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Review Article

Adventures in Multivalency
The Harry S. Fischer Memorial Lecture
CMR 2005; Evian, France

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ABSTRACT: This review discusses multivalency in the context of drug discovery, specifically the discovery of new diagnostic imaging and related agents. The aim is to draw attention to the powerful role that multivalency plays throughout research involving molecular biology, in general, and much of biochemically targeted contrast agent research, in particular. Two examples from the author's laboratory are described. We created small (~5 kDa) peptide 'dimers' composed of two different, chemically linked peptides. The monomer peptides both bound to the same target protein with $K_d \approx 100$ s nM, while the heterodimers had sub-nM K_d values. Biological activity was evident in the heterodimers where none or very little existed in homodimers, monomers or monomer mixtures. Two different tyrosine kinases (KDR and C-Met) and four peptide families produced consistent results: multivalent heterodimers were uniquely different. The second example begins with making two micron ultrasound bubbles coated with the peptide, TKPPR (a Tuftsin antagonist) as a negative control for bubbles targeted with angiogenesis target-binding peptides. Unexpected binding of a 'negative' control, (TKPPR)-targeted bubble to endothelial cells expressing angiogenesis targets, led to the surprising result that TKPPR, only when multimerized, binds avidly, specifically and actively to neuropilin-1, a VEGF co-receptor. VEGF is the primary stimulator of angiogenesis. Tuftsin is a small peptide (TKPR) derived from IgG that binds to macrophages during inflammation, and has been studied for over 30 years. The receptor has never been cloned. The results led to new conclusions about Tuftsin, neuropilin-1 and the purpose, up to now unknown, of exon 8 in VEGF. Multivalency can be used rationally to solve practical problems in drug discovery. When targeting larger structures, multivalency is frequently unavoidable, and can lead to unpredictable and useful biochemical information, as well as to new drug candidates. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: multivalency; contrast agents; molecular imaging; targeted imaging; angiogenesis

INTRODUCTION

The valency of a microscopic entity is the number of separate connections that it makes with another microscopic entity. The term as I use it here applies to atoms, molecules, proteins, cells and various forms of nanoparticulates (1). A multivalent entity is therefore one able to connect to another entity through multiple separate attachments.

Nature's use of multivalency is ubiquitous. Starting with the simplest double or triple bond in the gasses we breathe, oxygen (O=O) and nitrogen (N≡N), chemistry is shot through with multivalency. The bond in nitrogen is twice the strength of the one in oxygen, for example. In Fig. 1, we see a familiar example from a science that supports nuclear and MR imaging: chelate chemistry. It demonstrates how the quantitative power generated by multivalent interactions mounts with increased valency. In biology, examples include influenza viral attachment to cells via trimeric hemagglutinin A to sialic acid (protein to small molecule), neutrophil extravasation (oligosaccharide to protein), *E. coli* infection of the renal system (protein–peptide) and macrophage binding to pathogens (protein–sugar, e.g the mannose units of the Fc portion of pathogen-binding IgG) (1).

While I am defining terms, an adventure is defined as a chance occurrence; an enterprise involving the uncertain or unknown (2).

This paper will review two ongoing projects with the aim to illustrate the power of multivalency in the field of biochemically targeted diagnostic imaging agents, and also some adventurous aspects of the subject.

BIOCHEMICALLY TARGETED IMAGING AGENTS

Most, if not all, new imaging agents now in the discovery phase are designed to seek out and bind to biochemicals

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Abbreviations use: KDR or VEGFR-2, kinase domain receptor; VEGF, vascular endothelial growth factor.

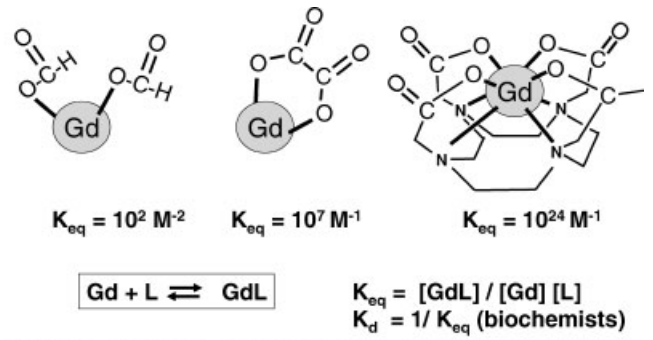

Figure 1. Demonstration of the power of multivalency in the case of chelating ligands. The energy of the interaction multiplies dramatically as the binders become more multivalent.

