Unsulfated DTPA- and DOTA-CCK analogs as specific high-affinity ligands for CCK-B receptor-expressing human and rat tissues in vitro and in vivo

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Abstract. Receptors for regulatory peptides such as somatostatin or vasoactive intestinal polypeptide are expressed by a number of human neoplasms and can be visualized in vivo with peptide receptor scintigraphy. Recently, the CCK-B receptor, which binds both gastrin and cholecystokinin with high affinity, was shown using in vitro methods to be overexpressed in a number of human tumor tissues, including medullary thyroid carcinomas, small cell lung cancers, astrocytomas, gastrointestinal tumors, and stromal ovarian cancers. In the present study, we have designed novel, unsulfated CCK octapeptide analogs linked to the metal chelating DTPA and DOTA, and have tested them for their binding affinity to CCK-B receptor-positive tissue from human tumors: The most potent compounds assayed were DTPA-[Nle^{28,31}]-CCK(26–33) (MP2286) and DTPA-[D-Asp^{26},Nle^{28,31}]-CCK(26–33) (MP2288) with an IC_{50} of 1.5 n*M*. For comparison, analogs with C-terminal DTPA, such as [Nle^{28,31},Aphe^{33}(*p*-NH-DTPA)]-CCK(26–33) and CCK-(26–33)-NH$(CH_2)_2$ NH-DTPA, had an IC_{50} of >100 n*M*. DOTA-[D-Asp^{26},Nle^{28,31}]-CCK(26–33) had an IC_{50} of 3.9 n*M*. The compounds were selective for CCK-B receptors as they did not bind with high affinity to CCK-A receptors expressed in human tumors (meningiomas or gastroenteropancreatic tumors). In vivo rat biodistribution studies with indium-111 labeled MP2286 and MP2288 showed that the primary mode of clearance was renal, and the primary sites of uptake (% ID/g 24 h p.i.) were kidneys (0.270 and 0.262, respectively) and the gastrointestinal tract. The CCK-B receptor-expressing gastric mucosa showed specific in vivo accumulation of ^{111}In-labeled MP2288 which could be blocked in the presence of excess unlabeled MP2288. ^{111}In-labeled MP2286 and MP2288 were also found to be stable in human plasma whereas both compounds were degraded in urine (>40% after 3 h at 37°C). The affinity, specificity, biodistribution, and stability of these two DTPA-CCK analogs indicate that these compounds have substantial promise for use in the in vivo visualization of CCK-B receptor-expressing tumors.

Key words: Cholecystokinin-B receptors – ∧Metal chelating ligands – Cholecystokinin – Octapeptide – Biodistribution – Tumor targeting

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Introduction

The successful application of radiolabeled somatostatin and vasoactive intestinal peptide analogs for the in vivo imaging of receptor-expressing tumors [1, 2] has stimulated the search for other disease-associated peptide receptors [3, 4]. Peptides which can be used to target abundant peptide receptors, particularly those which are selectively expressed, may be especially useful as tools in nuclear medicine for the development of new diagnostic imaging agents and/or radiotherapeutics. Recently, we have evaluated cholecystokinin (CCK)-A and CCK-B receptors in the normal human gastrointestinal tract [5] as well as in a variety of human tumors [6, 7]. It was observed that specific tumor types frequently express CCK-B receptors, namely medullary thyroid carcinomas (MTCs), small-cell lung cancers (SCLCs), astrocytomas, stromal ovarian tumors, and some gastroenteropancreatic tumors [7]. We were able to design an iodinated CCK analog, the nonsulfated ^{125}I[D-Tyr-Gly, Nle^{28,31}]-CCK(26–33), which retained high affinity in the nanomolar range and showed high specificity for human CCK-B receptors [7], as a potential radioligand for in vivo scintigraphy. However, for routine use in diagnostic nuclear medicine, it would be preferable to have CCK analogs linked to a chelator, such as DTPA or DOTA, which can then be labeled with indium-111 or other clinically useful radioisotopes. This approach would represent further progress, as observed previously with Octreoscan [1].

In the present study, we therefore designed and synthesized a number of CCK derivatives linked to DTPA or DOTA. These compounds were tested for their affinity for CCK-A and -B receptors expressed by human tumors, using in vitro receptor autoradiography. The compounds with the highest binding affinities were further tested in vitro for their stability in human plasma and urine and in vivo for their biodistribution behavior in normal rats.

Materials and methods

General method for the solid phase synthesis of CCK analogs

Solid phase peptide synthesis was performed using an Applied Biosystems Model 432 A Synergy Peptide synthesizer using Fmoc (9-fluorenylmethoxycarbonyl) strategy [8]. Instrument protocol required resin containing 25 mmol of Fmoc-amine content and 75 mmol of subsequent Fmoc-protected amino acids activated by a combination of *N*-hydroxylbenzotriazole (HOBt)/(2-(1-H benzotriazol-1-yl)-1,1,1,3-tetramethyluronium hexafluorophosphate (HBTU). Three letter codes for common amino acids are used. All the standard Fmoc-protected amino acids were purchased commercially unless otherwise stated. The unusual amino acids used have the following abbreviations: *p*-aminophenylalanine, Aphe; 2,3-diaminopropionic acid, β-Dpr. Fmoc-(*p*-Alloc)-Aphe-OH was prepared from Fmoc-Aphe-OH and allyloxycarbonyl chloride by standard procedures.

Rink amide resin was used for the synthesis of C-terminal carboxamide formation unless otherwise stated. After the synthesis was completed, the peptides were cleaved from the resin and deprotected using a mixture comprising trifluoroacetic acid:phenol:thioanisole:water (85:5:5:5) for 6–10 h at room temperature. The products were precipitated by *t*-butyl methyl ether and centrifuged. The peptide-resin mixture was washed with *t*-butyl methyl ether and centrifuged 5–6 times to remove residual cleavage mixture. Acetonitrile:water (2:3) was added to the residue and filtered to remove the resin. The filtrate containing the crude peptide was lyophilized and pure peptides were obtained by preparative liquid chromatography. Molecular weight determination was done by mass spectrometry operating in electrospray mode (ESI).

