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Virtual Screening Targeting the Urokinase Receptor, Biochemical and Cell-Based Studies, Synthesis, Pharmacokinetic Characterization, and Effect on Breast Tumor Metastasis

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Abstract

Virtual screening targeting the urokinase receptor (uPAR) led to (3R)-4-cyclohexyl-3-(hexahydrobenzo[d][1,3]dioxol-5-yl)-N-((hexahydrobenzo[d][1,3]dioxol-5-yl)methyl)butan-1-aminium **1** (IPR-1) and 4-(4-((3,5-dimethylcyclohexyl)carbamoyl)-2-(4-isopropylcyclohexyl)pyrazolidin-3-yl)piperidin-1-ium **3** (IPR-69). Synthesis of an analog of **1**,

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namely **2** (IPR-9), and **3** led to breast MDA-MB-231 invasion, migration and adhesion assays with IC₅₀ near 30 μ M. Both compounds blocked angiogenesis with IC₅₀ of 3 μ M. Compounds **2** and **3** inhibited cell growth with IC₅₀ of 6 and 18 μ M and induced apoptosis. Biochemical assays revealed lead-like properties for **3**, but not **2**. Compound **3** administered orally reached peak concentration of nearly 40 μ M with a half-life of about 2 hours. In NOD-SCID mice inoculated with breast TMD-231 cells in their mammary fat pads, compound **3** showed a 20% reduction in tumor volumes and less extensive metastasis was observed for the treated mice. The suitable pharmacokinetic properties of **3** and the encouraging preliminary results in metastasis make it an ideal starting point for next generation compounds.

Introduction

When a primary tumor metastasizes, the prospects for survival become substantially worse, resulting in approximately 90% death in patients.¹ Metastasis occurs when cells from the primary tumor travel to distant sites and form new colonies. Cells escape from the primary tumors primarily through the circulatory system. These cells gain access to the vasculature as a result of multiple complex processes that involve invasion, migration, adhesion, and angiogenesis. A small number of cells that survive the voyage through the circulatory system arrive at new organ sites. Adhesion to and recognition of those sites in the endothelium is followed by re-entry through a process known as extravasation and result in the formation of a new tumor colony.

The urokinase receptor (uPAR) is a cell surface GPI-anchored protein that has been widely implicated with promoting metastasis. The receptor enhances pericellular proteolysis by serving as a docking site to the urokinase-type plasminogen activator (uPA), triggering a cascade of proteolytic events that include activation of plasminogen and matrix metalloproteinases (MMPs). The receptor also activates other cell signaling through lateral interactions with cell surface receptors that include integrins,² receptor tyrosine kinases (RTKs),^{3, 4} and G-protein coupled receptors (GPCRs). The dual roles of the urokinase system in promoting degradation of the ECM *and* signaling has implicated the receptor with almost every step of tumor formation and progression, including tumorigenesis,⁵ cell proliferation,^{6, 7} cell migration and adhesion,⁸ angiogenesis,^{9, 10} and intravasation/extravasation.¹¹

Towards the goal of discovering compounds that modulate interactions of uPAR and block invasion and metastasis *in vivo*, we used a docking-based virtual screening to identify small molecules that bind to uPAR. We describe the successful outcome of this search, and the initial biological evaluations of the two most promising structures from this effort.

Results

Structure-Based Virtual Screening by Docking Compounds to uPAR

Analysis of the three dimensional structure of uPAR revealed a hydrophobic pocket that accommodates the growth-factor domain (GFD) of its serine proteinase ligand uPA (PDB ID: 2FD6). The 300,000 compounds in the ChemDiv library were docked into the pocket using the computer program AutoDock4. The resulting complexes were scored with several scoring functions that include ChemScore,¹² Gold,¹³ AutoDock,¹⁴ and DOCK.¹⁵ The top ranking 200 compounds for each scoring function were visualized, and clustered by chemical similarity. About 50 compounds were selected among the top compounds from each scoring function. A total of 210 compounds were acquired from ChemDiv for biochemical evaluation.

Biochemical Assessment of Compound Binding to uPAR

The design of a fluorescent probe was guided by the three-dimensional structure of uPAR in complex with peptide AE147.¹⁶ As this peptide binds at the same site that is occupied by the growth factor-like domain of uPA, it provides the basis for the development of a competition assay to assess binding to this site. Fluorescein was introduced to the N-terminus of AE147. The resulting peptide, AE147-FAM, binds to uPAR with high affinity with an estimated K_D of 120 nM, and shows similar potency to the EGF-like domain of uPA (Fig. 1A).¹⁶ All 210 compounds were screened at an initial concentration of 50 μ M for displacement of AE147-FAM peptide. Eleven compounds gave polarization readings that were five times that of the standard deviation of the control peptide (Fig. 1B). Secondary concentration-dependent study for these eleven compounds was performed. Among them, two compounds (Scheme 1) showed concentration-dependent inhibition (Fig. 1C). The resulting inhibition curves were used to determine an inhibition constant, K_i , for each. The K_i value for (3R)-4-cyclohexyl-3-(hexahydrobenzo[d][1,3]dioxol-5-yl)-N-((hexahydrobenzo[d][1,3]dioxol-5-yl)methyl)butan-1-aminium **1** (IPR-1) was 18 μ M, while the K_i value for 4-(4-((3,5-dimethylcyclohexyl)carbamoyl)-2-(4-isopropylcyclohexyl)pyrazolidin-3-yl)piperidin-1-ium **3** (IPR-69) was 30 μ M (Fig. 1C).

Cellular Assays to Probe Effect on Metastasis

We tested whether the two active compounds affected invasion, adhesion and migration in cell culture in a concentration-dependent study (Fig. 2). For invasion, the Transwell (or Boyden) chamber assay was used.¹⁷ The assay uses a chamber with two compartments that are separated by a microporous membrane known as Matrigel consisting of components of the ECM such as collagen, laminin and fibronectin.¹⁷ Invasive tumor cells degrade the Matrigel and migrate through the membrane to the lower compartment. Instead of **1**, we tested a more soluble analog **2** (IPR-9). Compound **2** had an inhibition constant (20 μ M) similar to that of **1**. Both **2** and **3** substantially impaired MDA-MB-231 invasion (Fig. 2) with IC_{50} of 30 μ M for both compounds (Scheme 2).

To assess whether compounds inhibit cell migration, the same Boyden chamber apparatus is used except that the porous membrane is not coated with the Matrigel layer. Compounds **2** and **3** significantly blocked migration of MDA-MB-231 across the membrane (Fig 2A). The IC_{50} values (43 μ M for **2** and 40 μ M for **3**) were somewhat larger than the values observed for invasion (Fig. 2).

The effect of compounds on cell attachment (adhesion) to wells pre-coated with fibronectin was evaluated using an assay we have described previously.¹⁸ Similar assays have evaluated extensively uPAR-mediated cell attachment to ECM components mediated by integrin.¹⁹⁻²¹ Both **2** and **3** inhibited adhesion in a concentration-dependent manner with IC_{50} values of 45 μ M (Fig. 2).

Angiogenesis

The formation of new blood vessels from pre-existing ones is known as angiogenesis, which is vital for tumor growth, invasion, and metastasis. The effect of **2** and **3** on angiogenesis was assessed in human umbilical vein endothelial cells (HUVEC) in a Matrigel-based tube formation assay.²² The formation of these tubes is reminiscent of capillary formation in a tumor during angiogenesis. Both compounds inhibited tube formation in Matrigel in a concentration-dependent manner (Fig. 3A and 3B). As illustrated from the images in Fig. 3A and 3B, less tube formation is observed with increasing concentration of compound for both **2** and **3**. The extent of tube formation is quantified as shown by the histogram plots in Figs. 3A and 3B and afforded an estimation of IC_{50} . Interestingly, both **2** and **3** showed greater

potency in blocking angiogenesis by nearly an order of magnitude ($IC_{50} = 3 \mu M$) in comparison with invasion.