in vivo. Most of these targeted imaging agents consist of three elements: a biochemical binder (sometimes called a 'ligand'), an entity that is capable of being detected in the image (sometimes called a 'reporter'), and a mechanism to combine these two elements such as a chemical linking moiety. Nuclear medicine has been creating biochemically targeted agents for decades because the radioactive elements are detected in images with the high sensitivity required for biochemistry (i.e. $\leq$ nM or better). The other commonly used modalities tend to approach the problem of scarce biochemical receptors relative to their sensitivity by clustering many reporting entities [e.g. gas bubbles for ultrasound (US), paramagnetic gadolinium (Gd) chelates, and superparamagnetic iron (Fe) oxide] into nano- and macrostructures that also contain biochemical binders. This strategy can be made very efficient with numerous reporters per bound biochemical target, and it is used in many of the abstracts herein. Even viruses and nanotubes have been converted to diagnostic imaging agents (3,4). One unifying characteristic of such structures is that they are inherently multivalent with respect to presentation of the binding ligand to the biochemical target. This makes the study of multivalency effects highly relevant, in general, and important in any particular example of its use. I will discuss two examples that bracket a range of size and valency, using peptides which are targeted to angiogenesis and tumor receptors.

HETERODIMERS

Angiogenesis, the process by which tissues generate new blood vessels, is a well-validated cancer therapeutic target, and is relevant in other diseases with an inflammatory component, such as rheumatoid arthritis and arterial plaque formation. In angiogenesis, cells in need of a blood supply secrete ligands that stimulate blood vessels' endothelial cells to bud and migrate toward the cells secreting the ligands, forming new capillaries. Drugs that bind to the natural angiogenesis ligands and receptors need to be powerful binders to compete with the natural binding reactions *in vivo*. The primary ligand secreted by the cells, vascular endothelial growth factor (VEGF), binds to kinase domain receptor (KDR or VEGFR-2) on endothelial cells with K_d values in the 1 nM range. Concentrations of VEGF *in vivo* during angiogenesis are also very high relative to corresponding normal tissues.

For some applications, monoclonal antibodies can be used as binders. They can routinely be made with the required nM binding K_d, compete with endogenous ligands, and be biologically active. However, antibodies are large, 180 kDa proteins that circulate for days after intravenous administration. As imaging agents, this feature causes a high background that can decrease image contrast, slow excretion with attendant toxicity risks, and slow extravasation, a rate-limiting process when the target is on the abluminal side of the capillary. There are also extra regulatory, safety and manufacturing hurdles with biologic drugs. Antibody fragments can be made much smaller (ScFv$<$25 kDa), but in so doing they tend to lose their strong binding ability. Antibodies rely, after all, on bivalency in binding.

Small molecule drugs ($\leq$1 kDa) can sometimes be created with the desired binding and pharmacokinetic properties, but these are generally made via very expensive and time-consuming medicinal chemistry campaigns, and when successful, only occasionally do they have a structure suitable for chemical linking to a diagnostic reporter. If a neutral atom or region of the molecule not involved in the binding is available for binding to a reporter, the $\leq$1 kDa size still means that that many diagnostic labels of real use, such as Tc chelates $\sim$0.4 kDa, are large enough to destroy the binding specificity and/or binding strength. A practical solution would be to create molecules like polypeptides and oligosaccharides with sizes of $\sim$5–10 kDa. These are

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Harry W. Fischer (1921–1998) was a pioneer in contrast media research and co-founder of the CMR, together with Dr. Lasser. The Harry Fischer Memorial Award was established as a recognition to outstanding researchers in the field of contrast media, with funds managed and distributed by the RSNA. Last year, Dr. Michael F. Tweedle was the recipient of this prestigious award.

Table 1. K_d values for some of the better peptides binding to KDR. Rows 1 and 2 are from round one phage libraries, while row 3 is from a round two library. Data from Shrivastava (7)

Monomeric peptide	K_d (nM)
Ac-GDSRVCWEDSWGGEVCFRYDPGGGKNH$_2$	69
Ac-AGPKWCEEDWYYCMITGTGGGKNH$_2$	280
Ac-AGPTWCEDDWYYCWLFGTGGGKNH$_2$	3

large enough to tolerate labeling, but small enough to be rapidly excreted and pass through endothelial cell fenestrae to extravascular tissues like small molecule drugs. Specific binding peptides can also be discovered *de novo* within large, diverse 'libraries' using phage display technology (5). The binding strength of *de novo* examples has, however, tended to be low like the smaller *de novo* antibody fragments ($K_d \sim 100$–1000 nM), requiring lengthy optimization campaigns.

