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Single-Molecule Force Measurement Guides the Design of Multivalent Ligand with Picomolar Affinity

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Abstract

Interaction of multiple entities and receptors, or multivalency is widely applied to achieve high affinity ligands for diagnostic and therapeutic purpose. However, lack of knowledge on receptor

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distribution in living subjects remains a challenge for rational structure design. Here, we developed a force measurement platform to probe the distribution and distance of the cell surface vascular endothelial growth factor receptors (VEGFR) in live cells, and used this to assess the geometry of appropriate linkers for distinct multivalent binding modes. A tetravalent lead ZD-4, which was developed from an antitumor drug ZD6474 (Vandetanib) with combined hybrid binding effects, yielded about 2000-fold improvement in the binding affinity to VEGFR with IC_{50} value of 25 pM. We confirmed the improved affinity by the associated increase of tumor uptake in the VEGFR-targeting positron emission tomography (PET) imaging using U87 tumor xenograft mouse model. It showed that radiolabelled ZD-4 resulted in 12 times increase in the tumor uptake than radiolabelled parent drug ZD6474. Our study suggests that combining statistical and chelate effect determined by receptor-receptor distance on live cells for the magnification of multivalent binding is effective. The force measurement platform we describe would be also amenable to other cell-surface multivalent ligand design.

Graphical Abstract

Efficient hybrid multivalent design leads to highly increased tumor uptake.

Keywords

atomic force microscopy; ligand design; multivalency; PET imaging; VEGFR

Multivalency governs many biological interactions and has been widely applied to synthetic compounds in support of targeted molecular imaging and drug therapy.^[1] A number of multivalent binding mechanisms could be used to acquire high binding affinity, including chelate effect, statistical effect, steric stabilization, subsite binding and receptor clustering.^[2] The type of multivalent binding is largely determined by the selection of linkers tethering active entities.^[3] Although proper architectural design to support multiple bindings plays a key role for the affinity, length of the linker largely determines the statistical or the chelate effect in the interaction (Scheme 1). In terms of multivalent ligands targeting cell-surface receptors however, lack of knowledge on receptor distribution and distance in live cells often impedes the rational selection of linkers and scaffold architectures to fit into the relevant binding mechanisms for construction of multivalent ligands.

Atomic force microscopy (AFM), by the virtue of its spatial resolution and its ability to function in aqueous systems, has emerged as a powerful tool to probe biological systems in their native state at a nanoscale resolution.^[4] In this study, we developed a force measurement platform, based on AFM to tackle the challenge in spatial information of cell-surface receptors. Specifically, the interaction of drug compound, ZD6474 (vandetanib; Caprelsa from AstraZeneca), with its targeting to vascular endothelial growth factor receptors (VEGFR) in live human umbilical vein endothelial cells (HUVECs) was studied. For the force measurement, the molecular probe ZD6474 was first conjugated with thioctic acid (Figure 1a) and then attached to a gold-coated AFM tip by means of a strong Au-S bond (Figure 1b). The ligand-functionalized tip was brought into contact with the surface of the HUVECs where the binding of ZD6474/VEGFR took place. The tip was then withdrawn from the surface, pulling ligand out of the binding pocket. The force required to separate the ligand from its specific binding site, the “ligand dissociation force” was determined. As shown in Figure 1, thiol modified polyethylene glycol (Thiol PEG) was used as a competing molecule to reduce the number of ZD6474 probe attached to the AFM tip, which could reduce the number of binding events even at a molecular level during each contact by changing the ratio between the ZD6474 probe and the thiol PEG.

For comparison, AFM tips functionalized with 100% ZD6474 probe and 5% ZD6474 probe diluted with thiol PEG were used for the force measurement. As shown in Figure 2, both AFM height images and adhesion force mapping images were recorded. Interestingly, the protruding area in the force mapping, which represented the specific binding, seemed to be non-uniformly distributed across the native cell surface when comparing the height and force mapping images (Figure 2a-b, c-d). The specific dissociation events were more concentrated along the cellular periphery (Figure S1 in SI). It indicated more concentrated VEGFR distribution at the cell periphery, which is in agreement with independent immunolabeling observations.^[5] In view of this heterogeneous distribution of VEGFR, the AFM force measurement was mostly focused on the cell periphery where there appeared to be abundant expression of the receptors.