Method A. For the incorporation of DTPA (diethylene triamine penta-acetic acid), the N-terminal Fmoc-protecting group was removed in the synthesizer. Tri-*t*-butyl-DTPA (75 mmol) was placed at the appropriate location in the synthesizer and activated similar to other amino acids for coupling [9]. Cleavage, deprotection, and isolation of the peptides were carried out as outlined above.

Method B. Incorporation of *p*-(DTPA-NH)-Phe at the C-terminus: The peptide synthesis was carried out as described above with the incorporation of Fmoc-(*p*-Alloc)-Aphe-OH at position 8 and *t*-Boc-Asp(OtBu)-OH at the N-terminus. The resin containing the protected octapeptide was treated with $Pd(PPh_3)_4$ in chloroform:acetic acid:N-methyl morpholine (37:2:1) to remove the allyloxycarbonyl protecting group [10]. The resin was transferred back to the synthesizer and condensed with tri-*t*-butyl-DTPA as described in method A. At the end of the synthesis, MP-2336 was isolated according to the general method described above.

Method C. Incorporation of DTPA at the C-terminus: The protected peptide containing *t*-Boc-Asp(OtBu)-OH at the N-terminus was prepared according to the standard protocol above using diaminoethane-trityl resin. At the end of the synthesis, the protected peptide was obtained from the resin using 3×2 ml of 1% trifluoroacetic acid in methylene chloride. Trifluoroacetic acid was neutralized with 5 ml of 5% triethylamine, evaporated to dryness, and dissolved in 1 ml of *N*,*N*-dimethylformamide (DMF). To this solution, 75 mmol of tri-*t*-butyl-DTPA anhydride in DMF (prepared from 75 mmol of tri-*t*-butyl-DTPA and 75 mmol of dicyclohexylcarbodiimide in 1 ml of DMF) was added, and the solution was shaken for 2–3 h. DMF was removed under reduced pressure. Deprotection and isolation of MP-2312 was carried out as described in general methods.

Method D. Incorporation of DOTA at the N-terminus: An identical procedure to that described in method A was followed for the incorporation of DOTA at the N-terminus. Tri-*t*-butyl DOTA was placed in the synthesizer instead of tri-*t*-butyl-DTPA, and the activation was carried out in a similar manner. Tri-*t*-butyl DOTA was synthesized internally by a modification of the procedure of Mishra et al. [11]. At the end of the synthesis, MP-2354 was isolated according to the general method described above.

The structures of the synthesized CCK analogs are shown in Table 1. The various structural modifications were chosen in order (1) to improve the peptide stability by modifying or replacing those amino acids most susceptible to facilitate peptide degradation, (2) to identify an adequate location to attach a chelator molecule without loss of receptor affinity.

Evaluation of binding affinities with in vitro receptor autoradiography

Human tumors known from previous studies [7] to express either CCK-A or CCK-B receptors were used. All tissues were frozen immediately after surgical resection and stored at –70°C. Receptor autoradiography was performed on 10- and 20-μm thick cryostat (Leitz 1720, Rockleigh, N.J.) sections of the tissue samples, mounted on microscope slides, and then stored at –20°C for at least 3 days to improve adhesion of the tissue to the slide, as described elsewhere [7]. Each tissue underwent receptor autoradiographic processing with ^{125}I-D-Tyr-Gly-Asp-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-Phe-amide (^{125}I-CCK), a radioligand identifying both CCK-A and -B receptors, as described previously [5, 7]. The sections were preincubated in 50 mmol/l Tris-HCl, 130 mmol/l NaCl, 4.7 mmol/l KCl, 5 mmol/l MgCl, 1 mmol/l ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′,-tetra-acetic acid, and 0.5% bovine serum albumin (BSA), pH 7.4 (preincubation solution), for 30 min at 25°C. The slides were then incubated in a solution containing the same medium as the preincubation solution minus the BSA, and the following compounds were added: 0.025% bacitracin, 1 mmol/l dithiothreitol, 2 μg/ml chymostatin, 4 μg/ml leupeptin, pH 6.5, and the radioligand, 45 pmol/l ^{125}I-CCK (2000 Ci/mmol; Anawa). The slides were incubated at room temperature with the radioligand for 150 min. Increasing amounts of nonradioactive, sulfated CCK-8, gastrin, or each of the newly designed DTPA- or DOTA-CCK analogs were added to the incubation medium to generate competitive inhibition curves. On completion of the incubation, the slides were washed 6 times for 15 min each in ice-cold preincubation solution, pH 7.4. The slides were rinsed twice in ice-cold distilled water for 5 s each. They were then dried under a stream of cold air at 4°C, apposed to ^{3}H-hyperfilms, and exposed for 1–7 days in X-ray cassettes.

The same protocol was used for the CCK-A and CCK-B receptor analysis of the rat stomach, jejunum, ileum, and colon [5].

The autoradiographs were quantified using a computer-assisted image-processing system, as described elsewhere [12]. Tissue standards for iodinated compounds (Amersham, Little Chalfont, UK) were used for this purpose.

The selection of tumors expressing the adequate CCK receptor was performed as reported previously [7]. Tumors were considered as expressing CCK-A receptors when the ^{125}I-CCK analog was fully displaced by 50 n*M* sulfated CCK-8 but not displaced by 50 n*M* gastrin. Conversely, tumors were considered as expressing CCK-B receptors when the ^{125}I-CCK ligand was fully displaced by nanomolar concentrations of sulfated CCK-8 and gastrin. Tumors expressing concomitantly CCK-A and -B receptors were not used in this study.

The two MP2286 and MP2288 analogs used in displacement studies were labeled with ^{115}In as follows: The ^{115}In-peptide complex was prepared by reacting MP2288 or MP2286 (70 nmol) with $^{115}InCl_3$ (105 nmol) in 200 µl of 0.005 *N* HCl for 30 min at room temperature. The solution was lyophilized to dryness and redissolved in 5 m*M* $NaHCO_3$ and analyzed by reverse phase high-performance liquid chromatography (HPLC) and mass spectrometry. The analysis indicated that the peptide was >99% ^{115}In-complex.

