Discovery of New Small Molecules Targeting the Vitronectin-Binding Site of the Urokinase Receptor That Block Cancer Cell Invasion

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Abstract

Besides focusing urokinase (uPA) proteolytic activity on the cell membrane, the uPA receptor (uPAR) is able to bind vitronectin, via a direct binding site. Furthermore, uPAR interacts with other cell surface receptors, such as integrins, receptor tyrosine kinases, and chemotaxis receptors, triggering cell-signaling pathways that promote tumor progression. The ability of uPAR to coordinate binding and degradation of extracellular matrix (ECM) and cell signaling makes it an attractive therapeutic target in cancer. We used structure-based virtual screening (SB-VS) to search for small molecules targeting the uPAR-binding site for vitronectin. Forty-one compounds were identified and tested on uPAR-negative HEK-293 epithelial cells transfected with uPAR (uPAR-293 cells), using the parental cell line transfected with the empty vector (V-293 cells) as a control. Compounds 6 and 37 selectively inhibited uPAR-293 cell adhesion to vitronectin and the resulting changes in cell morphology and signal transduction, without exerting any effect on V-293 cells. Compounds 6 and 37 inhibited uPAR-293 cell binding to vitronectin with IC50 values of 3.6 and 1.2 μmol/L, respectively. Compounds 6 and 37 targeted S88 and R91, key residues for uPAR binding to vitronectin but also for uPAR interaction with the fMLF family of chemotaxis receptors (fMLF-Rs). As a consequence, compounds 6 and 37 impaired uPAR-293 cell migration toward fetal calf serum (FCS), uPA, and fMLF, likely by inhibiting the interaction between uPAR and FPR1, the high affinity fMLF-R. Both compounds blocked in vitro ECM invasion of several cancer cell types, thus representing new promising leads for pharmaceuticals in cancer.

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Introduction

The urokinase (uPA)-mediated plasminogen activation system, which generates the potent serine-protease plasmin, is involved in various pathologic processes, including angiogenesis, inflammation, wound healing, and metastasis (1, 2).

The key molecule of this system, the uPA receptor (uPAR), is anchored to the plasma membrane by a glycosylphosphatidylinositol moiety and is formed by three homologous domains (DI, DII, and DIII, from the N-terminus). The uPA-binding site is located in the DI domain, but the full-length molecule is required for an efficient binding (3, 4). uPAR enhances pericellular proteolysis by serving as a docking site to uPA, thus triggering a cascade of proteolytic events that leads to the active degradation of extracellular matrix (ECM) components (5).

Despite the lack of a transmembrane domain, uPAR can activate intracellular signaling through lateral interactions with other cell surface receptors, such as integrins, receptor tyrosine kinases, and G-protein–coupled chemotaxis receptors (6).

uPAR ability to regulate integrin activity plays a key role in cell adhesion, migration, proliferation, and survival (7, 8). Recently, integrin-binding sites have been identified in uPAR domain DII (residues 130–142; ref. 9) and in uPAR domain DIII (residues 240–248; ref. 10). Moreover, uPAR cross-talk with EGF receptor (EGFR) is extensive and may regulate the shift from tumor cell dormancy to proliferation (11, 12).

uPAR interaction with receptors for fMet-Leu-Phe (fMLF-Rs; ref. 13) is required for both uPA- and fMLF-dependent cell migration and occurs through a chemotactic domain located in the DI–DII linker region, the SRSRY sequence (amino acids, 88–92; ref. 14). Because a soluble cleaved form of uPAR, exposing at the N-terminus of the SRSRY sequence is a ligand for fMLF-Rs (15), it has been proposed that uPA binding to uPAR determines a conformational modification of the receptor with the...
exposure of the chemotactic SRSRY domain that, in turn, binds and activates members of the fMLF-R family, thus inducing chemotaxis (16).

Furthermore, uPAR itself is an adhesion receptor; indeed, it binds vitronectin, an abundant component of provisional ECM (17, 18). uPAR interactions with integrins and vitronectin are positively regulated by uPA (19, 20), and both uPA and vitronectin can induce uPAR-mediated cytoskeletal reorganization and cell migration (21, 22).

Toward the goal of discovering uPAR-inhibitory compounds, we focused on uPAR binding to vitronectin. Indeed, it has been recently reported that uPAR promotes metastasis of human malignancies by engaging vitronectin through the activation of a cell signaling to Rac-1 (23). In epithelial cell lines, uPAR induces phenotypic changes consistent with hypoxia-induced epithelial–mesenchymal transition (EMT), through a direct binding to vitronectin (24). Abnormal uPAR levels, like occur in cancer, may encourage EMT through vitronectin binding, thus facilitating tumor invasion and metastasis (25).

The X-ray structure of the ternary complex between uPAR, the aminoterminal fragment of uPA (ATF) and the somatomedin B domain of vitronectin (SBM) has been recently determined (25). There is now evidence that uPAR–vitronectin interaction is entirely mediated by a composite epitope exposed on the DI/DII interface of uPAR (residues R30, W32, S56, R58, I63, S65, S88, R91, R116, and Q114; ref. 27).

Therefore, we used structure-based virtual screening (SB-VS; ref. 28) of the National Cancer Institute (NCI, National Health Institutes, Bethesda, MD) Diversity Set II to identify small molecules targeting this site and disrupting uPAR binding to vitronectin.

Here, we describe the successful outcome of this search and the initial biologic evaluation of the two most promising compounds from this effort.

Materials and Methods

Computational chemistry

Molecular modeling and graphics manipulations were conducted using Maestro software (Maestro, version 9.2; Schrödinger, LLC) and PyMOL packages (29) running on an E4 Computer Engineering E1080 workstation provided of a Intel Core i7-930 Quad-Core processor.

