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Hybrid Bombesin Analogues: Combining an Agonist and an Antagonist in Defined Distances for Optimized Tumor Targeting

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Supporting Information

ABSTRACT: Radiolabeled hybrid ligands with defined distances between an agonist and an antagonist for the gastrin-releasing peptide receptor were found to have excellent tumor-targeting properties. Oligoprolines served as rigid scaffolds that allowed for tailoring distances of 10, 20, and 30 Å between the recognition elements. In vitro and in vivo studies revealed that the hybrid ligand with a distance of 20 Å between the recognition elements exhibits the highest yet observed tumor cell uptake and retention time in prostate cancer cells.

Peptidic, radiolabeled ligands that bind to receptors overexpressed on tumor cells are attractive for molecular imaging and targeted therapy.[1] The gastrin-releasing peptide receptor (GRP-R) is one of the most interesting targets since it is overexpressed on human prostate adenocarcinoma, the most frequently diagnosed cancer among men in developed countries.[2,3] GRP-R can be targeted both by agonists and antagonists that belong to the family of bombesin peptides.[4−6] Whereas antagonists bind to GRP-R and remain on the surface of tumor cells, typical agonists bind to GRP-R and are then internalized into the cells.[4−8] Among the largest challenges for effective targeting in general is the development of radiolabeled ligands that are (a) effectively taken up by the tumor, either by binding to the cell surface or by internalization, (b) not washed out of the tumor, and (c) have high tumor specificity.[1] Toward these goals the use of multivalent ligands is an attractive approach since the entropic penalty for binding more than one binding element is minimized.[9] Several multivalent ligands have been developed for tumor targeting that combine either identical recognition elements or elements that bind to two different receptors.[10,11] Typical cores for multivalent ligands have a high degree of conformational flexibility and therefore do not allow for a precise control of the distance between the recognition elements.[10,11] We envisioned linking an agonist together with an antagonist for GRP-R on a radiolabeled rigid scaffold in defined distances as an attractive approach toward improved targeting ligands. Such hybrid ligands[12] may not only combine the potency of agonists (internalization) with that of antagonists (high cell surface binding), but also profit from an optimal distance between the recognition elements and therefore lead to higher tumor cell uptake and a longer retention within the tumor.

Within this manuscript we present radiolabeled oligoprolines functionalized with bombesin derived antagonists and agonists in distances of 10, 20, and 30 Å as targeting ligands for GRP-R (**1**−**3**, Figure 1). We demonstrate in vitro and in vivo that the hybrid ligand **2** with a distance of 20 Å between the recognition elements has superior tumor cell uptake properties compared to mono- and bivalent analogues as well as hybrids with shorter or longer spacings between the agonist and antagonist.

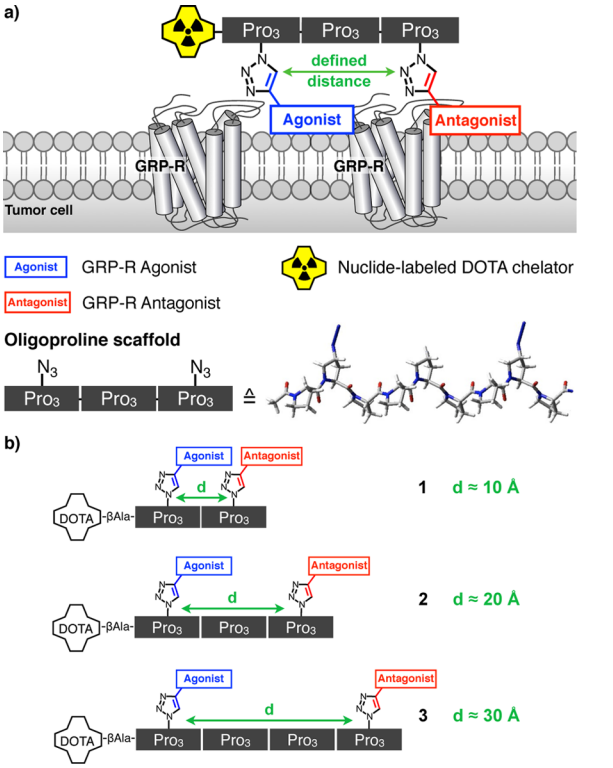

Figure 1. (a) General concept of oligoproline-based hybrid ligands for GRP-R targeting. (b) Hybrid ligands **1**−**3**.