Effect on Cell Proliferation and Mechanism of Cell Killing

An MTT assay revealed that **2** and **3** inhibited cell proliferation with IC_{50} of 17 and 6 μM , respectively (Fig. 4A and 4C). To gain insight into the mechanism by which these compounds are inhibiting growth, a flow cytometry analysis with annexin V-FITC and PI staining was performed. The level of apoptotic cells in MDA-MB-231 was assessed by the percentage of Annexin V-positive/PI-negative cells present after exposure of MDA-MB-231 cells to increasing concentration of **3** for 24 h (Fig. 4D). At a concentration of 50 μM , **3** induced significant apoptosis as evidenced by a 51% and 21% increase in apoptotic cells (Annexin V positive/PI-negative) and necrotic cells (Annexin V-positive/PI-positive), respectively. A similar analysis for **2** indicated that the compound promoted cell death via both necrosis and apoptosis as illustrated in Fig. 4B. At a concentration of 50 μM , 26% of cells were necrotic (Annexin V positive/PI-positive), a 22% increase over control. 46% of cells were apoptotic (Annexin V positive/PI-negative), compared with 4% of control.

Compounds **2** and **3** Block MMP-9 Activity in MDA-MB-231 cells

To degrade collagen within the ECM, malignant cells unleash a series of extracellular proteases known as matrix metalloproteinases (MMPs). Inhibition of MMP activity with small molecules has been a cornerstone of efforts to develop drugs that block tumor invasion and metastasis.²³ The gelatinases (MMP-2 and MMP-9) in particular have been strongly associated with invasion and metastasis. We assessed whether **2** and **3** impaired MMP-9 (gelatinase B) activity. When exposed to **2** and **3** at increasing concentration, MDA-MB-231 cells showed a concentration-dependent reduction in MMP-9 activity (Fig. 5A and 5B). IC_{50} for inhibition of MMP-9 activity by **2** and **3** were estimated at 25 μM , which corresponded well with the IC_{50} for inhibition of invasion that were observed from the Boyden chamber study. At 50 μM , **3** nearly completely abrogated MMP-9 activity with 93% inhibition of MMP-9 activity.

Cell Signaling

In light of the previously reported role of uPAR in signaling, the effect of **1**, **2**, and **3** on signaling was studied by Western blot analysis. Following exposure of the compounds at a concentration of 100 μM to MDA-MB-231 cells for 30 minutes, immunoblotting revealed significant impairment of MAPK phosphorylation by **2** and **3** (Fig. 6A). Compound **3** also abrogated phosphorylation, but the effects were weaker than **2** and its parent compound (Fig. 6A). A concentration-dependent study was subsequently carried out for **1** (Fig. 6B). The results confirmed that the compound completely abrogated phosphorylation of MAPK, with an IC_{50} estimated at approximately 20 μM . The effect of the compound was also studied in two other signaling pathways including FAK and Src. FAK is constitutively associated with β -integrin subunits of integrin receptors. The compound did not show any significant effect on FAK phosphorylation compared with DMSO. Even less effect was observed on phosphorylation of Src, which is upstream of MAPK (Fig. 6B). This suggests that **1** is unlikely to impair integrin signaling. The effect of the compounds on MAPK and the lack of effect on FAK and Src signaling suggest that the compounds are not promiscuous and their effects on metastasis can be attributed to the targeting of specific signaling pathways.

Synthesis of **2** and **3**

The synthesis of N-1 substituted pyrazole **3** followed the route developed by scientists at Abbott Laboratories. The pyrazole core was prepared by condensation of 1,3-dicarbonyl enol ethers with a variety of hydrazines. The yield was good to excellent in all cases.²⁴

Thus, commercially available N-Boc isonipecotic acid **4** was converted to known β -keto ester **6** through a simple two-step sequence (coupling of Meldrum's acid followed by ethanolysis).²⁵ Alternatively, condensation of the acid chloride of **6** with potassium ethyl malonate in the presence of a magnesium chloride-triethylamine base system also gave **7**.²⁶ With the requisite β -keto ester **7** in hand, the formation of pyrazole core was explored with commercially available 4-isopropyl phenylhydrazine. The amide bond formation followed by the removal of N-Boc gave the desired N-1 substituted pyrazole **3**.

The synthesis of a secondary benzyl amine **2**, used 1,4-conjugate addition and reductive amination. Thus, Knoevenagel condensation between commercially available phenylacetaldehyde and diethyl malonate using a catalytic amount of piperidine and acetic acid in refluxing toluene gave **11**, then subjected to the conjugate addition conditions developed by Bosch, where excess of CuCl promoted an efficient conjugate addition of Grignard reagents. With the desired Michael adduct **12** to aldehyde **15** was achieved by a sequence of conventional functional group manipulation; hydrolysis, decarboxylation, reduction, and oxidation. The reductive amination between aldehyde **15** and commercially available 4-(dimethylamino)benzylamine dihydrochloride gave the desired secondary amine **2** as a racemate.

***In vitro* Studies for Insights into PK Properties of 3**

Inhibition of the hERG channel is undesirable. Checking for hERG blockage is an integral step in the drug discovery cycle. We employ a method that is well-suited for high-throughput measurements based on fluorescence polarization (FP) as implemented in the Predictor Assay by Invitrogen (Carlsbad, CA). This assay is based on the principle that a fluorescent tracer upon binding to the hERG channel will induce an increase in the FP signal that will diminish when an inhibitor displaces the interaction between the tracer and the hERG channel. Data generated with this assay was shown to correlate well with patch-clamp assays measurements.²⁷ We performed FP measurements for **2** and **3** using an EnVision (PerkinElmer) plate reader at an excitation of 540 nm and emission at 573 nm. As shown in Fig. 7A, **1** and its derivative blocked the channel at all three concentrations considered (1, 10 and 25 μ M). In fact, these two compounds showed even greater potency than the well-known channel blocker E-4031.²⁸ Compound **3**, on the other hand, exhibited lower levels of hERG blockage. At the highest concentration of 25 μ M, which is near its biological activity in tumor cells, 60% blockage is detected.

Another source of toxicity for drugs is cytochrome P450s (CYPs) inhibition. CYPs detoxify harmful compounds and catalyze key reactions in the formation of endogenous compounds such as hormones and steroids.²⁹ Studies have shown that 90% of drugs are metabolized by at least one of the seven known CYP isoforms (CYP-1A2, 2C9, 2C18, 2C19, 2D6, 2E1 and 3A4). We assayed CYPs metabolism using a fluorescence-based assay,³⁰ recently implemented by Invitrogen (Carlsbad, CA). Compounds **1**, **2** and **3** were evaluated for inhibition of CYP2C9. As a positive control, the CYP2C9 inhibitor sulphaphenazole inhibited the metabolism of the fluorescent substrate by ~69% at 5 μ M (Fig. 7B). Little inhibition was observed for **1** and **2** at 1 μ M, but more significant inhibition at concentrations that are comparable to their biological activity. At 25 μ M, these compounds inhibit the enzyme by nearly 80%, comparable to the levels seen for sulphaphenazole, suggesting potential toxicity *in vivo*. Compound **3**, on the other hand revealed little inhibition of CYP2C9 (Fig. 7B). Even at 25 μ M, only 20% inhibition is detected, comparable to what we have observed for FDA-approved drugs.³¹

Finally, we assessed DNA binding by these three compounds. DNA binding is often a reflection of non-specificity, which can lead to adverse side effects. We employ a well-established Fluorescent Intercalator Displacement assay³² that measures displacement of

ethidium bromide, a known intercalator of DNA. In this assay, ethidium bromide (EB) bound to the salmon sperm DNA. DNA binding was not strongly affected at the three concentrations of **1**, **2**, and **3** that were tested. A 30% displacement of ethidium bromide was observed for **1** and **2**, while **3** showed less than 10% displacement at 25 μ M (Fig. 7C).

