Identification and stabilization of a highly selective gastrin-releasing peptide receptor agonist

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The gastrin-releasing peptide receptor (GRPR) is part of the bombesin receptor family and a well-known target in cancer diagnosis and therapy. In the last decade, promising results have been achieved by using peptide-drug conjugates, which allow selective targeting of GRPR expressing tumor cells. Most ligands, however, have been antagonists even though agonists can lead to higher tumor uptake owing to their internalization. So far, only a few studies focused on the identification of small GRPR-selective agonists that are metabolically stable. Here, we developed novel bombesin analogs with high selectivity for the GRPR and improved blood plasma stability. The most promising analog [D-Phe⁶, β-Ala¹¹, NMe-Ala¹³, Nle¹⁴]Bn(6–14) displays an activity of 0.3nM at the GRPR, a more than 4000-fold selectivity over the other two bombesin receptors and more than 75% stability in human blood plasma after 24 hours. This analog is proposed as a promising drug shuttle for the intracellular delivery of different payloads in targeted tumor therapy approaches.

KEYWORDS
bombesin, GPCR, metabolic stabilization, peptide-drug shuttle, tumor targeting

1 \ | INTRODUCTION

G protein-coupled receptors (GPCRs) have evolved recently as great targets in cancer therapy and diagnosis due to their high overexpression in malignant cells.¹ The gastrin-releasing peptide receptor (GRPR, BB2) forms together with the neuromedin B receptor (NMBR, BB1) and the bombesin receptor subtype-3 (BRS-3, BB3), the bombesin receptor family, which gained special interest in the field of tumor targeting. The GRPR is one of the most overexpressed GPCRs in various cancers such as small lung cell,²-⁴ prostate,⁵,⁶ head/neck squamous cell,⁷  colon,⁸ glioblastoma,⁹ and breast cancer.¹⁰,¹¹ These findings initiated the development of small peptides derived from the 14 amino acid bombesin peptides, which fully activate all three receptors. These findings initiated the development of small peptides derived from the 14 amino acid bombesin peptides, which fully activate all three receptors. The last nine C-terminal amino acids are essential for the activity and share high similarity with those found in the endogenous ligands neuromedin B (NMB) and gastrin-releasing peptide (GRP). However, crucial for the development of peptide-drug conjugates is their high selectivity for the overexpressed receptor to prevent adverse side effects due to an unspecific accumulation and subsequent damage of healthy tissues. Owing to the small size of the peptides and straightforward synthesis, various different payloads...
like nanoparticles, quantum dots, cytotoxic moieties, photosensitizers, or siRNA have been attached to various bombesin peptides and were successfully delivered to tumor cells. Further, promising results have been achieved with bombesin analogs for the imaging of prostate cancer.

The majority of the developed peptide carriers have been antagonists because they can target the GRPR without inducing the biological downstream responses in healthy tissues. A recent example for this approach is the conjugate [α-Phe6, NMe-Gly12, Sta13, Leu14]Bn(6-14), which showed great results in the selective delivery of doxorubicin-filled nanoparticles. Even though antagonists are discussed to provide better pharmacodynamics, the cellular uptake mechanism of the cargo at the tumor site is highly dependent on the payload itself. In contrast, the use of agonists lead to a receptor-mediated internalization into intracellular vesicles and thus facilitates a cargo-independent cellular uptake. Since the GRPR is subsequently separated in the endosome and recycles back to the cell membrane, this process can be repeated and may lead to an active intracellular accumulation of the peptide-drug conjugate. In the past, derivatives such as [Nle14]BBN(7-14) or Bn(6-14), modified with radiotracers, were proposed as suitable conjugates for the detection of GRPR expressing tumors. Even though these conjugates showed great tumor uptake, they were found to be metabolically instable, which resulted in a fast wash out. The rapid metabolic degradation of bombesin-based conjugates and their low selectivity profile impact their drug delivery capacity and limit medical application. Thus, we developed GRPR-selective bombesin analogs with sufficient blood plasma stability and demonstrated the intracellular delivery of a fluorophore-payload. The derivatives with the highest activity, greatest selectivity, and optimal metabolic stability can be considered as a drug shuttle for targeted tumor treatment.

2 MATERIALS AND METHODS

2.1 Materials

All N-α-Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBT), ethyl 2-cyano-2-(hydroxymino)acetate (Oxyma), and N,N′-disopropylcarbodimide (DIC) were purchased from Iris Biotech (Marktredwitz, Germany), and the NovaSyn TGR R resin and O-(7-azbenzotriazolyl)-tetramethyluronium hexafluorophosphate (HATU) were supplied from Novabiochem (Darmstadt, Germany). 6-Carboxytetramethylrhodamine (TAMRA) was purchased from ChemPep, Inc (Wellington, Florida), and acetonitrile (ACN) was sourced from VWR (Darmstadt, Germany). Dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Biosolve ( Valkenswaard, The Netherlands). Piperidine, triisopropylsilane (TIS), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, Missouri). Diethyl ether was from Merck (Darmstadt, Germany). The automated synthesis was performed with a SYRO I peptide synthesizer from MultiSynTech (Witten, Germany).

2.2 Solid phase peptide synthesis

All bombesin derivatives were synthesized by solid phase peptide synthesis (SPPS) using fluorenylmethoxycarbonyl (Fmoc)/tBu strategy at a 15 μmol scale. A NovaSynTGR R resin was used to generate an amidated C-terminus. Automated SPPS was performed on a SYRO I peptide synthesizer using eightfold molar excess of N-α-Fmoc-protected amino acids, ethyl 2-cyano-2-(hydroxymino)acetate (Oxyma), and N,N′-disopropylcarbodiimide (DIC) in DMF. All automated coupling steps were carried out twice with a reaction time of 40 minutes. Fmoc protecting groups were cleaved using 40% piperidine in DMF (v/v) for 3 minutes and 20% piperidine in DMF (v/v) for 10 minutes.