To create small peptide binders to KDR, we used phage display to an Fc fusion–KDR construct, in collaboration with the Dyax Corporation (5). The peptide binders generated were mostly single loop peptides (<20-mers) with K_d values from 1 μM to tens of nM affinity for KDR (first two rows of Table 1). It is worth noting that phage display uses an M13 virus that attaches to its target via a pentavalent presentation of the expressed peptides. When the peptides are then synthesized for testing they lose the size and especially the multivalency of the phage presentation. A second library (new viral particles) created around the consensus sequences from the first library produced monomeric peptides with K_d as low as 3 nM (row 3 of Table 1; the $GGGKNH_2$ is added as a linker and has little or no effect on binding).

While 3 nM is an admirable K_d, it was found that very few examples in this range of K_d occurred and that none of these examples were able to compete effectively with the natural ligand, VEGF, except at concentrations ≥100 nM. We noted, however, that the peptides tended to belong to one of two consensus sequence families and that these two families did not compete with one another for KDR binding. This led us to conclude that we might increase affinity and effectiveness by creating multimers.

To test this hypothesis we made tetrameric constructs using biotinylated conjugates of the binding peptides. Biotin is a small molecule (MW = 144 Da) that is derivatizable and binds to avidin (MW = 60 kDa) with a stoichiometry of 4 biotin:1 avidin, and with a first $K_d \approx 10^{-15}$ M. We used this well-known binding pair as a scaffold to make easily manipulated tetramers. Several of our peptides were biotinylated and then bound to avidin with the desired stoichiometry. We made two series of tetramers: homotetramers and heterotetramers. The analytical assay was measured binding to cells that had been transfected (genetically altered) to over-express KDR vs binding to control cells that had been mock-transfected and did not over-express KDR. The results are shown in Fig. 2. The pairs of bars in the graph are transfected on the right of each pair and mock-transfected on the left. The tetramer tested in each case is described below the bar with a number for each of three peptides used to construct it. For example, from left to right, the first tetramer contained 0 nonbinding peptides, 4 binding peptides-1, and 0 binding peptides-2. The next tetramer to the right contained 1 nonbinder, 3 binder-1 and 0 binder-2. Looking at the family of homotetramers (left most four tetramers) we can see that binding to the KDR-expressing cells increased as the number of copies of the binder-1 increased from 1 to 4, and that the same trend with lower magnitude was observed for the weaker binder-2. Interestingly, when we made heterotetramers with peptides from two families that did not compete with

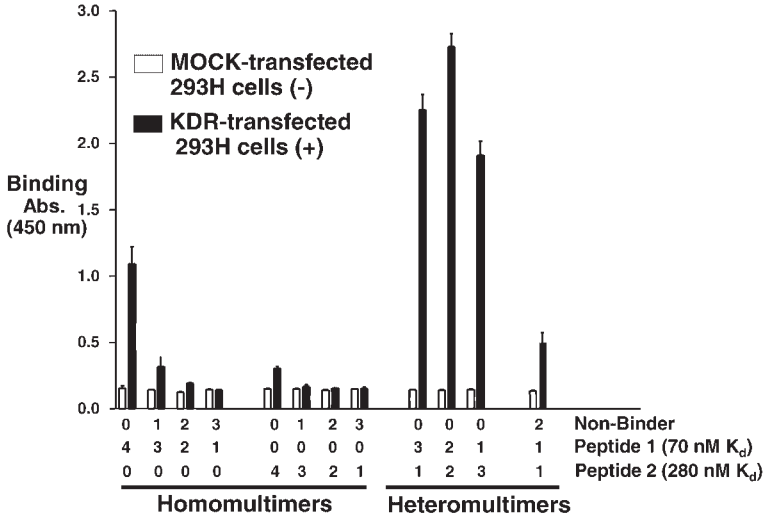


Figure 2. Cell binding results for homo and heterotetramers of biotinylated peptides with avidin (7). In the homotetramer series binding increases with more binding vs nonbinding peptides in the tetramer. Heterotetramers show much stronger binding than homotetramers.

