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Challenges for drug discovery - a case study of urokinase receptor inhibition

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Abstract

Urokinase receptor (uPAR) is a widely recognized target for potential treatment of cancer. The development of uPAR inhibitors has been going on for over a decade. Despite the identification and validation of many highly potent hits using screening or medicinal approaches, none of them has been moved further along the drug discovery pipeline. The development of uPAR inhibitors exemplifies several challenges now faced by drug discovery. These include 1) hydrophobicity and thus poor bioavailability of the inhibitors from screening approaches; 2) specificity of the inhibitor, where a peptidyl inhibitor causes conformational change of the receptor; 3) species specificity, where some inhibitors developed based on the human receptor do not inhibit the murine receptor and thus cannot be validated in mouse models. The recently determined crystal structures of uPAR in complex with its ligand or inhibitor not only provide the structural insight to understand these challenges but also offer a potential solution for further inhibitor development and thus illustrate the importance of structural information in facilitating drug discovery.

Drug discovery was historically driven by serendipity and by screening approaches [1]. The last decade has witnessed an increasing implementation of screening approaches in drug discovery due to the advent of high throughput screening (HTS) technology. For many pharmaceutical companies, HTS has become an essential component to identify drug leads [2]. Robotic compound handling and assaying facilities now perform various tasks such as retrieval of compounds from storage, sample preparation and plating, and the running of assays of ever-increasing complexity. Such automated systems can handle tens of thousands of compounds per day. As a result, HTS has become a focus in the drug discovery process. Herein we present the drug development of urokinase receptor as an example to illustrate several challenges still faced by drug discovery, and suggest that the availability of crystal structure of the target protein can greatly facilitate the drug discovery.

Urokinase receptor as a target for the treatment of cancer

Urokinase-type plasminogen activator (uPA) is one of the two primary endogenous systems that mediate plasminogen activation into plasmin [3]. The cellular receptor of uPA, uPAR, binds with both zymogen uPA (pro-uPA) and activated uPA with a high affinity ($K_D < 1\text{nM}$), promotes the activation of pro-uPA, and localizes the proteolytic activities of uPA onto cell surface [4,5]. Plasmin generated by uPA has broad proteolytic specificity, capable of directly degrading several extracellular matrix proteins and activating the matrix metalloproteases

(MMPs), collagenase and stromolysin-1, from their zymogen forms (pro-MMPs), thus implicating uPA-uPAR's role in cell adhesion and migration. Besides this role of promoting protease activity, uPAR can also affects migration, adhesion, differentiation and proliferation through intracellular signaling. Lacking transmembrane and cytosolic segments in its protein structure, uPAR is believed to carry out its signal transduction role by interacting with an array of extracellular partners, including vitronectin (VN), several integrins, G-protein-coupled receptors, and uPAR-associated protein [6]. Important regulators of the uPA-uPAR system include plasminogen activator inhibitor 1 (PAI1), an inhibitor of uPA, and α 2-antiplasmin, an inhibitor of solution phase plasmin. Other regulators of the uPA-uPAR system include α 2-macroglobulin receptor and low-density lipoprotein (LDL)-receptor-related protein (LRP) that can interact with both PAI1 and uPAR [7]. LRP can recognize the uPAR-uPA-PAI1 complex and induce the internalization of the complex, leading to the cytosolic degradation of uPA, but not uPAR, which is then recycled back to cell surface.

The critical role of uPA/uPAR in tumor biology is widely recognized [6,⁸⁻¹⁰]. uPAR is overexpressed in a variety of tumor cell lines. At the tissue level, uPAR is also found to have significantly higher concentrations in several human malignant tumors compared with the corresponding normal tissues. Higher uPAR expression can also commonly be found in tumor-associated stromal cells, including macrophages, mast cells, and endothelial cells [11]. Patients with lung cancer [12], ovarian [13], breast and colorectal cancer [14], as well as other type of cancers, have higher plasma levels of soluble uPAR than in healthy individuals. High levels of uPAR in plasma or tumor tissue lysates were found to be a marker of poor prognosis in many human tumor cell types [15,16]. Over-expression of uPAR in human neuroglioma [17], osteosarcoma [18], epidermoid carcinoma [19] and rat mammary cancer cells [20] resulted in increased tumorigenicity and tumor invasiveness in vitro and in vivo.

