested with human α-thrombin, compounds 3a and 3k were significantly less potent in inhibiting platelet aggregation compared with that of YC-1 (\( p<0.05 \) at 50 μM and \( p<0.001 \) at 100 μM, Figure 4A and 4C). There were no significant differences between 3a and 3k at either concentration. These results suggested that compounds 3a and 3k may have significantly less side effects in potential internal bleeding in cancer patients.

Cytotoxicity of 3a and 3k in MDA-MB-231 cells. To investigate whether the effect of 3a and 3k on HIF-1α inhibition is due to cytotoxicity, we performed an in vitro cytotoxicity study on MDA-MB-231 cells using the SRB assay. As indicated in Table I, both compounds 3a and 3k exhibited very weak cytotoxicity with IC\(_{50}\) values of approximately 10 μM. It should be noted that in the HRE-
luciferase reporter assay, the IC\textsubscript{50} values were determined to be approximately 0.6 to 2.2 \(\mu\)M for 3a and 3k, respectively. These data suggest that specificity of each drug for the HIF pathway occurs at lower \(\mu\)M levels than is estimated using the general SRB assay. Effective inhibition of other HIF-dependent activities in the cell independent of cell proliferation and/or cell survival may be even more relevant to survival from cancer, including effects on tumor cell migratory potential since HIF-1\(\alpha\) is a known potent promoter of tumor metastasis (38).

3a and 3k Significantly Inhibit Breast Cancer Cell Migration. The \textit{in vitro} scratch assay or wound-healing assay has been adopted to conveniently measure the migration of homogenous cell populations (24). As shown in Figure 5, compounds 3a and 3k significantly inhibited the closing of the scratch edge in MDA-MB-231 cells in a time dependent manner at concentrations of 3 \(\mu\)M. Each drug exhibited a better capability of attenuating cancer cell migration as YC-1, which was used as the positive control in this assay.

3a and 3k Have Reasonable Metabolic Stability in Liver Microsomes. \textit{In vitro} liver microsomal stability assay is an efficient way to predict \textit{in vivo} hepatic clearance and to establish the metabolic profiles of a drug candidate prior to conducting expensive \textit{in vivo} pharmacokinetic studies. To gain insight into the metabolic stability of these compounds, we performed an \textit{in vitro} hepatic stability study using liver microsomes of mouse, rat and human origin. As shown in Table II and Figure 6A-B, compounds 3a and 3k showed species-specific differences in metabolic stability. In human liver microsomes, 3a and 3k had a half-life time of 77 min and 51 min, respectively. In contrast in mouse microsomes, 3a and 3k had much shorter half-life of 17 and 13 min, respectively. In rat liver microsomes, 3a and 3k demonstrated half-life of 18 and 12 min, respectively. In summary, 3a and 3k demonstrate excellent metabolic stability in human microsomes.

Aqueous solubility of 3a, 3k, and YC-1. Aqueous solubility is an important factor that can affect the absorption of a drug. We determined the aqueous solubility of YC-1, 3a and 3k using a minituarized shake-flask method and LC-MS/MS.
quantification. As shown in Table III, the aqueous solubility of 3k was 1.7 and 1.2 μg/mL in buffer pH 7.0 and water, respectively, which is comparable with that of YC-1 (0.9 and 1.2 μg/mL in buffer pH 7.0 and water, respectively). However, the aqueous solubility for 3a determined in this parallel experiment was 0.22 μg/mL in pH 7.0 buffer and 0.16 μg/mL in water. The results suggest that compound 3k, which is a more potent HIF-1α inhibitor than YC-1, also has a better aqueous solubility than 3a, and is slightly more soluble than YC-1.

