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Synthesis and *in vitro* characterization of radioiodinatable benzodiazepines selective for type 1 and 2 cholecystokinin receptors

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Abstract

Radiolabeled antagonists of specific peptide receptors identify a higher number of receptor binding sites than agonists and may thus be preferable for *in vivo* tumor targeting. In this study, two novel radioiodinated 1,4-benzodiazepines, (*S*)-1-(3-iodophenyl)-3-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)urea (**7**) and (*R*)-1-(3-iodophenyl)-3-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)urea (**9**), were developed. They were characterized *in vitro* as high affinity selective antagonists at cholecystokinin type 1 and 2 (CCK_1 and CCK_2) receptors using receptor binding, calcium mobilization, and internalization studies. Their binding to human tumor tissues was assessed with *in vitro* receptor autoradiography and compared with an established peptidic CCK agonist radioligand. The ^{125}I-labeled CCK_1 receptor-selective compound **9** often revealed a substantially higher amount of CCK_1 receptor binding sites in tumors than the agonist ^{125}I-CCK. Conversely, the radioiodinated CCK_2 receptor-selective compound **7** showed generally weaker tumor binding than ^{125}I-CCK. In conclusion, compound **9** is an excellent radioiodinated non-peptidic antagonist ligand for direct and selective labeling of CCK_1 receptors *in vitro*. Moreover, it represents a suitable candidate to test antagonist binding to CCK_1 receptor-expressing tumors *in vivo*.

Introduction

Radiolabeled peptide receptor ligands used for targeted imaging or therapy of peptide receptor-expressing tumors represent an emerging class of radiopharmaceuticals.[1, 2] The clinically best-established examples are somatostatin receptor ligands, which are highly effective in scintigraphic imaging and radiotherapy of somatostatin receptor-expressing gastroenteropancreatic neuroendocrine tumors.[3, 4] Up to now, radiolabeled receptor agonists have primarily been used for these applications, based on the concept that internalization of the radioligand-receptor complex into tumor cells that follows agonist binding to the receptor is an important prerequisite for accumulation of radioactivity within tumors. Recently, however, it was recognized that radiolabeled somatostatin receptor antagonists, which internalize poorly, result in stronger and longer-lasting uptake of radioactivity in tumors *in*

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vivo compared with agonists.[5] A major molecular basis for this is considered to be the ability of somatostatin receptor antagonists to bind with high affinity to a larger fraction of somatostatin receptors than agonists.[5] As one important determinant of the success of *in vivo* tumor targeting is high uptake of radioactivity in the tumor area, somatostatin receptor antagonists may therefore be preferable to agonists for these applications.

The discovery in the somatostatin receptor field that radiolabeled antagonists show better tumor targeting characteristics than agonists has significantly increased the interest in radiolabeled peptide receptor antagonists in general with respect to their usefulness for *in vivo* targeting.[6] It has subsequently been shown also for bombesin receptor-expressing tumors that radiolabeled bombesin antagonists perform better than agonists in terms of radioactivity uptake in tumors *in vivo*.[7] The same may well be true for antagonists of other peptide receptors, as indeed a number of receptors, such as corticotropin releasing factor, histamine, muscarinic cholinergic, and cholecystokinin (CCK) receptors, have been shown *in vitro* to exhibit more binding sites for antagonists than for agonists.[8–11]

Radiolabeled CCK receptor antagonists are particularly interesting candidates to test for their tumor binding capabilities. First, CCK receptors are expressed in a variety of clinically important cancers: for instance, CCK_2 receptors show high levels of expression in medullary thyroid carcinomas, small cell lung cancer, and gastrointestinal stromal tumors (GIST), whereas CCK_1 receptors are overexpressed, to a lower degree, in GIST, ileal carcinoid tumors, leiomyosarcomas, and meningiomas.[12–14] The high CCK_2 receptor expression in medullary thyroid carcinomas has already led to successful clinical applications with radiolabeled agonists, and the promising initial results have stimulated ongoing research for new, improved CCK_2 receptor radioligands for clinical applications.[12, 15] Second, there is a long history of CCK receptor antagonist development.[16, 17] A large number of non-peptidyl antagonists have been designed on the basis of various chemical classes. Benzodiazepines represent one group, comprising highly potent and selective CCK_1 and CCK_2 receptor antagonists.[16, 18, 19] Of particular interest, benzodiazepines have been shown to act at an allosteric site in the helical bundle region within CCK_1 receptors, binding to a receptor domain different from the orthosteric binding site for the natural ligand CCK-8.[20, 21]

Therefore, the aims of the present study were to prepare radioiodinated benzodiazepine antagonist ligands selective for the CCK_1 and CCK_2 receptors, pharmacologically and functionally characterize these, and assess their *in vitro* tumor-binding properties relative to a radioiodinated peptidyl CCK receptor agonist. The ^{125}I-labeled benzodiazepine antagonist that expressed selectivity for the CCK_2 receptor was analogous to the 3-iodo-phenyl derivative of methyl-1,4-benzodiazepine described by Bock et al. in 1993.[22] The ^{125}I-labeled benzodiazepine antagonist that expressed selectivity for the CCK_1 receptor was structurally similar to this compound, except incorporating the opposite stereochemistry of the 3-position side chain, with precedent recognized by Bock et al. [22] and by extensive structure-activity data published by that group.[23] The binding behavior of the ^{125}I-labeled compounds in original human tumor tissues was quantitatively analyzed in comparison with that of the agonist radioligand ^{125}I-CCK using *in vitro* receptor autoradiography.

Results

Chemistry

Synthesis of 3-amino-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one[24] and its resolution has been reported previously.[25, 26] Reaction of the amine **2** with 3-iodophenyl isocyanate in methylene chloride resulted in (*R*)-1-(3-iodophenyl)-3-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)urea (**3**) (Scheme 1). In a similar way (*S*)-1-(3-iodophenyl)-3-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-

yl)urea (**5**) was obtained by reaction of amine **4** with 3-iodophenyl isocyanate. Treatment of **3** with hexamethyldistannane gave (*R*)-1-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)-3-(3(trimethylstannyl)phenyl)urea (**6**) mediated by catalysis of [dichlorobis(triphenylphosphine)palladium (II)] in 1.4-dioxane at 60°C. Similarly, the urea **5** was converted into (*S*)-1-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)-3-(3 (trimetylstannyl)phenyl)urea (**8**). Radioiodinated **7** was synthesized via reaction of urea **6** with Na^{125}**I** mediated by iodo-beads in a solvent mixture of acetonitrile/methanol/dimethysulfoxide/water (1:1:1:2; v:v:v:v) diluted in 0.1% trifluoroacetic acid (see the experimental procedure). Similarly, stannylated urea **8** was converted into radioiodinated **9**.

Receptor binding and biological activity studies

Binding of the radioiodinated compounds **7** and **9** to CCK receptors was characterized in receptor-bearing membrane preparations. Binding conditions were established to ensure reaching binding equilibrium. This required incubation for 60 min at room temperature. Non-saturable binding represented less than 15 % of total cpm bound for both radioligands.

Increasing amounts of radiodinated compounds were incubated with a constant amount of membranes bearing CCK_1 or CCK_2 receptors to determine the dissociation constant K_d (Figure 1). The calculated pK_d values for compound **9** at CCK_1 receptor and compound **7** at CCK_2 receptor were 8.94 ± 0.14 and 8.53 ± 0.05, respectively.

