Cholecystokinin-B/Gastrin Receptor Binding Peptides: Preclinical Development and Evaluation of Their Diagnostic and Therapeutic Potential

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Abstract

The high sensitivity of pentagastrin stimulation in detecting primary or metastatic medullary thyroid cancer (MTC) suggests widespread expression of the corresponding receptor type on human MTC. Indeed, autoradiographic studies demonstrated cholecystokinin (CCK)-B/gastrin receptors not only in >90% of MTCs but in a high percentage of small cell lung cancers and potentially a variety of gastrointestinal adenocarcinomas. In a pilot study, we have demonstrated the feasibility of radiolabeled gastrin-I to target CCK-B receptor-expressing tissues in vivo and patients (T. M. Behr et al., Eur. J. Nucl. Med., 25: 424–430, 1998). The aim of the present study was to systematically optimize, in a preclinical model, suitable radioligands for targeting CCK-B receptors in vivo.

For this purpose, a variety of CCK/gastrin-related peptides, all having in common the COOH-terminal CCK-receptor binding tetrapeptide sequence Trp-Met-Asp-PheNH₂ or derivatives thereof, were studied. They were radioiodiated by the Iodogen or Bolton-Hunter procedures. The peptides tested were members of the gastrin- or cholecystokinin families or possessed characteristics of both, which differ by the intramolecular position of a tyrosyl moiety (occurring in native or sulfated form). Their stability and affinity were studied in vitro and in vivo; their biodistribution and therapeutic efficacy were tested in nude mice bearing s.c. human MTC xenografts. Diethylene-triamine-pentaacetate derivatives of suitable peptides were synthesized, evaluated, and labeled with ¹¹¹In.

Introduction

The development of regulatory peptides as tools to visualize and, more recently, to treat malignant tumors has been an important focus of interest over the past years (1–3). The successful development of sufficiently stable, radiolabeled somatostatin analogues, such as ¹²³I-Tyr₃-, or ¹¹¹In-DTPA₃-D-Phe¹-octreotide for diagnostic purposes (1, 2), as well as the introduction of ⁶⁷Tb-DTPA- or ⁹⁰Y-tetraazacyclododecanet₄-N₄,N₄,N₄,N₄-tetra-acetate-conjugates (3) for therapeutic applications, has opened new horizons in nuclear oncology (3–5). More recently, other regulatory peptides, such as vasoactive intestinal polypeptide (6), substance P (7), or gastrin-releasing peptide/bombesin derivatives (8), have emerged as potentially useful candidates for in vivo scintigraphy and radioprobe research.

Whereas somatostatin receptor scintigraphy has proven as

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³ The abbreviations used are: DTPA, diethylene-triamine-pentaacetate; CCK, cholecystokinin; MTC, medullary thyroid cancer; HPLC, high-performance liquid chromatography; MTD, maximum tolerated dose; % ID/g, percentage of injected dose per gram; p.i., post injection.
valuable tool for the staging of gastroenteropancreatic tumors (e.g., carcinoids), its sensitivity and accuracy in other neoplasms, such as MTC or small cell lung cancer, is limited (9–11). Because of the outstanding diagnostic accuracy of the pentagastrin test in detecting the presence, persistence, or recurrence of malignant C cells, even far below a size detectable by conventional morphological imaging methods, we postulated the expression of the corresponding receptor type in human MTC (12). Indeed, receptor autoradiographic studies by Reubi and Waser (13) had demonstrated the expression and presence of CCK-B/gastrin receptors not only in >90% of MTCs but in a high percentage of other tumor types, such as small cell lung cancer, stromal ovarian cancers, and astrocytomas as well (14). Earlier studies suggested an even more widespread occurrence of gastrin receptors, e.g., in colon, pancreatic, or stomach cancers (15). Therefore, we undertook pilot experiments in nude mice bearing human MTC xenografts and were able to demonstrate the feasibility of radiolabeled gastrin-I to target CCK-B receptor-expressing tumors, as well as CCK receptor-expressing normal organs (e.g., the stomach) in vivo in animals and patients (12).

However, a variety of problems remained to be solved, such as the molecular characteristics, which render the peptide as an optimal candidate for in vivo targeting of CCK-B receptor-expressing tumors, considering in vivo stability, affinity to and selectivity for the CCK-B receptor, or the potentially unfavorable accretion in normal organs (such as the liver, bowel, or kidney). The aim of the present study was, therefore, to develop and optimize, in a preclinical model, suitable radioligands for targeting CCK receptors in vivo.

Materials and Methods

Peptides. The peptides as listed in Table 1 were investigated in the present study. They belong either to the gastrin or cholecystokinin “super” families or possess characteristics of both (16). Both families differ by the location of their tyrosyl moiety. These peptides were commercially obtained from Sigma Chemie (Deisenhofen, Germany), ICN Biomedicals (Eschwege, Germany), or Bachem Biochemicals (Heidelberg, Germany), or sulfated CCK-8 (Bachem Biochemicals) were dissolved in 1 ml of 0.05 M sodium phosphate (pH 7.4). One hundred µl of this solution were mixed with 100 µCi of sodium iodide in 0.1 M NaOH from New England Nuclear DuPont (Brussels, Belgium and North Billerica, MA). Radioiodination by the Iodogen method was performed essentially as described earlier (12). Briefly, the peptide in 0.05 M NH4OH was transferred into an Iodogen-coated glass vial (100 µg of Iodogen coating the inner surface of a 2-ml vial) with a magnetic stirbar placed inside. Five hundred µl of 0.5 M sodium phosphate buffer (pH 7.4) were added. The vial was placed on a magnetic stirrer, and the activity was added in 500 µl of 0.05 M sodium phosphate (pH 7.4; up to 200–250 mCi/mg of peptide). After a stirring time of 5–10 min, the radioiodinated peptide was purified from unreacted iodine by gel filtration chromatography on a PD-10 column (Sephadex G-25 preequilibrated in a 15 × 50-mm polypropylene column, which was purchased from Sigma Chemie). The column was eluted with 0.05 M PBS buffer (pH 7.4), collecting 10 drops/fraction. Fractions containing the labeled peptide were combined and were subsequently filtered through a sterile Millipore-G filter (pore size, 0.22 µm; Millipore, Molsheim, France). Radiochemical purity was shown by HPLC (Nucleosil 120–3 C18, 250 × 4 mm, Marchery-Nagel; solvent A: 0.1% trifluoroacetic acid in water; solvent B: AcCN; flow rate 0.5 ml/min; gradient: 0–5 min 100% solvent A, 0% solvent B; 5–15 min 20% solvent A, 80% solvent B).