The N-α-Fmoc-protected amino acids O-methyl-l-homoserine (Hse (Me)), beta-(2-thienyl)-l-alanine (Tha), 2-aminoisobutyric acid (Aib), and N-methyl-l-alanine (NMe-A) were manually introduced, using a fivefold molar excess of 1-hydroxybenzotriazole (HOBT), DIC, and the respective amino acid in DMF at room temperature for at least 4 hours. For the synthesis of fluorescently labeled conjugates, a three-unit ethyleneglycol spacer (EG3) was coupled manually to the N-terminus with 2 Eq. of Fmoc-NH-PEG3-COOH and 1.9 Eq. of O-(7-azbenzotriazolyl)-tetramethyluronium hexafluorophosphate (HATU). N,N-Diisopropylethylamine (DIEPA) was added in twofold molar excess and the reaction was performed for approximately 18 hours. Fmoc was cleaved using 30% piperidine for 10 minutes twice and 6-carboxy tetramethylrhodamine (TAMRA) was coupled in twofold molar excess with 1.9 Eq. HATU and 2 Eq. DIEPA. The peptides were cleaved from the resin and simultaneously deprotected by incubation with trifluoroacetic acid (TFA), triisopropyl silane (TIS), and aqua dest (90:2.5:2.5, v/v/v) for 3 hours followed by precipitation using ice-cold diethyl ether. Purification was performed by preparative RP-HPLC applying a linear binary gradient system of eluent A (0.1% TFA in water, v/v) and eluent B (0.08% TFA in ACN, v/v). UV absorption was measured at λ = 220 nm. The correct identity and purity of the synthesized peptides were confirmed by analytical RP-HPLC, MALDI-ToF-MS (UltraflexII, Bruker), and ESI-MS (HCT, Bruker) and are summarized in Table 1.

**TABLE 1** Analytical data of synthesized peptides with indicated amino acid substitutions

| No. | Sequence | MWmono | Signal | ACN | Purity |
|-----|----------|--------|--------|-----|--------|
| 1   | GNLWATGHFM | 1131.5 | 1132.6 | 38.8 | >95    |
| 2   | GNHWAVGLHM | 2857.5 | 2858.4 | 31.1 | >95    |
| 3   | NQWAVGLHM | 1053.5 | 1054.5 | 26.3 | >95    |
| 4   | fQWAVbHFX | 1116.6 | 1117.6 | 31.3 | >95    |
| 5   | zWAVbHFX | 952.5 | 953.5 | 31.7 | >95    |
| 6   | aQWAVbHFX | 1040.6 | 1041.5 | 29.0 | >95    |
| 7   | fQWAAbHFX | 1088.6 | 1089.6 | 39.0 | >95    |
| 8   | zWAAbHFX | 924.5 | 925.5 | 29.0 | >95    |
| 9   | aQWAAbHFX | 1012.6 | 1013.5 | 26.5 | >95    |
| 10  | fQWAVbHLX | 1082.6 | 1083.6 | 30.5 | >95    |
| 11  | fQWAVbHAX | 1040.6 | 1041.6 | 26.3 | >95    |

*The N-terminal amino acids 1-17 of GRP.

aRP-HPLC performed on Phenomenex Aerus Peptide 3.6u XB-C18 (250 mm × 4.6 mm, 3.6 μm, 100 Å), flow rate: 1.55 mL/min.
### 2.3 | Biological methods

#### 2.3.1 | Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, Dulbecco's phosphate buffered saline (DPBS), Ham's F12, Hank's balanced salt solution (HBSS), and trypsin/EDTA were purchased from Lonza (Basel, Switzerland). Fetal calf serum (FCS) was obtained from Biochrom (Berlin, Germany). G418-sulfate and poly-D-lysine were obtained from Merck (Darmstadt, Germany). Opti-MEM was purchased from Life Technologies (Carlsbad, California), and Hoechst 33342, enalaprilat, sarcubitrilat, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), probenecid, Pluronic F-127, LB-medium, and Spin-X filter tube were sourced from Sigma-Aldrich (St. Louis, Missouri). The Fluo2-AM dye was purchased from Abcam (Cambridge, UK), and the 8-well ibiTreat μ-slides were obtained from IBIDI (Martinsried, Germany). Competent *Escherichia coli* DH5α cells and lipofectamine 2000 were supplied from Invitrogen (Carlsbad, California). HEK293 and PC3 cells were purchased from DSMZ (Braunschweig, Germany) and cultivated in cell culture flasks of TPP (Trasadingen, Switzerland). The plasmids were purchased from OriGene (Rockville, Maryland), and the plasmid purification kit PureYield was supplied from Promega (Madison, Wisconsin). Black μCLEAR CELLSTAR 96-well plates from Greiner Bio-One (Kremsmünster, Austria) and Ca2+ flux measurements were performed with a FlexStation 3 from Molecular Devices (San Jose, California). Fluorescence microscopy studies were performed with a Zeiss microscope (Oberkochen, Germany).

#### 2.3.2 | Stable transfection

The plasmids (pCMV6_NMBR-tGFP, pCMV6_GRPR-tGFP, and pCMV6_BRS-3-tGFP) were amplified using *E. coli* DH5α cells and purified with a PureYield Kit. Correct constructs have been confirmed by sequencing. HEK293 cells were stably transfected with the plasmids using Lipofectamine 2000 and selected with G418.