Competition-binding curves were utilized to examine the relative affinities of each of the compounds (Figure 2, Table 1). Both compounds bound with high affinity and exhibited selectivity for the two subtypes of CCK receptors. Each of the benzodiazepines competed for binding in a concentration-dependent manner, with curves that were parallel to each other, but that reflected differences in affinity of 50- to 500-fold (Figure 2). Calculated pK_i values from homologous competition-binding assays shown in Table 1 were consistent with the pK_d values determined in the saturation binding assays. Of particular interest, CCK, the natural peptide ligand for these receptors, displaced only a portion of the saturable radioligand binding, with competition-binding curves not parallel to those for the benzodiazepines. This may reflect different structural determinants for binding of peptides and benzodiazepines at these receptors.[20, 21, 27]

We then attempted to evaluate the effect of the novel benzodiazepine compounds on receptor function. With a range of tested concentrations from 10 pM to 10 μM, neither compound **3** nor compound **5** could stimulate any calcium response in CCK_1 or CCK_2 receptor-bearing cells. Additionally, pre-incubation of the cells with the benzodiazepine compounds inhibited the calcium responses stimulated by CCK peptide in a concentration-dependent manner at both CCK_1 and CCK_2 receptors (Figure 3). Therefore, compounds **3** and **5** represent antagonists acting at the CCK receptors.

Furthermore, we monitored the effects of compounds **3** and **5** binding to CCK_2 and CCK_1 receptors, respectively, on receptor internalization by tracking fluorescently-tagged CCK_1 and CCK_2 receptors expressed in CHO cells. Compound **5** did not influence the internalization of CCK_1 receptors. On the other hand, compound **3** binding to CCK_2 receptors was surprisingly followed by receptor internalization (Figure 4A and B). The agonist CCK-8 was able to stimulate internalization of both CCK_1 and CCK_2 receptors into endocytic compartments as is typical of agonists. Of note, the fluorescently-tagged CCK_1 and CCK_2 receptors were localized in endosomal structures within 10 min of CCK-8 stimulation. Kinetics of internalization were similar for yellow fluorescent protein (YFP)-tagged CCK_2 receptor after occupation with CCK-8 or compound **3**; for CCK_1 receptors, the kinetics were as expected (Figure 4C and D).

Binding of the ^{125}I-radiolabelled compounds **7** and **9** to human tissues was tested in a series of CCK_1 and CCK_2 receptor-expressing human tumors as well as in human gallbladder using *in vitro* receptor autoradiography. We compared the results obtained with compounds **7** and **9** with those using a radiolabeled CCK analog (Table 2). Compound **9** labeled CCK_1 receptor-expressing tissues very well. It showed strong binding to human gallbladders (Figure 5). Moreover, it labeled CCK_1 receptor-expressing tumors such as meningiomas and GIST extremely well, revealing even more binding sites than were detected with the ^{125}I-CCK radioligand (Figure 5, Table 2). Compound **9** bound with high affinity to all these tissues and was displaced completely and with high affinity by the CCK_1 receptor-selective benzodiazepine antagonist, compound **10** (L-364,71818,[31]). Cold CCK-8 displaced compound **9** also with high affinity, however incompletely, whereas gastrin was inactive (Figures 5 and 6). Of note, some of the CCK_2 receptor-expressing medullary thyroid carcinomas were also labeled, although in general weakly, with compound **9** (Table 2). While this binding could be displaced by the corresponding cold compound **5**, there was no high affinity displacement observed with established CCK_1 or CCK_2 receptor-selective analogs; the compound **9** labeling of these medullary thyroid carcinomas can therefore not be conclusively assigned to one of the two established subtypes of CCK receptors.

Compound **7** labeled CCK_2 receptor-expressing tumor tissues. However, the signal intensity varied substantially among the various tumors. CCK_2 receptor-expressing GIST and leiomyomas were often well labeled. High affinity binding was observed with the corresponding cold ligand **3**. Cold CCK-8 displaced the radioligand from the majority of labeled sites with high affinity (Figures 5 and 6). Curiously, CCK_2 receptor-expressing medullary thyroid carcinomas, as well as a subset of GIST and leiomyomas, were extremely poorly labeled with compound **7** when compared with the apparent amounts of CCK_2 receptors identified with ^{125}I-CCK in each of these tumors (Table 2). Binding affinity of the corresponding cold ligand **3** was moderately high, and cold CCK-8 only poorly displaced compound **7** binding. Typical CCK_2 receptor pharmacology was not observed in these tumors. CCK_1 receptor expressing-tumors were not labeled with compound **7**, whereas CCK_1 receptor expressing-gallbladders were weakly labeled with this radioligand (Table 2). These binding sites could, however, not be displaced by selective CCK_1 or CCK_2 analogs.

Discussion and Conclusions

It was recently recognized in the somatostatin receptor field that radiolabeled antagonists show stronger binding to receptor-expressing tumors than agonists *in vivo* and may thus be preferable for clinical applications.[5] In the present study, it was for the first time evaluated if also CCK receptor antagonists may exhibit increased binding compared with an agonist to tumoral CCK_1 and CCK_2 receptors, which represent clinically important targets as well.[12, 15] For this purpose, two radioiodinated 1,4-benzodiazepine compounds, **7** and **9**, were developed based on previous extensive structure-activity data.[16, 17, 22–24] The ligands were characterized by high affinity and selectivity for, as well as antagonistic actions at CCK_1 and CCK_2 receptors in receptor-bearing membrane preparations. The CCK_1 receptor-selective compound **9** showed excellent *in vitro* binding to tumor tissues, often identifying a substantially higher amount of CCK_1 receptors than the radioiodinated agonist CCK. By contrast, the CCK_2 receptor-selective compound **7** performed less well than ^{125}I-CCK, showing generally weaker tumor binding.

The two radioiodinated compounds **9** and **7** exhibited good pharmacological characteristics at CCK_1 and CCK_2 receptors, respectively, in terms of specificity, affinity, and selectivity. Several lines of evidence of specific CCK receptor binding of the compounds were provided. Both compounds showed saturable binding to CCK receptor-bearing membranes. Moreover, in competition-binding assays, compound **9** binding to CCK_1 receptor-expressing membranes and tissues was displaced by the universal CCK receptor ligand, CCK-8, as well as by the

CCK_1 receptor-selective benzodiazepine ligand, compound **10**, and compound **7** binding to CCK_2 receptor-expressing membranes and tissues was also displaced by CCK-8. The affinities of compounds **9** and **7** at CCK_1 and CCK_2 receptors, respectively, were both high, with Ki values in the low nanomolar concentration range. Compounds **9** and **7** also showed good selectivity for CCK_1 and CCK_2 receptor subtypes, respectively, with compound **7** exhibiting two orders of magnitude lower affinity for CCK_1 receptors than for CCK_2 receptors, and compound **9** binding with fifty times lower affinity to CCK_2 receptors than to CCK_1 receptors.

It is noteworthy that both compounds did not show typical competitive binding with CCK-8 at CCK receptors. In particular, compound **9** binding to CCK_1 receptors was not fully displaced by CCK-8. Likewise, displacement of compound **7** binding to CCK_2 receptor-expressing tissues by CCK-8 was often poor. This non-competitive binding of the benzodiazepine analogues and CCK-8 at CCK receptors may be explained by binding to different domains within the receptor. Indeed, other benzodiazepines have been demonstrated to represent allosteric ligands at CCK_1 receptors, binding to a CCK_1 receptor domain distinct from the orthosteric binding site for the natural ligand.[20, 21]

Evidence of the antagonistic features of compounds **3** and **5** at CCK receptors was provided by two different functional assays. Both ligands did not stimulate calcium mobilization themselves, but completely inhibited the CCK-8-stimulated calcium response in CCK receptor-bearing cells. Furthermore, binding of the CCK_1 receptor-selective compound **5** did not trigger CCK_1 receptor internalization. Interestingly, binding of the CCK_2 receptor-selective compound **3** to CCK_2 receptors was, however, followed by internalization. Indeed, it has been recognized that some CCK receptor antagonists are not neutral antagonists but stimulate receptor internalization. This was previously found for several CCK_1 receptor antagonists[29] and is now demonstrated for the first time also for a CCK_2 receptor antagonist. Apparently, although having no effect on G protein coupling and subsequent intracellular signaling, binding of specific antagonists can lead to a conformational change or can stabilize a conformation of both CCK_1 and CCK_2 receptors exposing domains that mediate internalization. Of note, for a hypothetical receptor-targeted tumor therapy with antagonists, efficient internalization of the radioligand-receptor complex into tumor cells could be a favorable feature. It may allow accumulation of radioactivity close to the tumor cell nucleus, i.e. the actual target, and, thus, the use of isotopes with a very short tissue penetration range, such as Auger emitters like ^{123}I.[30]