Radioiodination by the Bolton-Hunter procedure was performed essentially as described previously (17). Briefly, 2.5 mg of crystalline succinimidyl-3-(4-hydroxyphenyl)-propionate (Sigma Chemie) were dissolved in 10 ml of 0.25 M PBS (pH 7.5). One hundred µl of this solution were mixed with 100 µCi of sodium iodide (Na131I or Na125I) in 400 µl of 0.25 M PBS (pH 7.5) and 2 mg of chloramine-T (Sigma Chemie). The reaction was stopped after 10 s by the addition of 1.2 mg of sodium metabisulfite in 600 µl of 0.05 M PBS (pH 7.5). After the addition of 200 µl of dimethylformamide, the radioiodinated Bolton-Hunter reagent was extracted with two 500-µl portions of benzene and recovered by evaporating the solvent under a gentle stream of dry nitrogen. The peptide (100 µg) was added in 200 µl of 0.1 M sodium borate (pH 8.5) in an ice bath. After vortexing the mixture for 15 min in the ice bath, the reaction mixture was separated on a Sephadex G-25 Superfine column (1 × 55 cm). The radiochemical purity was shown by HPLC as described above.

Synthesis of DTPA-Derivatives and Labeling with 111In. Five mg of minigastrin (obtained from Bachem Biochemicals, Heidelberg, Germany), its [d-Leu1]-derivative (Genosys, Cambridge, United Kingdom), or sulfated CCK-8 (Bachem Biochemicals) were dissolved in 1 ml of 0.05 M aqueous NH4OH at concentrations between 1 and 10 mg/ml.

Radioiodination of the Peptides. Iodine-131 and iodine-125 were purchased as sodium iodide in 0.1 M NaOH from New England Nuclear DuPont (Brussels, Belgium and North Billerica, MA). Radioiodination by the Iodogen method was performed essentially as described earlier (12). Briefly, the peptide in 0.05 M NH4OH was transferred into an Iodogen-coated glass vial (100 µg of Iodogen coating the inner surface of a 2-ml vial) with a magnetic stirbar placed inside. Five hundred µl of 0.5 M sodium phosphate buffer (pH 7.4) were added. The vial was placed on a magnetic stirrer, and the activity was added in 500 µl of 0.05 M sodium phosphate (pH 7.4; up to 200–250 mCi/mg of peptide). After a stirring time of 5–10 min, the radioiodinated peptide was purified from unreacted iodine by gel filtration chromatography on a PD-10 column (Sephadex G-25 preequilibrated in a 15 × 50-mm polypropylene column, which was purchased from Sigma Chemie). The column was eluted with 0.05 M PBS buffer (pH 7.4), collecting 10 drops/fraction. Fractions containing the labeled peptide were combined and were subsequently filtered through a sterile Millipore-G filter (pore size, 0.22 µm; Millipore, Molsheim, France). Radiochemical purity was shown by HPLC (Nucleosil 120–3 C18, 250 × 4 mm, Marchery-Nagel; solvent A: 0.1% trifluoroacetic acid in water; solvent B: AcCN; flow rate 0.5 ml/min; gradient: 0–5 min 100% solvent A, 0% solvent B; 5–15 min 20% solvent A, 80% solvent B)
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<th>Peptide</th>
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<th>Label(^b)</th>
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<td>Big gastrin</td>
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<td>pGlu-Leu-Gly-Pro-Gln-Gly-Pro-Pro-His-Leu-Val-Ala-Asp-Pro-Ser-Lys*-Lys*-</td>
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<td>F(^{(+))}, BH(^{(+))})</td>
<td>(\sim 10^{-8}) M</td>
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<td>Minigastrin</td>
<td>13</td>
<td><em>Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr</em>-Gly-Trp-Met-Asp-PheNH(_2)</td>
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<td><em>D-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr</em>-Gly-Trp-Met-Asp-PheNH(_2)</td>
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<td><strong>Cholecystokinin derivatives</strong></td>
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<td>pGlu-Gln-Asp-Tyr*-Thr-Gly-Trp-Met-Asp-PheNH(_2)</td>
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<td><strong>Peptides with common features of both, gastrin, and CCK</strong></td>
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<td>DTPA-minigastrin or its [α-Leu(^1)] analogue</td>
<td>13</td>
<td>DTPA-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr*-Gly-Trp-Met-Asp-PheNH(_2)</td>
<td>(^{111})In(^{(*)})</td>
<td>(\sim 10^{-9}) M</td>
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<td>DTPA-sCCK-8</td>
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<td>DTPA-Asp-Tyr-Met-Gly-Trp-Met-Asp-PheNH(_2)</td>
<td>(^{111})In(^{(*)})</td>
<td>ND</td>
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</table>

\(^a\) * potential attachment sites of the radiiodinated Bolton-Hunter moieties; \(^\ddagger\), tyrosyl moieties that may be radioidinated by the Iodogen procedure.

\(^\ddagger\) I, Iodogen; BH, Bolton-Hunter radiiodination; ND, not done.
Serum Stability Testing in Vitro. For the assessment of the serum stability of the various radiolabeled peptides in vitro, the radiolabeled peptides (typically 200 μCi, 1 μg) were added to 2 ml of serum from a healthy donor at 37°C. Conventional low pressure gel filtration chromatography [on a Sephadex S-300-HR (Sigma Chemie) column, dimensions 1 x 100 cm] and/or size exclusion HPLC (Bio-Sil SEC-250 column, 300 x 7.8 mm; Bio-Rad Laboratories, Richmond, CA) were performed immediately after the serum addition or after incubation periods of 1, 4, and 24 h at 37°C, as well as of 1, 4, and 24 h at 2°C (20). Detection of the radiopeptides and their metabolic products was performed with an UV detector at a wavelength of 280 nm, as well as by γ scintillation counting.