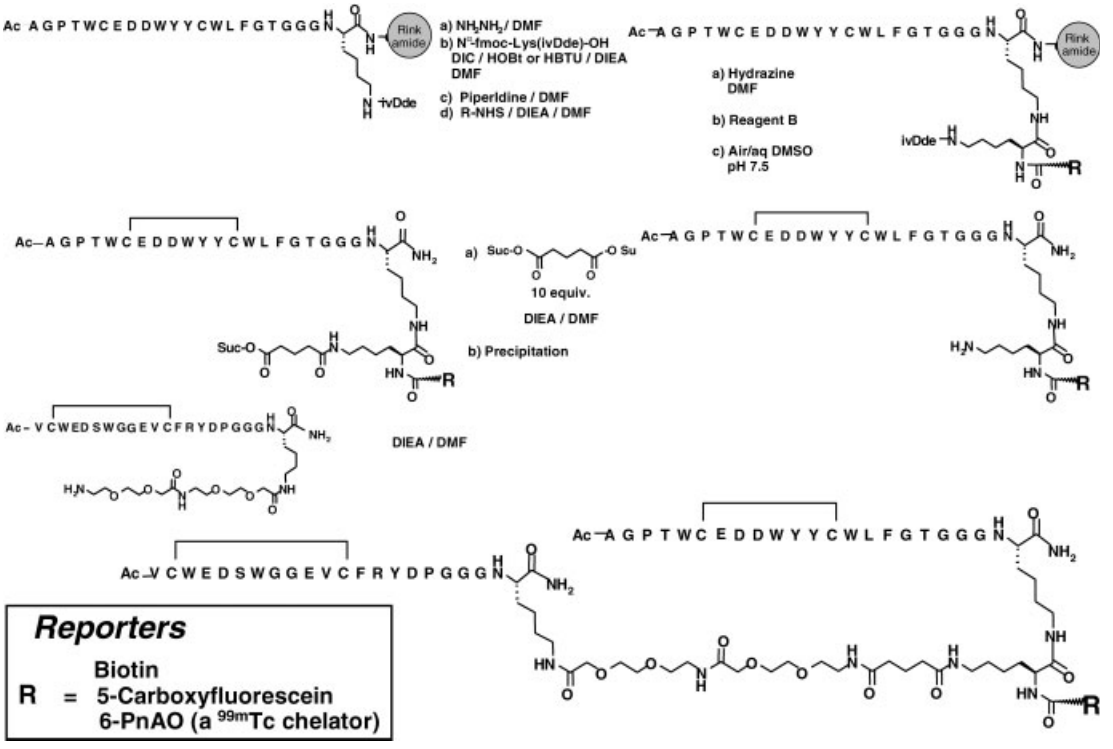


Figure 3. Synthetic route to the peptide dimers, after Pillai (6).

one another, the multivalency effect was dramatically increased. Noting that even heterotetramers with two nonbinders possessed significant binding, we decided to create synthetically accessible heterodimers. These, we reasoned would be a strong enough starting point for optimization and still remain at the 5 kDa size. The structure and synthetic route to these constructs are exemplified in Fig. 3, as published by Pillai (6). While these molecules are unsymmetrical, and therefore technically are not dimers, we will retain the 'dimer' nomenclature for simplicity.

Binding results are shown in Table 2. Remarkably, the heterodimers demonstrated *100–300 fold enhancement* even over the homodimers. They produced sub-nM K_d values to the KDR target. These values rival good antibody binding while being <3% of the antibody's molecular weight. Moreover, the heterodimers, but not monomers or homodimers, actively interfered with autophosphorylation of KDR by VEGF in cultured endothelial cells (Table 3) with IC50 values at or near their K_d values, as published by Shrivastava (7) (structures are disclosed in Shrivastava's paper). We also tried mixtures of both monomers unconnected by the chemical links, and they were inactive like the homodimers.

Table 2. K_d values of peptides linked as in Fig. 3, demonstrating the advantage of heterodimerization. Data from Shrivastava (7)

Peptide	K_d (nM)	
Monomer 4	3.2	K_d by fluorescence polarization for monomers, SPR (Biacore) for dimers
Dimer 4 + 4	4.8	
Monomer 1	70	100 – 300 fold enhancement
Dimer 1 + 1	185	
Monomer 2	280	
Dimer 1 + 2	0.60	
Dimer 1 + 4	0.55	

In addition, activity assays were positive for the ability of the test compounds to inhibit endothelial cell migration stimulated by VEGF. In a consistent pattern, heterodimer 1 + 4 ($K_d = 0.5$ nM) had an IC50 of 1 nM in this assay, while monomer 4 ($K_d = 3.2$ nM) showed an IC50 of 800 nM. Taken together, the results indicate that heterodimerization confers much more than extra binding

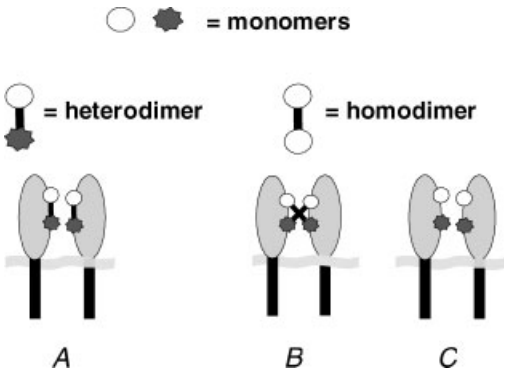
Table 3. IC50 values for homo- and heterodimers. The assay is western blot-based, detecting autophosphorylation of KDR in cultured endothelial cells when VEGF is added along with the different potential inhibitors. Data from Shrivastava (7)

	1 + 4	4 + 4	1 + 1	VEGF
IC50 (nM)	0.5	>100	>100	
Binding K_d (nM)	0.5	4.8	185	1 (VEGF)
	Heterodimer	Homodimers		Control

strength. It also adds biological activity. Finally, 12 recombinant target proteins, both similar and dissimilar to KDR, were tested against the monomers and heterodimers, all with negative binding. This rules out nonspecific binding.