Protein preparation

The X-ray coordinates of human uPAR–ATF–SBM ternary complex (PDB code: 3BT1; ref. 27) were extracted from the Protein Data Bank (30). The structure was then prepared using the Protein Preparation Wizard of the Schrödinger graphical interface Maestro. Hydrogen atoms were added to the protein consistent with the neutral physiologic pH (7.0). Thus, the R and S side chains were cationic, whereas the E and D side chain carboxylates were anionic. The protonation and flip states of the imidazole rings of the hydrogen (H) residues were adjusted together with the side chain amides of N and Q residues and the OH and SH orientations to optimize such interactions. X-ray water molecules and ATF and SMB ligands were removed during protein preparation, the last step of which was energy minimization of the entire structure. The minimization was terminated when the root mean square deviation (rmsd) of the heavy atoms in the energy-minimized structure relative to the starting (X-ray) coordinates exceeded 0.3 Å. This ensures that the integrity of the X-ray structure is preserved in further modeling studies while eliminating potential stereochemical short contacts that may exist in the protein structure. Furthermore, this process also ensures that the hydrogen atoms are placed in optimized geometries.

Ligand preparation and filtering

Three-dimensional (3D) structures of NCI Diversity Set II (1,364 compounds) were extracted by the website (31) and prepared using LigPrep software v2.5 (LigPrep, version 2.5; Schrödinger, LLC) with Epik to expand protonation and tautomeric states at 7.0 ± 2.0 pH units. In post-LigPrep steps, unspecified stereoisomers were retained up to 4 low-energy stereoisomers and sample 5/6-membered rings retained up to one conformation per ligand as default parameters suggested in Maestro virtual screening workflow. High-energy ionization/tautomer states were removed from the generated conformations. So, about 2,000 structures including stereoisomers, tautomers, and ionization states were ready to be submitted to the subsequent docking runs.

SB-VS protocol

The Grid-based Ligand Docking from Energetics (GLIDE) virtual screening application in Schrödinger Molecular Modeling Suite was used to screen compounds using two levels of docking precision. A modified version of the Chemscore function is used by GLIDE to assign a score to each ligand in all poses. In the first step, GLIDE was run in standard precision mode. The 20% of the top-scoring ligands (272 compounds) were kept and redocked using the GLIDE extra precision mode, which similarly retained the 20% of the best-ranked compounds (54 compounds). The extra precision–docking procedure of GLIDE, which incorporates a more accurate, finer-grained docking algorithm, was designed to eliminate false-positives that survive the standard precision stage. After visual inspection of the top-ranked compounds, 41 hits were chosen for biologic evaluation. Flexible docking was allowed in all stages and default parameters from the Virtual Screening Workflow were used in all docking studies, in addition to the aforementioned modifications to the percentage of compounds entering each stage. All final scores and poses came from GLIDE extra precision. The grid for docking studies was chosen sufficiently large to enclose all residues involved in the vitronectin–uPAR interactions within a cubic box of dimensions 46Å × 46Å × 46Å. The enclosing box was centered on the vitronectin-binding site setting the bounding box with the sizes of 14 Å × 14 Å × 14 Å. A van der Waals radius scaling factor of 0.80 for atoms with a partial
atomic charge (absolute value) less than 0.15 was used to soften the potential for nonpolar parts of the receptor. Compounds 6 and 37 identified by SB-VS were flexibly docked using the same protein grid prepared for virtual screening protocol. Ten poses were collected for each ligand and ranked according to predicted GLIDE extra precision score.

**Chemical inhibitors**

All compounds were obtained from the NCI/DTP Open Chemical Repository (32). The compounds identified by Virtual Screening were dissolved in dimethyl sulfoxide (DMSO) and stored at –20°C, at a concentration of 0.01 mol/L.

**Cell cultures and transfections**

The uPAR-negative (17) human embryonic kidney cell line HEK-293 [American Type Culture Collection (ATCC)-certified from LGC Standards] was grown in Dulbecco’s modified Eagle medium (DMEM; Gibco) supplemented with 10% FBS. The sarcoma-derived HT1080, the PC3 prostate cancer, the HCT colon cancer, and the MDAMB231 breast cancer cell lines (ATCC-certified from LGC Standards) were grown in DMEM (Gibco) supplemented with 10% FBS. Authentication of cell lines was conducted by ATCC using a short-tandem repeat assay. Upon receipt, all cell lines were stored in liquid nitrogen and passaged for less than 6 months before use in this study.

uPAR cDNA was cloned in a pcDNA3 vector with resistance to Geneticin (Invitrogen), and the resulting plasmid was named uPAR-pcDNA3. HEK-293 cells were stably transfected with uPAR-pcDNA3 or with the empty vector pcDNA3, as described previously (14).

**Western blot analysis**

Cells were lysed in PBS (0.08 mol/L NaCl, 0.002 mol/L KCl, 0.0115 mol/L Na2HPO4, and 0.002 mol/L KH2PO4, pH 7.2) containing 1% Triton X-100, in the presence of a protease inhibitor cocktail containing AEBSF, Aprotinin, Bestatin, E-64, Leupeptin, and Pepstatin A (Sigma-Aldrich) and a phosphatase inhibitor cocktail containing micromycin LR, cantharidin, and bromotetramisole (Sigma-Aldrich). Protein concentration of lysates was determined using a colorimetric assay (Bio-Rad). Equal amounts of protein were subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) filters (Millipore). Membranes were incubated for 20 hours at 4°C with the 399 polyclonal anti-uPAR antibody (American Diagnostica), mouse monoclonal anti-p-Erk or rabbit polyclonal anti-Erk2 antibodies (Santa Cruz Biotechnology), or with nonimmune immunoglobulins (Ig; Jackson ImmunoResearch), as a negative control. Filters were further incubated for 30 minutes at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies (Bio-Rad). The reaction was revealed by an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences).

**Cell adhesion assay**

The adhesion assays were conducted on 96-well flat-bottomed plates for cell (Nunc). Wells were coated with 1 μg of vitronectin (Becton Dickinson Biosciences), fibronectin (Roche), or with 100 mL of heat-denatured 1% bovine serum albumin (BSA) in PBS, as a negative control, and incubated overnight at 4°C. The plates were then blocked 1 hour at room temperature with 1% heat-denatured BSA in PBS. Cells were detached with trypsin, resuspended in DMEM containing 10% FBS and incubated for 1 hour at 37°C, 5% CO2, to allow receptor recovery. Cells were then washed with serum-free DMEM, counted, distributed into the wells at a density of 105 cells per well, and incubated for 1 hour at 37°C in the presence of inhibitors, or DMSO, as a vehicle control. Attached cells were mixed with 3% paraformaldehyde in PBS for 10 minutes and then incubated with 2% methanol for 10 minutes. Cells were then stained for 10 minutes with 0.5% crystal violet in 20% methanol. Stain was eluted by 0.1 mol/L sodium citrate in 50% ethanol, pH 4.2, and the absorbance at 540 nm was measured by a spectrophotometer.