For redox experiments, the labeled peptides were incubated for 1 h at 37°C in 1 M H2O2, or for 48 h in 1 M DTT (21). Subsequent HPLC analysis was performed as described above (Nucleosil 120-3 C18, 250 x 4 mm; Marchery-Nagel).

Affinity/IC50 Determination. The 50% inhibitory concentrations (IC50s) of the various radiolabeled peptides were determined by a procedure modified from Schmidberger et al. (22). Briefly, single-cell suspensions of TT cells (Ref. 23; for details, see below) in Ham’s F12K medium (ICN Biomedicals, Eschwege, Germany), supplemented with 0.1% NaN3, were incubated for 1 h in polystyrene cups at room temperature with the samples were spun down, the pellets were washed three times with ice-cold Ham’s F12K (0.1% NaN3), and were counted in the scintillation counter. The data were plotted as receptor binding, parallel groups of mice were given either 1 μg of radiolabeled peptide alone or 1 μg of the labeled peptide, supplemented with 1 μg of unlabeled peptide.

Experimental Radiopeptide Therapy. Tumor sizes were determined by caliper measurement in three dimensions immediately before therapy and at weekly intervals thereafter. Tumor volumes were estimated by multiplying the product of the three perpendicular diameters by 0.5, assuming an elliptical geometry (24). Tumors were either left untreated (controls), were given unlabeled gastrin-I, or were injected with a single dose of 131I-labeled gastrin-I or gastrin-I fragment 1–14. Ten to 20 animals were studied in each group. Body weight was recorded weekly, and survival was monitored (24). The MTD was defined as the highest possible activity under the respective conditions that did not result in any animal deaths, with the next, 10–20% higher dose level resulting in at least 10% of the animals dying (24). Total and differential WBC and platelet counts were determined on the day of therapy and at weekly intervals thereafter. Seventy-five-μl heparinized specimens were collected by retroorbital bleeding. The samples were counted on a Technicon H3 Auto-Analyzer (Bayer-Diagnostik, Munich, Germany). Blood urea nitrogen and creatinine as indicators of renal function, as well as glutamate oxaloacetate transaminase and alkaline phosphatase as liver function parameters were determined at the same time points as the blood counts. Means ± SDs were calculated for each group (24).

Pilot Human Biodistribution Study. A normal volunteer (T. M. B.) was injected over a 5-min period with 5 mCi of 111In-labeled DTPA-minigastrin (~10 μg). Whole-body scans were acquired over a 48-h period, using a Picker Prism 2000 double-headed gamma camera.

Statistical Analysis. Differences in the tissue uptake values and the biodistribution of the radioligands in various tissues were statistically analyzed with the Student’s t test for unpaired data, as described previously (25). Pairwise comparisons were performed with the Wilcoxon Rank-Sum test (26). Differences in the therapeutic efficacy between the treatment modalities were analyzed by assuming an exponential tumor growth pattern; nonlinear regression analysis based on asymptotic approximation was used as described earlier (27).

Results

Radioiodination, Chemical and Serum Stability, and Affinity of the Various Peptides in Vitro. The peptides listed in Table 1 were investigated. They belong either to the gastrin or cholecystokinin “super” families or possess characteristics of both (16). Both families differ by the location of their tyrosyl...
moiety, with one amino acid in the gastrin family (usually Gly) or two amino acids in the CCK family (Met-Gly or Thr-Gly) between this very tyrosine moiety and the COOH-terminal receptor binding sequence Trp-Met-Asp-PheNH₂. Cionin can be phylogenetically regarded as an ancestor of both CCK and gastrin, because it bears two tyrosyl residues (one in each position). Finally, the structures of des-BOC-pentagastrin and CCK fragment 30–33 (“CCK-4”) is basically confined to the receptor binding sequence common to both the gastrin and CCK families. Two peptides, lacking this (complete) receptor binding tetrapeptide, served as irrelevant controls: gastrin-I fragment 1–14 and [Tyr(SO₃H)₂]⁺ CCK 26–29 (Table 1). These peptides were radioiodinated by the Iodogen or Bolton-Hunter procedures as indicated, dependent upon the availability of a nonsubstituted tyrosyl moiety (iodogen) or free amino group (Bolton-Hunter). HPLC analysis of the radioiodinated peptides revealed a single peak (≥95% of the total activity) for Bolton-Hunter-labeled peptides in contrast to several (up to four) closely related peaks for their Iodogen-labeled analogues. Earlier studies had shown that the methionin moieties of CCK or gastrin derivatives are readily susceptible to oxidation, even under rather mildly oxidizing conditions, such as occurring during Iodogen radioiodination. Accordingly, iodination of [Leu₁³]-gastrin-I, lacking any Met residues, led to a single (≥95% of the total activity) radioiodinated peptide.

To further study the role of methionin oxidation, redox experiments were undertaken by incubating the labeled peptides for 1 h at 37°C in 10% H₂O₂, or for 48 h in 100 mM DTT (21). As had been observed earlier (21), HPLC analysis showed nearly complete interconversion of the various peptide peaks to a homogenous product each under these respective conditions. However, pilot receptor binding studies failed to show any major influence of the methionin oxidation on the receptor binding affinities (data not shown), which is in accordance with earlier reports (28). Furthermore, pilot studies comparing the biodistribution of big-gastrin, minigastrin, or nsCCK-8 labeled by the Iodogen (¹³¹I) versus Bolton-Hunter (¹²⁵I) procedures intraindividually did not show any significant difference between the biological behavior of both labels (data not shown). Therefore, the radiolabeled peptides were used for the subsequent studies without further consideration of partially oxidized Met residues.