A second tyrosine kinase, C-Met (high affinity receptor for hepatocyte growth factor) was used to generate heterodimers from its own phage display hits. The system behaved the same way as the KDR system. Monomers with 880 and 220 nM K_d were combined to produce a heterodimer with 0.7 nM(7). The method worked with two different proteins and four families of peptides with 100% reliability. Therefore, KDR is not exceptional as a receptor, although C-Met and KDR are both tyrosine kinases. The structure of the heterodimers proved to be chemically tolerant as well. The linker is unsymmetrical, but switching the peptides between the two different ends of the linker had little effect on binding, nor did substituting a variety of labels (5-carboxyfluorescein, biotin, chelates) at the position indicated in Fig.3. Surprisingly, the linker length also was not important between 20 and 40 atoms.

Taking the facts together, we can generate a hypothesis for the binding of the heterodimers to KDR. This is, of course, speculative, but is consistent with the facts as they are known. Tyrosine kinases are known to dimerize *in vivo* during activation. In fact the activating ligand, VEGF, is itself a dimer that appears to bind between two tyrosine kinase monomer units (8). We hypothesize that the probable mode of binding is for the heterodimer to bind to only one monomer unit of KDR, thus preventing this critical dimerization–VEGF–binding event (diagram A in Scheme 1). If the heterodimer were binding across the KDR dimer (diagram B in Scheme 1), then we would reasonably expect monomer mixtures of both peptides and homodimers with low K_d values to be more closely competitive with heterodimers and with VEGF in activation assays.



Scheme 1. Binding of heterodimers to the tyrosine kinase, KDR. A is the probable binding mode for heterodimers to KDR.

Since the molecules in question are not very sensitive to labeling, and are small, highly active binders, the primary goal of the study was achieved. The molecules generated are small enough to deserve the nickname, 'microbodies'. Although not antibody-derived, the concept 'microbodies' seems appropriate, as they are divalent but smaller than the smallest single chain variable region antibody fragments. Apparently, at least as far as tyrosine kinases are concerned, the procedure used works in a general way. It is possible that the phenomenon could be even more generalized to proteins other than tyrosine kinases.

MULTIVALENCY IN A LARGE PARTICLE

The largest particles (insoluble nano- to macrostructures) used in diagnostic imaging are the 2 μm diameter bubbles used as contrast agents in ultrasound imaging. The inside of the bubble is filled with a gas that is insoluble in water. The structure of the shell varies from phospholipids to biodegradable synthetics and cross-linked proteins, but

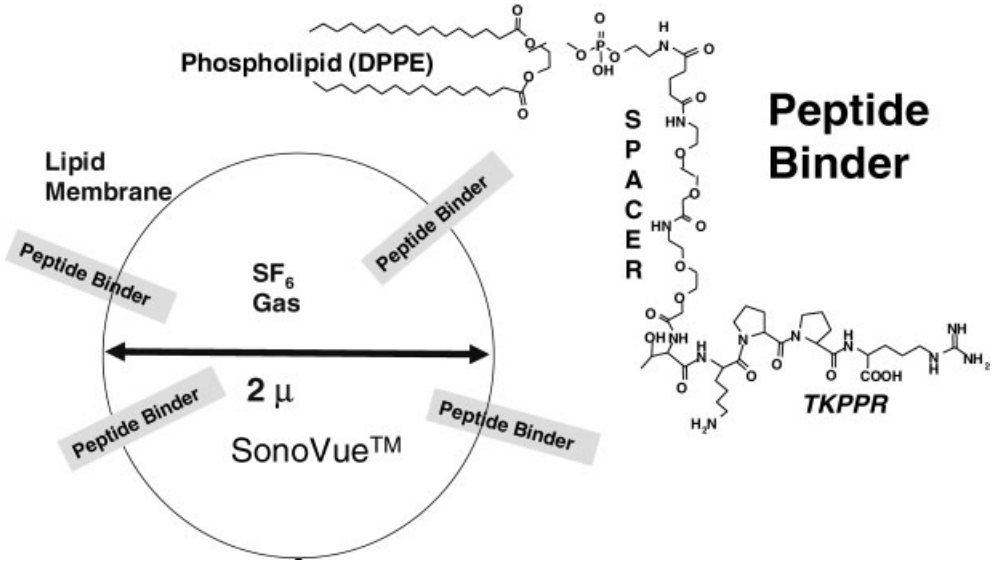


Figure 4. Structure of TKPPR-derivatized, 2 μm, lipid monolayer bubbles.

each of them can be derivatized to expose binders on their surface. In our case we derivatized the product, SonoVue®, a commercial phospholipid shell bubble. To attach peptides we created phospholipid–linker–peptide conjugates, as reported in von Wronski (9). Our objective was to create a bubble targeted to angiogenesis. As part of the program, we needed a peptide that did not bind to angiogenesis targets. We chose one from our stock that we had in quantity, TKPPR.