To decipher the dissociating force of ZD6474/VEGFR, histograms were analyzed from statistics of the dissociation force measured in Figure 2c and 2d. For the group associated with the 100%-tip, the histogram revealed a unimodal distribution of the dissociating forces with a maxima at 728 ± 16 pN (Figure 2e). This showed an ensemble result of multiple binding events between ZD6474 and VEGFR that occurred in the contact experiments. Since the AFM tip was saturated with the probe molecules, this force value was solely determined by the contact area of the AFM tip, which in turn confined the accessible number of receptors to the given contact area. When the probe coverage on the AFM tip was diluted, the accessible number of ligand molecules in the given contact area was dramatically reduced while the number of receptors remained unchanged. This would prominently decrease the number of binding events involved during each contact measurement. With the dilution to 5% of ZD6474, the histogram revealed a quartet distribution of the dissociation force with peak values at 66 ± 28 pN, 110 ± 12 pN, 156 ± 24 pN and 201 ± 17 pN (Figure 2f). As expected, the maximum dissociation force was reduced to 201 ± 17 pN compared to 728 ± 16 pN in the 100% tip. More importantly, each peak in the quartet distribution represented a different number of binding events occurring during the contact

measurements, and the difference between each pair of adjacent peaks was 45 ± 1 pN, which was in the range of the smallest peak value of 66 ± 28 pN. These results provided strong evidence that a single binding of ZD6474 to VEGFR was associated with a force of 45 ± 1 pN.

As mentioned above, the contact area of the AFM tip restricted the number of receptors accessed in the force measurements, and the number of receptors within the detecting area (N_{VEGFR}) could be readily calculated from the AFM force measurement as $N_{VEGFR} = F_{ensemble} / F_{single}$, where $F_{ensemble}$ was the ensemble of multiple dissociation forces obtained by the 100%-tip, and F_{single} was the dissociation force of ZD6474/VEGFR at a single-molecule level that was obtained by the 5%-tip. As determined from the data in Figure 2, the value of $F_{ensemble}$ was 728 ± 16 pN and F_{single} was 45 ± 1 pN, and consequently N_{VEGFR} was calculated to be 16 within the effective contact area of the AFM tip measuring about 250 nm^2 . Using a maximizing minimum algorithm, the maximal distance between the two adjacent VEGFR on cell periphery in live HUVECs was determined as about $48 \text{ \AA}$ (details in the SI). To our best knowledge, this is the first-time report of receptor distance in live cells measured by AFM.

With the actual distribution information of the receptors in hand, the lengths of the linkers can be rationally selected to design statistical and chelate bivalent binding. Scheme 2 presented a series of multivalent ligands that were used in this study of hybrid multivalent binding. All these compounds were prepared and characterized accordingly (see SI for details), and their binding affinity to VEGFR was measured by the IC_{50} values required to reduce the binding of the parent entity, ZD6474 (ZD-1), in the binding assay of ligand to cell surface receptors as previously described.^[6] The inhibitory binding curve was presented in Figure 3, and the measured IC_{50} values were summarized in Table 1. As designed, a linker with length of $\sim 14 \text{ \AA}$ that was greatly shorter than the proximal VEGFR distance in HUVECs, was used to tether ZD6474 entities and construct a bivalent ZD-2, in which the statistical effect was believed to be dominated in the binding. A second bivalent ZD-3 with linker of $\sim 47 \text{ \AA}$ that corresponded to the estimated distance of neighboring VEGFR was also constructed to assess the chelate effect. The IC_{50} of the monovalent ZD-1 was determined to be $47 \pm 3.0 \text{ nM}$, whereas IC_{50} of the bivalent designs ZD-2 and ZD-3 were found to be $1.8 \pm 0.2 \text{ nM}$ and $2.2 \pm 0.2 \text{ nM}$, respectively. The decreased IC_{50} of ZD-2 and ZD-3 thus represented an approximate 20-fold improvement in the affinity compared to that of ZD-1. It suggests the distinction of statistical and chelate effects in the bivalent binding by using the right lengths of linkers, and both statistical and chelate effects lead to increased receptor binding.