The right column of Table 1 shows the IC₅₀ values of the radioiodinated peptides obtained from cell binding studies. Interestingly, sulfated CCK and cionin analogues had the highest affinities (≤nm range), whereas desulfation or the complete removal of the NH₂-terminally located tyrosine-containing parts of the peptide (as is the case in des-BOC-pentagastrin or CCK-4) led to a loss of affinity by several orders of magnitude. Furthermore, Table 1 shows that the presence of an intact COOH-terminal Trp-Met-Asp-PheNH₂ sequence is crucial for receptor binding, whereas the methionin can be replaced by leucin or norleucin. Interestingly, similarly high affinities as seen for the sulfated CCK analogues were observed for some nonsulfated members of the gastrin family.

Stability testing of the various peptides was performed in human serum. At 37°C, all peptides had in vitro serum half-lives of several hours, but those peptides bearing NH₂-terminal pyrrol glutamic acid (pGlu) residues exhibited significantly longer half-lives (≥24 h at 37°C) than those with a free NH₂-terminal amino group (data not shown). As an example, Fig. 1 compares the size-exclusion gel-filtration chromatographic profiles of [¹³¹I]-labeled gastrin-I (bearing an NH₂-terminal pGlu moiety; Fig. 1a) versus minigastrin (having Leu as NH₂ terminus; Fig. 1b). The upper panels show the native radiolabeled peptides, the middle panels the respective peptides in serum at 2°C, and the lower panel at 37°C in serum. Fig. 1 shows that a small fraction of the peptides (≤10%) rapidly shifts to a higher molecular weight fraction upon short serum incubation at 2°C (fraction 30–40), suggesting binding to serum proteins, whereas the majority of the peptides remains unbound (fraction 37–52). Incubation at 37°C led to small molecular weight metabolites (fraction 65–90), coeluting with di-, tri-, or tetrapeptides (fraction 65–80) or monosodiumtetrionate (fragment 77–90). The comparison of Fig. 1, a and b, also shows much more rapid metabolic breakdown of minigastrin as compared with the pGlu-protected gastrin-I. Interestingly, also the replacement of the terminal methionin moiety in minigastrin by its d-isomer led to an improved serum stability (t₁/₂ = 8 h versus 45 min at 37°C).

**Biodistribution Studies of Various Peptides in Human MTC Xenograft-bearing Nude Mice.** Biodistribution studies of the various peptides listed in Table 1 were performed in TT xenograft-bearing nude mice. Fig. 2a shows the biodistribution patterns of those peptides essentially confined to the known receptor recognition region Trp-Met-Asp-PheNH₂ (such as CCK-4 and des-BOC-pentagastrin) and of the nonsulfated CCK analogues (nonsulfated CCK-8 and caerulein) at 1 h p.i. Because similar time kinetics were observed for most peptides studied, Fig. 3 shows the biodistribution of a typical member of the gastrin family over a 24-h period (differentiating into tissues known or expected to express CCK-B receptors (upper panel), organs involved in the excretion of the radiodeotide (middle panel), and pure “blood pool” organs (lower panel)), whereas Fig. 2b compares the tissue uptake of the most important tested peptides belonging to these families at 1 h p.i. quantitatively. This time point was chosen because it was typically the time point with the highest target-specific uptake (Fig. 3).

Accordingly, Fig. 2a compares the 1-h tissue uptake values of nsCCK-8 and nonsulfated caerulein to those of peptides essentially confined to the receptor-binding region, i.e., des-BOC-pentagastrin and CCK-4. Whereas the latter two showed hardly any stomach or tumor uptake, the uptake of nsCCK-8 and caerulein in these tissues was, although still rather low, significantly (P < 0.05) higher.

Fig. 2b shows the biodistribution pattern of typical sulfated members of the cholecystokinin family as well as of sulfated cionin as a peptide with characteristics common to both the CCK and gastrin families. Significantly higher uptake and longer retention times than with their nonsulfated analogues were observed with sulfated peptides in the tumor, stomach, gallbladder, and pancreas. However, significantly higher uptake occurred in the liver and bowel as well, whereas the renal accretion of sulfated compounds was significantly lower than with their nonsulfated analogues. Fig. 2b shows that all three sulfated compounds that conserve the Trp-Met-Asp-PheNH₂ sequence or its Nε analogue (i.e., sCCK-8, [Thr²⁸,Nε³¹]-sCCK-25–33, and cionin) had similar biodistribution patterns, whereas the uptake values of [Tyr(SO₃H)₂]⁺ CCK-26–29, lacking the recep-
Fig 1 Gel filtration chromatographic profiles of gastrin-I (a) and minigastrin (b), labeled with $^{131}$I according to the Iodogen procedure, on a Sephacryl S-300-HR column (1 $\times$ 100 cm). Upper panels, freshly labeled peptide each; middle panels, after serum incubation at 2°C; lower panels, after incubation at 37°C.

Because high affinity to the CCK-B receptor and good target organ (i.e., stomach and tumor) uptake were observed with both the sulfated CCK and the anionic pentaglutamate sequence containing gastrin analogues, we hypothesized that these negative charges may be involved in the receptor binding and affinity. Therefore, we examined the biodistribution of polar but neutral gastrin analogues, [Gln$^6$-$^{10}$]-gastrin-I and [Gln$^2$-$^6$]-minigastrin. Indeed, the tumor and stomach uptake were significantly ($P < 0.03$) lower than with gastrin-I or minigastrin, as was the case for the renal accretion as well (Fig. 2c). Again, no significant difference was observed between the biodistributions of both Gln$_n$-peptides (biodistribution data for [Gln$^6$-$^{10}$]-gastrin-I not shown). Whereas with members of the gastrin family, excellent visualization of the tumor and normal CCK-B receptor-expressing tissues was seen, the replacement of the pentaglutamate sequence, as in [Gln$^6$-$^{10}$]-gastrin-I, led to a significantly decreased CCK-B receptor-mediated tumor and stomach uptake (Fig. 2c).