#### 2.3.3 | Cell culture

All cell lines were maintained in T75 cell culture flasks at 37°C, 95% humidity, and 5% CO2 (standard conditions). HEK293 cells stably expressing the tGFP tagged NMBR, GRPR, or BRS-3 were grown in DMEM/HAM's F12 (1:1, v/v) containing 15% FBS (v/v) and 1.0 mg/mL G418. PC3 cells were cultures in RPMI 1640 medium containing 10% FCS (v/v). After cells reached full confluency, they were split in desired ratios from 1:2 to 1:12 into new cell culture flasks, filled with fresh medium for further cultivation, or seeded into cell culture vessels for assays.

#### 2.3.4 | Ca2+ mobilization assay

HEK293_NMBR-tGFP, HEK293_GRPR-tGFP, and HEK293_BRS-3-tGFP cells were seeded into black poly-D-lysine coated μCLEAR 96-well CELLSTAR plates at a density of 180 000 cells/well for the NMBR and GRPR-receptor and 220 000 cells/well for the BRS-3 and were incubated under standard conditions, overnight. At the following day, the medium was aspirated, and the cells were incubated for 40 minutes under standard conditions with Fluo-2-AM solution (2.3 μM Fluo-2 AM, 0.06% [v/v] Pluronic F-127 in assay buffer). The fluorescence dye solution was replaced by assay buffer (20 mM HEPES, 2.5 mM probenecid in HBSS, pH 7.5) and basal Ca2+ signal was measured for 20 seconds with a Flexstation 3 before ligand was added and Ca2+ response was determined for another 40 seconds ($A_{Ex} = 485 \text{ nm}, \lambda_{Em} = 525 \text{ nm}$). The x-fold over basal values were calculated, and the data were analyzed with GraphPad Prism 5.

### 2.3.5 | Live cell microscopy

Receptor internalization was investigated by using stably transfected HEK293 cells, which were seeded into 8-well ibiTreat μ-slides and incubated for 2 days at standard conditions. At the assay day, cells were starved for 30 minutes with 200 μL OptiMEM under standard conditions, and OptiMEM was subsequently replaced by 200 μL of OptiMEM containing 10⁻⁷ M peptide and stimulation was performed for 1 hour. Nuclei visualization was achieved with an addition of 1 μL of Hoechst 33342 (0.5 mg/mL) 30 minutes prior to image recording. Subsequently, cells were washed twice with OptiMEM to remove excessive peptide. Image recording was conducted directly after washing while cells were maintained in OptiMEM, using an Axio Observer. Z1 microscope equipped with an ApoTome Imaging System and a Heating Insert P Lab-Tek S1 unit (Zeiss, Oberkochen, Germany). Image processing was performed with AxioVision 3.1.

#### 2.3.6 | Blood plasma stability assay

Fluorescently labeled peptides were incubated in a concentration of 10⁻⁷ M in human blood plasma under constant shaking at 37°C. Samples (100 μL) were taken at desired time points, and plasma proteins were precipitated with an addition of 200 μL of ACN (5% TFA, v/v) at -20°C for at least 1 hour. After centrifugation with 21 000 rcf for 10 minutes, the supernatant was diluted 1:3 with aqua dest and transferred to a Costar Spin-X centrifuge filter tube (0.22 μm). After 30-minute centrifugation with 16 000 rcf, the fluorescence emission of the filtered peptide solutions was measured by RP-HPLC at $\lambda = 573 \text{ nm}$ using linear gradients of eluent B in A (eluent A = 0.1% TFA in water; eluent B = 0.08% TFA in ACN) on a Varian VarilTide RPC column (250 mm × 4.6 mm, 6 μm, 200 Å, Agilent, Santa Clara). Peak areas were normalized, and the half-life was calculated with GraphPad Prism 5.

### 3 | RESULTS

#### 3.1 | Design and synthesis of GRPR-selective peptides

We aimed for the development of a GRPR-selective peptide ligand with sufficient metabolic stability for a drug shuttle system. All analogs were
synthesized by a combination of automated and manual SPPS on a NovaSynTGR R resin generating a C-terminal amide. The native ligands NMB (1), GRP (2), a shortened bombesin derivative Bn(6-14) (3), and the universal ligand [D-Phe6, βAla13, Phe13, Nle14]Bn(6-14) (4) were synthesized as control peptides to validate the assay system. Analogs 5 to 7 were reported to be selective for the GRPR and therefore synthesized as controls.29 To further increase the selectivity, peptide chimeras were developed investigating potential additive effects of the selectivity gaining amino acid substitutions. Derivatives 5 and 7 were combined in peptide 8 containing the N-terminal truncation of one amino acid, the glutamic acid was cyclized to a pyroglutamic acid (Glp), and Val10 was substituted by Ala. Analog 9 contains also the Val to Ala substitution at position 10 but carries in addition an Ala at position 6 instead of the original D-Phe. Furthermore, we investigated position 13 by single amino substitutions because this position was reported to be highly important for the receptor activation. Since GRP (2) and Bn(6-14) (3) contain Leu at the penultimate position while NMB (1) features Phe, we expected to gain selectivity by exchanging the Phe13 in analog 4 by Leu13 (10). Additionally, we reduced the side chain at position 13 even further and introduced Ala (11). After the synthesis was completed, all analogs were analyzed by MALDI-TOF and ESI-MS to prove correct molecule identity. Homogeneity was measured by RP-HPLC and is summarized in Table 1.