***In vivo* PK Study of 3**

Compound **3** was administered to mice via a single oral gavage of 50 mg/kg using a formulation of 0.5% (w/v) of methylcellulose and 0.1% of tween-20. Blood (20 μ l) was taken from the mice at their tails at time intervals of 1, 2, 4, 12, 24, 36, and 48 hours (two time points from each of 22 mice³³) post injection. Blood plasma samples were prepared by centrifugation for quantification and HPLC-MS/MS analysis. Compound **3** was quantified as shown in Fig. 7D and the resulting PK parameters are provided in Fig. 7E. Compound **3** was detected in plasma at a maximum level of 40 μ M at $\sim$ 5 hours after administration. A half-life was estimated at 2 hours. These parameters suggest that **3** possesses suitable drug-like properties *in vivo* and sets the stage for further optimization of the potency of the compound.

Role of 3 in MDA-MB-231 Metastasis *in vivo*

The effect of **3** in blocking metastasis and growth in cell culture, along with its favorable *in vivo* pharmacokinetic properties prompted evaluation of its effect on metastasis *in vivo*. TMD-231 cells were inoculated into the mammary fat pad of female NOD/SCID mice. Dosing was initiated at day 18 post implantation. Animals were randomized and treated with vehicle or with **3** by daily oral gavage at a dose of 150 mg/kg (n = 11). Tumor volumes were determined by caliper measurements on a twice weekly basis, and calculated according to the formula $(\alpha^2 \times \beta)/2$, where α is the shorter and β is the longer of the two dimensions. Tumor volumes were determined by caliper measurements on a weekly basis. The study was conducted over a period of 68 days. The primary tumor in both control and treated mice grew substantially over the course of the study. Tumor volumes reached nearly 965 mm³ for untreated mice. For treated mice, tumor volumes reached an average of 779 mm³ at the end of the study, a 20% reduction (Fig. 8A).

Over the course of the study, several mice were sacrificed to determine whether breast tumor cells had metastasized to the lungs. At the end of the study, control and experimental animals were sacrificed and organs (lungs) were removed and evaluated for the presence of tumors. The number and size of metastasis in two to five fields per sample were calculated. A score of 4+ was given to a sample with highest metastasis index and relative metastasis in other samples are calculated (i.e., 1+, 2+, 3+) by a sample-blinded pathologist. In the untreated mice, 9 out of 11 mice had metastatic foci in the lungs (Score = 0). In contrast, only 3 out of 11 treated mice showed signs of metastatic lesions. The extent of metastasis in treated versus untreated was also different. Fewer of the treated mice developed substantial metastasis with a score >2 . For example, 5 of the untreated mice exhibited increased metastatic lesions (score >2), compared with only 3 of the treated mice (Fig. 8B). Finally, more of the untreated mice developed metastatic lesions with a score of 4 or greater (Fig. 8B). An illustration of the extent of metastasis to the lungs is shown in Fig. 8C with H&E staining images for control and treated mice.

Discussion

Throughout the metastatic process, malignant cells unleash a series of proteases that systematically degrade components of the ECM, not only to eventually gain access to the vasculature (extravasation), but also following attachment to new sites to create new colonies (intravasation). When a distant colony is established during metastasis, a constant supply of nutrients is needed to sustain the level of growth required for the tumor to

establish itself and begin the process of metastasis anew. This process, known as angiogenesis, also requires the contribution of proteases. uPAR plays an important role in these processes, not only by anchoring proteases at the cell surface, but also by engaging other receptors at the cell surface. Using virtual screening, a chemical library of nearly 300,000 molecules was screened for compounds that bind to uPAR. We identified active compounds by computation that were then shown experimentally to bind directly to uPAR.

Cellular studies revealed that two of these compounds, **2** and **3**, blocked invasion, migration, and adhesion of MDA-MB-231 breast cancer cells in cell culture. Both these compounds also impaired angiogenesis in a tube formation assays using HUVECs with an IC_{50} nearly an order of magnitude better ($IC_{50} = 3 \mu M$) than those observed in the invasion studies. Gel zymography analysis revealed that the compounds impaired MMP-9 activity in a concentration-dependent manner with IC_{50} that are comparable to those measured in the invasion studies. This was encouraging, since previous studies have shown that uPA promotes degradation of the ECM through activation of MMP-9 activity. Compounds **2** and **3** inhibited MDA-MB-231 proliferation ($IC_{50} = 17$ and $6 \mu M$, respectively), and subsequent flow cytometry analysis with annexin V staining revealed that both induced apoptosis, while **2** also caused significant necrosis. Signaling studies showed that both compounds impaired MAPK phosphorylation, but **1** was more potent. Compound **1** did not show any effect on FAK and Src signaling, suggesting that these compounds are unlikely affecting integrin-mediated signaling. Past studies have shown that uPAR can enhance signaling through integrins. The lack of effect of **1** on integrin signaling suggests that the uPA binding site is unlikely contributing to integrin signaling and that other sites on the receptor, such as the vitronectin binding site, are responsible for interaction with integrins.

A series of biochemical assays that included DNA binding, cytochrome P450 inhibition, and hERG K⁺ channel blockage provided insight into the drug-likeness properties of the compounds. Compound **3** exhibited the most favorable properties, showing no DNA binding, little inhibition of CYP2C9, and lower levels of hERG channel blockage. In contrast, **1** and its derivative **2**, showed significant impairment of CYP2C9 activity and even greater hERG K⁺ channel blockage than the well-known channel blocker E-4031. These results led us to focus on **3** and assess its PK properties *in vivo*. The compound was orally bioavailable, reaching concentrations of up to $40 \mu M$ with half-life of approximately 2 hours when administered. The compound was well tolerated at doses as high as 150 mg/kg. These promising results prompted efficacy studies to evaluate its anti-metastatic effect *in vivo* using a triple-negative TMD-231 (MDA-MB-231 variant) implanted in breast mammary fat pads. It was interesting that treated mice developed primary tumors that were on average 20% lower in size than untreated mice. In addition, the treated mice showed less metastasis to the lungs when compared with the untreated group.

It is likely that some of the anti-cancer effects of **3** are enhanced by off-targets. Given the effects of **3** on invasion and angiogenesis, we have explored the possibility that the compound may bind and inhibit MMP-9 or the vascular endothelial factor receptor (VEGFR), key mediators of invasion and angiogenesis. The compound was docked to the active site of MMP-9 and VEGFR2 (PDB codes 1GKC and 3EWH, respectively) using the Vina docking program. The resulting complexes along with the complex of **3** bound to uPAR were scored with the X-score scoring function, which we have recently shown to perform best in rank-ordering complexes. The resulting scores were -9.2, -9.7, and -9.7 for uPAR, MMP-9 and VEGFR2, respectively. While conclusive evidence will have to be obtained from biochemical studies, the similarity of these scores suggests that either MMP-9 or VEGFR2 may also be targets of the compound.

Cancer is a systems biology disease that involves multiple signaling pathways. Compounds with polypharmacology that target more than one pathway may lead to cancer therapeutics with greater efficacy. Therefore, off-targets that enhance the anti-cancer properties of a compound may be welcome, as long as they do not cause undue toxicity. Compound **3** seems to be well tolerated even at concentrations of 150 mg/kg and caused little toxicity, suggesting that off-targets are unlikely causing toxicity. The promising PK properties and the encouraging effects on tumor growth and metastasis *in vivo* suggest **3** as an excellent platform upon which to develop derivatives with greater efficacy *in vivo*.

Materials and Methods

Fluorescence Polarization Assay

Our fluorescence polarization assay used fluorescent AE147-FAM peptide. uPAR was titrated against the fluorescent AE147-FAM peptide and data were fit to a sigmoidal dose-dependent curve as the FP value increases to determine the K_d of binding using Sigmaplot (Systat Software Inc., CA). Inhibitor screens were carried out in triplicates using 500 nM uPAR, 100 nM AE147-FAM, and inhibitor concentrations ranging from 0.78 μ M to 100 μ M in 50 μ L volumes in black BD Falcon 384-well microplate. The compounds were serially diluted in DMSO then diluted in to 0.01% Triton X-100 in 1 $\times$ PBS buffer ensuring a final concentration of 2% DMSO (a concentration that did not affect peptide binding to uPAR). Polarized fluorescence intensities were measured immediately following addition of inhibitors to the protein-peptide mix at room temperature on an EnVision® Multilabel Plate Readers (PerkinElmer) with excitation and emission wavelengths of 485 and 530 nm, respectively.