**Cytotoxicity assay**

V-293 and uPAR-293 cells were plated, at 5 × 104 cells per well, in 96-well plates and grown for 24 hours. Cells were serum-starved overnight using DMEM containing 0.1% BSA, and then incubated for 1 and 24 hours at 37°C, 5% CO2 in the same medium containing the selected small molecules or DMSO, as a vehicle control. Of note, 20 μL/well of CellTiter 96 AQueous One Solution Reagent (Promega) was added and, after incubation at 37°C for 4 hours, the absorbance was determined by an ELISA reader (Bio-Rad) at a wavelength of 490 nm.

**Cell migration and invasion assays**

Cell migration assays were conducted in Boyden chambers using 8-μm pore size polystyrene plates (Millipore) filters (Millipore). Membranes were incubated for 20 hours at 4°C with the 399 polyclonal anti-uPAR antibody (American Diagnostica), mouse monoclonal anti-p-Erk or rabbit polyclonal anti-Erk2 antibodies (Santa Cruz Biotechnology), or with nonimmune immunoglobulins (Ig; Jackson ImmunoResearch), as a negative control. Filters were further incubated for 30 minutes at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies (Bio-Rad). The reaction was revealed by an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences). For the invasion assay, filters were coated with 50 μg/mL Matrigel (BD Biosciences) and incubated for 30 minutes at 37°C for gelling. A total of 2 × 105 cells, plated in the upper chamber in DMEM 0.1% BSA containing the selected small molecules or DMSO, as a vehicle control. Of note, 20 μL/well of CellTiter 96 AQueous One Solution Reagent (Promega) was added and, after incubation at 37°C for 4 hours, the absorbance was determined by an ELISA reader (Bio-Rad) at a wavelength of 490 nm.

## Cell migration and invasion assays

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At the end of both experiments, cells on the lower surface of the filter were fixed in ethanol, stained with hematoxylin, and counted at ×200 magnification (10 ×).
random fields/filter). Cell migration and invasion were expressed as a percentage increase over the control.

**Coimmunoprecipitation**

uPAR-293 cells (5 × 10⁶/sample) were plated in 100-mm dishes for 24 hours; then, the cells were incubated for 16 hours in DMEM 0.1% BSA containing the selected small molecules or DMSO, as a vehicle control, in serum-free medium. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 1% deoxycholate acid, 0.1% SDS, 1% Triton X-100, protease, and phosphatases inhibitors) and incubated with nonimmune serum and 10% protein A–sepharose for 2 hours at 4°C. After centrifugation, the supernatants were incubated with 2 μg/mL of the R4 monoclonal anti-uPAR antibody, kindly provided by Dr. G. Hoyer-Hansen (The Finsen Laboratory, Copenhagen, Denmark), or with nonimmune Igs, for 2 hours at 4°C and then with 10% protein A–sepharose for 30 minutes at room temperature. The immunoprecipitates were washed in RIPA buffer, subjected to 10% SDS-PAGE, and analyzed by Western blot analysis using a polyclonal antibody directed against the high-affinity fMLF receptor, FPR1 (Santa Cruz Biotechnology), or nonimmune Igs, as a negative control.

**Rac-1 pull-down assay**

uPAR-293 cells were starved for 24 hours and then plated on vitronectin-coated wells (5 mg/mL) for the indicated time, in the presence of compounds 6 and 37, or DMSO as a vehicle control. After a quick wash with ice-cold PBS, cells were lysed with glutathione S-transferase (GST)-FISH buffer [50 mmol/L Tris–HCl pH 7.4, 2 mmol/L MgCl₂, 1% NP-40, 10% glycerol, 100 mmol/L NaCl, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mg/mL aprotinin, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), and 2 mmol/L dithiothreitol (DTT)]. After 10 minutes at 4°C under agitation, cells were scraped and lysates were cleared by centrifugation in a precooled rotor. Five hundred microgram of total protein extract was mixed with 10 mg of GST-PAK-CRIB domain, which was mixed with 10 mg of GST-FISH buffer. The amounts of agitation. Beads were then rinsed three times rapidly with 1 mL of ice-cold GST-FISH buffer. The amounts of vitronectin-binding site contains some residues

**Binding of soluble uPAR to immobilized vitronectin**

High-binding plates with 9% flat-bottomed wells (Corning) were coated with 0.5 μg/well vitronectin diluted in PBS, or BSA as a negative control, and incubated at 4°C overnight. After a wash in PBS, residual binding sites were blocked with 200 μL of 1% BSA in PBS, for 1 hour at room temperature. Of note, 25 nmol/L biotinylated s-uPAR (diluted in PBS, 1 mg/mL BSA), alone or in the presence of compounds 6 and 37, was placed into coated wells. The plates were kept at 4°C for 1 hour, washed with PBS containing 0.1% Tween 20 and then peroxidase-labeled avidin (Amersham), diluted 1:1,500 in PBS, and 10 mg/mL BSA was added. After additional washings, the peroxidase substrate o-phenylenediamine (Sigma) was added and allowed to react for 3 minutes. The reactions were terminated with 1 mol/L H₂SO₄ and the product was quantified by measuring absorbance at 492 nm using an automated plate reader (Bio-Rad), as described previously (17). Results were expressed as a percentage decrease over the control, that is, the binding in the absence of compounds.

**Results**

**Identification by SB-VS of small molecules directed to the uPAR-binding site for vitronectin**

uPAR overexpression functions as a biomarker for cancer progression and metastasis in many forms of human malignancy (33). It has been recently proposed that uPAR can promote metastasis not only by a uPA-dependent mechanism but also through a direct binding to vitronectin followed by activation of a specific signal transduction (23–25).

To identify new potential small molecules capable of interfering in the vitronectin–uPAR interaction, we conducted a SB-VS experiment using the X-ray crystal structure of the human uPAR–ATF–SBM ternary complex (27) determined at 2.8 Å resolution (3BT1 in the Protein Data Bank).