3.2 Receptor selectivity investigation

The synthesized analogs were characterized with respect to receptor activation and selectivity by a Ca2+-mobilization assay (Table 2). To validate the assay setup, the native ligands NMB (1), GRP (2), a shortened bombesin analog Bn(6-14) (3), and the universal ligand (4) were tested. The NMB (1) exhibited equimolar potencies at the NMBR and the GRPR with an EC50 value 0.3nM whereas the BRS-3 was activated with an EC50 value of 215nM. The GRPR preferring peptide 2 activated the GRPR with an EC50 value of 0.6nM whereas the NMBR was activated with a slightly lower EC50 value of 15nM and the BRS-3 was hardly activated. Analogs 3 and 4 exhibited similar potencies with respect to NMBR and GRPR activation but only the universal ligand (4) activated the BRS-3 with an EC50 value of 5.5nM. Therefore, the universal ligand was used for normalization at each receptor. Peptide 5 exhibited slight selectivity for the GRPR due to a tenfold reduced potency at the NMBR and BRS-3 while the EC50 value at the GRPR was only slightly decreased compared with the universal ligand (4). The exchange of D-Phe6 by D-Ala in analog 6 had no effect on the activation properties at all three receptors. Substitution of D-Val8 to D-Ala (7) had no effect on the GRPR activation, whereas the potency at the NMBR and BRS-3 was slightly reduced. Derivative 8 bearing two modifications exhibited a tenfold loss of activity at the GRPR and an even stronger potency loss at the NMBR and BRS-3 resulting in a peptide with 150-fold selectivity towards the NMBR and more than 550-fold to the BRS-3. However, the combination of peptide 6 and 7 resulting in analog 9 did not provoke any significant increase in selectivity.

Since the combined analogs showed only slight selectivity for the GRPR mainly caused by a reduced NMBR and BRS-3 activity and not by a potency increase at the GRPR, we also investigated position 8 by single amino substitutions. Because GRP (2) and Bn(6-14) (3) contain a Leu at this position and exhibit a strongly reduced potency at the BRS-3, we exchanged the L-Phe13 in conjugate 4 by L-Leu (10). Surprisingly, this substitution had just minor effects on the potency at all three receptors. The exchange to D-Ala (11), however, led to a

**TABLE 2** Summarized receptor activation data of synthesized analogs

| No. | Sequence | EC50[nM] | Selectivity (X-fold) |
|-----|----------|----------|---------------------|
|     |          | NMBR     | GRPR    | BRS-3       | NMBR/GRPR | BRS-3/GRPR |
| 1   | GNLWATGHFM | 0.3 (9.56 ± 0.07) | 0.3 (9.63 ± 0.05) | 215 (6.67 ± 0.11) | 1 | 936 |
| 2   | kGNHWAVGHLM | 15 (7.81 ± 0.10) | 0.6 (9.23 ± 0.08) | ≥1000 (n.d.) | 26 | ≥1500 |
| 3   | NQWAVGHLM | 0.4 (9.40 ± 0.06) | 0.03 (10.6 ± 0.09) | ≥1000 (n.d.) | 14 | ≥8000 |
| 4   | fQWAVbHF | 0.7 (9.19 ± 0.08) | 0.05 (10.3 ± 0.03) | 5.5 (8.26 ± 0.11) | 4 | 110 |
| 5   | zWAVbHX | 6.9 (8.16 ± 0.10) | 0.1 (9.96 ± 0.12) | 50 (7.30 ± 0.09) | 63 | 456 |
| 6   | aQWAVbHF | 0.9 (9.06 ± 0.13) | 0.05 (10.3 ± 0.11) | 5.0 (8.30 ± 0.06) | 17 | 100 |
| 7   | fQWAhbHF | 2.3 (8.63 ± 0.09) | 0.08 (10.1 ± 0.10) | 15 (7.81 ± 0.08) | 29 | 194 |
| 8   | zWAhbHX | 79 (7.10 ± 0.09) | 0.5 (9.29 ± 0.11) | 295 (6.53 ± 0.08) | 153 | 568 |
| 9   | aQWAhbHF | 0.9 (9.06 ± 0.10) | 0.04 (10.4 ± 0.09) | 7.4 (8.13 ± 0.10) | 22 | 185 |
| 10  | fQWAVbHLX | 2.1 (8.68 ± 0.14) | 0.1 (10.1 ± 0.10) | 17 (7.75 ± 0.10) | 23 | 198 |
| 11  | fQWAVbHAX | ≥500 (n.d.) | 0.1 (9.9 ± 0.07) | ≥1000 (n.d.) | ≥4000 | ≥8000 |

Note. Data were obtained by Ca2+-mobilization assay using stably transfected HEK293_X-tGFP (X = NMBR, GRPR, BRS-3) cells. Values representing the mean of at least three independent experiments performed in duplicates. a = D-Ala; X = L-norleucine; z = pyroglamate; f = D-Phe; b = β-Ala.

Abbreviation: n.d.: not determined.

*The N-terminal amino acids 1-17 of GRP.
drastic loss of activity at the NMBR and BRS-3, while the potency at the GRPR was only slightly affected. This substitution led to a highly selective peptide with more than 4000-fold selectivity over the NMBR and more than 8000-fold towards the BRS-3 (Table 2 and Figure 1).

Next, fluorescence live cell imaging experiments were performed to examine receptor internalization. The unstimulated HEK293 cells stably expressing the tGFP tagged receptors demonstrated predominant localization of the receptor in the cell membrane. Stimulation with 100nM of NMB (1) or GRP (2), respectively, led to clear internalization of the NMBR and GRPR observable by the intracellular vesicles, whereas the BRS-3 was not affected after 1 h of stimulation. The universal ligand (3) was able to induce internalization of all three receptors whereas stimulation with the selective ligand [D-Phe⁶, βAla¹¹, Ala¹³, Nle¹⁴]Bn (6-14) (11) led only to the internalization of the GRPR while the NMBR and BRS-3 receptor were not affected (Figure 1B).