TKPPR was available because we had researched ^{99m}Tc(R-TKPPR) (10) (R is a Tc chelate) as a clinical diagnostic imaging agent targeting inflammation. TKPPR is an antagonist derived from TKPR, which was named Tuftsin by its discoverers. Tuftsin binds to a structurally uncharacterized Tuftsin receptor expressed on monocyte-derived macrophages. The receptor is thought to be involved in the primary events in inflammation. Probably for this reason, the diagnostic agent ^{99m}Tc(R-TKPPR) successfully enhanced the joints of rheumatoid arthritis patients. Tuftsin itself is a fragment of the Fc region of the IgG antibody family. While Tuftsin has been known and studied for over 30 years, the receptor has never been sequenced or cloned.

The structure of the targeted bubbles is shown in Fig. 4. The phospholipid constructs are readily incorporated into the bubbles by mixing them together, creating constructs with up to 5% of the surface phospholipids as the peptide-targeted versions. This translates to a possible valency of at least hundreds of bubble-attached binders when the targeted bubble binds to a cell. Of course, given the size and spherical shape of the bubble, only a fraction of the peptides on the bubble surface can bind to a cell simultaneously, but a large multivalent interaction is certainly likely.

Cultured endothelial cells express angiogenesis receptors. We therefore used a cultured human endothelial cell screen to test for binding of our targeted bubbles to angiogenesis receptors. Bubbles were added in suspension to the cultured cells that had grown adhered to the bottom of a transparent container. When the container was inverted the bubbles floated upward, insuring contact with the cells. After re-inversion and washing, light microscopy was sufficient to count the bubbles attached to the cells. Figure 5 shows light micrographs of human endothelial cell cultures after incubation with ultrasound bubbles. The underivatized bubbles (A) do not bind the cells, while the TKPPR-derivatized bubbles clearly do bind. Needless to say we were surprised by this very positive result with our 'negative' control. When the TKPPR bubbles were incubated with the cells along with free TKPPR peptide the binding disappeared [Fig. 5(C)], indicating that a specific binding interaction existed between the TKPPR bubbles and the cells. A competition experiment yielded an IC50 of 12.5 μM for the monomeric TKPPR competing with highly multimeric TKPPR bubbles. At this point the lead biochemist reasoned that, since he knew the cells were expressing angiogenesis targets, and that VEGF ended in the sequence, KPRR at the c terminal, VEGF might block the TKPPR bubble binding. He then successfully competed the TKPPR bubbles off the endothelial cells with VEGF, yielding an IC50 of 0.3 nM.

To track down the mechanism we ran a series of bioassays using mostly competition experiments. Table 4 records the results. The $(TKPPR)_2$ and $(TKPPR)_4$ entities are a synthetic homodimer and a homotetramer of TKPPR labeled with Oregon Green, a fluorescent dye that allows detection. These were synthesized and characterized by Raju (11). Recalling our multivalency results from the heterodimer project above allows us to understand the next results in rows 5–7. TKPR is the Tuftsin agonist, a

Table 4. K_d and IC50 results for TKPPR-targeted entities. Data from von Wronski (9)

No.	Compound	Target	Competitor	K_d	IC50	Method
1	Bubble	EC[a]	None		—	Light microscopy
2	TKPPR bubble	EC	None		—	Light microscopy
3	TKPPR bubble	EC	TKPPR	—	12.5 μM	Light microscopy
4	TKPPR bubble	EC	VEGF	—	0.3 nM	Light microscopy
5	TKPPR bubble	EC	TKPR	—	100 μM	Light microscopy
6	TKPPR bubble	EC	TKPPR	—	12.5 μM	Light microscopy
7	TKPPR bubble	EC	$(TKPPR)_4$	—	100 nM	Light microscopy
8	^{125}I-VEGF	NP-1/Fc	VEGF	—	1.5 nM	Radioactivity
9	^{125}I-VEGF	NP-1/Fc	TKPPR	—	30 μM	Radioactivity
10	^{125}I-VEGF	NP-1/Fc	$(TKPPR)_2$	—	3 μM	Radioactivity
11	^{125}I-VEGF	NP-1/Fc	$(TKPPR)_4$	—	100 nM	Radioactivity
12	$(TKPPR)_4$	KDR/Fc	—	None	—	Fluorescence polarization
13	$(TKPPR)_4$	NP-1/Fc	—	25–50 nM	—	Fluorescence polarization
14	^{125}I-VEGF	EC	VEGF	—	1 nM	Radioactivity
15	^{125}I-VEGF	EC	$(TKPPR)_4$	—	150 nM[b]	Radioactivity
16	^{125}I-VEGF	MB231 cells	VEGF	—	1 nM	Radioactivity
17	^{125}I-VEGF	MB231 cells	$(TKPPR)_4$	—	40 nM	Radioactivity
18	^{125}I-VEGF	MB231 cells	$(TKPPR)_2$	—	5–8 μM	Radioactivity