With availability of distinguished linkers to arise different multivalent bindings, the hybrid tetravalent ZD-4 was constructed to effectively combine the statistical and chelate effect in one structure for further optimization of binding affinity. As evaluated by the displacement/competition assay, ZD-4 showed unprecedentedly increased binding affinity with measured IC_{50} as low as $0.025 \pm 0.002 \text{ nM}$, which presents almost 2000-fold improvement over ZD-1, and nearly 100-fold increase compared to bivalent ZD-2 and ZD-3. As a comparison, another tetravalent ZD-5 that only used short linkers of $\sim 14 \text{ \AA}$ tethering the active entities, in which only a statistical effect would be expected to be visited in the multivalent binding, was

also study. Its IC_{50} was revealed 0.89 ± 0.08 nM, which showed only a 2-fold increase compared to the bivalent ligands and a 35-fold less potent than the hybrid ZD-4. The comparison of ZD-4 with ZD-5 strongly supports our proposition that optimized multivalent binding can be achieved by hybrid multivalent design.

Furthermore, the improved binding affinity of ZD-4 to VEGFR was investigated using *in vivo* VEGFR-targeting positron emission tomography (PET) imaging of U87 glioblastoma-bearing mice. Increased tumor uptake of the imaging tracer will be associated with improved VEGFR targeting.^[7] Upon radiolabelled with ^{64}Cu , the PET radiotracer ^{64}Cu -ZD4 (Scheme S1 in SI) with specific activity of 0.3–0.5 MBq/nmol was compared with its parent drug ZD6474 derived radiotracer ^{64}Cu -ZD6474 for their tumor uptake. Specifically, microPET/CT scans were performed on a U87 xenograft mouse model at 2 h, 6 h and 24 h after ~ 3.7 MBq (100 μCi) injection of ^{64}Cu -ZD4 or ^{64}Cu -ZD6474 ($n=6$). Representative whole-body 3D images at 24 h post injection (p.i.) are shown in Figure 4a. High tumor uptake of ^{64}Cu -ZD4 was observed as indicated by the arrow, and revealed ^{64}Cu -ZD4 to be a suitable PET probe for *in vivo* tumor VEGFR imaging. In comparison, the tumor uptake of ^{64}Cu -ZD6474 was revealed by the PET imaging to be much less. For each PET scan, regions of interest (ROIs) quantification analysis of tumor uptake was performed to obtain an imaging ROI-derived percentage of the injected radioactive dose per gram of tissue (%ID/g). From ROI analysis of decay-corrected PET images, the tumor uptake of ^{64}Cu -ZD4 probe was 6.16 ± 0.30 %ID/g as early as 2 h p.i., and increased to 7.24 ± 0.23 %ID/g and 7.90 ± 0.39 %ID/g at 6 h and 24 h p.i., respectively. For the parent monomer radiotracer, the tumor uptake of ^{64}Cu -ZD6474 was only 0.30 ± 0.11 %ID/g at 2 h, 0.66 ± 0.04 %ID/g at 6 h, and 0.46 ± 0.14 %ID/g at 24 h p.i.. The following histological study confirmed the abundant expression of VEGFR in the U87 tumor, which accounted for the high uptake of VEGFR-specific radiotracers. The *in vivo* PET imaging clearly showed that the improved binding of hybrid ZD-4 led to a significantly increased tumor uptake of ^{64}Cu -ZD4, which was 12-times higher than that of radiolabeled parent monomer ^{64}Cu -ZD6474 ($P < 0.0001$). The high tumor uptake and favorable pharmacological properties, suggests ^{64}Cu -ZD4 a suitable PET radiopharmaceutical. Promising radionuclide-based therapeutic products could be produced by incorporation into developed agents with appropriate radionuclides.