In vivo biodistribution in rats

The overall biodistribution properties of ^{111}In-labeled MP2286 and MP2288 were determined in rats. Peptide radiolabeling was performed in 25 m*M* NaOAc, 12.5 m*M* Na-ascorbate, pH 5.0. Typically, reactions were carried out using 1 mCi $^{111}InCl_3$ and 1 µg peptide in a total volume of 25 µl. After incubation for 15 min at room temperature the radiolabeled peptides were diluted to desired volumes for injection using sterile phosphate buffered saline (PBS) containing 5% ethanol. Radiolabeling efficiency was greater than 99%, as measured with reverse phase HPLC on a Novo-Pak C_{18} column, 3.9×150 mm (Waters), using a 15-min linear gradient of 0 to 70% solvent B (solvent A, 5% acetonitrile, 0.1% trifluoroacetic acid; solvent B, 90% acetonitrile, 0.1% trifluoroacetic acid). For each compound, nine normal female Sprague-Dawley rats (180–200 g) in groups of three were anesthetized with Metofane gas and injected via the jugular vein with 25 µCi of ^{111}In-labeled peptide. At 1, 4, and 24 h post injection, groups of rats were sacrificed and, after collection of a blood sample by cardiac puncture, tissue samples were removed for radioassay. The % injected dose per gram tissue was calculated against standard dilutions of the radiolabeled compounds.

In vivo blocking experiments

Animals were injected with ^{111}In-MP2288 prepared at high and low radio-specific activity to determine whether uptake into CCK-B receptor-expressing target tissues is specific. High specific activity (HSA), 1400 Ci/mmol ^{111}In-MP2288 was prepared as indicated above. The same radiolabeled material was used to prepare low specific activity peptide (~0.5 Ci/mmol) by addition of 0.5 mg of unlabeled peptide to 180 µCi of radiolabeled material. Two sets of three rats (fasted for 24 h) were anesthetized and then injected with 50 µCi of high or low radio-specific activity peptide. The animals were imaged by gamma scintigraphy at various times post injection. At 120 min post injection, the animals were sacrificed, and the gastrointestinal tract from the stomach to the distal large intestine was removed, rinsed with distilled water, positioned on paper, photographed, and then scintigraphed. Stomach tissue has been reported to express the highest density of CCK-B receptors [5] apart from the brain; therefore, after the above scintigraphic imaging, individual stomachs with contents were weighed and total radioactivity measured in a gamma counter. The stomachs were then cleaned and rinsed with distilled water and then reweighed and counted to determine the radioactivity associated specifically with tissue.

In vitro stability of CCK analogs in human urine and plasma

The stability of the analogs in urine was determined using 10× concentrated human urine prepared using a 10,000-dalton cutoff, YM10 Amicon membrane. Reactions contained 10 µl concentrated urine and radiolabeled peptide (see above), in a total volume of 100 µl of PBS, pH 7.2. Peptides were incubated for 3 h at 37°C and then analyzed by reverse phase HPLC on a Novo-Pak C_{18} column, 3.9×150 mm (Waters), using a 15-min linear gradient of 0 to 70% solvent B (solvent A, 5% acetonitrile, 0.1% trifluoroacetic acid; solvent B, 90% acetonitrile, 0.1% trifluoroacetic acid). Similar stability tests were performed using human plasma (Calbiochem). Three-hour incubations were as described above except that plasma was used in each 100 µl reaction.

Results

The binding affinities of nine nonsulfated, DTPA-linked CCK analogs and one DOTA-CCK analog were determined by measuring the displacement of receptor-bound ^{125}I-labeled CCK in tumor tissue sections expressing CCK-A and CCK-B receptors. The measured IC_{50} values are listed in Table 1. All of the tested compounds with an N-terminal DTPA (MP2247, MP2286, MP2288, MP2290, MP2296, MP2294) showed very high affinity binding to CCK-B receptors. The compound MP2354, an N-terminal-DOTA-CCK analog, also displayed very high affinity for CCK-B receptors. The compound MP2292, with L-Trp replaced with D-Trp, had a marked reduction in observed affinity. Also, CCK analogs with DTPA conjugated at or near the C-terminus (MP2336 and MP 2312, respectively) had very low binding affinity for the CCK-B receptor (Table 1). None of the synthesized compounds showed any significant affinity for the CCK-A receptor (Table 1). Figure 1 shows a displacement experiment with the various CCK analogs using a CCK-B receptor-expressing MTC and a CCK-A receptor-expressing meningioma, relative to sulfated CCK-8 and gastrin as control substances. Their rank order of displacement potencies corresponds closely to the values listed in Table 1. MP2288 and/or MP2286, which differ only by the presence of L-Asp (MP2286) or D-Asp at the N-terminus (MP2288), have very high binding affinities in all the tested tumor types expressing CCK-B receptors (MTCs, SCLCs, astrocytomas, gastroenteropancreatic tumors, stromal ovarian tumors). Furthermore, chelation of the DTPA ligand with ^{115}In in MP2288 or MP2286 does not affect the binding affinity for the CCK-B recep-

Table 1. Structures and binding properties (IC_{50}) of various DTPA- and DOTA-CCK analogs to human tumoral CCK-A and CCK-B receptors (substituted aminoacids are in italic)

Code Nr.	Synthesis method	Structures	Binding properties	
			CCK-B-R IC_{50} (n*M*)	CCK-A-R IC_{50} (n*M*)
MP2247	A	DTPA-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2	2.3	>100
MP2286	A	DTPA-Asp-Tyr-*Nle*-Gly-Trp-*Nle*-Asp-Phe-NH_2	1.5	>100
MP2288	A	DTPA-*D*Asp-Tyr-*Nle*-Gly-Trp-*Nle*-Asp-Phe-NH_2	1.5	>100
MP2290	A	DTPA-*D*Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2	2.8	>100
MP2296	A	DTPA-Asp-Tyr-Thr-Gly-Trp-*Nle*-Asp-Phe-NH_2	4.5	>100
MP2294	A	*Dpr*-(β-DTPA)-Tyr-*Nle*-Gly-Trp-*Nle*-Asp-Phe-NH_2	3.5	>100
MP2292	A	DTPA-*D*Asp-Tyr-Nle-Gly-*D*Trp-Nle-Asp-Phe-NH_2	42.0	>100
MP2312	B	Asp-Tyr-*Nle*-Gly-Trp-*Nle*-Asp-*p*(DTPA-NH)Phe-NH_2	>100	>100
MP2336	C	Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH-$(CH_2)_2$-NH-DTPA	>100	>100
MP2354	D	DOTA-*D*Asp-Tyr-*Nle*-Gly-Trp-*Nle*-Asp-Phe-NH_2	3.9	>100