[a]Cultured endothelial cell.
[b]Half blocked.

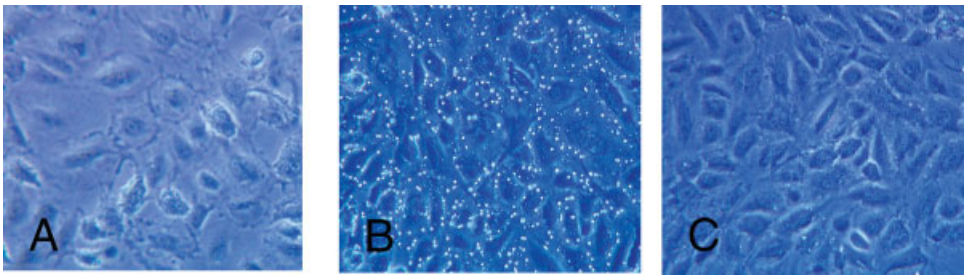


Figure 5. Light micrographs of cultured human endothelial cells incubated with ultrasound bubbles (9). (a) Underivatized bubbles; (b) TKPPR-derivatized bubbles; (c) TKPPR-derivatized bubbles mixed with 100 μM TKPPR (9). Binding in (b), but not (a) and (c), demonstrates that TKPPR-derivatized bubbles are specifically binding to the endothelial cells.

weaker binder than the antagonist, TKPPR. It is weaker also at inhibiting TKPPR bubble binding to the endothelial cells, while the tetramer of the same peptide has an IC50 125 times lower.

The question of what target the TKPPR multimers were binding to was approached by running a series of additional competitive inhibition studies, all of which were negative: BSA, heparin, IgG, bFGF, PIGF-1, IL-1, the cyclic peptide CTKPPRC, and finally $VEGF_{121}$. The negative results suggested, again, a specific reaction.

The $VEGF_{121}$ experiment was especially illuminating because $VEGF_{121}$ differs from VEGF (meaning $VEGF_{165}$ herein) in that it lacks exon 7, which is the exon that codes for the region of VEGF associated with binding of that protein to its co-receptor neuropilin-1 (NP-1). So NP-1 appeared to be a candidate for the specific binding site of the multivalent TKPPR-labeled entities. To validate this hypothesis radiolabeled VEGF was allowed to bind to NP-1/Fc fusions in the presence of potential inhibitors (rows 8–11 in Table 4). The results speak for themselves, as the reaction is significantly inhibited at concentrations as low as 100 nM for the tetramer. The tetramer was then used to confirm NP-1 binding directly using fluorescence polarization to detect binding of $(TKPPR)_4$ to NP-1/Fc and KDR/Fc fusions in solution. It bound strongly to NP-1/Fc (25–50 nM) and not detectably to KDR/Fc (rows 12 and 13 in Table 4).

NP-1, by the way, is a multidomain protein interacting with the VEGF dimer which binds to the KDR dimer—more multivalency. A biological relationship between angiogenesis and inflammation is indeed known to exist. Notably, both endothelial cells undergoing angiogenesis and monocyte-derived macrophages in primary inflammation express NP-1.

It is always possible that in our experiments adventitious binding, having no biological significance,

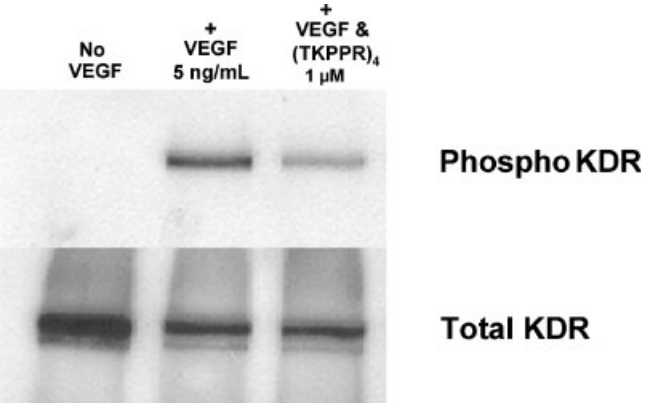


Figure 6. Western blot analysis of extracts of KDR-expressing endothelial cells showing relative autophosphorylation of KDR in the presence of VEGF and VEGF plus $(TKPPR)_4$(9). The tetramer blocks the reaction. See Raju (11) for synthesis of the tetramer.