Down-regulation of uPAR expression by using anti-gene techniques (antisense, ribozyme, DNzyme and siRNA) has been shown to reverse invasive behavior, induce substantial tumor regression, or even total inhibition of tumor metastasis of several cancers including squamous cell carcinoma, glioblastoma, colon, prostate, and non-small cell lung cancer [10]. Recombinant soluble uPAR inhibited the invasiveness of ovarian cancer cells by competing with surface uPAR for ligand binding [21]. Adenovirus-mediated delivery of ATF, an uPAR-uPA antagonist, suppresses angiogenesis-dependent tumor growth, lung dissemination and liver metastasis in mice [22]. Another uPAR antagonist, the GFD domain of uPA fused with the Fc portion of human IgG, inhibits angiogenesis and primary tumor growth in syngeneic mice in vivo [23]. An anti-uPAR mAb has shown remarkably efficacy inhibiting primary tumor growth, peritoneal invasion and liver metastasis of an orthotopically transplanted pancreatic carcinoma cell line [24]. Gene deletion of uPAR seems not to affect normal physical development, normal reproduction, normal thrombosis, vascular remodeling, and angiogenesis as shown in a mouse gene knockout study in which uPAR-null mice were born and survived to adulthood with no overt phenotypic abnormalities [25]. These findings suggest that uPAR inhibitors might be useful therapeutically for the prevention of invasion and metastasis of certain human cancers.

Barriers to therapeutic intervention: Hydrophobicity of the current small molecular uPAR inhibitors

Since the discovery of uPAR [26-28], several classes of uPAR inhibitors have been developed, including monoclonal antibodies [24,29], recombinant fusion proteins [30-32], peptidyl inhibitors [33-38], and small molecule inhibitors [39]. In this paper, we will focus on the small molecular inhibitors including peptidyl inhibitors.

Two kinds of small molecular peptidyl uPAR inhibitors have been developed including linear peptides and cyclic peptides. A bacteriophage peptide display library was used to generate peptides that inhibit the uPAR-uPA interaction [33]. This resulted in a large number of 15-mer inhibitory peptides with IC₅₀-values in the range from 10 nM to 10 μ M for the uPAR-uPA interaction. A 17-mer peptide, AEPMPHSLNFSQYLWYT, displayed the highest binding affinity towards uPAR. The functional important segment of the peptide was further narrowed down to SLNFSQYLWS [40]. Further studies on structure-activity relationships and affinity maturation by combinatorial chemistry identified a novel 9-mer inhibitory peptide denoted as AE105 (D-Cha-F-s-r-Y-L-W-S, where capital letters denote amino acid in L-configuration, lower case letters denote D-configuration amino acids, and Cha represents *cyclo*-L-hexylalanine) and has a K_D of ~0.4 nM for uPAR binding [34,35]. This peptide competes with ATF (amino terminal fragment, uPA 1-143) binding to uPAR-expressing cells with an IC₅₀ of 10 nM. Recently, the peptide was also coupled to an α -emitter (²¹³Bi) through a K-G-S-G-G spacer, and the labeled AE105 probe (²¹³Bi-P-P4D) showed 17 nM IC₅₀ to human ovarian cancer cells OVMZ-6 and specific uptake to this tumor implanted in nude mice in vivo [41].

Magdolen and co-workers [36-38] developed a cyclic peptidyl uPAR inhibitor based on the fact that residues in a beta-hairpin loop (18-32) of uPA is the major binding determinant of its receptor [4]. After extensive synthesis and testing, they obtained a cyclic peptide, cyclo21;29 [D-Cys21]-uPA21_30[S21C;H29C], that showed an IC₅₀ of 40 nM toward uPAR [38]. Both the cyclic structure (maintained by the Cys21-Cys29 disulfide bond) and the chirality of D-Cys21 are important for the high affinity binding of uPAR. The cyclic peptide was shown to reduce the overall tumor burden of an orthotopic xenograft of human ovarian cystadenomacarcinoma cell line in nude mice [42].

Although peptides tend to have short half-lives in plasma, the above mentioned peptide inhibitors are quite stable in plasma. Even so, small molecular organic compounds are still a major goal for inhibitor development. Several small molecule organic inhibitors for uPAR were identified by traditional screening of chemical libraries (for a review, see [39]). Some inhibitors have IC₅₀ values in the lower nanomolar range. However, no further development/evaluation of these inhibitors can be found in the literature. A common characteristic of all these peptidyl and small molecule inhibitors is that they are all quite hydrophobic, and thus, may have poor bioavailability.