Proliferation Assay

The procedure consisted of culturing cells at 37°C in 10% FBS-DMEM medium containing various amounts of compound. 5 mM compound stock in 100 % DMSO was 1:50 diluted in medium, filtered, and serially diluted in 96-well plate. Then seeded cells were incubated for 3 days. Viable cells were quantified by MTT assay at absorbance of 570 and 630 nm.

Invasion and Migration Assays

This was performed using BD BioCoat Matrigel Invasion Chamber (cat. 354480, BD Biosciences, Bedford, MA). The undersurface of the inserts were coated with 30 μ g/ml of fibronectin (Sigma, F2006) in PBS at 4 °C overnight. The inserts were washed with PBS once. 0.5 ml of serum-free medium was separately added to the upper and lower chambers to equilibrate the Matrigel invasion chambers at 37 °C, 5% CO₂ for 2 hours. After starvation with serum-free DMEM for 4 hours, subconfluent MDA-MB-231 cells were trypsinized and resuspended in 0.1% FBS DMEM. 5×10^4 cells in 500 μ l of 0.1% FBS DMEM containing various compounds or 1.0% DMSO (as control) were added to the upper chambers. 500 μ l of 10% FBS DMEM containing the same concentration of the same compound or DMSO were added to the lower chambers. We incubated the invasion chambers for 3 hours at 37 °C, 5% CO₂. Non-invaded cells were removed from the upper chamber with a cotton swab. The invaded cells were fixed with 100% methanol and then stained with Hematoxylin Stain Harris Modified Method (Fisher, SH30-500D). We washed the filters with water 3 times. Filters were air dried and the invaded cells were counted in ten randomly selected microscopic fields ($\times 200$ magnification). The experiment was performed in triplicate per group and shown by mean $\pm$ SE.

Gelatin Zymography

MDA-MB-231 cells were treated with different concentrations of uPAR compounds in serum free medium for 24h, the conditioned medium were collected, concentrated by Amicon Ultra centrifugal filter units (Millipore, #UFC500324), proteins were normalized and electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (10%) containing 1 mg/ml gelatin. After electrophoresis, the gel was washed twice in 50 mM Tris-HCl (pH 7.6) containing 5 mM CaCl₂ and 2.5% Triton X-100 for 30 minutes at room temperature and incubated in buffer that contained 50 mM Tris-HCl (pH 7.6), 200 mM NaCl, 10 mM CaCl₂ at 37°C for 36h. Then, the gels were stained with 0.05% Coomassie brilliant blue (CBB) and destained with 30% methanol in 10% acetic acid. Areas of gelatinolytic degradation appeared as transparent bands on the blue stained background of the gel. Data were quantified using Image J.

Adhesion Assay

Quantitative cell adhesion assays were carried out in non-tissue culture treated 96-well microtiter plates (Evergreen Scientific, Los Angeles, CA) which were coated with 15 µg/ml fibronectin (Sigma) for 1 h at room temperature. Fibronectin coated and uncoated control wells were blocked for 1 h with 3% heat-denatured bovine serum albumin (BSA) at 37°C. Cells were split 1 day prior to the experiment to achieve a subconfluent culture. Briefly, MDA-MB-231 cells were collected with trypsin, quenched with soybean trypsin inhibitor (Calbiochem), washed twice with serum-free medium, and 2×10^4 cells in 100 µl serum-free medium containing various compounds were added to each well, quadruplicate per group and incubated for 90 min at 37°C. The wells were washed and the number of adherent cells was quantified by crystal violet staining at 570 nm.^{34, 35} The results were shown by means $\pm$ SE.

Angiogenesis Assay

Matrigel assays were performed as previously described with minor modifications.³⁶ Early-passage (2-3) cord blood-derived endothelial cells were seeded onto 96-well tissue culture plates coated with 40 µL Matrigel (BD Biosciences) at a cell density of 7500 cells per well. Cells were observed every 2 hours by visual microscopy with an inverted microscope at 40 $\times$ magnification for capillary-like formation. The percentage of the tube formation to the vehicle control group was calculated for each compound treated group.

Cloning, Expression and Purification of uPAR

We have successfully cloned, expressed and purified uPAR. From 1 L of culture, we express nearly 12 mg of protein, which will be sufficient to conduct the experiments that we propose in this study. Briefly, a truncated, soluble form of human uPAR (suPAR, amino acids 1 – 283) was expressed in stably transfected *Drosophila* S2 cells using the *Drosophila* Expression System (Invitrogen). suPAR was obtained by a two-step purification process. The conditioned culture medium was filtered (0.45 µm) and loaded onto a DEAE Sephadex column equilibrated with 20 mM Tris (pH 7.4). The protein was eluted with a gradient of 0 – 1 M NaCl in 20 mM Tris (pH 7.4). suPAR containing fractions were then pooled, concentrated, and filtered prior to RP-HPLC using a semi-preparative (10 $\times$ 25 cm) C8 column. A 1 ml aliquot of the concentrated protein was loaded onto the column at initial gradient conditions of 95% eluent A and 5% eluent B, where eluent A was 0.1% TFA/100% H₂O and eluent B was 70:30 acetonitrile/0.00085 MeCN/H₂O/TFA. The protein elutes using a gradient of 5% eluent A to 95% eluent B over 46 minutes at a flow rate of 4 ml/min. Under these conditions, the protein eluted as a single sharp peak (t_R = 29 min). SDS page analysis of the uPAR containing fractions showed a single band at the expected mass of 60 kDa.

Surface plasmon resonance confirmed that the purified uPAR was capable of binding to uPA. The identity of the 60 kDa band was established by immunoblot (not shown).

Apoptosis Assay

MDA-MB-231 cells were cultured in DMEM medium supplemented with 10% FBS, 100 IU/ml penicillin and 50 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C and grew to 80 – 90% confluence in P-60 mm plates. Then treated with various amounts of compounds or 1% DMSO (as control) for different time points. Collected the supernatants and washed the cells with 1× PBS twice and collected. The cells were detached by using cellstripper (Mediatech Inc, VA), and washed twice with cold 1× PBS and resuspended in 1× binding buffer (Cat. PNN1001, Invitrogen Corporation, Camarillo, CA) at a concentration of 1× 10⁶ cells / ml. Transferred 100 µl of the solution (1× 10⁵ cells) to a 5 ml culture tube. Added 5 µl of Recombinant Human Annexin V FITC conjugate (Cat. ANNEXINV01, Invitrogen) and 5 µl of propidium iodide solution (Cat. P4864, Sigma-Aldrich, St. Louis, MO). Gently vortexed the cells and incubated for 15 min at room (25 °C) in the dark. Added 400 µl of 1 × binding buffer to each tube. Analyzed by flow cytometry (FACScan).

Immunoblots

MDA-MB-231 cells treated with 100 µM of compound(s) and 1% DMSO for 30 min. Harvested cell lysates in RIPA buffer were supplemented with protease inhibitors and phosphatase inhibitors. Under non-reducing condition equal amounts of protein were loaded per lane and separated in 4-12% NuPAGE gel (Invitrogen) at 200 V for 60 minutes, then transferred to nitrocellulose membrane at 30 V for one hour using XCell II blot module. For immunoblotting, the first antibody was Phosph-p44/42 MAPK (Thr202/Tyr204) (D13.14.4E) rabbit mAb (#4370, Cell Signaling Technology, Inc., Danvers, MA) 1:2000 or P44/42 MAP Kinase (137F5) rabbit mAb (#4695, Cell Signaling Technology, Inc., Danvers, MA) 1:500 in 5% non-fat milk-TBST at 4 °C overnight. Secondary antibody consisted of goat anti-rabbit IgG HRP 1:3000 in 5% non-fat milk-TBST at room for 1 hour. The protein bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (WBKLS0100, Millipore, Billerica, MA).