Before starting the search, the binding site for vitronectin was identified on the uPAR crystal structure; it is exposed on the DI/DII interface of the receptor and comprises residues R30, W32, R58, I63, S56, R88, R91, R116, and Q114 (27). Among these, W32, R58, I63, R91, and Y92 were identified as key residues for uPAR/vitronectin binding by alanine-scanning mutagenesis (18, 25–27). In details, residues F13, Y28, and D22 of vitronectin form an open pocket to bind R91 of uPAR. Y27 and Y28 of vitronectin insert into a large cavity of uPAR, showing shape complementarity of this interface. The phenyl ring of Y28 contacts uPAR residues I63 and W32, mainly through hydrophobic interactions. Interestingly, the vitronectin-binding site contains some residues included in the uPAR chemotactic sequence (SRSRY; amino acids, 88–92), required for cell migration toward fMLF and uPA (14–16).

Docking simulations were conducted through the Glide software (available from Schrödinger LLC; refs. 34, 35), selecting the NCI Diversity Set II as virtual library of compounds. Then, GLIDE standard precision mode docked each chemical structure into the uPAR:
vitronectin–binding site retaining the 20% of the top-scoring ligands. The resulting 272 compounds were then redocked and scored with Glide extra precision to estimate binding affinity and rank the ligands. The resulting 54 top-ranked compounds were analyzed by visual inspection because it has broadly shown that docking scoring functions are often more successful at predicting a binding pose than the actual binding affinity (36).

Therefore, compounds were prioritized by taking into account their binding mode as well as the overall match among binding modes of all the stereoisomers, tautomers, and ionization states of each compound and, second, their docking score. As for the visual inspection, compounds were checked for a good protein–ligand complementarity. Moreover, ligands able to make interactions with residues known to be important by mutagenesis studies or because they interact with known substrates such as vitronectin or fMLF were prioritized.

To promote the selection of structurally diverse compounds, potential hits were grouped into chemical classes by visual inspection, and one molecule was selected for each class.

Finally, compounds were checked for ready sample availability from the compound provider, and 41 structurally diverse compounds (Fig. 1) were requested and tested in a cell-based assay.

**Four compounds selected by SB-VS inhibited uPAR-transfected epithelial cell binding to vitronectin**

Human embryonic kidney (HEK-293) cells, unlike most cancer cells, do not express uPAR or uPA endogenously (14, 17, 18, 23). Therefore, as a model to mimic the effects of uPAR overexpression in cancer cells and to investigate the ability of SB-VS–selected compounds to inhibit uPAR-mediated cell binding to vitronectin, HEK-293 cells were stably transfected with a human uPAR cDNA (uPAR-293 cells) or with an empty vector (V-293 cells).

uPAR expression was then evaluated by Western blot analysis, using an anti-uPAR polyclonal antibody, in uPAR-293 and V-293 cell lysates. uPAR-293 cells expressed high levels of full-length uPAR, whereas V-293 cells were uPAR-negative (Fig. 2A).

In uPAR-293 cells, uPAR and integrins form stable complexes that both inhibit the native integrin-mediated
cell adhesion to fibronectin and promote adhesion to vitronectin, via the direct vitronectin-binding site on uPAR (14, 17). Therefore, we evaluated uPAR-293 cell adhesion to fibronectin and vitronectin, using the uPAR-negative V-293 cell line as a control. As expected, uPAR-293 cells showed a strongly increased cell adhesion to vitronectin, as compared with V-293 cells; on the contrary, the native cell adhesion to fibronectin was inhibited by uPAR overexpression in uPAR-293 cells, as compared with V-293 cells (Fig. 2B).

To identify potential molecules able to inhibit uPAR binding to vitronectin, uPAR-293 cells were subjected to adhesion experiments on vitronectin-coated wells in the presence of 41 SB-VS-selected compounds (50 μmol/L) or DMSO, as a negative control. Although compounds 9–12 and 14, increased cell adhesion to vitronectin, only 4 compounds significantly inhibited uPAR-293 cell adhesion to vitronectin; namely, compounds 6 [4,4′-dimethyl-[1,1′-biphenyl]-2,2′,5,5′-tetraol], 13 [1-(phenanthren-9-yl)guanidine], 24 [8-((4-tert-butyl)phenoxymethyl)-1,3-dimethyl-1H-purine-2,6(3H,9H)-dione], and 37 [piperidin-2-yl][2-(trifluoromethyl)-6-(4-(trifluoromethyl)phenyl)pyridin-4-yl]methanol] (Fig. 2C).

Figure 2. Increased cell adhesion to vitronectin in uPAR-transfected HEK-293 cells can be inhibited by SB-VS-selected compounds. A, HEK-293 cells were transfected with uPAR cDNA (uPAR-293) or with empty vector (V-293) and their lysates were analyzed by 10% SDS-PAGE and Western blot analysis with a polyclonal anti-uPAR antibody. uPAR-293 cells expressed high amounts of uPAR. B, V-293 (■) and uPAR-293 (▲) cells were plated onto vitronectin (VN)- and fibronectin (FN)-coated wells for 1 hour, attached cells were fixed and stained with crystal violet; the stain was eluted and the absorbance (OD) at 540 nm was measured by a spectrophotometer. Values represent the mean ± SD of 3 experiments carried out in triplicate (∗, P < 0.05). uPAR-293 cells showed increased cell adhesion to vitronectin and decreased cell adhesion to fibronectin, as compared with V-293 cells. C, uPAR-293 cells were plated on vitronectin-coated wells for 1 hour in the presence of 41 compounds identified by SB-VS (50 μmol/L) or DMSO (○), as a vehicle control. Attached cells were fixed and stained with crystal violet; the stain was eluted and the absorbance (OD) at 540 nm was measured by a spectrophotometer. Values represent the mean ± SD of 3 experiments carried out in triplicate (∗, P < 0.05). Four compounds (6, 14, 24, and 37) significantly inhibited uPAR-293 cell adhesion to vitronectin.