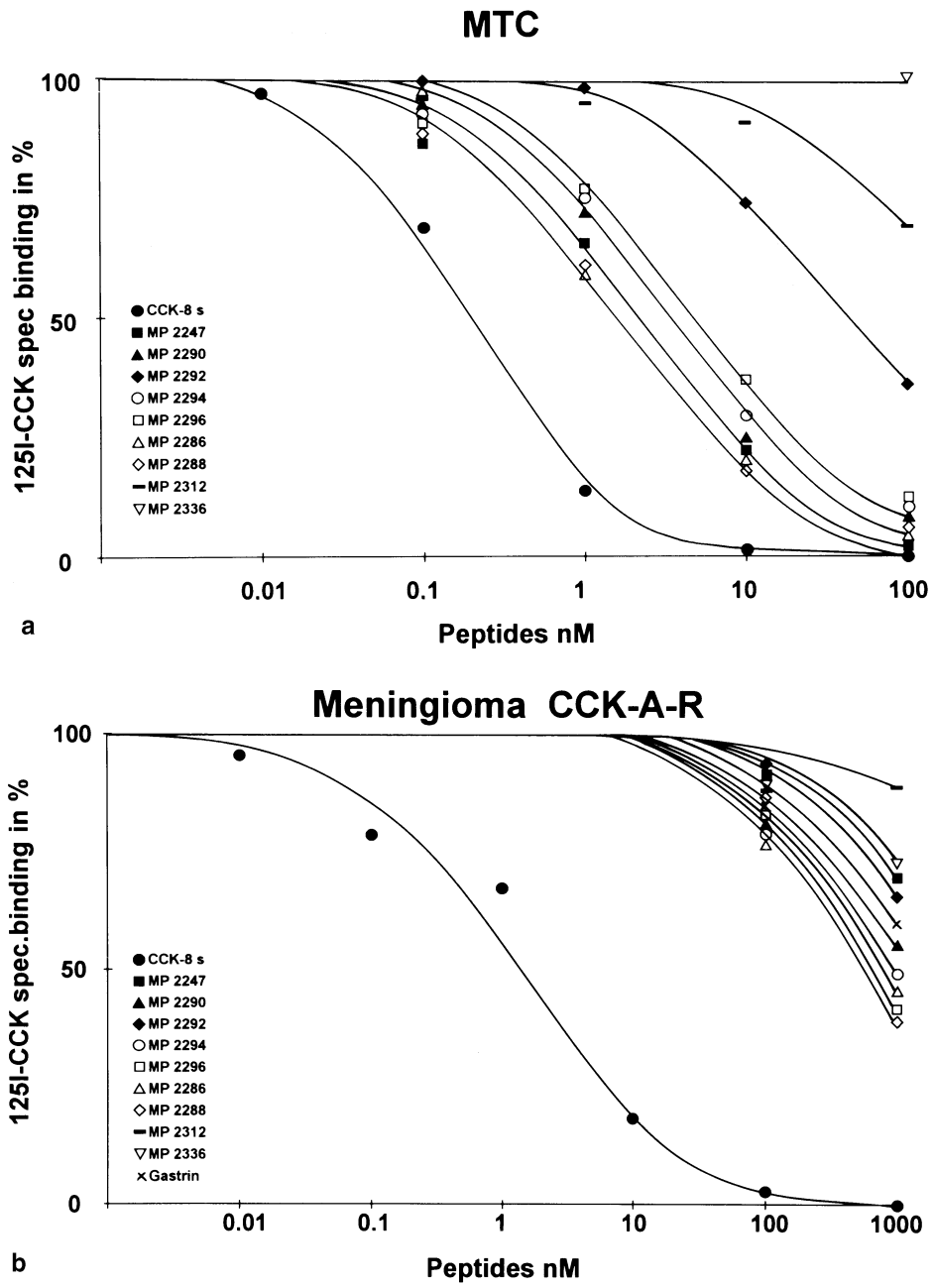

Fig. 1A, B. Competition experiments using ^{125}I-CCK as radioligand with increasing concentrations of sulfated CCK-8 or gastrin as controls, and increasing concentrations of the nine different DTPA-CCK analogs (listed in Table 1). **A** Tissue sections from a CCK-B receptor-expressing MTC; **B** tissue sections from a CCK-A receptor-expressing meningioma. Each tissue section was incubated with 45 pmol/l ^{125}I-CCK. Each *point* represents the radioligand binding to tumor tissue as determined by absorbance in autoradiographs. Nonspecific binding was subtracted from all values

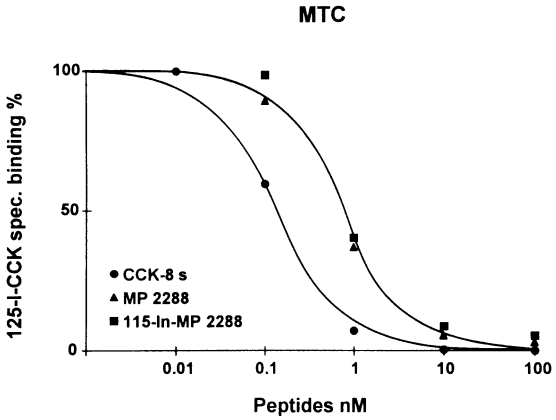

Fig. 2. Competition experiment with sulfated CCK-8 (control), MP2288, and ^{115}In-MP2288 in a CCK-B receptor-expressing MTC. Same experimental conditions as described in Fig. 1

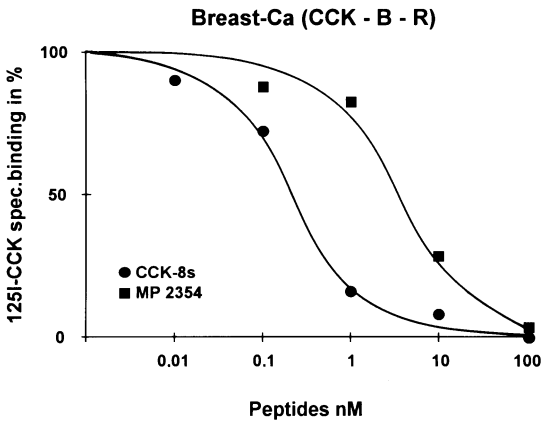

Fig. 3. Competition experiment with sulfated CCK-8 and the DOTA analog MP2354 in a CCK-B receptor-expressing breast cancer. Same conditions as in Fig. 1

tor, as seen in a CCK-B receptor-expressing MTC (Fig. 2). Substitution of the DTPA chelator with DOTA also had no major effect on binding affinity, as demonstrated by the IC_{50} value determined for MP2354 using a breast tumor sample expressing CCK-B receptors (Table 1, Fig. 3).