Fibronectin (Sigma, F2006) 30 µg/ml was coated in 6-well plates overnight. MDA-MB-231 serum-starved for 4 hours and then seeded in FN coated 6-well plates and treated with 5, 10, 20 and 40 µM compound(s) and 1% DMSO for 30 min, cells were lysated in RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. Then immunoblotting with phospho-p44/42 MAPK (Thr202/Tyr204), p44/42 MAPK; phospho-FAK (Tyr397), FAK; phospho-Src family (Tyr416), Src, was carried respectively.

Synthesis

All chemicals were purchased from either Aldrich or Acros and used as received. Column chromatography was carried out with silica gel (25-63 µ). Mass spectra were measured on an Agilent 6520 Accurate Mass Q-TOF instrument. ¹H NMR spectra were recorded in CDCl₃ or Methanol-d₄ on a Bruker 500 MHz spectrometer. Chemical shifts are reported using residual CHCl₃ or MeOH as internal references. All compounds that were evaluated in biological assays had >95% purity using HPLC.

1-(tert-butoxycarbonyl)piperidine-4-carboxylic acid (4)—To a stirred solution of isonipecotic acid (77.4 mmol, 10.0 g) and potassium carbonate (154.8 mmol, 21.4 g) in water (150 mL) at 0°C, was added dropwise a solution of di-*t*-butyldicarbonate (77.4 mmol, 16.9 g) in THF (150 mL). The reaction mixture was gradually warmed to r.t. and stirred overnight. The solvents were evaporated and the residue was dissolved in DCM. DCM layer

was washed with 1N HCl (3×100 mL), water, dried over sodium sulfate, and concentrated in vacuo to give pure **4** (13.03 g, 75%) as a white powder. ^1H NMR (500 MHz, CDCl_3) δ 4.02 (br s, 2H), 2.85 (t, $J = 11.5$ Hz, 2H), 2.49 (m, 1H), 1.90 (d, $J = 11.5$ Hz, 2H), 1.65 (m, 2H), 1.45 (s, 9H); ^{13}C NMR (125 MHz, CDCl_3) δ 180.1, 154.7, 79.7, 40.7, 28.3, 27.6. MS calc'd for $\text{C}_{11}\text{H}_{18}\text{NO}_4$ ($\text{M}-\text{H}$) $^-$ 228.1241, found 228.1240.

tert-butyl-4-(2,2-dimethyl-4,6-dioxo-1,3-dioxane-5-carbonyl)piperidine-1-carboxylate (5)—To a stirred solution of **4** (56.8 mmol, 13.03 g) and DMAP (5.68 mmol, 694 mg) in DCM (10 mL) at 0°C , were added DCC (62.5 mmol, 12.9 g) and 2,2-dimethyl-1,3-dioxane-4,6-dione (62.5 mmol, 9.00 g) sequentially. The reaction mixture was gradually warmed to r.t. and stirred overnight. Reaction was filtered and washed with DCM. The resultant orange solution was concentrated in vacuo. Product was used directly without isolation.

tert-butyl-4-(3-ethoxy-3-oxopropanoyl)piperidine-1-carboxylate (6)—To **5**, was added abs. ethanol (200 mL) and the solution was refluxed for 48h. The solution was concentrated in vacuo and purified by flash chromatography (DCM) to give **3** as a reddish oil (14.36 g, 85%). ^1H NMR (500 MHz, CDCl_3) δ 12.09 (s, 0.14H, enol OH), 4.89 (s, 0.14H enol C-H), 4.13 (q, $J = 7$ Hz, 2H), 4.10-3.96 (m, 2H), 3.42 (s, 2H), 2.81-2.67 (m, 2H), 2.62-2.52 (m, 1H), 1.85-1.71 (m, 2H), 1.55-1.43 (m, 2H), 1.39 (s, 9H), 1.21 (t, $J = 7$ Hz, 3H); ^{13}C NMR (125 MHz, CDCl_3) δ 204.0, 180.2 (enol), 172.7 (enol), 167.0, 154.4, 87.52, 79.51, 61.3, 48.5, 47.1, 28.2, 27.1, 13.9; $R_f = 0.2$ (DCM). HRMS calc'd for $\text{C}_{15}\text{H}_{26}\text{NO}_5$ ($\text{M}+\text{H}$) $^+$ 300.1805, found 300.1808.

(E,Z)-tert-butyl-4-(3-ethoxy-2(ethoxycarbonyl)acryloyl)piperidine-1-carboxylate (7)—Under argon, **6** (47.9 mmol, 14.36 g), triethyl orthoformate (143.7 mmol, 24 mL), and acetic anhydride (95.8, 9 mL) were mixed and refluxed at 100°C for 48h. Low-boiling impurities were evaporated off and the crude product was purified by flash chromatography (DCM) to give **7**, as a yellow-colored oil (14.92 g, 88%). $R_f = 0.22$ (1% MeOH/DCM). ^1H NMR (500 MHz, CDCl_3) δ 7.59 (s, 0.54 H, minor), 7.52 (s, 1H, major), 4.24 (q, $J = 7.1$ Hz, 2H), 4.21-4.09 (m, 7H), 4.08-3.92 (m, 4H), 3.09-3.01 (m, 0.58H, minor), 2.95-2.87 (m, 1H, major), 2.83-2.67 (m, 4H), 1.85-1.67 (m, 4H), 1.58-1.47 (m, 4H), 1.41 (s, 19H), 1.37-1.26 (m, 9H), 1.23 (t, $J = 7.1$ Hz, 6H); ^{13}C NMR (125 MHz, CDCl_3) δ major isomer: 201.7, 165.7, 165.3, 162.3, 154.61, 112.6, 79.3, 72.2, 60.5, 48.0, 45.4, 28.3, 27.2, 15.2, 14.2; minor isomer 199.6, 165.2, 154.59, 112.9, 72.7, 60.7, 28.0, 15.1, 14.1; MS calc'd for $\text{C}_{18}\text{H}_{30}\text{NO}_6$ ($\text{M}+\text{H}$) $^+$ 356.2068, found 356.2067.

tert-butyl-4-(4(ethoxycarbonyl)-1-(4-isopropylphenyl)-1H-pyrazol-5-yl)piperidine-1-carboxylate (8)—Free hydrazine was prepared from HCl salt by washing with saturated sodium bicarbonate solution and extracting with DCM. DCM was removed in vacuo. To a stirred solution of free 4-isopropyl phenyl hydrazine (21.7 mmol, 3.26 g) in abs. ethanol (100 mL), was added **7** (19.7 mmol, 7.00 g) in abs. ethanol (100 mL). The reaction was refluxed at 100°C for 48h. Ethanol was removed in vacuo and the crude reddish-brown residue was purified by flash chromatography (1% MeOH/DCM) to give **5** (6.27 g, 72%) as a reddish-brown oil. $R_f = 0.22$ (1% MeOH/DCM) ^1H NMR (500 MHz, CDCl_3) δ 8.00 (s, 1H), 7.33 (d, $J = 10$ Hz, 2H), 7.22 (d, $J = 10$ Hz, 2H), 4.29 (q, $J = 7.1$ Hz, 2H), 4.13-4.01 (m, 2H), 3.14-3.05 (m, 1H), 3.03-2.94 (m, 1H), 2.66-2.51 (m, 2H), 2.31-2.20 (m, 2H), 1.50-1.30 (m, 2H), 1.43 (s, 9H), 1.35 (t, $J = 7$ Hz, 3H), 1.28 (d, $J = 7$ Hz, 6H); ^{13}C NMR (125 MHz, CDCl_3) δ 163.2, 154.7, 150.3, 149.6, 142.8, 137.0, 127.3, 126.3, 112.0, 79.3, 60.0, 47.2, 35.1, 33.8, 28.4, 27.2, 23.8, 14.3; .