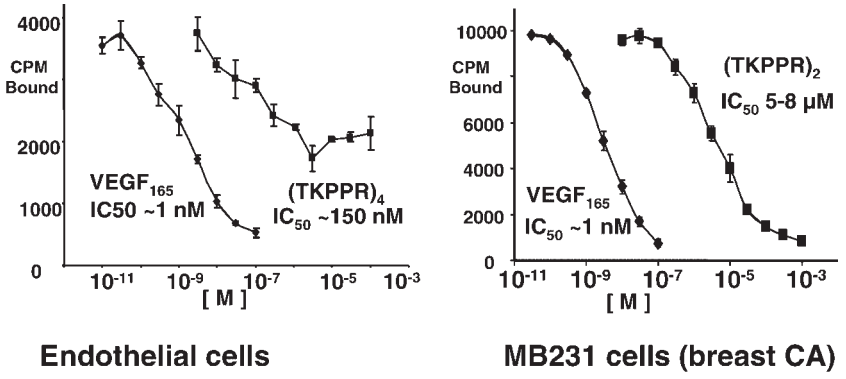


Figure 7. Multimers of TKPPR partially block VEGF binding to endothelial cells expressing KDR and NP-1 and fully block VEGF binding to MB231 breast cancer cells that contain NP-1 but not KDR. [Data for $(TKPPR)_4$ blocking MB231 cells in Table 4, row 17 (9)].

could have been generated to some random crevice of a protein. To check for biological activity autophosphorylation experiments were run, as described in the heterodimer project above. Figure 6 shows that $(TKPPR)_4$ partially, but not fully, blocks VEGF-stimulated autophosphorylation in KDR-expressing endothelial cells. Clearly, this positive result confirms a biologically significant active binding. The partial, rather than complete blocking of the activity is also reasonably justified. NP-1 acts as a co-receptor for VEGF binding to KDR. In the presence of NP-1, VEGF binding with and activation of KDR are greatly enhanced. $(TKPPR)_4$ interfered with this enhanced KDR activation, leaving only the basal level of KDR activation by VEGF.

To confirm the nature of the partial blocking, further experiments on binding to cells were conducted. Two cell types were subjected to VEGF binding experiments interfered with by competitors. Experimental results are shown in rows 14–18 of Table 4 and in Fig. 7. The endothelial cells differ in a crucial way from the MB231 breast cancer cells. The endothelial cells have both KDR and NP-1 expression, while the MB231 cells have *only* NP-1 expression—no KDR. VEGF can therefore bind to the endothelial cells using both receptors and to MB231 cells only through NP-1. In MB231 cells VEGF binding to the cells could be completely blocked by the TKPPR multimers that bind to NP-1, while in the endothelial cells, only half of the VEGF binding to the cells could be blocked. Partial blocking makes biological sense if the multimeric TKPPR constructs are binding to NP-1 and not to KDR.

It is known that the exon 7 in VEGF is responsible for NP-1 binding, but the purpose of exon 8, which codes for the amino acids, CDKPRR, has never been documented. We believe that our experiments suggest that exon 8 of VEGF is involved in NP-1 binding, and that the related peptides, TKPPR and its multimers, interfere with that binding. It is indeed possible that most, if not all of the activity of Tuftsin is accounted for by NP-1. It is in fact possible that the Tuftsin receptor, in whole or part, *is* NP-1.

CONCLUSIONS

My aim was to draw attention to the powerful role that multivalency plays throughout research involving molecular biology, in general, and much of biochemically targeted contrast agent research, in particular. Mother Nature uses it ubiquitously but our experience with it in new molecule creation, while certainly not new, is still innocent. In these two examples we saw two rather surprising discoveries. The first, in relatively small molecules, was that more than 100-fold gains in binding strength and biological activity are achieved reliably in peptide–kinase binding by connecting two noncompeting, but weak *de novo*-derived peptide binders. Multivalency here was used rationally to solve a practical problem in drug discovery. In the second example, unavoidable multivalency in targeted US bubble research led us to an accidental finding, and thence to discover the probable identity of a very old protein. In addition, our control molecule, the TKPPR bubble, is now a novel drug candidate as an angiogenesis-targeted US contrast agent. Multivalency is ripe to yield not only new drugs, but also new biochemical information.

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