Accordingly, Table 2 summarizes the mean target:background (i.e., radiolocalization) ratios of the two most important CCK-B receptor-expressing tissues (i.e., stomach and tumor). Table 2 shows that the best stomach:nontumor ratios were obtained with members of the gastrin family, especially gastrin-I, its [Leu$^{15}$] analogue, or minigastrin, because of their lower accretion in CCK-A-expressing abdominal organs, best tumor:nontumor ratios were obtained with members of the gastrin family, especially gastrin-I, its [Leu$^{15}$] analogue, or minigastrin.

The specificity of receptor-targeted uptake in various tissues was assessed by comparing the biodistribution of 1 µg of radiiodinated gastrin-I to the 1000-fold higher peptide amount by coinjecting 1 mg of unlabeled gastrin-I (Fig. 4). The uptake...
Fig. 2 Biodistribution of various CCK-B receptor binding peptides at 1 h p.i. a, comparison of the tissue uptake values of radioiodinated nsCCK-8, caerulein, des-BOC-pentagastrin, and CCK-4. b, comparison of the tissue uptake values of radioiodinated sCCK-8, [Thr^{28}, Nle^{31}]sCCK-25-33, cionin, and [Tyr(SO_{3}H)^{2}]sCCK-26-29. c, comparison of the 1-h tissue uptake values of radioiodinated big-gastrin, gastrin-I, [Leu^{15}]-gastrin-I, minigastrin, and [Gln^{42-6}]minigastrin. tu, tumor; lu, lung; li, liver; spl, spleen; ki, kidney; sto, stomach; pa, pancreas; ga, gallbladder; bw, bowel; br, brain; ad, adrenal gland; mu, muscle; bo, bone; bl, blood. Bars, SD.
in the tumor, stomach, and pancreas was significantly lower at the 1-mg saturation dose. Interestingly, despite receptor blocking, the gallbladder uptake was still quite substantial, indicating that this uptake may indeed be due to nonspecific biliary excretion. Surprisingly, the renal accretion was lowered under receptor saturation conditions as well (P < 0.05), whereas the initial uptake of their nonsulfated analogues was ~2-fold higher (Fig. 5; compare upper and middle panels), which holds true for members of the gastrin family (lower panel) as well. Interestingly, the retention of peptides bearing an NH2-terminal pGlu moiety was significantly longer (dashed lines in Fig. 5; e.g., caerulein as nonsulfated CCK analogue; gastrin-I, its [Leu15] analogue, or fragment 1-14 in the gastrin family) than with “open-ended” peptides (various dotted lines in Fig. 5). Finally, big-gastrin, being the only peptide with two positively charged lysine groups and having an NH2-terminal pGlu moiety, had the highest renal uptake and longest retention of all peptides studied.

**Initial Therapeutic Experiments with 131I-Labeled Gastrin Analogues.** Because of the encouraging “diagnostic” results of CCK-B receptor targeting, we undertook initial experiments to assess whether radiolabeled gastrin may be therapeutically useful. To determine the maximum tolerated dose and dose-limiting organs, varying amounts of gastrin-I or its fragment 1–14 were injected into groups of 10 animals each, starting at 2 mCi, and increasing in 10 to 20% steps. Myelotoxicity was found to be dose limiting (blood count nadirs at 1–2 weeks p.i., complete recovery within 2–3 weeks) with both 131I-labeled peptides, and the maximum tolerated activity was reached at 4.5 mCi, corresponding to a blood dose of ~3.5 Gy (12). No sign of second-organ toxicity was observed; values for blood urea nitrogen and creatinine, as well as the liver enzymes, remained within normal limits. Tumor growth was retarded significantly with 131I-gastrin-I (P = 0.03) as compared with untreated controls, animals given the same amount of unlabeled peptide, or with animals given 131I-labeled fragment 1–14 as irrelevant peptide (Fig. 6).

**Development of 111In-Labeled CCK-B Receptor Binding Peptides.** On the basis of the results presented thus far, gastrin analogues containing the highly anionic Glu6 sequence, together with the receptor binding COOH-terminal tetrapeptide Trp-Met-Asp-PheNH2, seem to combine high affinity and selectivity for the CCK-B receptor with a relatively low renal accretion. Therefore, minigastrin and its [d-Leu1] analogue were chosen as basis to develop an 111In-labeled CCK-B receptor imaging agent for potential clinical purposes. As described in more detail in “Materials and Methods,” a DTPA-derivative was synthesized. To assess the biodistribution properties of Pan-CCK ligands, a DTPA-derivative of sCCK-8 was created as well. HPLC analysis demonstrated a single peptide each, which could be labeled with 111In at a specific activity of ~0.5 mCi/mg with >98% radiochemical purity without requiring additional postlabeling purification. The biodistribution of 111In-DTPA-minigastrin in TT-bearing nude mice is shown in Fig. 7. The peptide showed maximum tumor uptake of 5.0 ± 1.2%ID/g and a stomach uptake of 9.4 ± 2.7%ID/g at 1 h p.i. It
Table 2  Mean target/background ratios (i.e., radiolocalization indices) at 1 h p.i. for the most important CCK-B receptor-expressing tissues (i.e., the tumor and stomach) for the various radioiodinated peptides tested