Identification of metabolites of 3k in human liver microsomes. In vitro metabolite identification studies were conducted in human liver microsomes to identify metabolism-labile sites in 3k. We chose 3k for this study because it has better drug-like profiles, such as enhanced HIF-1α expression inhibition and a better aqueous solubility than 3a. LC-MS/MS was used for metabolite identification on the basis of mass shifts relative to the parent 3k. We first performed Q1 Scan and precursor ion scan as well as neutral loss to identify all possible metabolites. Then, we used products ion scan to examine all peaks of interest to obtain structural information. Figure 6C shows the MRM chromatography of 3k with its six metabolites (M1-M6) after incubating for 2h. Because the hydroxyl groups are highly polar, all the metabolites had shorter retention times than the parent compound 3k. Compound 3k has three sets of major metabolites as indicated in Figure 6C-H, with m/z of 358 (M1, M2), 374
The product ions (M3, M4, M6), and 390 (M5), respectively. The product ion spectrum of 3k (Figure 6D) had an abundant product ion m/z 91 (benzyl moiety). The major and most abundant metabolites are M1 and M2, which are isomers (m/z 358, mass shift +16 Da, mono-hydroxylation) (Figure 6E and 6F). Based on the fragment pattern of M1 (m/z 162.0), we tentatively assigned the hydroxylation to the amide NH. Based on the fragment pattern of M2 (m/z 107, and 134), we tentatively assigned the hydroxylation to the benzylic CH2 in the part B (Figure 1A) of 3k. However, we were unable to unambiguously establish the absolute structure at this stage due to the difficulty in obtaining sufficient amount of this metabolite needed for NMR analysis. M3, M4, and M6 (m/z 374, mass shifts +32 Da, di-hydroxylation) (Figure 6G), and M5 (mixture of a few trihydroxylated metabolite, m/z 390, mass shifts +48 Da, tri-hydroxylation) (Figure 6H), had the fragment ions with m/z 91 and 240. Overall, we detected six metabolites based on the mass shift and the metabolic pathway is summarized on Figure 7A.

Species-specific metabolism of 3k. The metabolite kinetics were determined by treating liver microsomes from human, mouse, and rat with 3k (0.5 μM) for 2h. The parent compound 3k and the six metabolites were monitored (Figure 7B-E). The half-lives of 3k in liver microsomes from different species varied over a wide range from 12-51 min (Figure 7E, Table II). The relative amount of each metabolite is presented in Figure 8A-C. Interestingly, we found that mono-hydroxylation (M2) was predominant only in human (Figure 8A). The dihydroxylated metabolite (M6) was found to be more abundant in rat than that of human and mouse (Figure 8B and C). We increased the concentration of 3k from 0.5 to 50 μM and incubated with 1 mg/mL human liver microsome which is to saturate the microsomal enzymes and maximize metabolite production (Figure 8D). We can clearly see that M1 (mono-hydroxylation) is the major metabolite followed by M4 and M2 (isomer of M1). The di-hydroxylated products (M3) and tri-hydroxylated products (M5) are much less compared to M1 probably due to the lower efficiency in converting mono-hydroxylation metabolite to di- and tri-hydroxylated products.

Determination of plasma and liver microsome protein binding for 3k. The therapeutic efficiency of a drug may be influenced by the extent it binds to the plasma proteins. Usually the unbound fraction of the drug exhibits pharmacological effects.
and can be metabolized or excreted. A drug with a high degree of protein-binding (>95%) generally has a lower therapeutic index and is prone to having high toxicity due to drug-drug interactions, which alter protein binding levels. To assess the protein binding of compound 3k, we performed a protein binding assay. As indicated in Table IV, compound 3k showed medium protein binding (58.7% and 61.4% in mouse plasma and mouse liver microsomes, 60.4% and 75.8% in rat plasma and rat liver microsomal protein, and 63.4% and 64% in human plasma and human liver microsomes) for both plasma and liver microsome proteins in all tested species. The results from protein binding assay suggest that compound 3k may have reasonable therapeutic index and relatively low risk of toxicity due to drug-drug interactions.

Figure 5. 3a and 3k inhibit in vitro migration of breast cancer cells. MDA-MB-231 cells (n=3) were incubated with serum-free medium with 3 μM of 3a, 3k or YC-1. (A) representative images of cell monolayer after incubation for the indicated times. The white scale bar in each image represents 1000 μm. (B) column graph of cell migration distance after 32 h incubation (compared to the 0 h cell edge). Results were displayed with mean±SEM. *p<0.05 versus DMSO vehicle control group.