### 3.3 Blood plasma stability investigation

Metabolic stability is an essential criterion for the medical application of peptide-drug conjugates. Therefore, a fluorescently labeled version of [D-Phe⁶, βAla¹¹, Ala¹³, Nle¹⁴]Bn(6-14) (11) was synthesized by introducing a 6-carboxytetramethylrhodamine to an oligo ethylene glycol linker that was attached to the N-terminus of peptide 11 yielding 11T (Table 3). This conjugate was then tested in a blood plasma stability assay. We observed that the original peak with a retention time of 16.3 min decreased over time and was completely degraded after 1 h revealing a half-life of 14.2 min (Figure 2A,D). The arising

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**FIGURE 1** Selectivity profiling of [D-Phe⁶, βAla¹¹, Ala¹³, Nle¹⁴]Bn(6-14) compared with native ligands and the universal ligand. (A) Concentration-response curves from Ca²⁺-flux assay testing the selectivity of [D-Phe⁶, βAla¹¹, Ala¹³, Nle¹⁴]Bn(6-14) (11) on stably transfected HEK293_X-tGFP (X = NMBR, GRPR, BRS-3) cells in comparison to native ligands (NMB, 1; GRP, 2) and the universal ligand (4). Sigmoidal curves were obtained by stimulation with increasing concentrations of peptide. Data were normalized to universal response (bottom value = 0%, top value = 100%) and the standard error of mean (SEM) is indicated at each data point, which represents the mean, obtained in at least two independent experiments, each performed in duplicates. (B) Receptor internalization studies of 11 in comparison to native ligands (1, 2) and the universal ligand (4) using HEK293 cells stably expressing the NMBR, GRPR or BRS-3 receptor fused to tGFP (green). Cells were stimulated with 100 nM ligand for 1 h at 37°C. Cell nuclei were stained with Hoechst 33342 (blue) and the universal ligand was used as positive control. Scale bar = 10 μm.
### TABLE 3
Analytical data of stabilized peptide analogs with indicated amino acid substitutions

| No. | Sequence | MW<sub>mono</sub> | Signal (M + H)<sup>+</sup> | ACN | Purity % |
|-----|----------|------------------|-----------------|-----|--------|
| 12  | fQWAVbHA | 1040.6           | 1041.6          | 19.2| >95    |
| 13  | fQWAVbAX | 1040.6           | 1041.5          | 27.1| >95    |
| 14  | fQWAVbHA | 1042.5           | 1043.5          | 27.6| >95    |
| 15  | fQWAVbTha-AX | 1056.5 | 1057.6          | 33.5| >95    |
| 16  | fQWAVbH-Aib-X | 1054.6 | 1055.6          | 27.6| >95    |
| 17  | fQWAVbH-NMe-A-X | 1054.6 | 1055.6          | 26.8| >95    |
| 11T | TAMRA-EG₃-fQWAVbHAX | 1655.8 | 1656.8          | 39.5| >95    |
| 12T | TAMRA-EG₃-fQWAVbHAX | 1655.8 | 1656.9          | 38.6| >95    |
| 13T | TAMRA-EG₃-fQWAVbHAX | 1655.8 | 1656.8          | 38.1| >95    |
| 14T | TAMRA-EG₃-fQWAVbHA | 1657.8 | 1658.8          | 35.8| >95    |
| 15T | TAMRA-EG₃-fQWAVb-Tha-AX | 1671.8 | 1672.8          | 44.2| >95    |
| 16T | TAMRA-EG₃-fQWAVbH-Aib-X | 1669.8 | 1670.8          | 38.8| >95    |
| 17T | TAMRA-EG₃-fQWAVbH-NMe-A-X | 1669.8 | 1670.8          | 38.1| >95    |

Tha = β-(2-thienyl)-L-alanine; Aib = 2-aminoisobutyric acid; Hse (Me) = O-methyl-L-homoserine; X = L-norleucine; NMe-A = N-methyl-L-alanine; * RP-HPLC performed on Phenomenex Aeris Peptide 3.6u XB-C18 (250 mm × 4.6 mm, 3.6 μm, 100 Å), flow rate: 1.55 mL/min

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**FIGURE 2** Blood plasma stability investigation of conjugate 11T. (A) Fluorescence RP-HPLC chromatograms of 11T after blood plasma incubation using a Agilent VariTide RPC column and a linear gradient of 25% to 55% (v/v) eluent B (ACN + 0.08% TFA (v/v) in eluent A (H₂O + 0.1% TFA (v/v) over 30 min. (B) MALDI-Tof-MS spectra of individual collected RP-HPLC peaks shown in A. (C) Sequences of the original and cleaved products with calculated mono isotopic masses. (D) Plotted peak area of the original conjugate 11T (red) and the resulting cleavage products (green and blue) with their corresponding retention times. Data points represent mean ± SEM of at least two independent experiments.
second peak at a retention time of 14.7 min was analyzed by MALDI-ToF-MS and was correlated to the cleavage product TAMRA-EG3-IQWAVbH-OH with a mass of 1472.1 Da. These findings demonstrated rapid degradation of \(11T\) by cleavage of the peptide bond between His\(^{12}\) and Ala\(^{13}\) (Figure 2B,C). After 6 hours of incubation, a third peak arose disclosing a second cleavage site, which has been identified as cleavage product by the detected mass signal of \(m/z\) 909.4 correlating to the cleavage between Gln\(^7\) and Trp\(^{10}\) (Figure 2).