In summary, we successfully demonstrated an AFM force measurement platform that was applied to guide the efficient design of VEGFR-targeting hybrid ZD-4 from drug ZD6474. Compound ZD-4 exhibited picomolar receptor affinity, increased tumor uptake and favorable pharmacological properties, which showed high potential in diagnostic imaging and therapeutic treatment of VEGFR-related diseases. Our study suggested a useful strategy to design ligands with dramatic increased binding affinity from known drug by effectively combining various multivalent binding mechanisms. And the force measurement platform that was capable of probing the actual distribution of cell-surface receptors could greatly facilitate the rational design of multivalent drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Structures of the ZD6474 probe and thiol PEG that were used in the functionalization of ATM tips. (b) Schematic drawing for AFM force measurement of ZD6474 binding to VEGFR. Tips functionalized by various concentrations of ZD6474 probe diluted with thiol PEG, such as the 100%-tip and 5%-tip depicted in (b) were prepared.

Figure 2.

The force measurement of ZD6474 to VEGFR in live HUVECs. Measurements that used AFM tips functionalized with 100% (a, c, e) and 5% (b, d, f) concentration of ZD6474 probe are shown and compared: (a, b), 3D height images of the cells obtained during the AFM force measurement; (c, d), Adhesion force mapping presented as 3D images corresponding to the height images in each group; (e, f), Histograms of ZD6474/VEGFR specific dissociative forces obtained from statistics of the area presented as the specific binding. 1024 histogram bins were used.

Figure 3.

Competition binding curves of compounds ZD1–5 to HUVECs. In the binding assay, ^{64}Cu -ZD6474 was used as binding probe and the ZD compound from the design was individually supplemented as competition agent. The IC_{50} was calculated from the competitive binding curve. Data shown are represented as mean $\pm$ SD ($n = 6$ per group).

Figure 4.

Whole-body microPET/CT image of U87 glioblastoma-bearing mice. (a) Representative PET/CT images of the tumor-bearing mice were displayed 24 h p.i. of ^{64}Cu -ZD6474 and ^{64}Cu -ZD4 radiotracers. Tumor is indicated by a white arrowhead. (b) Comparison of decay-corrected ROI analysis of ^{64}Cu -ZD6474 and ^{64}Cu -ZD4 in tumor. Data shown are represented as mean $\pm$ SD ($n = 6$ per group, $P < 0.0001$). (c) The imaged U87 glioblastoma xenograft was histologically studied using haematoxylin and eosin (H&E) and VEGFR2-antibody immunohistochemistry (VEGFR2 IHC) staining.

Scheme 1.

Two effects that govern bivalent binding to cell-surface receptors: (a) statistical effect, arisen from increased local concentration of active entities tethered by short linker in bivalent ligands; (b) chelate effect, arisen from simultaneously binding multiple receptors by active entities tethered by long linker in bivalent.

Scheme 2.

Chemical structure of compounds ZD1–5. The parent ligand entities were highlighted in red.

Table 1.Linker length and IC_{50} of compounds ZD1–5.

Compounds	Valency	Linker Length ^[a] [Å]	IC_{50} ^[b] [nM]
ZD-1	1	n/a	47 ± 3.0
ZD-2	2	~14	1.8 ± 0.2
ZD-3	2	~47	2.2 ± 0.2
ZD-4	4	~47 and ~14	0.025 ± 0.002
ZD-5	4	~14	0.89 ± 0.08

^[a] Linker length is calculated by distance measurement using Chem3D Ultra 8.0.^[b] IC_{50} is the concentration of an inhibitor required to reduce the specific binding by 50%.