Prediction of the in vivo clearance of 3k in human, rat, and mouse. As shown in Table IV, compound 3k showed moderate protein binding activity in both plasma protein and liver microsome assays. With the medium protein binding (60.4% and 75.8% in rat plasma protein and rat liver microsomal protein, and 63.4% and 64% in human plasma and human liver microsomes), compound 3k was predicted to have medium hepatic clearances (44.1 ml/min/kg) in rat and low hepatic clearances due to longer half-life in human (12.2 ml/min/kg), respectively. Likewise, metabolic stability studies and in vitro protein binding further suggested that humans are likely to have a medium hepatic extraction ratio (0.58) while rat will have a relatively high extraction ratio (0.8).
Figure 6. Metabolic stability of 3a (A) and 3k (B) in liver microsomes from mouse, rat, and human. Substrate (0.5 μM) was incubated at 37 °C with 1mg/ml microsomal protein from different species, sampled at different times from 0 to 120 min, and analyzed by LC-MS/MS. Metabolite identification (C-H). 3k (50 μM) was incubated with 1mg/ml human liver microsomes for 2 h. (C), chromatography. (D), MS/MS spectrum of parent 3k. Five major MS/MS spectra were identified in M1 (E), M2 (F), M3, M4, M6 (G), and M5 (H).
Discussion

YC-1 is a well-established HIF inhibitor that has been widely used as a standard compound to target the HIF-1 pathway in multiple pre-clinical studies (18, 34, 35, 39-43). However, YC-1 has limited anticancer potency and, more relevant to patients, may also cause a serious side-effect of uncontrolled bleeding in humans, based on data from platelet aggregation assays (20-22). To overcome these limitations of YC-1, we designed and synthesized novel benzimidazole analogs as highly potent HIF-inhibitors with the goal of identifying compounds that effectively inhibit HIF-1α protein expression, while having improved toxicity profiles with reasonable drug-like properties.

Starting from the chemical structure of YC-1, a successful virtual screening was performed and several hits were found, with the compound G3 being identified as potentially more potent than YC-1. Based on G3, we designed and synthesized thirteen new analogs. Through a high throughput HRE-luciferase assay to measure total HIF transcriptional output, we discovered that 3a and 3k are the most potent HIF-1α inhibitors in this series. Consistent with HIF-luciferase assay results,
western blot analysis further confirmed that both 3a and 3k are more effective than YC-1 in the inhibition of HIF-1α expression at normoxia. Notably, compounds 3a and 3k significantly inhibited the migration of MDA-MB-231 cells in a time- and dose-dependent manner, with comparable capability of attenuating cancer cell migration as YC-1. Platelet aggregation and in vitro cytotoxicity assays revealed lower toxicity profiles for 3a and 3k compared to YC-1, especially for thrombin-mediated platelet aggregation. Compound 3k also had favorable drug-like properties, including long half-life on human liver microsomes, reasonable protein binding levels, and good aqueous solubility. In addition, the metabolic pathway of 3k and kinetics of its metabolites have been characterized and identified. These collective observations predict a significant clinical advantage of compounds 3a and 3k over YC-1 and further suggest that 3a and 3k have the potential of being...

Table IV. Plasma protein binding and prediction of in vivo clearance of 3k from in vitro data in mouse, rat, and human. Hepatic blood flow were reported from Davies and Morris (44).

<table>
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N.A., not available.

Figure 8. Comparison of metabolic profiles of 3k in species. Substrate (0.5 μM) was incubated with 1 mg/ml microsomal protein of human (A), mouse (B), and rat (C). The spectra were obtained from LC-MS/MS after 120 min incubation of substrate with liver microsomes. Kinetics of metabolites in human liver microsome (D). 3k (50 μM) was incubated with 1 mg/ml microsomal proteins. Samples at various time points were analyzed by LC-MS/MS.
developed in the future to be more effective and less toxic therapeutics for the treatment of a variety of cancers in which the HIFs are known to be over-expressed. The in vivo pharmacokinetic study for compound 3k is currently in progress and will be reported in the future.

In summary, we have developed a highly potent inhibitor against HIF expression and activity, compound 3k, which also exhibits relatively low cytotoxicity and favorable drug-like and in vitro pharmacokinetic properties. In summary, compound 3k appears to have a more favorable potential than YC-1 and could be developed into a novel HIF-1α inhibitor for patients suffering from a variety of cancer types.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

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References