MP2286 and MP2288 were investigated further for their biodistribution properties in normal rats and for their stability in human urine and blood plasma. Tables 2 and 3 and Fig. 4 show the biodistribution of these two compounds as the mean percentages of the injected dose (% ID) per gram tissue. For both compounds tested, the blood and peripheral soft tissues such as kidneys, muscle, spleen, heart, and small intestine showed very similar localization and low retention patterns. Blood clearance was rapid for both compounds over the 24-h time course studied. The bone (femur) tissue also showed a very similar pattern of localization and very low level of retention for both compounds. MP2286 cleared slightly more rapidly from the liver, pancreas, and the lungs, compared with MP2288. The stomach and contents indicated a higher value at 60 min post injection for

Table 2. Percent injected dose of ^{111}In-MP2288 per gram of tissue sample (% ID/g±SE; *n*=3)

Tissue	Time post injection		
	1 h	4 h	24 h
Blood	0.062±0.008	0.014±0.001	0.004±0.000
Liver	0.086±0.009	0.099±0.013	0.042±0.002
Kidneys	0.402±0.035	0.261±0.016	0.262±0.009
Skeletal muscle	0.013±0.001	0.004±0.000	0.004±0.000
Bone	0.028±0.003	0.013±0.001	0.014±0.001
Spleen	0.027±0.001	0.019±0.002	0.021±0.001
Heart	0.022±0.003	0.006±0.000	0.005±0.000
Lung	0.053±0.010	0.025±0.007	0.019±0.008
Pancreas	0.050±0.003	0.047±0.010	0.027±0.003
Stomach[a]	0.303±0.106	0.079±0.024	0.099±0.030
Small intestines[a]	0.101±0.029	0.026±0.007	0.019±0.005
Large intestines[a]	0.141±0.086	0.123±0.035	0.151±0.065

[a] Includes contents

Table 3. Percent injected dose of ^{111}In-MP2286 per gram of tissue sample (% ID/g±SE; *n*=3)

Tissue	Time post injection		
	1 h	4 h	24 h
Blood	0.066±0.008	0.010±0.000	0.005±0.000
Liver	0.043±0.004	0.029±0.001	0.023±0.001
Kidneys	0.428±0.023	0.331±0.008	0.270±0.015
Skeletal muscle	0.014±0.001	0.004±0.000	0.004±0.000
Bone	0.032±0.004	0.012±0.001	0.011±0.000
Spleen	0.030±0.002	0.022±0.001	0.022±0.001
Heart	0.023±0.002	0.006±0.000	0.004±0.000
Lung	0.050±0.002	0.014±0.001	0.008±0.000
Pancreas	0.039±0.002	0.023±0.001	0.017±0.002
Stomach[a]	0.083±0.004	0.123±0.027	0.063±0.007
Small intestines[a]	0.108±0.030	0.038±0.007	0.014±0.002
Large intestines[a]	1.231±0.353	0.412±0.146	0.166±0.040

[a] Includes contents

MP2288, but at 4 and 24 h post injection the two compounds distributed in a similar pattern. The uptake into the large intestines showed a greater discrepancy between the two compounds, with MP2286 showing almost ninefold greater accumulation than MP2288 at 1 h post injection. However, at 24 h post injection the distributions into this organ were nearly identical (Tables 2, 3). The uptake into the large intestines was determined without removal of contents, and the difference between MP2288 and MP2286 may in part be due to variability in intestinal fecal content. In separate studies, animals fasted for 24 h displayed much less uptake variability in the large intestines (data not shown). Urinary excretion was rapid for both compounds and not significantly different for MP2286 compared with MP2288 (84%±2.7% vs 77%±7.6%), and fecal excretion was low (6.2%±1.8% for MP2288 vs 7.2%±2.6% for MP2286).

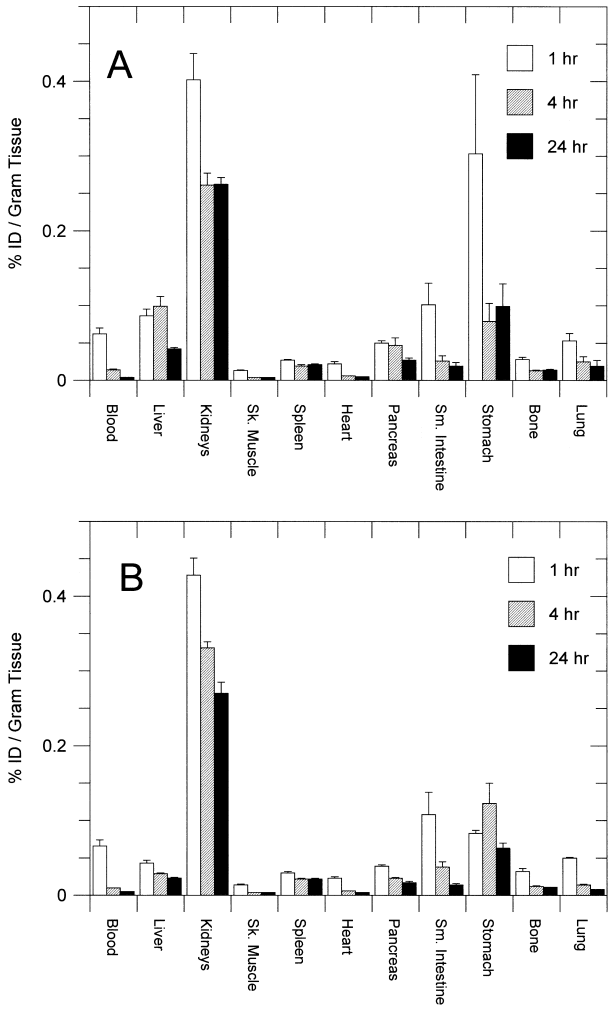

Fig. 4. Biodistribution of ^{111}In-MP2288 (**A**) and ^{111}In-MP2286 (**B**) in normal Sprague-Dawley rats at 1, 4, and 24 h post injection (see text for details). *Error bars* indicate standard error (n = 3)