| Peptide (family) | Blood Lung Liver Pancreas Gall-bladder Bowel Kidney Blood Lung Liver Pancreas Gall-bladder Bowel Kidney |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Gastrin derivatives |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Big-gastrin       | 1.2              | 1.4              | 1.2              | 1.1              | 0.4              | 4.7              | 0.1              | 2.1              | 2.0              | 3.0              | 2.8              | 1.2              | 12.2             | 0.2              |
| Gastrin-I         | 4.9              | 2.8              | 5.8              | 1.7              | 0.1              | 4.4              | 0.4              | 9.8              | 5.3              | 11.5             | 3.4              | 0.2              | 8.9              | 0.9              |
| [Leu^{13}]Gastrin-I | 3.7              | 3.0              | 7.0              | 2.5              | 0.1              | 4.4              | 0.4              | 8.5              | 8.1              | 22.0             | 6.7              | 0.2              | 16.9             | 1.5              |
| [Gln_{6}^{27}]-Gastrin-I | 1.1              | 1.2              | 0.7              | 1.3              | 0.2              | 1.3              | 0.7              | 6.5              | 3.4              | 19.0             | 4.0              | 0.5              | 2.9              | 2.3              |
| Minigastrin       | 5.1              | 2.6              | 6.2              | 2.7              | 0.1              | 7.5              | 1.4              | 10.4             | 5.2              | 6.1              | 5.5              | 0.3              | 15.1             | 2.8              |
| [Glu_{5}^{27}]-Minigastrin | 1.2              | 1.2              | 0.8              | 1.5              | 0.2              | 1.4              | 0.7              | 6.9              | 3.5              | 2.5              | 4.5              | 0.5              | 4.0              | 2.0              |
| Gastrin-I fragment 11-17 | 2.0              | 2.3              | 3.8              | 4.9              | 0.2              | 2.4              | 0.6              | 3.1              | 4.3              | 6.4              | 8.3              | 0.4              | 4.4              | 1.2              |
| Gastrin-I fragment 1-14 | 1.1              | 1.0              | 1.9              | 0.8              | 0.1              | 2.1              | 0.2              | 2.9              | 2.8              | 5.2              | 2.2              | 0.1              | 5.9              | 0.5              |
| Cholecystokinin derivatives |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Caerulein         | 1.6              | 1.5              | 3.1              | 1.7              | 0.2              | 3.5              | 0.5              | 2.9              | 2.7              | 5.7              | 3.0              | 0.3              | 6.4              | 0.9              |
| [Thr^{28}, Nle^{31}]-sCCK-25-33 | 2.4              | 7.2              | 1.2              | 1.1              | <0.1             | 1.1              | 3.0              | 10.5             | 35.9             | 6.0              | 6.1              | 0.1              | 5.3              | 10.5             |
| sCCK-8            | 2.7              | 8.5              | 1.4              | 1.5              | <0.1             | 1.2              | 2.5              | 10.5             | 35.9             | 6.0              | 6.1              | 0.1              | 5.3              | 10.5             |
| nsCCK-8           | 2.5              | 2.1              | 3.6              | 5.0              | 0.2              | 2.3              | 0.6              | 6.3              | 5.1              | 8.4              | 11.6             | 0.5              | 5.4              | 1.4              |
| [Tyr(SO_{3}H)^{27}] CCK 26-29 | 1.3              | 4.3              | 0.6              | 1.5              | 0.1              | 1.2              | 1.2              | 2.6              | 8.7              | 1.2              | 2.9              | 0.2              | 2.3              | 2.4              |
| Peptides with common features of both, gastrin, and CCK |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Cionin            | 3.6              | 8.3              | 1.3              | 1.4              | 0.1              | 1.3              | 2.6              | 17.8             | 28.0             | 6.2              | 4.6              | 0.4              | 6.2              | 12.4             |
| des-BOC-pentagastrin | 1.5              | 1.0              | 1.9              | 1.2              | 0.1              | 0.9              | 0.9              | 3.6              | 2.5              | 4.8              | 2.9              | 0.2              | 2.3              | 0.5              |
| CCK fragment 30-33 (CCK-4) | 1.1              | 0.7              | 1.7              | 0.9              | 0.1              | 1.1              | 0.2              | 2.4              | 1.5              | 3.6              | 2.0              | 0.2              | 2.3              | 0.4              |

Fig. 4  Specificity of CCK-B receptor binding.  

a, comparison of the biodistribution of 1 μg of 111In-labeled gastrin-I versus 1 μg of radioiodinated gastrin, supplemented with 1 mg of the unlabeled peptide or 1 μg of fragment 1–14 (lacking the receptor binding tetrapeptide sequence) to assess the tissues with receptor-specific versus those with nonspecific uptake. *, P < 0.05; **, P < 0.01.  

b, external scintigraphy comparing 1 μg of 111In-labeled gastrin-I (left) versus the 1 mg of blocking dose (right).  

Bars, SD.

had a rapid clearance from the blood and blood-pool organs (lungs, liver, spleen, muscle, and bone) with mainly renal (maximum kidney uptake, ~45%ID/g) and some biliary excretion (transient gallbladder uptake with activity transfer into the bowel). In contrast to radioiodinated minigastrin, a prolonged retention in the adrenals was observed with its 111In-labeled derivative. No significant difference was observed between 111In-DTPA-minigastrin and its [D-Leu^{1}]-analogue (data not shown).  

Distribution of 111In-DTPA-Minigastrin in a Healthy Human Volunteer. A healthy human volunteer (T. M. B.) was investigated with 111In-DTPA-minigastrin. A peptide amount of 10 μg (labeled with 5 mCi of 111In) was slowly injected i.v., which caused a short episode of pentagastrin stim-
Fig. 5 Comparison of the biokinetics of various peptides in the kidneys for non-sulfated (upper panel), or sulfated CCK analogues (middle panel), or members of the gastrin family (lower panel). Solid lines, peptides containing cationic amino acids (lysine); dashed lines, peptides with an NH₂-terminal pGlu moiety; various dotted lines, peptides lacking both of these characteristics. Top, --□--, nsCCK-8; --○--, caerulein; --△--, des-BOC-pentagastrin; --Δ--, CCK-4. Middle, --×--, sCCK-8; --+--, [Thr₂⁸, Nle₃]sCCK-25-33; --*--, cionin; ----, [Tyr(SO₃H)₂]⁶⁶-CCK-26-29. Bottom, ---I---, big-gastrin; --△--, gastrin-I; --▼--, [Leu⁵]¹⁵-gastrin-I; --○--, [Gln⁵₂-6⁶]-minigastrin; --△--, gastrin-I fragment, 1–14.