5-(1-(tert-butoxycarbonyl)piperidin-4-yl)-1-(4-isopropylphenyl)-1H-pyrazole-4-carboxylic acid (9)—To a stirred solution of **8** (14.2 mmol, 6.27 g) in 95% ethanol (35 mL), was added a 2.0 M NaOH solution (35 mL). The reaction mixture was refluxed at 70°C for 20h. Ethanol was removed in vacuo and the resulting solid was acidified to pH 2 at 0°C using 1M HCl. The reddish-brown solid was filtered off and washed with cold water to give **9** (3.98 g, 68%). ¹H NMR (500 MHz, CDCl₃) δ 8.07 (s, 1H), 7.40-7.20 (m, 4H), 4.32-3.98 (m, 2H), 3.14 (app t, J = 12 Hz, 1H), 3.05-2.94 (m, 1H), 2.71-2.52 (m, 2H), 2.37-2.20 (m, 2H), 1.65-1.52 (m, 3H), 1.47 (s, 9H), 1.31 (d, J = 7 Hz, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 168.2, 154.8, 150.6, 150.4, 143.5, 136.8, 127.3, 126.3, 111.1, 79.5, 35.1, 33.8, 28.5, 28.4, 27.3, 23.8. HRMS calc'd for C₂₃H₃₂N₃O₄ (M+H)⁺ 414.2387, found 414.2385.

tert-butyl-4-(4-((3,5-dimethylphenyl)carbamoyl)-1-(4-isopropylphenyl)-1H-pyrazol-5-yl)piperidine-1-carboxylate (10)—To a stirred solution of **9** (3.05 mmol, 1.26 g) and DMAP (0.30 mmol, 37 mg) in DCM (10 mL) at 0°C, was added DCC (3.35 mmol, 691 mg) and 3,5-dimethyl aniline (3.35 mmol, 483 mg) sequentially. The reaction was gradually warmed to r.t., and ran for 48h. DCM was removed in vacuo and the crude mixture was purified by flash chromatography (30% EA/Hex) to give **10** (745 mg, 47%) as a brownish solid. ¹H NMR (500 MHz, CDCl₃) δ 7.85 (s, 1H), 7.69 (s, 1H), 7.33 (d, J = 8.3 Hz, 2H), 7.24 (d, J = 8.3 Hz, 2H), 7.20 (s, 1H), 6.76 (s, 1H), 4.25-3.97 (m, 2H), 3.16 (app t, J = 12 Hz, 1H), 3.00 (app p, J = 7 Hz, 1H), 2.71-2.48 (m, 2H), 2.30 (s, 6H), 2.26-2.17 (m, 2H), 1.67-1.56 (m, 2H), 1.42 (s, 9H), 1.29 (d, J = 7 Hz, 6H). ¹³C NMR (125 MHz, CDCl₃) δ 161.6, 154.8, 150.42, 148.9, 138.8, 138.5, 137.7, 137.2, 127.3, 126.5, 126.1, 117.9, 115.9, 79.3, 35.1, 33.9, 30.3, 29.4, 28.4, 23.9, 21.4; *R*_f = 0.33 (30% EA/Hex). MS calc'd for C₃₁H₃₉N₄O₃ (M-H)⁻ 515.3028, found 515.3030.

N-(3,5-dimethylphenyl)-1-(4-isopropylphenyl)-5-(piperidin-4-yl)-1H-pyrazole-4-carboxamide (3)—To a stirred solution of **10** (1.44 mmol, 745 mg) in DCM (5 mL) at 0°C, was added TFA (5 mL). The reaction mixture was warmed to r.t. and stirred for 1h. The solvents were removed in vacuo. The organic residue was re-dissolved in DCM. The organic layer was washed with sat. sodium bicarbonate, brine, and dried over MgSO₄. The solvent was removed in vacuo to yield **3** (590 mg, 98%) as a brown solid. ¹H NMR (500 MHz, CDCl₃) δ 7.86 (s, 1H), 7.49 (s, 1H), 7.38 (d, J = 8.3 Hz, 2H), 7.25 (d, J = 8.3 Hz, 2H), 6.79 (s, 1H), 3.34-3.27 (m, 2H), 3.26-3.19 (m, 1H), 3.09-2.95 (m, 1H), 2.72 (app t, J = 13.2 Hz, 2H), 2.46 (qd, J = 13, 9.7, 3.7 Hz, 2H), 2.32 (s, 6H), 1.74 (d, J = 15 Hz, 2H), 1.32 (d, J = 7 Hz, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 161.8, 150.3, 149.1, 138.73, 138.69, 137.8, 137.0, 127.4, 126.2, 126.1, 118.0, 115.8, 46.2, 34.9, 33.9, 30.2, 23.8, 21.4. MS calc'd for C₂₆H₃₃N₄O (M+H)⁺ 417.2649, found 417.2646.

diethyl 2-(benzo[d][1,3]dioxol-5-ylmethylene)malonate (11)—To a solution of diethyl malonate (31.2 mmol, 5.0 g) and piperonal (37.4, 5.61 g) in toluene (80 mL), was added piperidine (3.12 mmol, 0.308 mL) followed by acetic acid (3.12 mmol, 0.179 mL). The reaction was refluxed at 150°C under Dean-Stark conditions for 24h. The reaction mixture was purified by flash chromatography (20% EA/Hex) to give an inseparable mixture of **8** and aldehyde (3.83 g, 42%) as a clear oil. TLC *R*_f = 0.31 (20% EA/Hex). ¹H NMR showed **11** constituted 86% of the mixture (3.29 g, 36%). ¹H NMR (500 MHz, CDCl₃) δ 7.61 (s, 1H), 7.01-6.98 (m, 1H), 6.96-6.94 (m, 1H), 6.82-6.78 (m, 1H), 6.00 (s, 2H), 4.36 (q, J = 7.1 Hz, 2H), 4.28 (q, J = 7.1 Hz, 2H), 1.32 (2t, J = 7.2 Hz, 6H); ¹³C NMR (126 MHz, CDCl₃) δ 190.2, 166.9, 164.2, 149.8, 148.2, 141.6, 128.5, 126.9, 126.1, 124.0, 108.5, 108.3, 108.3, 106.8, 102.0, 101.6, 61.6, 61.4, 14.1, 13.8. HRMS calc'd for C₁₅H₁₇O₆ (M+H)⁺ 293.1020, found 293.1025.

(+) Diethyl 2-(1-(benzo[d][1,3]dioxol-5-yl)-2-phenylethyl)malonate (12)—Under argon, **11** (9.61 mmol, 2.81 g) in dry Et₂O (30 mL) was added slowly via cannula to a suspension of CuCl (0.481 mmol, 48 mg) and benzyl magnesium chloride (11.5 mmol, 5.75 mL) at -78°C. The mixture was stirred while gradually raising the temperature to r.t. overnight. Sat. NH₄Cl was added. The aqueous layer was extracted with Et₂O (3x). The combined organic extracts were washed with brine and dried over MgSO₄. The crude residue was purified by flash chromatography (10% EA/Hex) to give **12** (2.26 g, 98%) as a yellowish oil. The product was directly used for the following step without further purification. MS calc'd for C₂₂H₂₅O₆ (M+H)⁺ 385.1646, found 385.1654

(+) 3-(benzo[d][1,3]dioxol-5-yl)-4-phenylbutanoic acid (13)—To a stirred solution of **12** (5.37 mmol, 2.06 g) in MeOH/H₂O (1:1, 40 mL) was added a 10% KOH solution (40 mL) in one portion. The reaction mixture was stirred at r.t. for 24h. MeOH was removed in vacuo and the water layer was acidified to pH 2 with 6N HCl at 0°C. The precipitate was collected by vacuum filtration. The crude solid was dissolved in *p*-xylene (30 mL) and refluxed overnight at 170°C. The crude mixture was purified by flash chromatography (10% EA/Hex) to yield **13** (1.42 g, 93%) as a white solid. TLC *R_f* = 0.17 (10% EA/Hex). ¹H NMR (500 MHz, CDCl₃) δ 7.28-7.14 (m, 3H), 7.09-7.03 (m, 2H), 6.73-6.65 (m, 2H), 6.61-6.55 (m, 1H), 5.92 (s, 2H), 3.38-3.28 (m, 1H), 2.91-2.82 (m, 2H), 2.70-2.54 (m, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 177.1, 147.6, 146.2, 139.3, 137.0, 129.2, 128.3, 126.3, 120.6, 108.2, 107.7, 100.9, 43.4, 43.1, 39.9;. MS calc'd for C₁₇H₁₇O₄ (M+H)⁺ 285.1121, found 285.1116.