### 3.4 Identification of the responsible peptidase

Since the cleavage of the peptide bond between His\(^{12}\) and Ala\(^{13}\) in peptide \(11\) emerged as the main half-life determining step, we aimed to identify the responsible endopeptidase. According to the cleavage site, two different protease inhibitors (sarcubitrilat and enalaprilat) were added to the blood plasma prior to peptide incubation (Figure 3). The addition of 1\(\mu\)M sarcubitrilat, which is known to inhibit the neutral endopeptidase (NEP, EC 3.4.24.11) had no effect on the stability of the peptide. However, 1\(\mu\)M enalaprilat inhibiting the angiotensin converting enzyme (ACE, EC 3.4.15.1) caused a significant increase in half-life of \(11T\) due to the delayed appearance of the peak at 14.7 minutes. This indicates that ACE is responsible for the cleavage of the His\(^{12}\)-Ala\(^{13}\) peptide bond in human blood plasma causing a very short half-life of 14.4 minutes. Inhibition of ACE by enalaprilat led to an increase in half-life of more than 45 h promoting the hypothesis that stabilization of this bond will result in a derivative with greatly increased metabolic stability.

### 3.5 Rational stabilization of the most selective analog

After the predominant cleavage site and the responsible peptidase were identified, we used multiple rational approaches to improve blood plasma stability and support the translation into medical application. This was expected to be challenging because the selectivity-gaining amino acid substitution in \([D-Phe^6, \beta-Ala^{11}, Ala^{13}, Nle^{14}]Bn(6-14)\) \((11)\) is in close proximity to the main cleavage side and most likely part of the ACE recognition site. Key amino acids were substituted by unnatural amino acids, which were chosen to be related to the original residues to maintain high selectivity while reducing recognition by the ACE to increase the metabolic stability. As first approach, the amino acids at positions 12 and 13 were exchanged by their corresponding \(D\)-isomers. This led to peptide \(12\) bearing \(D\)-Ala\(^{12}\) and analog \(13\) with \(D\)-His\(^{12}\). Next, in peptide \(14\), Nle\(^{14}\) was replaced by O-methyl-L-homoserine to increase the C-terminal hydrophilicity and potentially impair the recognition by the ACE, which needs primarily hydrophilic C-terminal residues. Furthermore, \(\beta\)-(2-thienyl)-L-alanine was introduced instead of the histidine at position 12 \((15)\), and Ala\(^{13}\) was substituted by 2-aminoisobutyric acid \((16)\). Finally, the peptide bond between the His\(^{12}\) and Ala\(^{13}\) was N-methylated to prevent degradation and increase thereby metabolic stability. To determine the stability, TAMRA labeled conjugates \((11T-17T)\) were synthesized, and unlabeled analogs \((12-17)\) were used for the receptor activation and selectivity experiments. All derivatives were analyzed with respect to their correct molecule identity and homogeneity were determined by mass spectrometry and RP-HPLC (Table 3).

### 3.6 Increased metabolic stability while GRPR selectivity is retained

The rationally modified analogs were tested in Ca\(^{2+}\)-mobilization assays to determine whether selectivity and GRPR activity were retained while the blood plasma stability has been increased. Peptides \(12\) and \(13\) containing the \(D\)-isomer substitutions at position 12 and 13 were found to be completely inactive at the NMBR and BRS-3. Additionally, analog \(12\) exhibited almost a complete loss of activity at the GRPR, while peptide \(13\) displayed a more than 100-fold reduced activity at the GRPR with an \(EC_{50}\) value of 33nM (Table 4). Even though both peptides showed an unsatisfactory receptor activation profile, a clear improvement in stability has been observed with half-lives of more than 100 hours for the respective conjugates \(12T\) and \(13T\).

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**Figure 3**: Blood plasma stability assays with different protease inhibitors. (A, B) Fluorescence RP-HPLC chromatograms of TAMRA-EG3-[D-Phe\(^6\), \(\beta\)-Ala\(^{11}\), Ala\(^{13}\), Nle\(^{14}\)]Bn(6-14) \((11)\) after blood plasma incubation with 1\(\mu\)M sarcubitrilat (A) and 1\(\mu\)M enalaprilat (B) using an Agilent VariTide RPC column and a linear gradient of 25% to 55% (v/v) eluent B (ACN + 0.08% TFA (v/v) in eluent A (H\(_2\)O + 0.1% TFA (v/v) over 30 min. (C) Normalized peak area of the original conjugate \(11T\) with and without peptidase inhibitors. Data points represent mean ± SEM of at least two independent experiments.
Next, analog 14 containing O-methyl-L-homoserine at position 14 displayed no NMBR and BRS-3 activity at 1μM stimulation while the potency at the GRPR was just slightly reduced. The corresponding TAMRA conjugate 14T showed good stability in blood plasma (t1/2 = 38 h). Peptide 15 containing a β-(2-thieryl)-L-alanine instead of histidine at position 12 displayed a half-life of 43.7 minutes and thus a three-fold increase compared with the initial peptide [D-Phe6, β-Ala11, Ala13, Nle14]Bn(6-14) (11) but the selectivity profile was maintained (Table 4 and Figure 4). The EC50 value at GRPR was slightly reduced to 3.9nM, and the activity at the NMBR and BRS-3 was still detectable. The exchange of the selectivity determining Ala13 by 2-aminoisobutyric acid (16) revealed an even better selectivity profile for the GRPR than the peptide 11. Next, to the increased stability of to 4.6 hours, the activity at the NMBR and BRS-3 receptor was completely abolished, and the EC50 value of 0.2nM was not altered. In the last approach, N-methylation was introduced between position 12 and 13, leading to analog 17. This modification led to a highly selective GRPR preferring peptide with an EC50 value of 0.3nM and no NMBR and BRS-3 activity. Additionally, the corresponding TAMRA derivative (17T) displayed a half-life of 88.6 hours and accordingly high metabolic stability in human blood plasma (Table 4 and Figure 4).