Recently we introduced azidoproline (Azp) containing oligoprolines as conformationally well-defined molecular scaffolds that can be easily functionalized in defined spatial orientations.[13] In aqueous environments they adopt the highly symmetric polyproline II (PPII) helix in which every third residue is stacked on top of each other in a distance of ∼10 Å.[13,14] The functionalization pattern of this molecular scaffold can be

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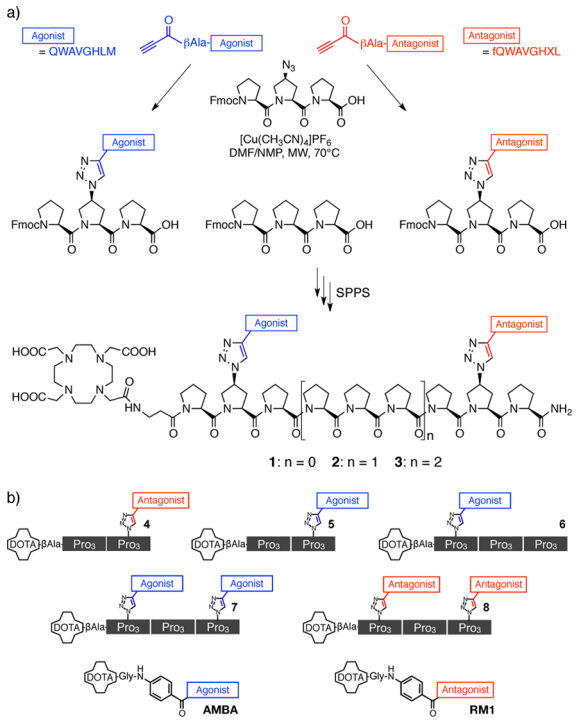
easily fine-tuned by a modular chemical synthesis. In addition, oligoproline derivatives are very soluble in water under physiological conditions. Oligoprolines were therefore envisioned to be ideally suited scaffolds for the synthesis of hybrid ligands bearing an agonist and an antagonist of GRP-R in defined distances from each other. The agonist AMBA[15] and the antagonist RM1[5,16] are among the best so far developed radiolabeled ligands for targeting GRP-R (Scheme 1b). Their

Scheme 1. (a) General Synthesis of the Oligoproline-Based Ligands 1−8 and (b) Schematic Representation of Monovalent Ligands 4−6, Bivalent Ligands 7 and 8, and RM1 and AMBA



recognition elements QWAVGHLM and fQWAVGHXL (X = Statin), respectively, were therefore chosen for the synthesis and evaluation of hybrid ligands for GRP-R.[17] The chelator DOTA[18] was chosen since it is an established tight binder for radionuclides such as ^{68}Ga and ^{177}Lu for in vitro and in vivo studies.[19]

For the synthesis of the hybrid ligands **1−3**, the alkinylated and side chain protected agonist and antagonist (Scheme 1a) were prepared by regular solid phase peptide synthesis (SPPS) and conjugated with the Azp containing trimer Fmoc-Pro-(4*S*)Azp-Pro-OH using Cu(I)-catalyzed Huisgen's 1,3-dipolar cycloaddition reactions ("click reaction").[20] The resulting building blocks were then assembled by SPPS with the unfunctionalized building block Fmoc-Pro-Pro-Pro-OH to the desired length. Each of the coupling steps proceeded with satisfactory yields using HATU/^{i}Pr$_2$NEt as coupling reagent and piperidine in DMF for the Fmoc deprotections. After coupling of β-alanine as a short spacer and the chelator DOTA, the hybrids **1−3** were released from the Rink amide resin along with all side chain protecting groups under acidic conditions.[21] The same modular synthetic strategy was used to prepare the conjugates **4−8** that bear either a single antagonist (**4**), agonist (**5** and **6**), two agonists (**7**) or two antagonists (**8**) on the oligoproline scaffold (Scheme 1b). These ligands were designed as mono- and homobivalent reference compounds to the hybrids **1−3** and to probe the influence of the oligoproline scaffold. CD-spectroscopic analyses of such oligoproline-bombesin conjugates confirmed that they adopt as expected the PPII conformation in water.[21]

To evaluate the tumor cell uptake properties of the ligands **1−8** in vitro, GRP-R overexpressing human prostate adenocarcinoma cells (PC-3) were used. Each of the hybrid, mono-, and homobivalent ligands was labeled with the β^-- and γ-emitter ^{177}Lu ($t_{1/2}$ = 6.7 d) to allow for a quantitative analysis of their tumor cell uptake properties after incubation with PC-3 cells.[21] In addition, to evaluate the importance of the covalent linkage between the agonist and the antagonist, an experiment was performed in which an equimolar mixture of the monovalent ligands **4** and **5** was incubated with the PC-3 cells.