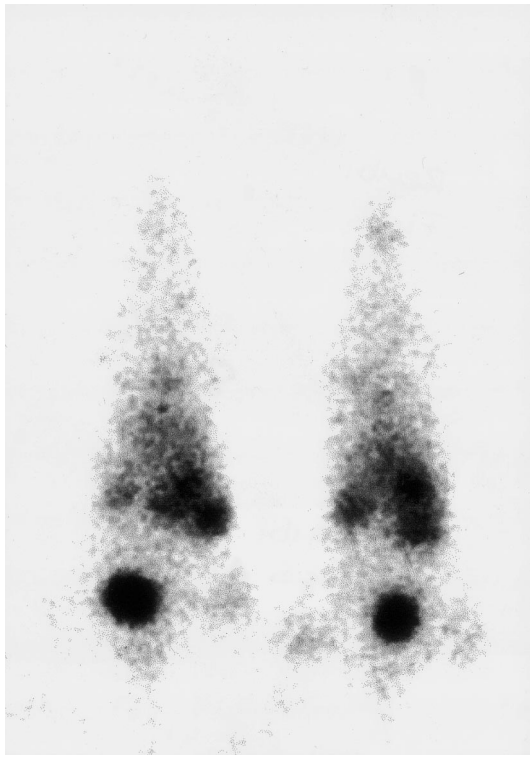

Fig. 5. Scintigrams of in vivo biodistribution of ^{111}In-MP2288 at high specific activity (1400 Ci/mmol) in two rats. Images were obtained at 90 min post injection. Strong labeling of the bladder and both kidneys is observed, suggesting rapid and predominantly renal clearance. Dorsal view

The overall recovery was nearly identical (93%±0.3% for MP2286 vs. 86%±5.3% for MP2288). Scintigraphic images of the biodistribution of MP2288 taken at 90 min p.i. in 24-h fasted rats are shown in Fig. 5 and underscore the rapid renal clearance and low-level uptake into most tissues.

To further evaluate the specificity of in vivo uptake of the labeled CCK-8 analogs into CCK-B receptor-expressing tissues of the gastrointestinal tract, a receptor blocking study was performed using 24-h fasted normal rats injected with ^{111}In-MP2288 radiolabeled at high or low specific activities (1400 Ci/mmol vs 0.5 Ci/mmol) to distinguish regions of nonspecific binding from regions which display specific CCK receptors. In a preliminary study with high specific activity ^{111}In-MP2288, fasted animals were imaged by scintigraphy at 15-min intervals over a 4-h time span. It was found that between 1 and 3 h a majority of the radioligand had cleared from most tissues but that significant uptake was still apparent in the gut region. We therefore sacrificed animals at 120 min post injection for evaluation. In order to map the entire region of the gastrointestinal tract for possible receptor expression, the whole gastrointestinal tract from the stomach to the distal large intestines was removed intact, photographed, and then scintigraphed (Fig. 6A and B). Figure 6B shows the ex vivo scintigraphy of the resected gastrointestinal tract from three rats. The stomachs displayed high uptake of radiolabeled peptide, corresponding to specific binding, since it was blocked in the presence of excess cold peptide (using low specific activity MP2288). The rest of the gastrointestinal tract did not show tracer uptake, except for an area which forms the boundaries of the duodenum and jejunum, where a strong, albeit apparently nonspecific uptake, was observed. Radioactivity associated with either the proximal or distal regions of the small intestines was not affected by the blocking dose (data not shown). In agreement with the above-described specific in vivo labeling of the stomach, in vitro receptor autoradiography revealed a high density of CCK-B receptors in the rat gastric mucosa, whereas ileum and colon did not show measurable amounts of CCK-B receptors, but only a moderate number of CCK-A receptors in the smooth muscles (Fig. 6C and D). The stomach tissue was examined further by determining total radioactivity of the intact organ, before and after removal of contents, with and without receptor blocking conditions. Specific in vivo binding to stomach tissue was only apparent after removal of