Based on their good pharmacological and antagonistic characteristics, compounds **7** and **9** represented suitable radioligands to test for their binding properties in human tumor tissues as compared with radiolabeled agonists. Unexpectedly, the tumor binding of these two compounds differed in fundamental ways. On the one hand, the CCK_1 receptor-selective compound **9** performed quite well: It often identified considerably more, in fact up to almost six times more, CCK_1 receptor binding sites in tissues than the radiolabeled agonist ^{125}I-CCK. Of particular interest, this was especially the case for human tumors with low or moderate ^{125}I-CCK binding, but not for normal human tissue, i.e. the gallbladder. This was fully in line with previous findings for somatostatin receptor antagonists.[5] In contrast, the radiolabeled CCK_2 receptor antagonist compound **7** did consistently label not larger, but in fact considerably smaller fractions of CCK_2 receptor binding sites in tumors than ^{125}I-CCK. Of note, in a minority of specimens, there was some saturable binding observed for both compounds that could not be assigned to either classical CCK_1 or CCK_2 receptors in competition-binding assays. In fact, it may not be surprising for synthetic, small ligands to find low affinity binding targets in complex tissues that exhibit a very large variety of molecular structures. However, given the large number of possibilities, it would go beyond the scope of the current project to specifically identify those binding sites.

The differences in the amounts of tumoral CCK receptor binding sites identified with radiolabeled antagonists and agonists may be explained by the concomitant expression of various CCK receptor states, where a different number of receptor states is available for antagonist and for agonist binding, analogous to somatostatin receptors.[5] Indeed, good evidence for this concept is available for the type 1 CCK receptor.[11, 19] The rat CCK_1 receptor was previously shown to comprise different affinity states for an agonist, where high and low affinity states were less abundant than a very low affinity state. All these states were, however, bound with high affinity by an antagonist, resulting in a larger fraction of CCK_1 receptor states with high affinity for the antagonist than for the agonist. Accordingly, radiolabeled CCK_1 receptor antagonists labeled considerably more CCK_1 receptor binding sites in the rat pancreas compared with CCK_1 receptor agonists.[31, 32] The present study indicates that the same is true for human CCK_1 receptors expressed in tumors. As for the CCK_2 receptor, different affinity states were also described.[19, 33] However, in contrast to the CCK_1 receptor, the majority of CCK_2 receptors represented states with high affinity for the agonist but variably lower affinities for antagonists, depending on the species.[19] Putative similar conditions of the human CCK_2 receptor may account for the superiority of the agonist ligand ^{125}I-CCK over the antagonist compound **7** in identifying CCK_2 receptors in human tissues.

Thus, compound **9** is, to our knowledge, the first published radioiodinated CCK receptor ligand selective for the subtype 1 receptor having acceptable pharmacological characteristics. The development of a benzodiazepine analogue that retains high CCK receptor affinity after introduction of a large iodine into this small molecule is indeed remarkable. ^{3}H- and ^{11}C-labeled benzodiazepines have been previously used for selective CCK_1 receptor labeling.[11, 19, 34] Up to now, however, the commonly-used radioiodinated CCK peptides that recognize both CCK_1 and CCK_2 receptors have remained the standard radioligands for CCK_1 receptors, as radioiodine shows more favorable characteristics than the other isotopes employed with respect to energy emission and half-life.[35] Compound **9** is, thus, a novel suitable tool for direct, selective identification of CCK_1 receptors not only *in vitro*, but in particular also *in vivo*.

To conclude, the present study demonstrates that a radioiodinated benzodiazepine acting as a CCK_1 receptor selective antagonist is an adequate radioligand *in vitro*. It can identify a higher number of CCK_1 receptor binding sites than a comparable agonist in human tumors. This provides the molecular basis that *in vivo* radiolabeled CCK_1 receptor antagonists could show better tumor targeting characteristics than agonists, analogous to somatostatin receptor antagonists.[5] With compound **9**, there is a suitable radioligand available to test this in an *in vivo* tumor model in follow-up studies. Conversely, based on the lower *in vitro* tumor binding of the CCK_2 receptor-selective compound **7** compared with the agonist, radiolabeled CCK_2 receptor antagonists appear at present not to be superior over agonists for *in vivo* tumor targeting.

Experimental Section

Ligand synthesis

General—Chemicals and solvents purchased from Aldrich or Fisher were used without further purification. Some dry solvents were made by MBRAUN MB-SPS Solvent Dispensing. Compounds such as *m*-iodophenyl isocyanate, hexamethyldistannane and bis-(triphenylphosphine)-palladium (II)-dichloride were purchased from Sigma and Aldrich. Melting points were taken in glass capillary tubes on a Unimelt, Thomas & Hoover apparatus and are uncorrected.

^{1}H and ^{13}C NMR spectra were obtained on an Oxford Varian VXR 300 MHz and on a Bruker 400 MHz avance NMR Spectrometer. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane, and spin multiplicities are given as s (singlet), d (doublet), dd

(double doublet), t (triplet), q (quartet), or m (multiplet). Polarities were obtained on AUTOPOL III Automatic Polarimeter. Mass spectra were obtained on a Bruker BioTOF II mass spectrometer. Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040–0.063 mm, Merck).

Purities of **3, 5, 6,** and **8** were over 98% based on analysis on HPLC column (Alltech Econosphere C18, 5u Column, 4.6×250mm) which was eluted with MeOH/water/Ammonium hydroxide (90:9:1) at a flow rate of 1 mL/min.

General method for the synthesis of iodinated ureas 3 and 5—The amines **2** or **4** (0.4 mmol) were mixed with 0.4 mmol 3-iodophenyl isocyanate in 10 mL methylene chloride at room temperature. After 2 h stirring the samples were quenched with water and the organic layer was separated. The same procedure was repeated with 3×10 mL methylene chloride. The collected organics were pooled and dried over anhydrous sodium sulfate. After evaporation of solvent the residue was separated on a gravity column using SiO_2 and eluted with a solvent mixture (methylene chloride/methanol/ammonium hydroxide: 95:4.5:0.5; v:v:v). Rf.: 0.2. The solvent was evaporated to dryness under reduced pressure and gave a solid which was recrystallized from ether.

(*R*)-1-(3-iodophenyl)-3-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)urea (**3**).

yield: 82.9%, white solid. mp. 172–174 °C, 173–175 °C.[22] $[\alpha]^{23} = -19.1$ (*c* 0.069, methanol). ^{1}H-NMR ($CDCl_3$): δ 8.0-6.7 (m, 15 H, Ar + urea-H), 5.6 (d, J = 7.91 Hz, 1H), 3.49 (s, 3H, aliphatic-H). MS (EIS) calcld for $C_{23}H_{19}IN_4NaO_2$, 533.05 (M+Na); found, 533.05.

(*S*)-1-(3-iodophenyl)-3-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)urea (**5**).

yield: 94.5%, white solid. mp. 171–173 °C. $[\alpha]^{23} = +50.5$ (*c* 0.122, methanol). ^{1}H-NMR ($CDCl_3$): δ 8.0-6.7 (m, 15 H, Ar + urea-H), 5.6 (d, J = 7.91 Hz, 1H), 3.49 (s, 3H, aliphatic-H). MS (EIS) calcld for $C_{23}H_{19}IN_4NaO_2$, 533.05 (M+Na); found, 533.05.