Two compounds specifically inhibited uPAR-mediated cell binding to vitronectin independently of cell cytotoxicity

To ensure that inhibition of uPAR-293 cell adhesion to vitronectin was not due to cell killing, the toxicity of the above selected compounds was measured by a cell viability assay. Serum-starved uPAR-293 cells were incubated for 1 and 24 hours with compounds 6, 13, 24, and 37, at the same concentration used in the adhesion assay (50 μmol/L), or with DMSO, as a control. Cell viability was then measured by a MTS-PES assay. Compounds 13 and 24 showed a significant level of cell toxicity (Fig. 3A), also confirmed on uPAR-negative V-293 cells (not shown), and were withdrawn from the study for their unspecific toxic effects. To show the specificity of uPAR as a target, the ability of compounds 6 and 37 to inhibit in vitro binding of recombinant suPAR to immobilized vitronectin was evaluated. Compounds 6 and 37 inhibited suPAR binding to vitronectin, albeit with a lower efficiency (Fig. 3B). Indeed, suPAR differs from membrane Glycosylphosphatidylinositol (GPI)-linked uPAR in the conformation and flexibility of the molecule, being the linker region between domains D1 and D2 less accessible (37). The possibility of
Figure 3. Two top-scoring molecules from the in silico screening show specificity for uPAR–vitronectin interaction and are not toxic to the cells.

A, serum-starved uPAR-293 cells were incubated for 1 and 24 hours with 50 μmol/L compound 6 [()], 13 [()], 24 [(), and 37 [()] or DMSO [()], as a vehicle control; cell survival was then evaluated by adding MTS-PES and, after incubation at 37 °C for 4 hours, the absorbance (optical density (OD)) was determined at 490 nm by a spectrophotometer. Values are the mean ± SD of 3 experiments carried out in triplicate (*, P < 0.05). Compounds 6 and 37 did not affect uPAR-293 cell viability, whereas compounds 13 and 24 were cytotoxic.

B, biotinylated suPAR was placed for 1 hour at 4 °C. Bound suPAR was revealed by HRP-avidin and OPD staining; the absorbance at 490 nm was measured by a spectrophotometer. Values of suPAR binding in BSA-coated wells were subtracted to obtain specific binding. Values represent the mean ± SD of 3 experiments carried out in triplicate (*, P < 0.05). Hundred percent values represent suPAR binding to vitronectin in the absence of compounds. C, V-293 [()] and uPAR-293 [()] cells were plated on vitronectin-coated wells in the presence of 50 μmol/L compounds 6 [()], 37 [()], or DMSO [()], as a vehicle control. Bound suPAR was revealed by HRP-avidin and OPD staining; the absorbance at 490 nm was measured by a spectrophotometer. Values represent the mean ± SD of 3 experiments carried out in triplicate (*, P < 0.05). Compounds 6 and 37 inhibited uPAR-293 cell adhesion to vitronectin without exerting any effect on uPAR-negative V-293 cells. D, uPAR-293 cells were plated on fibronectin (FN)- and vitronectin-coated wells in the presence of 50 μmol/L compounds 6 [()] or 37 [()] or DMSO [()], as a negative control. The attached cells were fixed and stained with crystal violet; the stain was eluted and the absorbance at 540 nm was measured by a spectrophotometer. Values represent the mean ± SD of 3 experiments carried out in triplicate (*, P < 0.05). Compounds 6 and 37 inhibited uPAR-293 cell adhesion to vitronectin without affecting their adhesion to fibronectin.

Conformational effects, imposed by the C-terminal hydrophobic group, is a widespread phenomenon among GPI-anchored proteins (37); therefore, we decided to study compounds efficiency in uPAR-293 cells, using the uPAR-negative V-293 cell line as a control. Compounds 6 and 37 inhibited uPAR-293 cell adhesion to vitronectin without affecting V-293 cell adhesion to the same substrate (Fig. 3C). Indeed, in the absence of uPAR, HEK-293 cell binding to vitronectin is mediated by specific integrins (38).

To further show the specificity of compounds 6 and 37 for uPAR–vitronectin interaction, their ability to inhibit uPAR-293 cell adhesion was evaluated both on vitronectin and fibronectin. Compounds 6 and 37 specifically inhibited uPAR-293 cell adhesion to vitronectin without affecting adhesion to fibronectin of the same cells (Fig. 3D).

Thus, compounds 6 and 37 are specific inhibitors of uPAR binding to vitronectin.

Compounds 6 and 37 inhibit uPAR-dependent cell binding to vitronectin in a dose-dependent manner with a submicromolar affinity

uPAR-923 cell adhesion to vitronectin was also evaluated in the presence of decreasing concentration of compounds 6 and 37, to evaluate the dose-dependency of their inhibitory activity. IC50 values of 6 and 37 were 3.6 and 1.2 μmol/L, respectively, as calculated by nonlinear
regression curves using the sigmoidal dose–response analysis of the GraphPad Prism software (Fig. 4A and B). $K_i$ of compounds 6 and 37 were 0.58 and 0.19 μmol/L, respectively, as calculated by the Cheng and Prusoff equation from EC50, using GraphPad Prism (39).

Thus, two novel small molecules specifically inhibiting uPAR-mediated cell binding to vitronectin were identified by SB-VS (Table 1).

**Compounds 6 and 37 abolished the changes in cell morphology and the signal transduction induced by uPAR-mediated cell adhesion to vitronectin**

uPAR triggers cell attachment to the matrix through a direct interaction with the SMB domain of vitronectin (27).

This initial adhesion is followed by engagement of integrins, changes in cell morphology, migration, and signal transduction (18, 23, 25).

When uPAR-293 cells were seeded on vitronectin, they underwent marked changes in morphology, including cell flattening and extensive lamellipodia formation. In the presence of compounds 6 and 37, at $2 \times IC_{50}$, uPAR-293 cells retained a rounded cell body, failed to form lamellipodia and only a round adhesion patch could be observed under the cell body (Fig. 5A).

The limited cell contact with matrix in the presence of compounds 6 and 37 determined a strongly reduced cell adhesion to vitronectin. Indeed, uPAR-293 cell adhesion to increasing concentration of vitronectin, in the absence of compounds 6 and 37, was significantly reduced compared to control conditions (Fig. 5B).