### Intracellular payload delivery

The intention of this study was the identification of a possible drug shuttle system for the intracellular delivery of different payloads. Therefore, the most promising analogs were investigated for their ability to deliver TAMRA molecules as payloads into endogenously GRPR expressing PC3 tumor cells and stably GRPR-tgfp transfected HEK293 cells (Figure 5). After 1 hour of stimulation with 1μM 11T, internalization of the GRPR (green) was observed in HEK293 cells as already found for the unlabeled peptide 11 (Figure 1). In addition to the internalized GRPR, TAMRA-fluorescence was detected in small intracellular vesicles, which were found to be partially co-localized with the receptor (Figure 5). Intracellular TAMRA fluorescence was also detected in PC3 cells after stimulation with 11T. Stimulation with the stabilized conjugates 14T, 16T, and 17T provided similar results for HEK293,GRPR cells as well as the for PC3 cells. All conjugates demonstrated uptake into intracellular vesicles supporting the concept of GRPR mediated delivery of certain payloads. Thus, these stabilized conjugates can be considered as targeting vectors for a GRPR based drug shuttle system.

### DISCUSSION

In the last decade, the bombesin receptor family evolved as a promising target for cancer diagnosis and therapy. Its potential is promoted by their strong overexpression on various tumor types. Many different payloads have been attached to a number of bombesin analogs and were successfully delivered to GRPR expressing cells. The majority of studies however was conducted with antagonistic bombesin derivatives like the GRPR-selective RM26 peptide, which was developed as a very promising candidate in clinical applications. The N-terminal modification with DOTA allowed the incorporation of different radionucleotides and thereby promoted its path towards clinical phase I for early detection and imaging of prostate cancer. Due to these promising results and their better pharmacodynamics, antagonists are
discussed to be favored over agonists. However, these studies mainly focused on tumor detection and the radionucleotide therapy while other targeted cancer therapy approaches were not investigated. Many anti-tumor drugs like cytotoxic agents or transcription factors have to reach intracellular compartments to facilitate their mode of action. Antagonistic peptide-drug conjugates cannot facilitate the required intracellular delivery. Agonists in contrast can activate their receptor selectively and induce the internalization of the peptide-receptor complex. After internalization, the receptor recycles back to the cell membrane while the ligand is trapped inside the cell. Because the receptor can be reactivated and internalized again, the peptide-drug conjugates are actively accumulated inside the cell and thereby lead to higher tumor accumulation values and longer retention times compared with antagonistic conjugates. Thus, different bombesin agonists as the shortened bombesin derivative Bn(7-14) or the universal ligand ([D-Tyr6, βAla11, Phe13, Nle14]Bn(6-14)) have been used as peptide-drug conjugates before but they possessed low selectivity for the GRPR or insufficient metabolic stability. Therefore, tumor targeting capacity and medical application are limited. In the past, further studies based on these peptides were carried out, which led to improved GRPR selectivity and metabolic stability and thereby increased tumor-targeting properties.

We aimed for the development of a highly GRPR-selective peptide agonist with excellent metabolic stability. First, we synthesized and tested based three known ligands (5-7) as starting point for our study. As all three control peptides were only slightly more selective than the starting peptide (4), we combined the individual amino acid substitutions to further increase the selectivity. In fact, the combination of peptide 5 and 7 yielded an analog with an increased selectivity for the GRPR. The more than 150-fold selectivity over the NMBR and more than 550-fold over the BRS-3 can mainly be explained by the importance of position 6 for the full activation of the NMBR and BRS-3 receptor because a tenfold drop in activity was noticed for the shortened peptide 5, while the GRPR was hardly affected. This is in agreement with the findings of Lin et al. which revealed the relevance of position 6 for full NMBR activation while it is not required for full GRPR activation. Our findings also suggest that the backbone at position 6 is more important for the activation of the NMBR and BRS-3 receptor than for the GRPR because the substitution of D-Phe6 to D-Ala6 (6) revealed no significant differences in the activation profile compared with the universal ligand (4). The additional substitution of Val10 by Ala10 increased the selectivity even further, even though the individual exchange led only to a minor selectivity increase mainly caused by a slightly reduced potency at the NMBR and BRS-3 receptor, which is in agreement with other studies.