Fig. 6 Therapeutic efficacy of ¹²⁵I-labeled gastrin-I at its MTD (4.5 mCi) as compared with untreated controls, animals given the same amount of unlabeled gastrin-I, or 4.5 mCi of fragment 1–14 (the difference between ¹²⁵I-gastrin-I and the other groups was statistically significant at P = 0.03). --□--, controls; --○--, unlabeled gastrin-I; --○--, 4.5 mCi ¹²⁵I-gastrin-I; --△--, 4.5 mCi ¹²⁵I-fragment 1–14.

ulation-like symptoms (mild nausea, cough, and “strange feeling” in the chest and abdomen), lasting for only a few minutes and resolving spontaneously. In accordance with the preclinical studies, receptor targeting was seen in physiologically CCK-B receptor-expressing tissues (mainly in the stomach but also the gallbladder; Fig. 9). Furthermore, a moderate uptake was seen in
the kidneys as excretory organs. The main route of excretion is renal, but a transient bowel activity seems to indicate some biliary excretion as well.

Discussion

The outstanding sensitivity of pentagastrin in detecting the presence of malignant C cells suggested the expression of a corresponding receptor type in human MTC (29, 30). Indeed, receptor autoradiographic studies by Reubi and co-workers (13, 14) demonstrated the presence of CCK-B/gastrin in >90% of MTCs but in a high percentage of other important tumor types, such as small cell lung cancer, stromal ovarian cancers, and astrocytomas as well. Although the expression of relevant amounts of CCK-B receptors in typical adenocarcinomas is a matter of debate, some investigators were able to demonstrate and characterize this receptor type on colon, gastric, and pancreatic cancers (15). Thus, there are ample reasons to regard CCK-B receptors as an attractive target for radiolabeled peptides for diagnostic and therapeutic purposes.

In a previous communication, we demonstrated the feasibility of radiolabeled gastrin-I to target CCK-B receptor-expressing tissues in animals and patients (12). However, a variety of problems remained to be solved, such as the molecular characteristics, which render the peptide as optimal candidate for in vivo targeting of CCK-B receptor-expressing tumors, considering in vivo stability, affinity to, and selectivity for the CCK-B receptor or the potentially unfavorable accretion in normal organs (e.g., the kidney). The aim of the present study was, therefore, to develop and optimize suitable radioligands for targeting CCK receptors in vivo.

Earlier studies had provided evidence for an unusual susceptibility of the methionin moieties of CCK and gastrin derivatives toward oxidation, e.g., during radioiodination. Our data seem to confirm this sensitivity, but in accordance to previous results (28), they also seem to confirm that (at least partial) oxidation of the Met residues does not affect the receptor-binding capabilities of these radioiodinated peptides. Accordingly, no significant differences in the biodistribution were noticed, whether using peptides labeled according to the Bolton-Hunter or the Iodogen procedures.

Because standard methods for determining the binding affinity of peptides either use quantitative receptor autoradiography in frozen tissue sections or cortex membrane preparations, our data obtained by a different methodology were only intended to provide rough estimates. This is the reason why only the approximate orders of magnitude are given in Table 1. Nevertheless, the values obtained are in good accordance to published values as far as available (31, 32). In accordance with data published previously (31, 32), our affinity/IC\text{50} results show that important requirements for a high affinity to the CCK-B receptor are the presence of the COOH-terminal tetrapeptide sequence Trp-Met-Asp-Phe\text{NH}_2 (where Met can be replaced by Nle or Leu), as well as either a sulfated tyrosine moiety (as is the case in sulfated CCK derivatives) or the presence of a Glu sequence adjacent to a nonsulfated Tyr residue (as is the case in the gastrin family). In contrast, nonsulfated CCK analogues showed a considerably decreased affinity, which is in accordance with published literature data as well (31, 32).

In contrast to the majority of regulatory peptides, CCK or gastrin derivatives seem to be fairly stable in serum. The major biological inactivation pathway is known to be renal filtration.
and enzymatic degradation (33). With half-lives of several hours, it did not seem to be necessary to introduce special stabilizing functional groups or molecular modifications, as had been the case in the development of somatostatin analogues (34). The protective effect of NH$_2$-terminal pGlu residues against serum peptidase action is a well-known phenomenon (34, 35) and could be demonstrated in this study as well. Although there are known “gastrin-binding” proteins in the serum (36), our data clearly suggest that the vast majority of CCK or gastrin analogues circulates as free peptides, which seems to be an important prerequisite for rapid receptor targeting and background clearance.

As has been expected from the interspecies similarity of peptides of the CCK/gastrin families and their receptors (30, 31), the biodistribution studies of human gastrin in nude mice showed specific binding to the tumor and most organs known to express CCK-B receptors. The lack of uptake in the brain [despite the known presence of CCK-B receptors (30)] is easily explained by the inability of the hydrophilic peptide to cross the blood-brain barrier. Furthermore, the biodistribution studies in MTC xenografted nude mice clearly demonstrate that the requirements of a peptide to yield reasonably good uptake in CCK-B receptor-expressing tissues, and in addition to yield sufficiently high target:background ratios, are to bear the COOH-terminal tetrapeptide sequence Trp-Met-Asp-PheNH$_2$ (where Met may be substituted by Leu or Nle) and to either contain a sulfated tyrosyl moiety or a highly anionic oligo(penta?):glutamate sequence in its NH$_2$-terminal neighborhood. It is a well-established phenomenon that a sulfated tyrosyl moiety is a crucial requirement for the affinity of a peptide to the CCK-A receptor, whereas in the case of CCK-B receptors, both sulfated CCK and (nonsulfated) gastrin derivatives have shown similar affinities (30, 31). Our hypothesis of the importance of the pentaglutamate sequence in the gastrin-related peptides for its receptor-binding properties (Fig. 10) is further supported by the comparably low affinity and poor receptor targeting properties of the two synthetic peptides, where the anionic Glu residues have been replaced by similarly polar but uncharged Gln moieties ([Gln$^5$-1$^6$]-gastrin-I and [Gln$^2$-6$^6$]-minigastrin; compare Fig. 2c).

The space-filling molecular model of gastrin derivatives (Fig. 10) shows the spacial neighborhood of the highly anionic oligo-glutamate sequence to the (in the case of members of the gastrin family, nonsulfated) tyrosyl residue. This is in accordance with our experimental data, indicating that the negative charges of the Glu$_x$ sequence may serve as surrogate of the anionic sulfate ester for mediating high-affinity binding and selectivity to the CCK-B receptor, whereas the presence of a sulfate ester itself is crucially necessary in this position for high-affinity binding to the CCK-A receptor (30, 31).