General procedure for the synthesis of stannylated ureas 6 and 8.[36]—To a double neck flask a solution of *m*-iodobenzodiazepine (74.8 mg, mmol) in degassed 1,4-dioxane (5 mL) was added 300 μL hexamethyldistannane (0.14 mmol) and 7 mg bis-(triphenylphosphine)-palladium (II)-dichloride. The solution was stirred at 60°C for overnight under nitrogen steam. After evaporation to dryness the crude mixture was separated by gravity column (SiO_2) using chloroform/methanol in a ratio of 97:3(v:v). Rf.: 0.43.

(*R*)-1-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)-3-(3 (trimetylstannyl)phenyl)urea (**6**).

Yield: 69.6%, white solid; mp. Decomposed > 175 °C.

^{1}H-NMR (d_6-DMSO): δ 8.8 (s, 1H), 7.5-6.79 (m, 14H), 5.0 (d, J=8.36 Hz, 1H), 3.1 (s, 3H), 0.0 (s, with Sn satellites, 9H). ^{13}C-NMR (d_6-DMSO): δ 167.6 (C=O), 165.8(C=O), 154.2, 142.6, 142.3, 139.6, 137.6, 132.06, 130.6, 129.4, 129.2, 128.7, 128.3, 128.2, 128.2, 124.5, 122.2, 117.6, 67.9 (NCH), 34.7 (NCH_3), −9.2 ($Sn(CH_3)_3$). MS (EIS) calcld for $C_{26}H_{29}N_4O_2Sn$, 549.13 (M+1); found, 549.49.

(*S*)-1-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-benzo[*e*][1,4]diazepin-3-yl)-3-(3 (trimetylstannyl)phenyl)urea (**8**).

Yield: 73%, white solid; mp. Decomposed >176 °C.

^{1}H-NMR (d_6-DMSO): δ 8.8 (s, 1H), 7.5-6.79 (m, 14H), 5.0 (d, J=8.36 Hz, 1H), 3.1 (s, 3H), 0.0 (s, with Sn satellites, 9H). ^{13}C-NMR (d_6-DMSO): δ 166.9 (C=O), 165.1(C=O), 153.4, 141.9, 141.5, 138.8, 136.8, 131.3, 129.8, 128.7, 128.4, 127.9, 127.6, 127.3, 123.7, 121.4, 122.2, 116.8, 67.1 (NCH), 34.01 (NCH_3), −10.2 ($Sn(CH_3)_3$). MS (EIS) calcld for $C_{26}H_{29}N_4O_2Sn$, 549.13 (M+1); found, 549.49.

Radio-iodination of benzodiazepines 7 and 9—Twenty μg of the precursor compound (**6** or **8**) was solubilized in 20 μL acetonitrile, 20 μL methanol, 20 μL dimethyl sulfoxide and 40 μL H_2O, and then diluted with 100 μL 0.1% trifluoroacetic acid. Radio-iodination was performed by incubating the above solution with the solid-phase oxidant, Iodo-beads (Pierce Chemical Co.), and 1 mCi $Na^{125}I$ for 15 seconds. The ^{125}I-labeled compounds **7** and **9** were purified by reversed-phase HPLC on an octadecylsilane column (Chrom Tech., Vydac C18, Catalog# 218TP54) to yield specific radioactivity of approximately 2000 Ci/mmol. The solvents were 0.1 % trifluoroacetic acid and acetonitrile, utilizing a flow rate of 1 ml/min and a gradient from 10 % to 60 % acetonitrile, increasing at 1 % per min starting 10 min into the run.

Cell culture

The Chinese hamster ovary (CHO) cell lines engineered to stably express the human CCK_1 receptor and human CCK_2 receptor[38] were used as sources of receptor for characterization of receptor binding and biological activity. Cells were grown in 150 mm tissue culture plastic dishes in Ham's F-12 medium supplemented with 5% Fetal Clone 2 (Hyclone Laboratories, Logan, UT) in an environment containing 5% CO_2 at 37°C. Cells were harvested for studies using non-enzymatic dissociation medium.

Membrane preparation

Particulate preparations enriched in plasma membranes were prepared from the CHO cell lines described above using the sucrose density gradient centrifugation method reported previously.[37] Membranes were suspended and homogenized in Krebs-Ringer-HEPES (KRH) medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 2 mM $CaCl_2$, and 1.2 mM $MgSO_4$) supplemented with 1 mM phenylmethylsulfonyl fluoride and 0.01% soybean trypsin inhibitor. Aliquots of membranes were stored at −80°C until use.

Receptor binding assays in membrane preparations

Saturation binding of compound **9** to CCK_1 receptor and compound **7** to CCK_2 receptor were done by incubating membranes (containing 5–10 μg of protein) from the receptor-bearing CHO cell lines with increasing amounts of compound **9** or compound **7** in KRH medium containing 0.2% bovine serum albumin and 0.01% soybean trypsin inhibitor at room temperature for 1 h. Non-specific binding was measured by analogous incubations containing a saturating concentration of the corresponding cold ligand (1 μM). Rapid separation of bound from free radioligand was performed with a Skatron cell harvester (Molecular Devices, Sunnyvale, CA), using receptor-binding filtermats. Bound radioactivity was quantified with a γ-spectrometer. Data were plotted using Prism 3.0. B_{max} and K_d were calculated using the LIGAND program of Munson and Rodbard.[39]

The specificity of binding of these agents to CCK_1 and CCK_2 receptors was also assessed with membranes from the receptor-bearing CHO cell lines in standard competition-binding assays. Compounds **7** and **9** were used as radioligands. Membranes containing 5–10 μg of protein were incubated with 10 pM radioligand (about 20,000 cpm) and increasing concentrations of non-radioactive ligands (0–1 μM) in KRH medium containing 0.2% bovine serum albumin and

0.01% soybean trypsin inhibitor for 1 h at room temperature to achieve steady state. Rapid separation of bound from free radioligand was performed with a Skatron cell harvester (Molecular Devices, Sunnyvale, CA), using receptor-binding filtermats. Bound radioactivity was quantified with a γ-spectrometer. Non-saturable binding was determined in the presence of 1 μM cold compound **5** for CCK_1 receptor or 1 μM cold compound **3** for CCK_2 receptor, and represented less than 15% of total binding in each case. Data were graphed using Prism version 3.0 (GraphPad Software, San Diego, CA) and were analyzed using the nonlinear least-square curve-fitting LIGAND program.[39] All assays were performed in duplicate or triplicate in at least three independent experiments.

Intracellular calcium mobilization

The effects of compounds **3** and **5** on CCK-stimulated intracellular calcium responses in receptor-bearing cells were investigated utilizing the well-established intracellular calcium assay.[37] CHO cells stably expressing human CCK_1 receptor or human CCK_2 receptor were detached from the dishes using non-enzymatic cell dissociation solution and washed with Ham's F-12 medium. The cells were re-suspended in serum-free Ham's F12 medium and incubated with 5 μM Fura-2 acetoxymethyl ester for 30 min at 37 °C. For each measurement, approximately 2 million cells were stimulated at 37 °C, and the fluorescence intensities were measured in a Fluoromax-3 spectrofluorometer (SPEX Industries, Edison, NJ) using a multigroup spectral acquisition profile. Emission data were collected over 300 s with 5 s intervals at an integration rate of 0.05 nm/s. Calcium responses were determined after excitation at 340 and 380 nm, with emission collected at 520 nm. The potential agonist effect was evaluated by stimulating the cells with compounds **3** and **5**, while the potential modulator effect was measured by pre-incubating the cells with increasing concentrations of **3** or **5** for 100 s before stimulating the cells with CCK. The data from three independent experiments were analyzed and plotted using the Prism 3.0 Program.