Barriers to therapeutic intervention: how to cross species boundaries

Remarkable species specificity exists in the uPA-uPAR interactions among human and other species. Binding of human soluble receptor with murine uPA is 68-fold weaker than that with human uPA. Similarly, interaction between murine receptor and human uPA is 46-fold weaker than that within murine pair [43]. In addition, the human uPAR inhibitors developed so far also show species specificity, e.g., the linear peptidyl inhibitors developed based on human uPAR did not bind to the murine uPAR or to the uPARs of three species of monkeys [4]. Since the efficacy of human uPAR inhibitors is commonly validated in mouse model systems, such species specificity of inhibitors presents a barrier for the uPAR inhibitor development.

Barriers to therapeutic intervention: inhibitors induced uPAR conformational change

The crystal structures of soluble uPAR in complex with ATF fragment of uPA [44], and with a peptidyl inhibitor (AE147) [35] were recently determined. Comparison of suPAR conformations between suPAR-ATF and suPAR-peptide (AE147) structures indicates that D1 domain of uPAR rotates by 20.5° if D2 and D3 domains in two uPAR structures are superimposed (Fig. 2). This demonstrates not only the inter-domain conformational flexibility

of uPAR, but also the possibility of inhibitor-induced uPAR conformational changes. Even though the peptide inhibitor is highly potent, such inhibitor-induced conformational perturbation on uPAR is not an ideal result for inhibitor development, because such changes may affect uPAR's interaction with other ligands, leading to lower specificity to the targeted function. uPAR is a surface receptor capable of interacting with an array of other proteins. Besides uPA, uPAR also interacts with several cell surface proteins (e.g., integrins and GPCR [45] or matrix proteins (e.g., vitronectin [46]). uPAR uses its central cavity to bind to its primary ligand (uPA) and its outer surface to interact with vitronectin [47]. The ligand occupancy on uPAR central cavity enhances the binding affinity between uPAR and vitronectin [48]. Therefore, an ideal inhibitor of uPAR-uPA interaction should occupy the central cavity, should not change the receptor conformation and, thus, should not affect uPAR interaction with its other ligand.

Structural insights for uPAR-uPA inhibitor development

uPAR-uPA interaction has been a target of intensive inhibitor development since the first report of high affinity antagonists in 1994 [33]. Several classes of potent inhibitors, including antibodies, peptides, or organic compounds, have been identified since then. However, most inhibitors do not move beyond the preclinical stage, and not many follow up reports can be found. Three barriers for uPAR-uPA inhibitor development are highlighted here. Recently determined crystal structures of uPAR complexes [44,⁴⁷,49] provide structural insight to these barriers, might offer solutions to overcome these barriers, and might revive the interest in the development of uPAR-uPA inhibitors.

It took years of effort to produce large quantities of homogeneous suPAR recombinant protein and to generate usable crystals of uPAR-ligand complexes [50,51]. Such capability will provide one of the methods to study inhibitor-induced uPAR conformational changes by crystallizing uPAR-inhibitor complexes and studying their crystal structures.

Human urokinase receptor (uPAR) is a glycosylated membrane protein composed of three homologous domains (D1, D2 and D3) and is linked to the cell membrane through a carboxyl-terminal glycosylphosphatidylinositol (GPI) anchor [43]. The crystal structure of uPAR-ATF complex shows (Fig. 3) that the three individual domains in uPAR are packed closely into each other, resulting in a unique cone-shaped central cavity surrounded by these three domains. The central cavity has a wide opening (25 Å) and significant depth (14 Å). uPA consists of three domains: a small N-terminal growth factor domain (GFD, 47 residues), a kringle domain, and a protease domain at its carboxy terminus. uPA inserts its GFD domain into the uPAR cavity and contacts with all three uPAR domains, whereas the kringle domain of urokinase has only few and apparently weak interactions with the receptor.

A distinguishing feature of the uPAR central cavity is a large hydrophobic patch on one side of the cavity (413 Å², Fig. 4), which is formed by the aliphatic hydrophobic residues from both D1 and D2 domains. Exposure of a large hydrophobic area to the aqueous environment is energetically disfavored. In the ligand-bound form, this hydrophobic patch is completely covered up by interaction with ATF. Thus, the uPA ligand might play an important role in stabilizing the receptor into the observed conformation [52]. The existence of this large hydrophobic area also explains why the small organic inhibitors discovered through HTS tend to be hydrophobic.