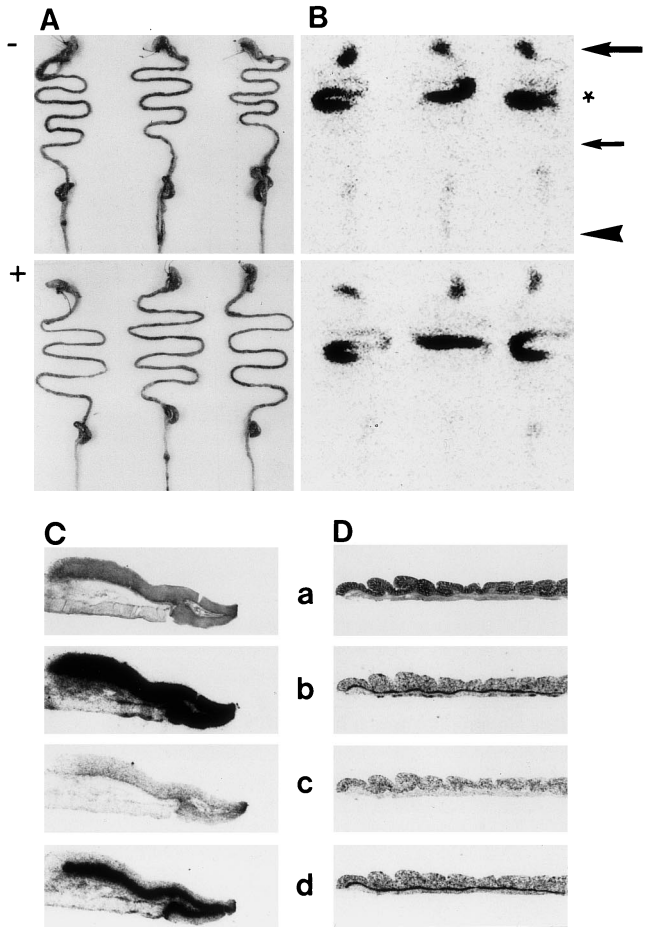

Fig. 6. A, B In vivo uptake of ^{111}In-MP2288 using high (–) and low (+) specific activity radiotracer (see Fig. 5 and text for additional details) measured in the corresponding excised gastrointestinal tracts of rats. **A** Photographs of the excised gastrointestinal tract. **B** Scintigrams of the excised gastrointestinal tract. *Large arrow*, stomach level; *small arrow*, ileum level; *arrowhead*, colon level; the star corresponds to a region of high nonspecific uptake (no blockade with excess cold peptide). The ^{111}In-MP2288 is only blocked with excess cold peptide at the stomach level, suggesting specific binding to CCK-B receptors. **C, D** In vitro CCK-receptor autoradiography of the rat stomach (**C**) and ileum (**D**). **a** Hematoxylin-eosin stained sections. **b** Autoradiographs showing total binding of ^{125}I-CCK, representing CCK-A and CCK-B receptors. **c** Autoradiographs showing nonspecific binding in the presence of 50 n*M* unlabeled sulfated CCK-8. **d** Autoradiographs showing non-specific binding in the presence of 50 n*M* unlabeled gastrin. The residual binding in **d** (in mucosa or muscles) represents CCK-A receptors only. The gastric mucosa has a high density of CCK-A and CCK-B receptors; the ileal mucosa has no CCK receptors, whereas the ileal muscles have only CCK-A receptors

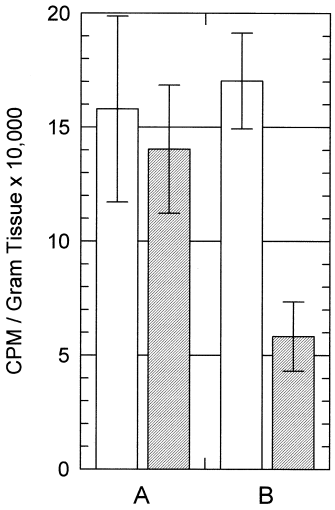

Fig. 7. In vivo uptake of high (*plain bars*) or low (*hatched bars*) specific activity radiotracer (1400 Ci/mmol and 0.5 Ci/mmol ^{111}In-MP2288 respectively) into stomach tissue before (**A**) and after (**B**) removal of contents. Radioactivity was measured by gamma counting and *error bars* indicated standard deviation (n = 3). An 1% injected dose corresponds to 425,000 cpm±4250 cpm. A strong blocking effect of excess cold peptide (= ^{111}In-MP2288 at low specific activity) is seen in the stomachs with their contents removed

the contents. Figure 7 shows that low specific activity MP2288 had a much reduced uptake compared with high specific activity MP2288, when measured in rat stomachs which were resected and then washed from their contents, confirming that specific CCK-B receptors can be specifically labeled in vivo with this CCK analog. The percent binding inhibition of low specific activity MP2288 compared with high specific activity MP2288 amounted to 65.6% (Fig. 7).

Preliminary HPLC evaluation of excreted urine from rats injected i.v. with ^{111}In-MP2286 or ^{111}In-MP2288 showed that there was no intact peptide present 1 h after tracer injection (data not shown). A first attempt to examine the nature of this degradation and at which level it occurred was made by determining in vitro the stability of the two compounds in both rat and human plasma and urine. Very little degradation of either ^{111}In-MP2286 or ^{111}In-MP2288 was observed in rat or human plasma after incubation for 3 h at 37°C (>90% intact peptide). The compounds were also stable when incubated in the presence of the low molecular weight filtrates of rat or human urine filtered through an Amicon YM10 membrane (10,000-dalton cutoff) (data not shown) or in buffered saline for 3 h at 37°C (Fig. 8). This shows that the observed degradation is not due to ligand instability due to temperature, pH effects, or the presence of metal competitors. In contrast, in similar incubations, the two tested CCK derivatives were partly degraded in unfiltered urine or in urine protein (retained fraction of tenfold concentrated urine filtered on YM10 membrane, Fig. 8). Both compounds showed identical decay rates with more than 50% intact peptide present after 3 h. Both compounds yielded similar but not identical degradation products as determined by HPLC (Fig. 9). This provides further evidence that the observed products are DTPA-linked peptide degradation products and not the result of ^{111}In transchelation into high molecular weight urine factors.

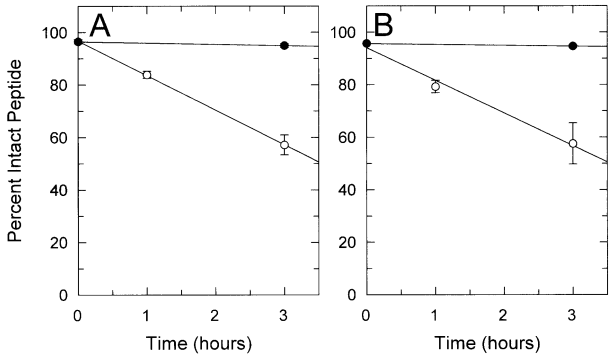

Fig. 8. In vitro degradation of ^{111}In-MP2288 (**A**) and ^{111}In-MP2286 (**B**) in human urine. Data obtained from integration of radiometric traces shown in Fig. 9 and graphed as percent intact peptide with respect to total eluted radioactivity. Saline controls incubated at 37°C for 3 h (●); and urine protein fraction incubated at 37°C for 3 h (○). *Error bars* indicate standard error (n = 3)

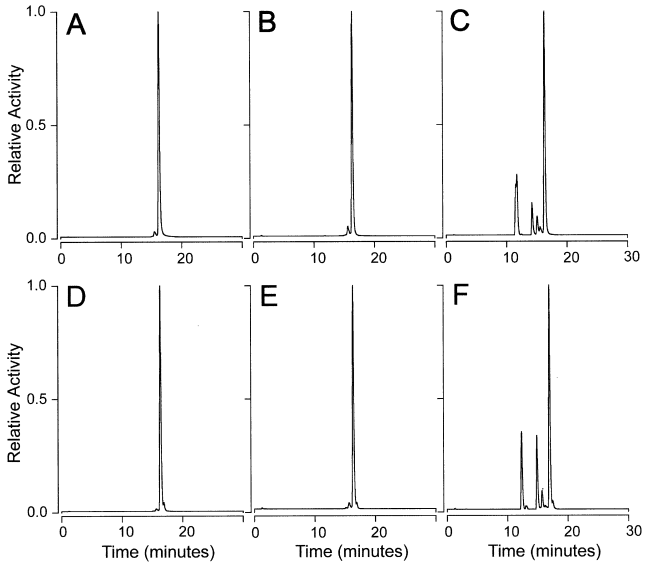

Fig. 9. In vitro degradation of ^{111}In-MP2288 (**A–C**) and ^{111}In-MP2286 (**D–F**) in human urine protein. Reverse-phase HPLC radiometric traces of control samples before incubation (**A, D**); saline control samples after 3 h incubation at 37°C (**B, E**); and samples incubated in saline with urine protein (**C, F**) after 3 h at 37°C (see methods for details). Secondary peaks in **C** and **F** indicate ^{111}In-DTPA-peptide-linked degradation products