(+) 3-(benzo[d][1,3]dioxol-5-yl)-4-phenylbutan-1-ol (14)—**13** (4.9 mmol, 1.39 g) in dry THF (50 mL) was added dropwise to a slurry of lithium aluminum hydride (9.8 mmol, 372 mg) in dry THF (30 mL) at 0°C over 30 min. The reaction mixture was stirred and gradually warmed to r.t. over 4h. The reaction was cooled again to 0°C and water was added until evolution of gas ceased. The resultant slurry was filtered over Celite washing with Et₂O. THF was removed in vacuo. The crude residue was purified by flash chromatography (30% EA/Hex) to yield **14** (1.14 g, 86%) as a clear oil. TLC *R_f* = 0.27 (30% EA/Hex) ¹H NMR (500 MHz, CDCl₃) δ 7.25-7.10 (m, 3H), 7.08-7.03 (m, 2H), 6.74-6.66 (m, 2H), 6.59-6.54 (m, 1H), 5.92 (s, 2H), 3.55-3.47 (m, 1H), 3.46-3.38 (m, 1H), 2.99-2.90 (m, 1H), 2.89-2.82 (m, 2H), 1.98-1.88 (m, 1H), 1.85-1.73 (m, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 147.6, 145.8, 140.2, 138.1, 129.1, 128.1, 125.9, 120.8, 108.0, 107.6, 100.7, 60.9, 44.1, 43.9, 38.3;. MS calc'd for C₁₇H₁₉O₃ (M+H)⁺ 271.1329, found 271.1326.

(+) 3-(benzo[d][1,3]dioxol-5-yl)-4-phenylbutanal (15)—To a stirred solution of **14** (4.04 mmol, 1.09 g), triethylamine (20.2 mmol, 2.8 mL), and DMSO (109 mmol, 7.7 mL) in DCM (20 mL), at 0°C, was added SO₃·Py (20.2 mmol, 3.21 g) portionwise over 5 min. The reaction mixture was stirred for 2h and subsequently warmed to r.t. Excess sodium bicarbonate was then added and the mixture stirred until all remaining SO₃·Py was consumed. The organic solution was diluted with DCM, washed with brine, dried over MgSO₄, and solvent was removed in vacuo. The crude residue was purified by flash chromatography (20% Et₂O/Hex) to give **15** (871 mg, 80%) as a yellowish oil. TLC *R_f* = 0.24 (20% Et₂O/Hex). ¹H NMR (500 MHz, CDCl₃) δ 9.59 (s, 1H), 7.29-7.15 (m, 3H), 7.09-7.03 (2H), 6.73-6.65 (m, 2H), 6.63-6.56 (m, 1H), 5.92 (s, 2H), 3.47-3.37 (m, 1H), 2.95-2.80 (m, 2H), 2.70 (d, *J* = 8.1 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 201.5, 147.8, 146.2, 139.2, 137.0, 129.1, 128.3, 126.3, 120.6, 108.2, 107.6, 100.9, 49.1, 43.4, 41.8;. MS calc'd for C₁₇H₁₅O₃ (M-H)⁻ 267.1027, found 267.1030.

4-(((3-(benzo[d][1,3]dioxol-5-yl)-4-phenylbutyl)amino)methyl)-N,N-dimethylaniline (3)—Under argon a flask was charged with **15** (36 mg, 0.13 mmol), THF (2 mL), and 4-(dimethylamino)benzylamine dihydrochloride (58 mg, 0.26 mmol).

Na(AcO)₃BH (55 mg, 0.26 mmol) was added with stirring at rt. After 20 h, 1M NaOH (2 mL) was added. The mixture was extracted with Et₂O (3 × 10 mL). The extract was dried over MgSO₄ and solvent removed in vacuo. The crude material was purified by column chromatography (2%-5% (10% NH₄OH/MeOH)/DCM) to give **1** (13 mg, 25%) as a colorless oil. TLC *R_f* = 0.18 (5% (10% NH₄OH/MeOH)/DCM). ¹H NMR (500 MHz, CDCl₃) δ 7.24-7.10 (m, 5H), 7.05-6.98 (m, 2H), 6.70-6.62 (m, 4H), 6.55-6.48 (m, 1H), 5.90 (d, *J* = 5 Hz, 2H), 3.69-3.52 (m, 2H), 2.91 (s, 6H), 2.82 (s, 3H), 2.53-2.45 (m, 2H), 2.00-1.77 (m, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 150.0, 147.5, 145.7, 140.1, 138.0, 129.5, 129.1, 128.0, 125.8, 120.8, 112.5, 108.0, 107.6, 100.7, 52.5, 46.4, 45.6, 43.9, 40.6, 34.8; MS calc'd for C₂₆H₃₁N₂O₂ (M+H)⁺ 403.2380, found 403.2385.

Pharmacokinetics of the Leads in Rodents

A method to quantify **1** in plasma was developed using HPLC-MS/MS (API 3200; Applied Biosystems). A 20 μL plasma sample was extracted with methyl tertiary butyl ether (MTBE) under neutral conditions (0.1 M phosphate buffer pH = 7.4) using temazepam as the internal standard. The extract is evaporated, reconstituted with mobile phase and injected into the HPLC. The HPLC column is a C-8 50 × 4.6 mm 5 μm column and separation of uPAR and temazepam occurred by a linear gradient mobile phase starting with acetonitrile: 0.1% formic acid (10:90) and changing to 80:20. The *m/z* Q1 and Q3 settings on the API 3200 for uPAR and temazepam are 418/297 and 301/255, respectively. The lower limit of quantification of **1** is 1 ng/mL using 20 μL of plasma. A standard response curve of **1** was created with concentrations that varied from 1 ng/mL to 1000 ng/mL using 300 ng/mL of temazepam for each sample.

In order to determine drug concentrations of the lead inhibitors in the pharmacological models, we first need to characterize the PK properties of the compounds in mice. The PK of **1** was determined in NOD/SCID mice following a single dose given PO. The study design used 3 mice per time point. Blood was collected at time intervals from 0 to 24 hours after dosing (8 time points), quantified by the procedure described above. Pharmacokinetic parameters for **1** including area under the curve (AUC), area under the moment curve (AUMC), and *t*_{1/2} were estimated using non-compartmental methods with Excel®. The maximum plasma concentration (*C*_{max}) and time of *C*_{max} (*t*_{max}) were obtained from the data. The AUC from zero to infinity (AUC_{0-∞}) was estimated from the AUC_{0-t} (time zero to the last quantifiable concentration *C*_{last}) and the AUC from *C*_{last} to infinity, *C*_{last}/*k*_{el}, where *k*_{el} is the rate constant of elimination. The AUMC_{0-∞} was estimated by an analogous manner. The systemic clearance (Cl/F, where F = bioavailability) of **1** was calculated from the dose and AUC_{0-∞}.