Receptor internalization studies

Receptor internalization studies were performed using CHO cells stably expressing YFP-tagged CCK receptor, as described earlier.[27] CHO-CCK-YFP cells grown on UV sterilized coverslips for at least 48 h were washed twice with phosphate buffered saline (PBS), pH 7.4, containing 0.08 mM $CaCl_2$ and 0.1 mM $MgCl_2$. The cells were then incubated with 100 nM of ligand as indicated (CCK-8 or compound **3** or compound **5**) at 4 °C for 90 min in PBS containing 0.08 mM $CaCl_2$ and 0.1 mM $MgCl_2$. After incubation, the cells were then washed with ice-cold PBS and incubated further with pre-warmed PBS at 37 °C for varying periods of time as indicated. At each time point, the cells were fixed with 2% paraformaldehyde, washed twice with PBS, and then mounted on slides using Vectashield (Vector Laboratories, Burlingame, CA). The cells surface and internal fluorescence was observed using a Zeiss LSM510 (Thornwood, NY) confocal microscope (pinhole diameter of 223 μm with a plan-apochromat 63 × 1.3 numerical aperture oil objective) configured to capture YFP emission (excitation at 488 nm by argon laser and emission through a 505 nm long pass filter). Background-subtracted images were assembled using Adobe Photoshop 7.0 (Adobe Systems, Mountain view, CA). Receptor on the cell surface as a percentage of cellular complement of receptor was quantified using Metamorph 6.32 (Molecular Devices, Sunnyvale, CA).

In vitro receptor autoradiography for CCK_1 or CCK_2 binding sites in human tissues

For *in vitro* receptor autoradiography, fresh frozen tissue samples obtained from surgical resection specimens were used. GIST, medullary thyroid carcinomas, leiomyomas, meningiomas, and gallbladders were investigated. The tissues originated either from samples investigated previously for peptide receptors collected in accordance with the required international ethical guidelines[14, 40–42] or from samples collected prospectively at the

Institute of Pathology of the University of Berne in agreement with international guidelines, including informed consent and approval by the Institutional Review Board.

In vitro receptor autoradiography to assess the expression of CCK_1 or CCK_2 receptor binding sites in human tissues was performed as described previously.[40] Briefly, 20μm-thick tissue sections mounted on glass slides were incubated with 45 pM of the radioligand ^{125}I-D-Tyr-Gly-[(Nle28,31)CCK-26–33] (^{125}I-CCK; specific activity: 2,000 Ci/mmol; Anawa, Wangen, Switzerland) either alone or in competition with 50 nM cold sulfated CCK-8 (Bachem, Bubendorf, Switzerland) or 50 nM cold gastrin (Bachem). Thus, CCK_2 receptors can be distinguished from CCK_1 receptors, as the former show a high affinity for both CCK-8 and gastrin, whereas the latter bind only CCK-8 with high affinity. The slides were then exposed to a radiation-sensitive film. The density of the CCK_2 receptor-specific signal on the film was quantified using a computer-assisted image processing system (Interfocus, Mering, Germany) and radioactive tissue standards (Autoradiographic [^{14}C] microscales, GE Healthcare, Little Chalfont, UK) containing known amounts of isotope, cross-calibrated to tissue-equivalent ligand concentrations.[43, 44]

In vitro receptor autoradiography for compound 7 and compound 9 binding sites

For autoradiography studies, the precursor compounds **6** and **8** were ^{125}I-radioiodinated with the chloramine T method and purified by reversed-phase (C-18) HPLC (Anawa, Wangen, Switzerland). Specific activity was 2,000 Ci/mmol. Receptor autoradiography was performed as described above for ^{125}I-CCK. Displacement experiments were performed with increasing concentrations of cold compounds **3**, **5**, **10**, gastrin, and CCK.

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Abbreviations

CHO
: Chinese hamster ovary cell line

CCK
: cholecystokinin

GIST
: gastrointestinal stromal tumors

KRH
: Krebs-Ringer's-HEPES medium

MTC
: medullary thyroid carcinoma

PBS
: phosphate-buffered saline

YFP
: yellow fluorescent protein

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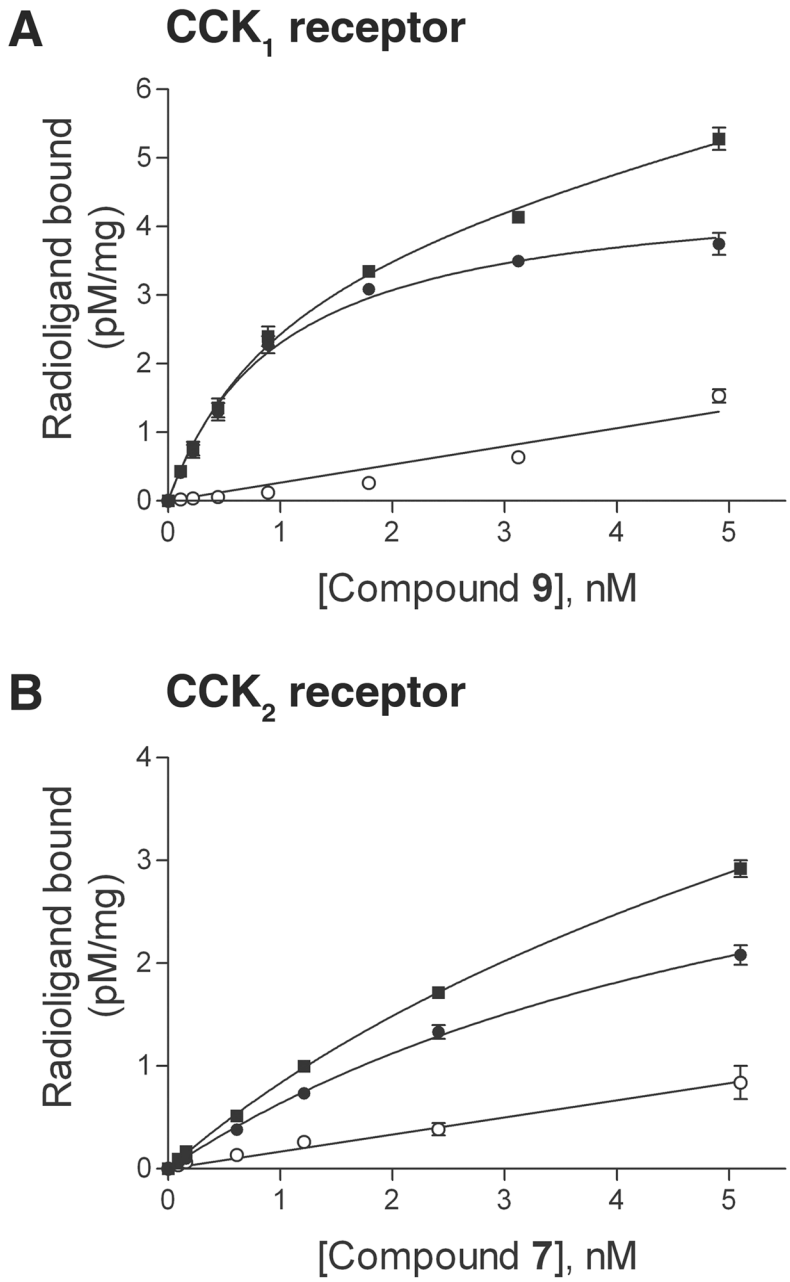


Figure 1.
Saturation binding of compound **9** at CCK_1 receptors and of compound **7** at CCK_2 receptors in receptor-bearing membrane preparations (■ total, ● saturable, ○ non-saturable). Results reflect means ± S.E.M. of data from 3 independent experiments.