**Table 1. The two low-micromolar uPAR-vitronectin inhibitors indentified by SB-VS**

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<th>Structure</th>
<th>NCI ID</th>
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<td><img src="image2.png" alt="Structure" /></td>
<td>305798</td>
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<td>1.23</td>
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**NOTE:** The second and third columns show compound structures, ID used throughout the text and NSC number. The fourth column shows the 4 diastereoisomers of compound 37. The fifth column shows the estimated free energy of binding (kcal/mol). The sixth and seventh columns show the $IC_{50}$ and $K_i$ values as measured in adhesion assays of uPAR-923 cell to vitronectin. The final column shows the predicted Log P value of each compound.
of inhibitors, showed a $K_D$ of 26 nmol/L, similar to that reported for in vitro uPAR-vitronectin binding (30 nmol/L; ref. 17); the addition of compounds 6 and 37, at their 2× $IC_{50}$, resulted in a significant loss of uPAR-293 cell binding to vitronectin (Fig. 5B).

After a direct binding to vitronectin, uPAR can activate extracellular signal–regulated kinases (ERK), most probably through its lateral interaction with integrins (18); therefore, we investigated whether compounds 6 and 37 were able to block ERK activation in response to vitronectin. Serum-starved uPAR-293 cells were plated on vitronectin-coated wells for 20 minutes in the presence of the 2 molecules, at 2× $IC_{50}$, or DMSO, as a negative control; then, cell lysates were analyzed by Western blot analysis with anti-phospho-ERK antibodies. Both compounds strongly reduced vitronectin-mediated ERK activation, as compared with DMSO-treated cells, in which a strong vitronectin-induced ERK activation was observed (Fig. 5C).

It has been reported that uPAR, through its binding to vitronectin, activates the small GTPase Rac-1, thus stimulating cell migration (23); indeed uPAR-293 cells plated on vitronectin show extensive lamellipodia formation (18, 23, 25; Fig. 5A, arrows). Therefore, we
investigated, by pull-down assays, whether compounds 6 and 37 were able to block Rac-1 activation in response to vitronectin. Serum-starved uPAR-293 cells were plated on vitronectin-coated wells for 20 minutes in the presence of both ligands, at 2 × IC$_{50}$, or DMSO, as a negative control. Active Rac-1 (Rac-1/GTP) was precipitated from cell lysates using the p21-binding domain (PBD) of its target, PAK1, bound to agarose beads; Rac-1/GTP was eluted from beads and estimated by Western blot analysis with a monoclonal anti-Rac-1 antibody. Both compounds strongly reduced vitronectin-mediated Rac-1 activation, as compared with DMSO-treated cells, in which Rac-1 was strongly activated (Fig. 5D).

Therefore, compounds 6 and 37 specifically inhibited uPAR-mediated cell binding to vitronectin and its downstream signals.

**Structural basis for uPAR inhibition by compounds 6 and 37**

To understand the structural basis of the binding of the newly discovered compounds 6 and 37 to uPAR, we scrutinized their binding poses by means of GLIDE program with the procedure described in the Materials and Methods. Figure 6 depicts the predicted binding modes of the 2 compounds in the vitronectin-binding site of uPAR.

All the top-ranked poses found by GLIDE for compound 6 show a twisted conformation of the ligand, which is stabilized by an intramolecular H-bond between the two phenolic OH groups. The molecule extends deep into the vitronectin-binding site of uPAR, making several H-bonds and hydrophobic interactions with key residues of the site. In particular, the OH group at position 2 is predicted to form 2 H-bonds with both S88 OH group and the guanidinyl Ne of R116. The OH group at position 5
also engages a H-bond with R91 side chain. Finally, the ligand 5′ OH group establishes a bidentate H-bond with the guanidinium group of R30. In addition, the uPAR/6 complex is further stabilized by hydrophobic contacts that involve the 4′-methyl group of the ligand and residues I63 and W32 of the receptor.

Interestingly, the docking results showed that, although with a different arrangement, the two phenyl rings of compound 6 nicely mimic the vitronectin residues Y27 and Y28, which in the crystal structure of the uPAR-ATF-SMB complex insert into a large cavity on uPAR’s surface making H-bonding and hydrophobic interactions (27).

It is also noteworthy that compound 6 interacts with R91 and S88, two key residues of the uPAR chemotactic epitope, which could interfere with the fMLF-R recognition and affect uPAR-dependent cell migration.

Because compound 37 has two chiral centers and the chemical sample from the NCI is a racemic mixture with undetermined diastereomeric ratio, we docked the 4 diastereomers (R,S)-37, (R,R)-37, (S,S)-37, and (S,R)-37 generated by LigPrep software (see Materials and Methods) into the uPAR-vitronectin-binding site and scored them by GLIDE extra precision. Whether one or all four of the diastereomers of compound 37 contributed to the inhibition is not entirely clear, as the top-ranked poses for each isomer within the vitronectin-binding site were not different and gave comparable G-scores (Table 1).

As depicted in Fig. 6B–E, one of the 4-CF₃ fluorine atoms of the diastereomers interacts tightly with the cationic terminus of both R30 and R116 side chains. The remaining 2 fluorine atoms of (R,S)-37 and (R,R)-37 also experience a slightly elongated H-bond with both S56 and S65 OH group. The 4-CF₃ phenyl ring of the 4 isomers forms hydrophobic contacts with W32 and I63 and is stabilized by edge-on-face aromatic stacking interactions with W32. The 2-CF₃ group of (R,S)-37, (R,R)-37 and (S,S)-37 is optimally oriented to make H-bonds with the cationic terminus of R91 side chain. On the contrary, the 2-CF₃ group of (S,R)-37 engages a H-bond with R58 residue. The F···H–NH₂ and F···H–O distances are below 4 Å, respectively, and the corresponding F···H–NH₂ and F···H–O angles are comprised between 107.0° and 151.0°. All these values decrease in the range considered acceptable for F···H–X (X = O, N, or S) H-bonds (36, 37). It has been argued that fluorine rarely engages in H-bonds in small-molecule X-ray crystal structures (40, 41). However, in protein pockets where ligands are immobilized by a variety of forces, they seem to be more common. F···X distances beyond 3.0 Å can be regarded as dipole–dipole interactions that most likely provide small stabilizing contributions (<1 kcal/mol) for the observed binding poses.