All ligands were taken up into the tumor cells. Blocking of the GRP-Rs on the PC-3 cells by an excess of the unlabeled agonist or antagonist confirmed that the cellular uptake of ligands **1−8** was as expected specific and receptor mediated. The surface-bound fraction and the internalized fraction of the ligands were then determined by an established protocol.[21] These experiments revealed remarkable properties of the oligoproline-based hybrid ligands (Table 1, Figure 2): (1) All hybrid ligands **1−3**

Table 1. PC-3 Cell Uptake of ^{177}Lu Labeled Ligands 1−8, AMBA, and RM1

entry	ligand	surface binding [%][a]	internalization [%][b]
1	**1**	17.2 ± 2.4	30.0 ± 5.0
2	**2**	11.0 ± 1.3	50.7 ± 2.8
3	**3**	6.5 ± 2.5	31.3 ± 0.6
4	**4**	19.5 ± 1.3	8.1 ± 0.4
5	**5**	2.5 ± 0.4	26.7 ± 1.2
6	**6**	2.1 ± 0.8	21.4 ± 9.8
7	**4** + **5**	8.0 ± 0.4	18.4 ± 0.3
8	**7**	2.5 ± 0.2	37.0 ± 1.0
9	**8**	21.1 ± 0.7	12.3 ± 0.9
10[c]	RM1	21.8 ± 0.9	4.7 ± 0.1
11[c]	AMBA	4.3 ± 0.3	29.0 ± 2.3

[a]Surface bound fraction of the ligand after 240 min. [b]Internalized fraction of the ligand after 240 min. [c]Values taken from ref 5.

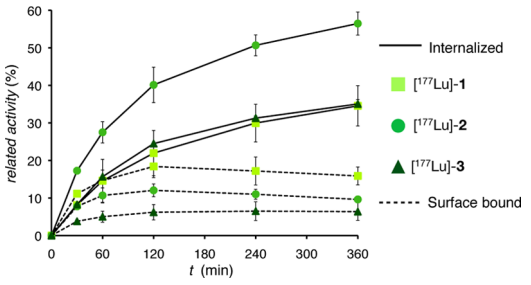


Figure 2. Time-dependent uptake of ^{177}Lu-labeled hybrid ligands **1−3** in PC-3 cells.

have a higher uptake into the tumor cells compared to not only the monovalent oligoproline-based controls (**4−6**), but also the established ligands RM1 and AMBA (Table 1, entries 1−6, 10 and 11).[22] (2) The monovalent ligands **4−6** have comparable uptake properties as AMBA and RM1 demonstrating that the oligoproline scaffold does not affect the uptake properties to a significant extent. The tumor cell uptake of the divalent agonist **7** and divalent antagonist **8** is higher compared to that of the monovalent ligands and in total comparable to those of hybrids **1**

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and **3**. (3) Among all ligands, hybrid **2** with a ~20 Å distance between the recognition elements stands out. More than 50% of **2** were internalized by the PC-3 cells, and 10% were bound on their cell surface after 4 h (Table 1, entry 2 and Figure 2). This is to the best of our knowledge the highest total cellular uptake yet reported for bombesin-derived radiolabeled ligands.[4−6,11a] It is twice as high as those of the monovalent ligands and significantly higher compared to those of the homodivalent ligands **7** and **8** with the same distance between the recognition elements. It is also higher than those of the other hybrid ligands **1** and **3** with shorter or longer distances between the recognition elements. Thus, not only the combination of an agonist with an antagonist but also the distance between them is critical for optimal tumor cell uptake.

A control experiment in which the tumor cell uptake of a mixture of the radiolabeled monovalent ligands **4** and **5** was examined (Table 1, entry 7) showed that the uptake of these noncovalently linked ligands was significantly lower compared to that of any of the hybrids and only comparable to those of the monovalent agonists or antagonists. Thus, the covalent linkage of the agonist and the antagonist in an optimal distance is critical for the high tumor cell uptake.

To evaluate whether hybrid ligand **2** exhibits also high tumor uptake in living organisms, we performed in vivo studies with nude mice in which PC-3 tumor xenografts had been implanted. For these studies, hybrid ligand **2** was labeled with the positron emitter ^{68}Ga ($t_{1/2}$ = 67.7 min) and was then injected into the mice. PET and PET/CT images that were recorded one hour after the injection showed a high uptake in the tumor (Figure 3a).