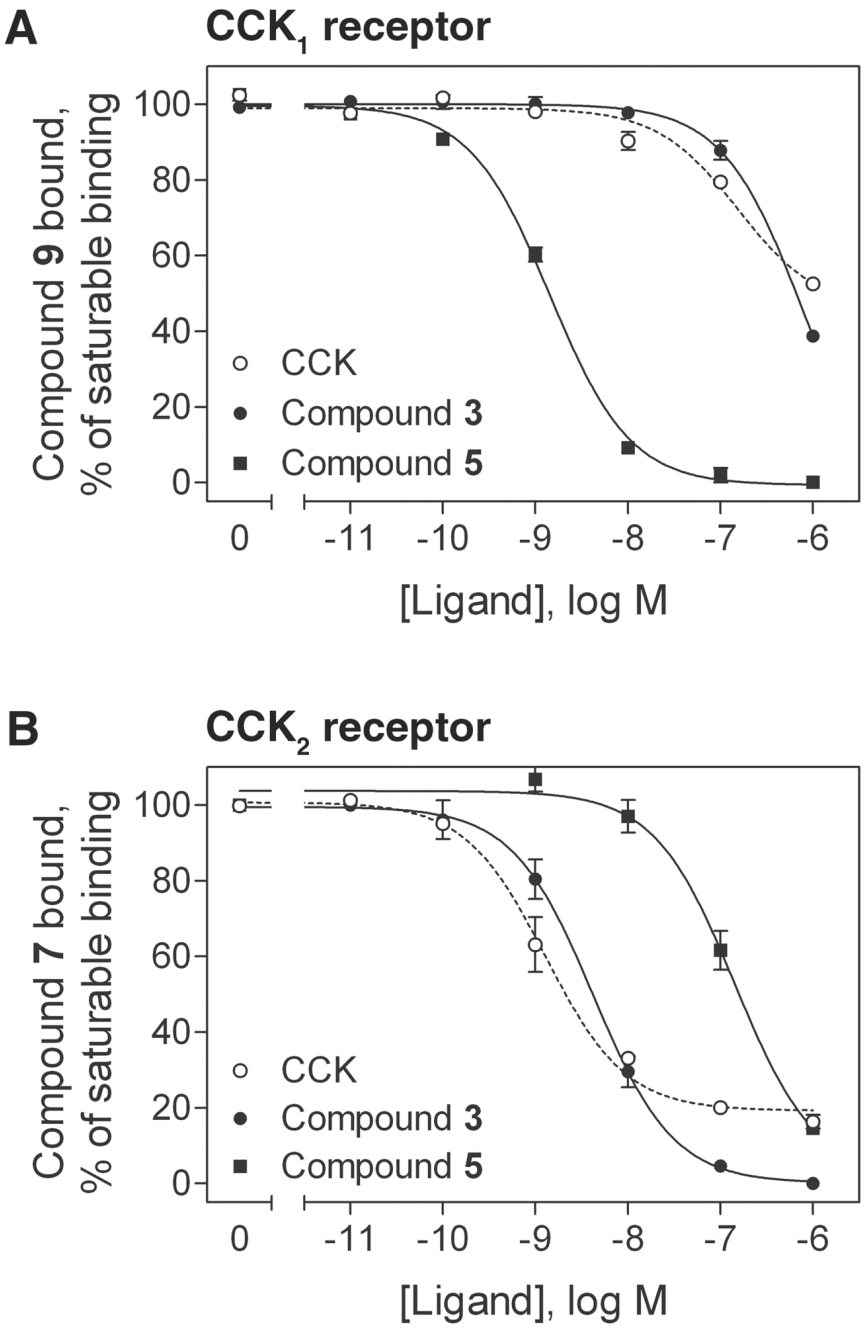


Figure 2.

Competition-binding curves for compound **9** at CCK_1 receptors (A) and for compound **7** at CCK_2 receptors (B) in receptor-bearing membrane preparations. Results reflect means ± S.E.M. of data from 3 independent experiments.

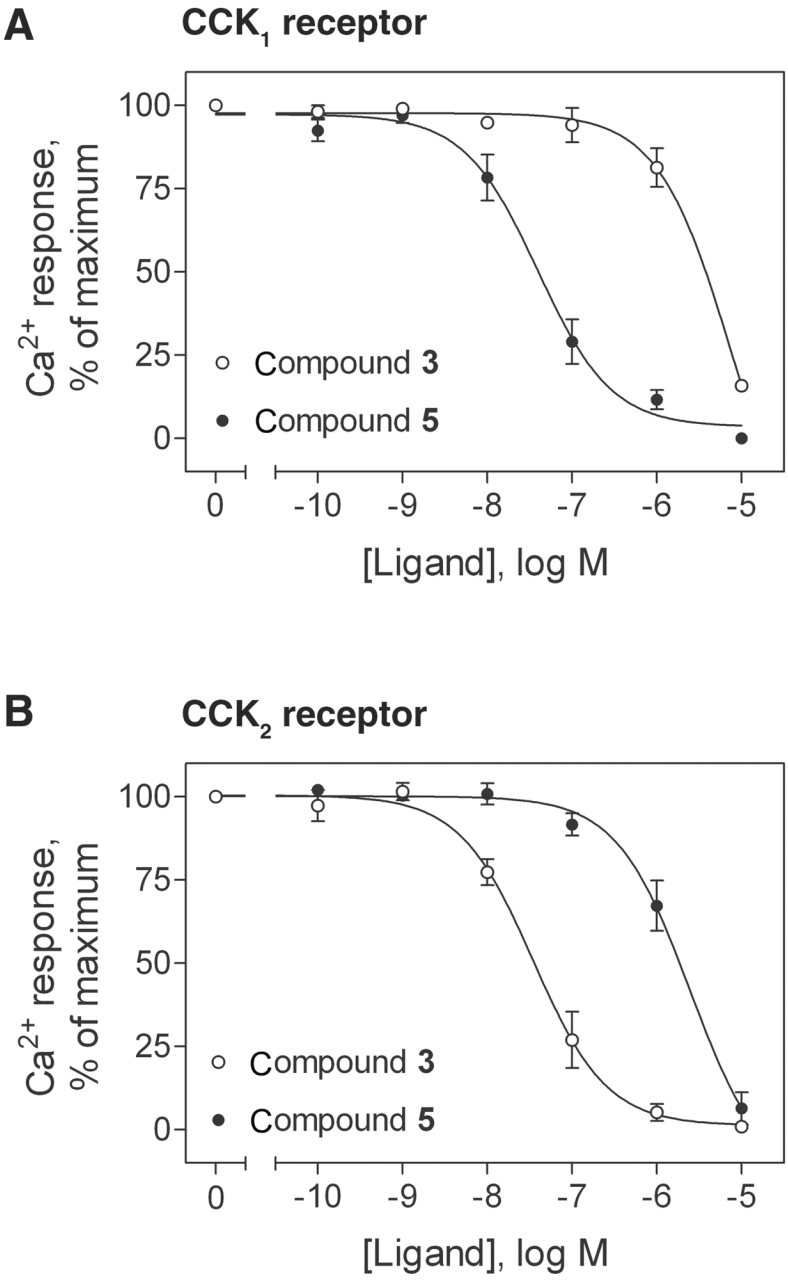


Figure 3.

Effects of compounds **3** and **5** on the biological activity of CCK receptors. CHO Cells stably expressing CCK_1 receptors (A) or CCK_2 receptors (B) were pre-incubated with increasing concentrations of compound **3** or **5** before stimulation by CCK-8 (final concentration 10^{-9} M). Results reflect means ± S.E.M. of data from 3 independent experiments.