The alcoholic OH group of (R,S)-37 and (R,R)-37 forms H-bonds with the R58 NH₂ group and the S112 CO backbone, whereas in (S,S)-37 and (S,R)-37 it forms H-bonds with the Q114 CO group and the R116 NH₂ group. With the exception of (R,R)-37, the protonated piperidine N1 of (R,S)-37, (S,S)-37, and (S,R)-37 donates a H-bond to Q114 CO side chain.

The predicted G-scores could suggest that the (R,S)-37 isomer binds slightly more strongly than the other ones. But further studies are needed to determine which isomer is the more active form for binding to uPAR.

From the docking results it seems evident that compound 37 entirely fills the vitronectin recognition pocket and engages numerous H-bonding and hydrophobic contacts with the receptor, explaining the slightly better affinity in comparison with compound 6 (Table 1). However, compound 37, differently from compound 6, contacts only partially the chemotactic SRSRY sequence interacting only with the R91 side chain.

**Compounds 6 and 37 inhibit the structural and functional interaction between uPAR and fMLF-Rs, thus blocking tumor cell invasion**

Membrane-bound uPAR functionally interacts with fMLF-Rs through its SRSRY sequence (residues 88–92) and this interaction is required for both fMLF- and uPA-dependent cell migration (14–16, 23, 42, 43). Therefore, we tested whether compounds 6 and 37 were able to affect uPAR-dependent cell migration, as they also target key residues of the uPAR chemotactic domain, namely S88 and R91.

To this aim, in vitro cell migration experiments were carried out on uPAR-293 cells. These cells acquire the ability to migrate toward uPA and fMLF by expressing uPAR; on the contrary, uPAR-negative V-293 cells do not migrate toward uPA and fMLF, albeit they express fMLF-Rs (14).

Both compounds strongly inhibited uPAR-293 cell migration toward FCS, whose main chemotactic component is vitronectin, as well as toward uPA and fMLF, when plated on collagen-coated membranes; as a control, V-293 cell migration toward FCS was not affected (Fig. 7A). As expected, given the ability of compounds 6 and 37 to inhibit uPAR-mediated cell adhesion to vitronectin, migration of uPAR-293 cells on membranes coated with vitronectin confirmed the results obtained with collagen-coated membranes (Fig. 7A).

Therefore, compounds 6 and 37, targeting R91 of uPAR, not only block cell adhesion to vitronectin, but also impair cell migration toward serum, uPA, and fMLF by specifically inhibiting the functional interaction between uPAR and fMLF-Rs.

To show that compounds 6 and 37 acted by inhibiting the structural interaction between uPAR and fMLF-Rs, coimmunoprecipitation experiments were carried out in uPAR-293 cells, constitutively expressing high levels of FPR1, the high affinity receptor for fMLF.

uPAR-293 cells were incubated for 20 hours in DMEM containing 0.1% BSA in the presence of compounds 6 and 37, at 2× IC₅₀, or DMSO, as a negative control. Cells lysates were incubated with the R4 monoclonal anti-uPAR antibody or with nonimmune Igs, as a control. Immunocomplexes were purified with protein-A sepharose and...
Compounds 6 and 37 inhibit the structural and functional interaction between uPAR and fMLF-Rs, thus blocking tumor cell invasion. A, V-293 cells and uPAR-293 cells were incubated with DMSO, as a vehicle control, or with compounds 6 and 37, at their IC50, plated in Boyden chambers coated with collagen (CG) or vitronectin (VN) and allowed to migrate toward FBS, the ATF or fMLF. Hundred percent values represent cell migration in the absence of chemoattractants. The values are the mean ± SD of 3 experiments carried out in triplicate (*, P < 0.05). The FBS-, ATF-, and fMLF-dependent uPAR-293 cell migration was strongly inhibited by compounds 6 and 37 both on collagen and vitronectin. B, serum-starved uPAR-293 cells were incubated for 16 hours with compounds 6 or 37 (INH), at 2 × IC50, or DMSO, as negative control. Cell lysates were immunoprecipitated with the R4 anti-uPAR monoclonal antibody or with nonimmune serum. The immunoprecipitated samples were electrophoresed on 10% SDs-PAGE and analyzed by Western blot analysis with an anti-FPR1 antibody (top right) or with nonimmune serum (top left). Nonimmunoprecipitated cell lysates were subjected to Western blot analysis with the anti-FPR1 antibody (bottom right) or with nonimmune serum (bottom left), as a loading control. uPAR-coimmunoprecipitates with FPR1; treatment with compounds 6 and 37 significantly reduced uPAR association to FPR1. C, highly invasive HT1080 sarcoma cells were treated with DMSO (−) or with compounds 6 or 37 (INH), at their 2 × IC50, and allowed to invade Matrigel, using as chemoattractants cell culture medium without serum (−FCS), to evaluate spontaneous invasion, or with 10% serum (+FBS). Invading cells on the lower side of the membrane were fixed, stained, and photographed; a representative image is shown. D, HT1080 sarcoma cells, HCT human colon cancer cells, PC3 human prostate cancer cells, and MDA-MB-231 human breast cancer cells were treated with DMSO (−) or with compounds 6 or 37, at their 2 × IC50, and allowed to invade Matrigel, using as chemoattractants cell culture medium without serum (−FCS) or with 10% serum (+FBS). Invading cells on the lower side of the membrane were fixed, stained, counted (magnification, ×200), and the results were expressed as a percentage of the invasion in the absence of chemoattractant (−FBS). The values are the mean ± SD of 3 experiments carried out in triplicate (*, P < 0.05). FBS-induced cancer cell invasiveness was significantly reduced by compounds 6 and 37.

In DMSO-treated uPAR-293 cells, immunoprecipitation with anti-uPAR antibodies and Western blot analysis with anti-FPR1 antibodies revealed a band corresponding to FPR1 that was absent in the same lysate immunoprecipitated with nonimmune Igs, thus indicating the existence of a structural interaction between uPAR and FPR1. This interaction was completely blocked by compound 6 and slightly reduced by compound 37. As a control, no bands were evidenced in uPAR-293 lysates immunoprecipitated with nonimmune Igs and subjected to Western blot analysis with anti-FPR1 antibodies.

The inhibition of uPAR–FPR1 interaction in cells treated with compounds 6 and 37 was specific, as evidenced by the presence of the same amount of FPR1 in all cell lysates tested by Western blot analysis with the anti-FPR1 antibody, as a loading control (Fig. 7B).