Discussion

The present study describes for the first time peptidic CCK analogs potentially suitable for scintigraphic investigations. These new DTPA- or DOTA-linked CCK analogs are highly specific for the CCK-B receptor and exhibit a nanomolar binding affinity. The compounds which bind with high affinity have the DTPA or DOTA moiety coupled to the N-terminal end of the CCK octapeptide whereas those which have low or no measurable affinity have the chelator molecule at the C-terminal end. The high specificity towards CCK-B receptors is determined by the presence of nonsulfated tyrosine. Indeed, it is well established [13] that a sulfated tyrosine in position 27 of CCK-33 is necessary for CCK and short CCK derivatives to retain high affinity for both CCK-A and CCK-B receptors. Replacement of sulfated tyrosine with tyrosine results in compounds that bind selectively to the CCK-B receptor. Moreover, the high-affinity binding observed in vitro for the two best compounds, MP2286 and MP2288, is retained when they are chelated with ^{115}In. An IC_{50} value below 2 nM of this category of compounds is considerably better than the binding affinity of the ^{115}In-DTPA-octreotide to somatostatin receptors [14]. As expected, the DOTA-containing CCK octapeptide analog (MP2354) also binds with high affinity.

In addition to their selective and high-affinity binding characteristics, the two DTPA-CCK compounds also appear to be quite stable in human plasma. Other compounds tested, MP2247 and MP2290 for example, were much less stable (data not shown). Increased plasma stability is probably due, in part, to the substitution of the two methionines in these latter compounds for norleucine (present in MP2286 and MP2288) [15]. The instability of ^{111}In-labeled MP2288 and MP2286 found in urine and in a urine protein fraction may be related to the fact that the kidney is the major place of inactivation of CCK and gastrin [16]; this appears to be due to enzymatic degradation, although further studies are necessary to determine the nature of the degradation products observed in Fig. 9. It is not clear what effect urine stability would have on the overall imaging properties of these compounds.

The results of the in vivo rat biodistribution studies showed that both MP2286 and MP2288 clear rapidly by renal excretion, and display low uptake/retention in the main peripheral soft tissues. These properties are desirable for receptor-targeted imaging agents [1], particularly when the receptor targets are located in the thoracic area (i.e., for the visualization of SCLCs and MTCs). Generally, the two compounds distributed in a similar manner in vivo when compared directly with each other. Their general pattern of distribution is not basically different from that of ^{111}In-DTPA-D-Phe-octreotide [17], except that their kidney retention is much lower.

Blocking studies, where the uptake of radiolabeled CCK was determined in the presence and absence of an excess amount of unlabeled peptide, illustrate the in vivo specificity of the imaging molecules. The blocking effect of excess cold peptide was evaluated on the gastrin target tissues of normal fasted rats because a CCK-B-receptor expressing animal tumor model was not available. This study shows that MP2288 specifically binds in vivo to the CCK-B receptors present in normal CCK target tissues. Significant uptake of high specific activity ^{111}In-MP2288 into stomach tissue was observed, and a substantial blocking effect on the uptake was observed using low specific activity ^{111}In-MP2288. This is explained by the fact that the stomach, in rat and human, is one of the organs with the highest levels of CCK-B re-

ceptor expression [5]. Conversely, the rat ileum and colon, which express only CCK-A receptors, did not show uptake of the CCK radioligands in vivo. There was also a narrow region of uptake in the duodenal/jejunal region of the small intestines. This uptake was observed using both high and low specific activity peptides, suggesting low affinity or nonspecific binding only. No uptake of MP2288 was observed scintigraphically in brain tissue, which is rich in CCK receptors, because such peptide analogs cannot cross the intact blood-brain barrier, at difference to the nonpeptide CCK-analogs [18, 19]. Similar results were obtained previously in somatostatin receptor studies in rats where the in vivo uptake of ^{123}I-octreotide and ^{111}In-DTPA octreotide [14] in somatostatin receptor-expressing targets, such as adrenals, could be blocked with unlabeled octreotide. Evidence for the specificity of the visualization process is therefore clearly given by the present data, suggesting that DTPA-CCK radioligands can label physiological CCK-B receptors in vivo, even in the absence of an adequate tumor model.

The present study shows that CCK analogs N-terminally linked to chelators retain high affinity and high selectivity for CCK-B receptors. These derivatives also have considerable stability in plasma and are rapidly cleared from the circulation through the kidneys. Moreover, it has been shown previously that CCK-B receptors can be efficiently internalized into cells upon CCK ligand binding [20], a mechanism which is thought to represent the basis for the in vivo accumulation of peptide radioligands in the cells expressing the corresponding peptide receptors. All the above-mentioned characteristics make these DTPA- and DOTA-CCK compounds highly promising as radioligands for the in vivo targeting of human tumors expressing CCK-B receptors, such as MTCs, SCLCs, astrocytomas, and stromal ovarian cancers, as well as some gastrointestinal, pancreatic, and breast tumors [7, 21, 22]. According to published incidence figures [6, 7], CCK-B receptor scintigraphy should identify the great majority (>90%) of MTCs, considerably more than are presently detected with Octreoscan [1, 23]. It also may be possible to use a cocktail of radiolabeled DTPA-octreotide and DTPA-CCK to amplify the scintigraphic signal in MTCs, since both somatostatin and CCK-B receptors are expressed concomitantly in many of these tumors [24]. Finally, since DOTA-CCK analogs also bind with high affinity to CCK-B receptors, radiotherapy may be possible using an yttrium-90 labeled peptide [25, 26].

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