Since the newly developed peptides activated the NMBR and BRS-3 still in a two- and three-digit nanomolar range, we investigated position 13, which was also identified to be important for the biological activity, uptake, and stability. Since NMB (1) features a Phe at this position and activates the BRS-3 receptor with an EC50 value of ca. 200nM, while the GRP (2) and Bn(6-14) (3) contain a Leu at this position and hardly activate the BRS-3, we expected a favored selectivity by an exchange of Phe13 to Leu13 (10). Surprisingly, this substitution led only to minor changes in the activation profile whereas the exchange by Ala led to a highly GRPR-selective analog.
This derivative features an excellent selectivity for the GRPR with more than 4000-fold over NMBR and more than 8000-fold over BRS-3. The almost completely abolished activity at the NMBR and BRS-3 and the only slightly altered potency at the GRPR were surprising because this position was found to be equally important in Ac-Bn(7-14) for NMBR and the GRPR activation.43

With this very selective peptide in hand, we investigated its blood plasma stability to improve its potential even further. Therefore, a fluorescently labeled version of the peptide was synthesized by conjugating a 6-carboxytetramethylrhodamine via a short three-unit ethylene glycol spacer to the N-terminus of 11, resulting in 11T. Even though multiple unnatural amino acids were incorporated into the peptide structure, a fast degradation of 11T was detectable with a half-life of 14.2 minutes. Analysis of the degradation product demonstrated fast peptide bond cleavage between His12 and Ala13 and between Gln7 and Trp5. Both cleavage sites were found in the original bombesin peptide as well as in various different antagonists.28,44,45 In addition to the cleavage site identification, we were also interested in resolving the responsible peptidase for the stability determining cleavage between His12 and Ala13. Because the angiotensin-converting enzyme (ACE, EC 3.4.15.1) and nephrilysin (NEP, EC 3.4.24.11) were identified as potent peptidases for Bn(7-14), we speculated that one of these two enzymes might also be involved in the cleavage of the newly developed peptide.46 Therefore, 11T was incubated with the ACE-inhibitor enalaprilat and the NEP-inhibitor sacubitril. In contrast to the NEP-Inhibitor, the ACE-inhibitor led to an increased half-life indicating the relevance of ACE for the stability of this bombesin derivative. Since this enzyme is highly abundant in the human blood and leads to a rapid degradation of the selective peptide, analog 11 had to be stabilized.47 Even though frequently used stabilization approaches as palmitoylation48,49 and PEGylation50 led in general to very good results, we aimed to stabilize the actual cleavage site by amino acid substitution to preserve the small molecular size allowing a maximized payload loading. This approach turned out to be fairly challenging due to the close proximity of the cleavage site to the selectivity gaining substitution at position 13. First, we separately exchanged the amino acids L-His12 and L-Ala13 by their isomeric counterparts, yielding highly stable derivative with half-lives of more than 100 hours. Unfortunately, both analogs displayed a strong potency loss at the GRPR. While analog 13 displayed with an EC50 value of 33nM a still reasonable potency, peptide 12 exhibited nearly no activity at the GRPR, which was not surprising because this position was shown before to be critical for GRPR activity and selectivity. Next, the C-terminal Nle was exchanged by the more hydrophilic Hse(Me) because the ACE-homolog ACE2 requires a hydrophobic or basic C-terminal amino acid for recognition.51 We expected to achieve a positive result with respect to receptor activation as the exchange of the original Met14 to Nle decreased the uptake into tumor cells in previous studies.24,42 In fact, the reintroduction of the more hydrophilic Hse(Me) at this position not only led to a more stable analog with a half-life of 38.1 hours but also abolished the NMBR and BRS-3 receptor activity completely whereas the potency change at the GRPR was negligible. These findings are partially in agreement with a recently published study in which the same exchange was performed in a 177Lu labeled Bn(6-14) conjugate. Even though no increase in blood plasma stability could be observed, the uptake into tumor xenografts was improved.52

Additionally, His12 was exchanged by Tha(15), which led only to a minor stabilization in blood plasma and resulted in a tenfold loss of activity at the GRPR. The introduced sulfur obviously hinders the correct conformation of the peptide and leads to a reduced potency. However, the exchange of Ala13 by Aib(16) resulted in a half-life of 4.6 hours, while selectivity for the GRPR as well as the potency was maintained. The mild improvement in stability and the conserved activation profile can be explained by the obvious similarities of Aib(16) to Ala(11) and Leu(10), which had no significant influence on the GRPR activation.

As a last modification, an N-methylated alanine was introduced to prevent peptide bond cleavage in blood plasma (17). This substitution yielded the most stable analog with a half-life of 88.9 hours, which also retained its GRPR activity and selectivity completely. This result is in accordance with the proposed binding mode, which suggested that the amide bond between His12 and Leu13 is not primarily important for formation of the internal hydrogen bonding network.43 The relevance of this position for the metabolic stability was already investigated by various groups, which exchanged the Leu13 in Bn(7-14) by the unnatural amino acid cyclohexylalanine (Cha) or by a statin group leading to a derivative with significantly improved stability in human blood plasma. However, these substitutions led also to reduced internalization rates into PC3 cells and binding affinities for the GRPR.53,54 In contrast to previously developed conjugates, the herein designed analogs feature not only subnanomolar activity and great selectivity for the GRPR, they also facilitate the uptake into GRPR expressing cells. We nicely demonstrated the successful uptake of TAMRA molecules into tumor cells by fluorescence microscopy proving the intracellular delivery of conjugated payloads. These analogs can be considered as universal targeting vectors for the selective delivery of bioactive molecules to GRPR expressing tumor cells.

5 | CONCLUSION

Here, we present the identification of a highly GRPR-selective agonist [D-Phe6, β-Ala11, Ala13, Nle14]Bn(6-14) with more than 4000-fold selectivity over the NMBR and more than 8000-fold over the BRS-3. Metabolic stability investigations revealed rapid degradation by the angiotensin-converting enzyme. Thus, this peptide was rationally stabilized by amino acid substitution leading to the derivative [D-Phe6, β-Ala11, NMe-Ala13, Nle14]Bn(6-14) with a half-life of 88.9 hours while GRPR activity and selectivity was maintained. Additional fluorescent microscopy studies demonstrated GRPR mediated uptake into PC3 cells. Therefore, this peptide can be considered as drug shuttle for the intracellular delivery of different payloads in a targeted tumor treatment.
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