A growing body of evidence suggests that the uPA–uPAR system promotes tumor metastasis by several
different mechanisms, and not solely through the breakdown of the ECM (30, 41). Among uPAR partners, the fMLF-R family of G-protein–coupled chemotaxis receptors is crucial in mediating cancer cell invasion and metastasis (42, 43). Therefore, we sought to investigate whether compounds 6 and 37 were able to inhibit the invasion through reconstituted basal membranes of the highly invasive human fibrosarcoma HT1080 cell line, constitutively expressing high uPAR levels (42). As shown in Fig. 7C, both compounds strongly reduced Matrigel invasion, as compared with DMSO-treated cells.

The same result was obtained on HCT human colon cancer cells, PC3 human prostate cancer cells, and on MDAMB231 breast cancer cells (Fig. 7D).

Therefore, compounds 6 and 37, two newly identified small-molecule inhibitors of uPAR, are able to block cancer cell invasion by targeting the direct uPAR binding to vitronectin and its structural and functional interaction with fMLF-Rs.

Discussion

uPAR expression and function have been implicated in nearly every step of tumor formation and progression (30, 44). Therefore, the identification of reagents with favorable pharmacokinetic characteristics capable of interfering with uPAR-mediated signaling is an area of great interest.

Many of the events of uPAR signaling are dependent on its binding to extracellular ligands, such as uPA and vitronectin. The main goal of this study was to identify small molecules that, by inhibiting uPAR binding to vitronectin, would block tumor cell migration and ECM invasion.

Our work was based on previous studies demonstrating that, although uPAR plays a pivotal role in the activation of protease cascades at the cell surface, it promotes metastasis of human malignancies mostly by engaging vitronectin through the activation of a cell signaling to Rac-1 (23, 24, 45) and by encouraging EMT (18, 24, 25).

We posited that inhibiting uPAR binding to vitronectin could be strongly effective in cancer therapy. Indeed, uPA–uPAR complex formation enhances pericellular proteolysis through the activation of plasminogen and, in turn, matrix metalloproteinases (MMP). However, recent experimental evidence indicates that some members of the MMP family behave as tumor-suppressor enzymes and may therefore be regarded as antitargets in cancer therapy (46). Moreover, small-molecule inhibitors of uPA binding to uPAR have been recently described (47, 48).

Another important advantage of our approach is the specificity of the target; indeed, uPAR binding to vitronectin takes place when uPAR is overexpressed (Fig. 2), a condition mostly of malignant tumors. Moreover, vitronectin, a circulating adhesive protein, becomes abundantly associated with ECM sites upon tissue remodeling, injury/repair, or under disease conditions (49).

Combined biologic and biochemical approaches identified the composite site of interaction between uPAR and vitronectin (26, 27). This information facilitated the in silico screening of compounds targeting the uPAR-binding site for vitronectin and the computational evaluation of the binding to the target protein. Through this approach, which has already been used to successfully identify inhibitors of Cdc25B dual specificity phosphatases (50), we identified 2 small molecules able to inhibit the uPAR-dependent cell binding to vitronectin and, when applied to cancer cells, to block ECM invasion.

Compounds 6 and 37 disrupted cell adhesion to vitronectin of uPAR-transfected HEK-293 cells, and stopped the activation of signals to ERK and Rac-1 in the same cells (Figs. 3 and 4). Cells that do not express uPAR, such as nontransfected HEK-293 cells, were insensitive to the effects of compounds 6 and 37.

Docking studies showed that both compounds make several H-bonds and hydrophobic interactions with many residues of the vitronectin-binding site of uPAR (Fig. 6). Compounds 6 deeply extends into the vitronectin-binding site of uPAR, nicely mimicking vitronectin itself; compounds 37 entirely fills the vitronectin recognition pocket of the receptor, explaining the slightly better affinity in comparison with compound 6 (Table 1).

Among others, compounds 6 and 37 also target residues comprised within the uPAR chemotactic domain (amino acids, 88–92), able to mediate uPAR interaction with the fMLF family of chemotaxis receptors (fMLF-Rs). Indeed, both compounds 6 and 37 not only blocked cell adhesion to vitronectin, but also impaired the uPAR and fMLF-Rs–dependent cell migration (Fig. 7A).

Compound 6, which interacts with R91 and S88, completely abolished also the structural interaction between uPAR and the high-affinity fMLF receptor, FPR1. Compound 37 which, differently from compound 6, partially contacts the chemotactic SRSRY sequence interacting only with the R91 side chain, slightly inhibited the structural interaction between uPAR and FPR (Fig. 7B), even though it exerted the same functional effect of compound 6 on cell migration. It is noteworthy that R91, the crucial residue in uPAR binding to vitronectin, as shown by Ala-scanning studies (18), is also, as shown by our results, the key residue in uPAR interaction with fMLF-Rs.

It was previously thought that, due to the large surfaces area involved in protein–protein interface (51), small molecules would not be optimal candidates for disruption of these interactions. More recently, it was concluded that “hotspots” might be responsible for the high-affinity protein–protein interaction (52). To the best of our knowledge, the disruption by a small molecule of uPAR binding to vitronectin and its consequence on intracellular signaling and biologic outcomes has not been already described, adding novelty to our approach.
Moreover, compounds 6 and 37 also inhibited uPAR interaction with fMLF-Rs that plays a fundamental role in tumor cell migration, invasion, and metastasis (18, 22, 23, 42, 43). Indeed, both compounds did not cause acute cancer cell death (Fig. 3A), but blocked ECM invasion by cells from several cancer types (Fig. 7C and D); thus, they may be effective in preventing the occurrence of metastasis.

In summary, this study used an integrated drug discovery pipeline consisting of molecular modeling approaches followed by experimental validation. We conducted a screening of a full diversity compound library based on predicted binding to the uPAR:vitronectin-binding site and identified two novel putative compounds for the treatment of cancer diseases. In addition, the functional selectivity and specificity of the selected compounds will allow for further insights in uPAR function and signaling.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

Discovery of New Small Molecules Targeting the Vitronectin-Binding Site of the Urokinase Receptor That Block Cancer Cell Invasion

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