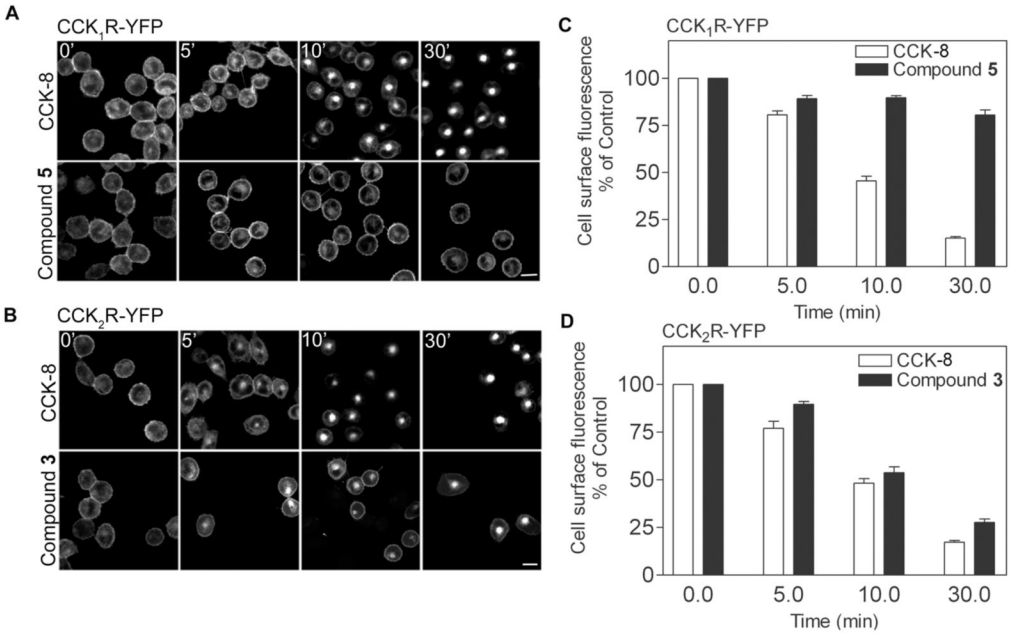


Figure 4.

Ligand-induced CCK receptor internalization. A, B: Representative confocal microscopic images are shown, demonstrating internalization of YFP-tagged CCK_1 receptors (A) and YFP-tagged CCK_2 receptors (B) expressed on CHO cells after their occupation with non-fluorescent ligands. Time points after stimulation are as noted. Scale bars = 25 μm. Compound **3** induced internalization of CCK_2 receptors like CCK-8 peptide. Conversely, CCK_1 receptor internalization was not stimulated by compound **5** binding, but by CCK-8 binding. C, D: Quantification of CCK receptors on the cell surface under these conditions. Results reflect means ± S.E.M. of data from 3 independent experiments.

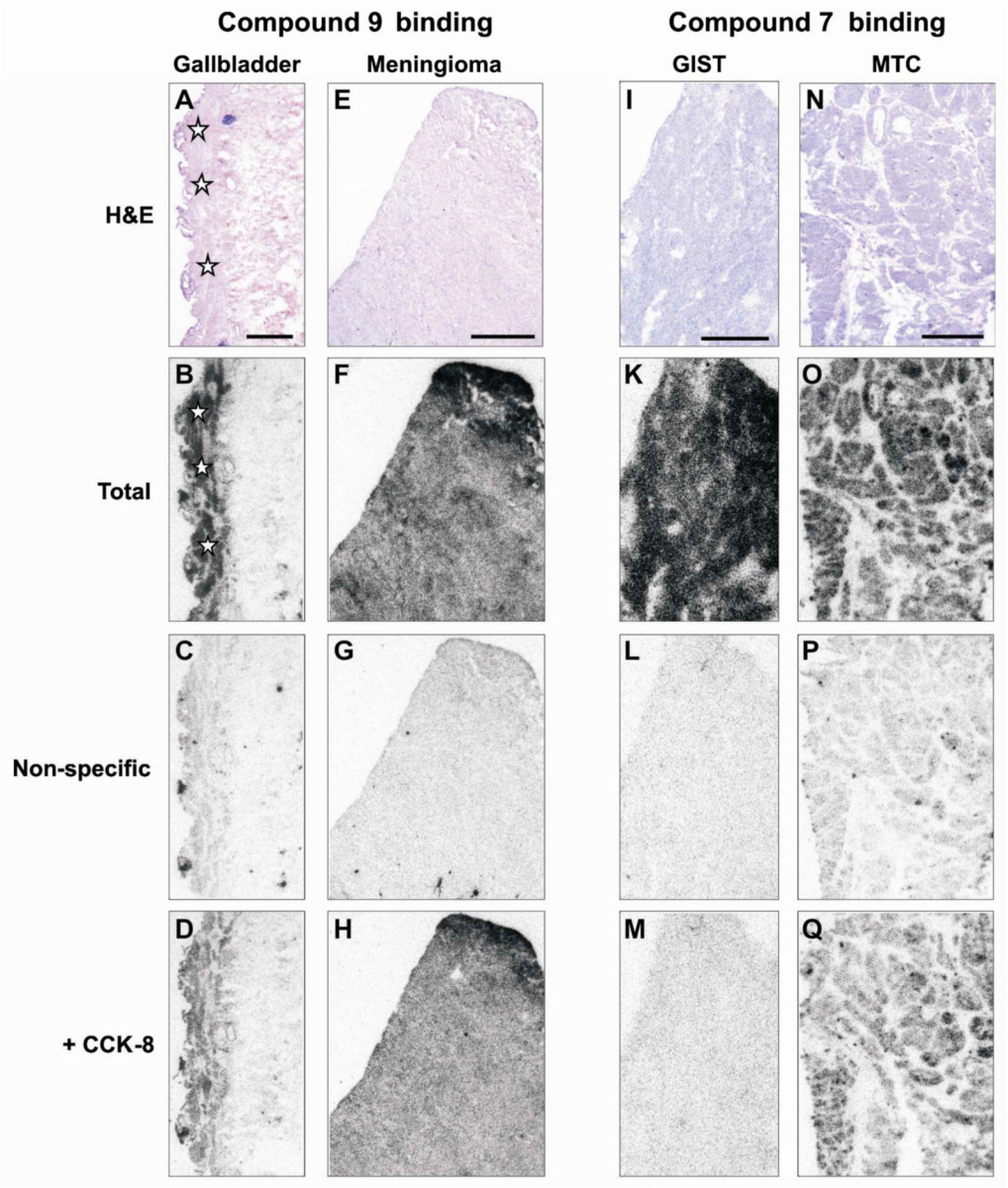


Figure 5.

In vitro receptor autoradiography on CCK_1 and CCK_2 receptor-expressing human tissues using compound **9** (columns 1 and 2) and compound **7** (columns 3 and 4) as radioligands. A, E, I, N: H&E-stained tissue sections showing a gallbladder wall (A) with the muscular layer (asterisks), a meninigoma (E), a GIST (I), and a medullary thyroid carcinoma (MTC; N). Bars = 1 mm. B, F, K, O: Autoradiograms showing total binding of compounds **9** and **7**. Strong binding of compound **9** to CCK_1 receptor-expressing tissues, i.e. the muscular layer of the gallbladder wall and the meningioma tissue (B, F). Likewise, strong binding of compound **7** to CCK_2 receptor-expressing tumor tissues (K, O). C, G, L, P: Autoradiograms showing non-specific compound **9** and compound **7** binding in the presence of cold compound **5** and cold compound **3**, respectively. The cold compounds displace the radioligands completely in all cases. D, H, M, Q: Autoradiograms showing compound **9** and compound **7** binding in presence of cold CCK-8. In both CCK_1 receptor-expressing tissues, there is incomplete displacement of compound **9** binding (D, H). In the CCK_2 receptor-expressing GIST, CCK-8 largely

displaces compound **7** (M), whereas displacement is incomplete in the medullary thyroid carcinoma (Q).