Mouse Xenograft Studies

NOD/SCID mice were obtained from the on-site breeding colony maintained by the In Vivo Therapeutics Core at the Indiana University Simon Cancer Center (IUSM, Indianapolis, IN) and maintained in pathogen-free conditions within the laboratory animal resources center (LARC) at the Indiana University School of Medicine according to an approved protocol by the Institutional Animal Care and Use Committee (IACUC) committee. TMD-231 cells (1 × 10⁶) were injected into the mammary fat pads (m.f.p.) of 4-6 week old mice. These cells have been shown previously to develop primary tumors and metastasize efficiently to the lungs when the primary tumors have been removed.³⁷ For metastasis studies, m.f.p. tumors were allowed to grow to ~100 mm³ mice. Mice were assigned to treatment group based on average tumor size/ weight. Following a recovery period of two days, mice received treatment of investigational drug (*n* = 15) or a PBS solvent control (*n* = 15). After four weeks mice were euthanized, the lungs were resected, fixed in formalin solution, sectioned, and stained with hematoxylin and eosin (H&E) for analysis.

The number and size of metastasis in two to five fields per sample were calculated and a score of 4+ was given to a sample with highest metastasis index and relative metastasis in other samples are calculated (i.e., 1+, 2+, 3+) by a sample-blinded pathologist. Tumor volume were be calculated as $\text{Length} * \text{Width}^2/2$ in millimeter

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Abbreviations

uPAR	urokinase receptor
uPA	urokinase-type plasminogen activator
suPAR	soluble urokinase receptor
MMP	matrix metalloproteinase
RTK	receptor tyrosine kinase
GPCR	G-protein coupled receptor
ECM	extracellular matrix
GFD	growth factor-like domain
HUVEC	human umbilical vein endothelial cells
MAPK	mitogen-activated protein kinase
FAK	focal adhesion kinase
DMSO	dimethyl sulfoxide
hERG	human Ether-à-go-go Related Gene
FP	fluorescence polarization
CYP	cytochrome P450
FDA	food and drug administration
EB	ethidium bromide
DNA	deoxyribonucleic acid
NOD/SCID	non-obese diabetic severe combined immunodeficient
PK	pharmacokinetic
PBS	phosphate buffered saline
Src	sarcoma
MDA-MB-231	MD Anderson Metastatic Breast
FBS	fetal bovine serum

DMEM	Dulbecco's Modified Eagle Medium
SDS	sodium dodecyl sulfate
CBB	Coomassie brilliant blue
BSA	bovine serum albumin
DEAE	Diethylaminoethyl cellulose
FITC	Fluorescein isothiocyanate
TBST	Tris-Buffered Saline and Tween 20
IB	immunoblot
NMR	nuclear magnetic resonance
DCM	dichloromethane
HRMS	high-resolution mass spectrometry
THF	tetrahydrofuran
PDB	protein databank
DMAP	4-Dimethylaminopyridine
DCC	N,N'-Dicyclohexylcarbodiimide
FAM	6-carboxyfluorescein
HPLC	high-performance liquid chromatography
GPI	Glycosylphosphatidylinositol
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
TMD-231	tumor-derived 231
AUC	area under the curve
AUMC	area under the moment curve
LARC	laboratory animal resources center
IACUC	Institutional animal care and use committee
HPLC	high-performance liquid chromatography
FN	fibronectin
ActD	actinomycin D

Figure 1. Biochemical evaluation of compounds. **(A)** Binding of fluorescent GFD (AE147-FAM) as a function of uPAR. Increasing concentration of suPAR protein were titrated against the fluorescent GFD-FAM peptide. **(B)** Fluorescence polarization for the top compounds that emerged from the computational screening **(C)** Abrogation of AE147-FAM binding to uPAR by **2** and **3** compounds inhibited in a concentration-dependent manner.

Figure 2. Effect on MDA-MB-231 metastasis. Concentration-dependent study of the effect of (A) Compound 1 and (B) Compound 3 on MDA-MB-231 invasion, migration and adhesion.

Figure 3. Effect of **1** and **3** tube formation in matrigel by HUVEC cells. Snapshots depicting formation of capillarity-like tubes of HUVEC after addition of DMSO (top left), and increasing concentration of (A) Compound **1** (top right and bottom) and (B) Compound **3**. Percentage of tube formation to the vehicle group was calculated for (A) compound **1** (B) compound **3** at various concentrations.

Figure 4. Inhibition of MDA-MB-231 proliferation and mechanism of cell killing. (A,C) MTT assay reveals that **1** and **3** inhibit MDA-MB-231 proliferation; (B,D) Flow cytometry analysis using Annexin V-FITC and PI staining.

Figure 5. Effect on ECM degradation. Gelatin zymography analysis for MDA-MB-231 with increasing concentration of (A) compound 1 and (B) compound 3.

Figure 6. Cell signaling study. **(A)** MDA-MB-231 treated with 100 μ M compound(s) for 30 min, then immunoblotted with phospho-p44/42 MAPK & p44/42 MAPK, respectively. **(B)** MDA-MB-231 treated with 5, 10, 20 and 40 μ M compound(s) for 30 min, then immunoblotted with phospho-p44/42 MAPK(Thr202/Tyr204), p44/42 MAPK; phospho-FAK (Tyr397), FAK; phospho-Src family (Tyr416), Src, respectively.

Figure 7. *in vivo* and *in vivo* PK parameters. **(A)** Percent inhibition of hERG K⁺. E-4031 is used as control. **(B)** Percent inhibition of CYP2C9 isozyme. Sulfaphenazole is used as control. **(C)** DNA binding of compounds using fluorescence emission (excitation: 530 nm and Emission: 615 nm) of Calf thymus DNA bound to ethidium bromide (DNA-EB) were measured. Actinomycin D (ActD), a known DNA intercalator is used as positive control. **(D)** PK analysis of **3** in female NOD/SCID mice (n = 1 per time point) dosed by oral gavage as a mixture of 50 mg/kg in 0.5% hydroxy-methyl-propyl-cellulose. **(E)** *In vivo* PK parameters.

Figure 8.

Effect of **3** on MDA-MB-231 tumor growth. (A) MDA-MB-231 cells were inoculated in the mammary fat pads of female NOD/SCID mice. Once the tumor volume reached 30 to 50 mm³, animals were randomized and treated with vehicle alone as control or 150 mg/kg of compound **3** three times a week for 10 weeks by oral gavage. Tumor volumes were determined by caliper measurements obtained weekly as described in Materials and Methods. (B) Semi-quantitative scoring system was used for the estimation of TMA scoring. Briefly, percentage of staining was categorized as “0” if there was no nuclear/cytoplasmic expression, “1” for up to 10% positive tumor nuclei/cells, “2” for 11–20% and until a maximum score of “10”. Intensity was scored as “1+,” “2+,” and “3+” for weak, moderate and strong staining respectively. (C) H&E staining images that illustrate metastasis in the lungs of animals.

Scheme 1.

Chemical structure of compounds that showed activity in the fluorescence polarization assay.

Scheme 2.

Reagents and Conditions: (a) K_2CO_3 , di-tert-butyl-di-carbonate, THF:H₂O, 0°C. (b) DMAP, DCC, 2,2-dimethyl-1,3-dioxane-4,6-dione, DCM, 0°C. (c) ethanol, reflux. (d) triethylorthoformate, acetic anhydride, reflux. (e) ethanol, 4-isopropyl phenyl hydrazine, reflux. (f) 2.0 M NaOH (aq), ethanol, 70°C. (g) DMAP, DCC, 3,5-dimethylaniline, DCM, 0°C. (h) trifluoroacetic acid:DCM, 0°C.

Scheme 3.

Reagents and Conditions: (a) cat. piperidine/AcOH toluene, reflux, diethyl malonate; (b) BnMgCl, CuCl, -78 °C to r.t.; (c) 10% KOH MeOH:H₂O (1:1) r.t.; (d) p-xylene, reflux; (e) LiAlH₄, THF 0 °C to r.t.; (f) Et₃N, DMSO, SO₃Py, DCM, 0 °C; (g) 4-(aminomethyl)-N,N-dimethylaniline hydrochloride, (h) Na(AcO)₃BH, THF, r.t.