The interface between uPAR and ATF can be classified into two regions based on the curvatures of uPAR surface. As illustrated in Fig. 5, region 1 is a large pocket formed mainly by uPAR D2 domain residues and accommodates several uPA GFD residues, especially Lys²³, Tyr²⁴, and Phe²⁵. This region is buried deep in the uPAR cavity and has both hydrophobic and polar interactions between uPA and its receptor. Region 2 of the uPAR-ATF interface is relatively

flat, formed mainly by the residues in uPAR D1 domain, and is hydrophobic in nature. This region interacts with the ATF hydrophobic residues, Phe²⁵, Ile²⁸ and Trp³⁰. Based on the structure of uPAR-ATF and the sequence of uPA (see Fig. 5), it can be seen that region 2 of uPAR (especially, Trp³⁰ and Gln⁴⁰ of uPA in this region) is responsible for the species specificity. The uPA residues in the region 1 are quite conserved among different species in sequence, and mostly likely, also on a structural level.

New opportunities in the development of uPAR inhibitors

The human uPAR inhibitors developed so far are from either screening or medicinal chemistry approaches and face various problems (see above). The recently determined crystal structures of uPAR in complex with uPA (1-135)[49] or with a peptidyl inhibitor (AE147) [44] provide new opportunities for the development of human uPAR inhibitors.

These structures facilitate the understanding of the structural origin of the species specificity and show that the screening method tends to generate hits toward the hydrophobic patch in the suPAR central cavity. This is consistent with a detailed analysis that shows HTS lead generation tends to produce hits that are high-molecular-weight and hydrophobic moieties, whereas high-quality lead compounds and the majority of drugs are generally low-molecular-weight, soluble entities [53,54].

Is it possible to develop small organic compounds to intervene with uPAR-uPA interaction and to overcome the above-mentioned barriers? We propose that the region 1 of uPAR (Fig. 5) be the targeting area for the design of small organic inhibitors to intervene with urokinase-receptor interaction and to overcome these barriers. A primary reason for this proposal is that the uPAR-uPA interaction at this region has both hydrophobic and hydrophilic characters and thus may allow inhibitors that are not too hydrophobic. In addition, inhibitors targeting at this area can bypass the species specificity problem, allowing the inhibitors developed based on human proteins can be validated in mouse models.

In summary, the case of uPAR inhibitor development provides an example to highlight the barriers and the challenges for drug discovery, especially for the screening approach, and suggest that the availability of crystal structures can greatly facilitate drug discovery.

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Figure 1.
Small molecule organic uPAR inhibitors that have IC₅₀ values in the low nanomolar range.

Figure 2.

Inhibitor-induced receptor conformational changes. On the left panel is the crystal structure of uPAR in complex with its native uPA ligand (only GFD domain of the ligand, in cyan, is shown in the figure). Three domains of uPAR (D1, lower right; D2, upper center; D3, lower left) form a central cavity to accommodate the uPA ligand. On the right panel is the structure of uPAR in complex with a peptidyl inhibitor (AE147, in cyan) showing the D1 (lower right) domain separated from the D3 (lower left) domain.

Figure 3.

Overall structure of uPAR in complex with its uPA ligand. uPAR is in surface representation in two views with three domains colored differently, while the amino-terminal fragment (ATF) of uPA is shown as a cyan ribbon. Three domains of uPAR pack closely to each other, resulting in a cavity surrounded by all three domains. The cavity has a wide opening of 25 Å and a depth of 14 Å. ATF inserts its growth factor domain into the uPAR cavity and contacts with all three uPAR domains, as shown in the top view picture on the right hand side.

Figure 4.

A large hydrophobic area exists in the uPAR central cavity. uPAR is in surface representation where carbon, oxygen and nitrogen atoms are colored in gray, red and blue, respectively.

Figure 5.

The uPAR-uPA interface can be classified into two regions (left and right circles). The receptor is in surface representation where carbon atoms of three domain of uPAR are colored orange, cyan and green, respectively. The uPA ligand (cyan) is in stick representation. Nitrogen atoms are colored in blue and oxygen atoms are in red. Region 1 includes uPA residues 23-KYF-25. Region 2 contains mainly uPA residues I28, W30, and N40.