The comparably weak performance of big-gastrin may be attributable to the presence of two lysine moieties, which on one hand may partially neutralize negative charges of the pentaglutamate sequence involved in receptor binding, and which, on the other hand, are known to contribute to the renal tubular reabsorption of peptides and low molecular weight proteins (37–39).

In accordance with their high affinity to the CCK-A receptor, which is almost ubiquitously present throughout the gastrointestinal tract (especially in the pancreatic acini and islets, the gallbladder, neurons, and smooth muscle cells throughout the bowel, as well as gastric mucosal cells; Ref. 40), sulfated CCK analogues (labeled with iodine or indium) showed high uptake not only in the stomach but also in the bowel, pancreas, liver, and gallbladder. We therefore postulate that for high affinity to, but also selectivity for, the CCK-B receptor, nonsulfated gastrin derivatives may be superior to CCK-A analogues, which have been proposed for CCK-B receptor visualization in vivo by Reubi et al. (41).

The main excretion pathways of gastrin and CCK-related peptides seem to be renal filtration and, to a lower extent, biliary excretion. Interestingly, the molecular structure of the various peptides was reflected by their renal handling as well. The highest renal accretion occurred with big-gastrin, which is the only peptide studied that bears positively charged lysine resi-
4 h p.i. 24 h p.i.

dues. The latter are well known to be involved in renal tubular reabsorption (see above; Ref. 39). In contrast, the sulfated, thus strongly anionic, CCK derivatives showed the lowest renal uptake and retention of all peptides studied. On the other hand, NH2-terminal pGlu moieties do not only seem to protect from serum peptidase digestion but also from lysosomal degradation in the renal tubuli, because peptides bearing such NH2-terminal pGlu moieties had longer activity retention in the kidneys than their "open-chained" counterparts (compare Fig. 5).

Because these data identify the amino acid sequence ... -(Glu)n-X-Tyr-(X)1-2-Trp-Met-Asp-PheNH2 (where X may be any neutral amino acid, such as Ala, Gly, or Thr; Met may be replaced by Leu or Nle; and n = 5, but other chain lengths remain to be tested) as the basic structure combining selectivity for and high affinity to the CCK-B receptor, we embarked on developing CCK-B receptor-targeting peptides that are suitable for labeling with radiometals, such as 111In. Such a peptide appears much more convenient for clinical purposes, because of the more favorable physical imaging characteristics of indium as compared with iodine and because of an anticipated easier labeling formulation. In addition, because indication for an internalization of CCK-B receptor ligands exists (42), residualizing radiolabels, such as radiometals, may be expected to improve tumor:nontumor ratios over rapidly released labels, such as conventional iodination (43). The DTPA-derivative of minigastrin tested in this study seems to fulfill all of these requirements. The animal biodistribution data show good tar-

Fig. 9 Targeting of CCK-B receptors in a 32-year-old healthy volunteer (T. M. B.). The 111In-DTPA-minigastrin whole-body scans at 4 h (left) and 24 h (right) clearly show uptake in the stomach and the gallbladder, as well as the kidneys. The main route of excretion is renal, but the transient bowel activity is indicative of some biliary excretion as well.

Fig. 10 The space-filling molecular model of gastrin-derivatives shows the spacial neighborhood of the highly anionic oligo-glutamate sequence to the (in the case of members of the gastrin family, nonsulfated) tyrosyl residue; our experimental data indicate (see text) that the negative charges of the Glu sequence may serve as surrogate of the anionic sulfate ester for mediating high-affinity binding and selectivity to the CCK-B receptor, whereas the presence of a sulfate ester itself is crucially necessary in this position for high-affinity binding to the CCK-A receptor (30, 31). The receptor-binding tetrapeptide Trp-Met-Asp-PheNH2 is seen on the left margin of this figure.
targeting of CCK-B receptor-expressing tissues (mainly tumor and stomach) and a more prolonged retention of the activity in these tissues than with iodine. Interestingly, in contrast with our observations with radioiodinated gastrin analogues, 111In-DTPA-minigastrin seems to exhibit some increased adrenal uptake. Future studies will have to address whether this is attributable to receptor-specific binding (as is the case for somatostatin analogues in rodent adrenals as well) or merely attributable to nonspecific processes.

In accordance with these preclinical data on the favorable targeting properties of 111In-DTPA-minigastrin, our initial human data confirm that this peptide is capable of targeting physiologically CCK-B receptor expelling organs (e.g., in the stomach or gallbladder). Probably, the renal accretion is mainly attributable to the peptide’s excretory pathway, although the CCK-B receptor-mediated uptake cannot be excluded, because recent data have clearly demonstrated CCK-B receptor expression in the kidney (44). Further clinical studies are warranted to show whether radiolabeled CCK-B receptor-binding peptides can improve the staging of actual MTCs or small cell lung cancer patients. This will be subject to future communications.

In summary, these data suggest that CCK/gastrin analogues may be a useful new class of receptor-binding peptides for diagnosis and therapy of CCK-B receptor-expressing tumors, such as MTCs or small cell lung cancers. This holds true even more, because no dependence of the quantitative amount of receptor expression upon the degree of tumor differentiation seems to exist for CCK-B receptors. This stands in clear contrast to somatostatin receptors, where in accordance to previous in vitro data (45), we were able to demonstrate in vivo the loss of somatostatin receptors in dedifferentiated and clinically more aggressive forms of MTC (11). Similar observations have been reported for the loss of somatostatin receptors in metastatic small cell lung cancer as well (9, 46). Nonsulfated gastrin derivatives may be preferable for scintigraphic purposes, because CCK-B receptor-mediated uptake cannot be excluded, because recent data have clearly demonstrated CCK-B receptor expression in the kidney (44). Further clinical studies are warranted to show whether radiolabeled CCK-B receptor-binding peptides can improve the staging of actual MTCs or small cell lung cancer patients. This will be subject to future communications.

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