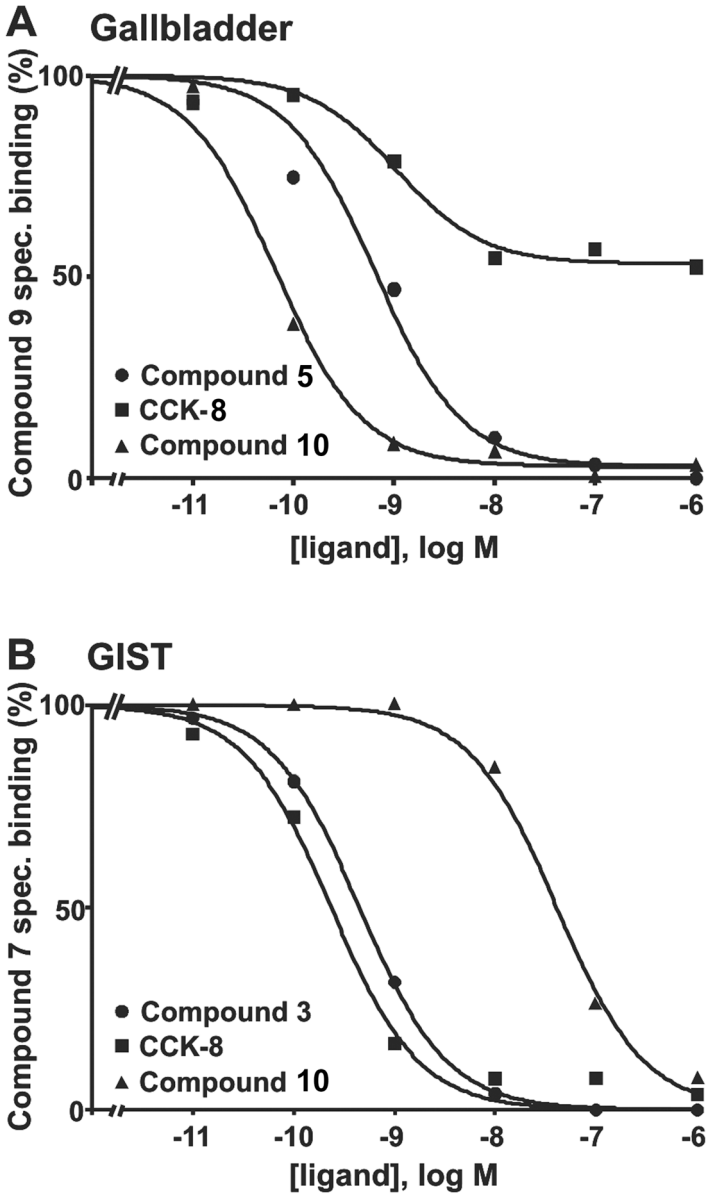
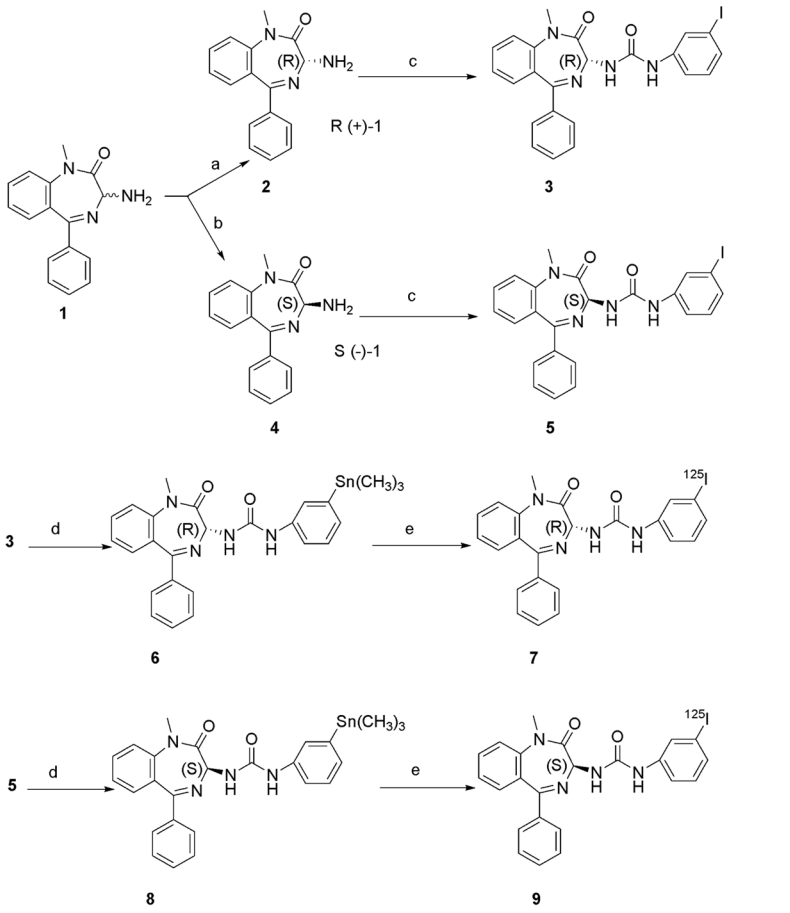


Figure 6.

Competition-binding experiments in a CCK_1 receptor-expressing gallbladder (A) and a CCK_2 receptor-expressing GIST (B). A: In the CCK_1 receptor-expressing gallbladder, compound **9** binding is displaced completely and with high affinity by the corresponding cold ligand compound **5** and by the CCK_1 receptor-selective benzodiazepine antagonist, compound **10**. CCK-8, however, displaces compound **9** incompletely. B: In the CCK_2 receptor-expressing GIST, compound **7** binding is displaced completely and with high affinity by compound **3** and by the natural CCK receptor ligand, CCK-8, but with low affinity by compound **10**.



a) i. (1S)-(+)-10-camphorsulfonic acid, CH_3CN/Et_2O; ii. 10% NaOH, CH_2Cl_2. b) Mother liquer from first step taken. i. (1R)-(-)-10-camphorsulfonic acid, CH_3CN/Et_2O, ii. NaOH, CH_2Cl_2. c) 3-iodophenyl isocyanate, in CH_2Cl_2 and r.t.; d) $(CH_3)_3Sn\text{-}Sn(CH_3)_3$, cat [Dichlorobis(triphenylphosphine)palladium (II)], 1,4-dioxane, 60 °C. e) $Na^{125}I$, Iodo-beads, 15 second.

Scheme 1.

Table 1

Binding characteristics of the CCK_1 receptor-selective radioligand **9** and the CCK_2 receptor-selective radioligand **7** in competition-binding assays with their ^{127}I-labeled nonradioactive homologs **5** and **3** (means ± S.E.M.).

	B_{max} (pmol/mg of protein)	pK_i	
		9	**7**
CCK_1 receptor	4.26 ± 0.45	9.00 ± 0.04	6.27 ± 0.02
CCK_2 receptor	6.24 ± 0.86	6.76 ± 0.14	8.43 ± 0.08

Table 2

Comparative quantitative *in vitro* receptor autoradiography in human tissues with ^{125}I-CCK, compound **9**, and compound **7** (binding density in dpm/mg tissue)

Tissue type and case number	^{125}I-CCK binding		Compound 9 binding	Compound 7 binding
	CCK_1 receptor	CCK_2 receptor		
GIST				
No. 1	939	10581	961	8684
No. 2	390	11811	1663	5627
No. 3	195	9986	1136	635
No. 4	7637	0	7597	0
No. 5	6882	5301	6945	2276
Medullary thyroid carcinomas				
No. 6	0	10919	2931	3358
No. 7	0	3936	1781	1295
No. 8	0	7027	829	1297
No. 9	0	9563	0	1120
No. 10	0	1730	0	602
No. 11	0	5164	0	1714
Meningiomas				
No. 12	2503	0	4499	0
No. 13	3226	0	12691	0
No. 14	1418	0	3148	0
No. 15	2323	0	5925	0
No. 16	8066	0	12739	nt[a]
No. 17	3771	0	4497	nt
Gallbladder (muscle)				
No. 18	8716	0	7688	1268
No. 19	6016	0	5983	970
No. 20	6190	0	6794	1225
No. 21	7416	0	7591	1457
No. 22	7533	0	8125	1363

[a] nt = not tested