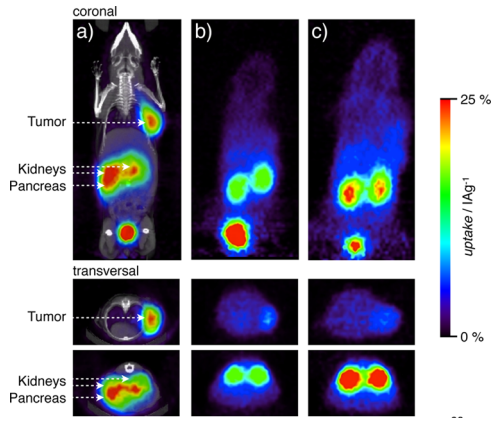

Figure 3. PET/CT (a) and PET (b and c) images of [^{68}Ga]-**2** in PC-3 tumor implanted mice 1 h after injection. 4 mm coronal and transversal slices: (a) unblocked, CT recorded on separate scanner; (b) blocked with excess of unlabeled agonist **5**; (c) blocked with excess of unlabeled antagonist **4**.

The tumor uptake is also in vivo receptor mediated as shown by experiments with mice in which the GRP-Rs were first blocked by an excess of either the unlabeled agonist **5** or antagonist **4** before injection of the radiolabeled ligand (Figure 3b and c). The tumor delineation was very good and the background radioactivity low. The only other organs that show uptake of the radiolabeled ligand are as expected from studies with other ligands: the pancreas that has high physiological GRP-R expression as well as the kidneys and the bladder through which the ligand is excreted. The high tumor uptake observed in the PET images was verified by additional quantitative biodistribution studies using the ^{177}Lu-labeled hybrid ligand **2**. These studies also revealed that the hybrid ligand is washed out of the tumor in the mice very slowly (Figure 4).

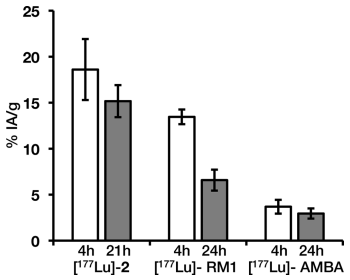

Figure 4. In vivo tumor uptake and retention of ^{177}Lu-labeled hybrid **2**, RM1, and AMBA determined by biodistribution studies in PC-3 tumor implanted mice (data of RM1 and AMBA are from ref 5).

The tumor uptake was 18.6 ± 3.3% injected activity per gram (IA g^{-1}) 4 h after the injection and was still 15.2 ± 1.7% IA g^{-1} after 21 h. This wash-out is significantly lower compared to that of the established antagonist RM1 where half of the ligand was washed out of the tumor within the same time period.[5] It is comparable to the wash-out of the agonist AMBA with which, however, the initial uptake after 4 h was only 3.7% IA g^{-1}.[5] This data underlines that the hybrid ligand **2** combines the best features of an agonist with those of an antagonist in vitro and in vivo: it has the low wash-out of an agonist and the high tumor uptake of an antagonist.

A detailed understanding of the importance of the distance between the agonistic and antagonistic recognition elements for the observed high synergistic effect is not trivial, in particular since the structure of GRP-R has not yet been elucidated and the understanding of the molecular mechanisms by which ligand-GPCR interactions trigger signaling is still limited.[23−25] In addition, it is not yet clear whether the agonist and the antagonist bind to the same site on the GRP-R.[16,26] Our experimental findings are in agreement with a mode of action that relies on the binding of one of the two recognition elements to the GRP-R and thereby predisposes the other into an optimal location to interact with another binding site. This could involve enhanced rebinding of the ligand with its second recognition element after dissociation within the same GRP-R.[27,9a] Alternatively, the hybrid ligand could bridge the distance between the binding sites of a GRP-R dimer.[25,28] Detailed pharmacological studies that will shed more light on the uptake mechanism of the agonist−antagonist hybrids are ongoing and will be reported in due course.

In conclusion, we showed that a radiolabeled hybrid ligand with a distance of 20 Å between an antagonist and an agonist for the GRP-R has excellent prostate tumor uptake properties. Comparison with other ligands revealed that not only the covalent linkage between the agonist and the antagonist but also the distance between them is critical for optimized tumor uptake. In vitro and in vivo studies showed that the hybrid ligand combines the high tumor uptake of the antagonist with the high internalization of the agonist, which results in a long lasting retention in the tumor. These features render hybrid ligands very attractive for tumor targeting. Since the oligoproline-based ligands are readily accessible by a modular synthesis, the concept of hybrid ligands with controlled distances between the recognition elements can be easily expanded to the targeting of other receptors, e.g., somatostatin and gastrin receptors that are

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overexpressed on neuroendocrine tumors, certain thyroid and lung cancers, respectively.

■ ASSOCIATED CONTENT

Supporting Information

Details on the synthesis, analytical data, and in vitro and in vivo studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(21) For experimental details, see the Supporting Information.

(22) Binding affinity studies showed that the hybrid ligands bind 2−4-fold tighter to GRP-R (**1**: K_d = 32 nM, **2**: K_d = 24 nM) than the monovalent antagonist (**4**: K_d = 100 nM) or agonist (**5**: 75 nM).

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