C-Terminal Heptapeptide of Gastrin Inhibits Astrocytomas Motility by Interacting with a New Gastrin Binding Site

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ABSTRACT

It is well known that the amidated C-terminal part of gastrin is crucial for its interaction with the classical seven transmembrane domain receptors CCK-1 or CCK-2. Nevertheless, over the past 10 years, several groups have characterized new binding sites using peptides related to gastrin (particularly glycine-extended forms of gastrin) on various tumoral and nontumoral cell lines. In the present study, we focused on the human astrocytic tumoral cell line U373. Although it has been described that gastrin was able to inhibit the motility of these cells, we were unable to detect any classical CCK/gastrin receptor.

On the other hand, by using the radiolabeled C-terminal heptapeptide of gastrin (125I-G-7), we evidenced a new binding site that possessed a pharmacological profile different from the classical CCK/gastrin receptors. This new gastrin binding site seemed to be coupled to G proteins and be implicated in c-Fos transcription gene. Moreover, we showed that G-7 was able to induce a strong inhibition of U373 cell migration, a crucial biological effect when we know that astrocytoma cells’ migration in brain parenchyma constitutes a major feature of malignancy in astrocytic tumors.

Gastrin and cholecystokinin (CCK), two members of a family of amidated peptide hormones characterized by an identical carboxyl-terminal pentapeptide sequence (-Gly-Trp-Met-Asp-Phe-NH2), are widely distributed throughout the central nervous system and the digestive tract (Dockray et al., 1989). On the basis of ligand binding and molecular biology studies, the receptors for the gastrin/CCK peptides family have been identified as belonging to two main classes, namely, the CCK-1 (CCK-A) and CCK-2 (CCK-B/gastrin) receptors (Moran et al., 1986; Jensen et al., 1989, 1994). These receptors have been cloned from brain, stomach, and pancreatic cells (Wank et al., 1992; Ito et al., 1993; Lee et al., 1993) as well as from blood cells (Oiry et al., 1997). Despite sharing 50% sequence identity, CCK-1 and CCK-2 receptors can be clearly identified by using a number of selective CCK agonists and antagonists (Noble et al., 1999). Both CCK-1 and CCK-2 receptors are members of the seven transmembrane G protein-coupled receptors. CCK-1 receptors predominate in the periphery and mediate actions such as pancreatic enzyme secretion, gall bladder contraction, and gut motility.

CCK-2 receptors are predominant in the central nervous system (Shulkes and Baldwin, 1997). These receptors are involved in a number of psychiatric disorders, including panic attacks (Bradwejn et al., 1990), anxiety, and perception of pain (Hughes et al., 1990). CCK and gastrin also regulate the growth of normal tissues as well as of gastrointestinal cancers (Townsend et al., 1986). Although CCK-2 receptors are frequently involved in medullary thyroid carcinomas, small cell lung cancers, astrocytomas, and stromal ovarian cancers, CCK-1 receptors are only rarely found in gastroenteropancreatic tumors, meningiomas, and some neuroblastomas (Reubi et al., 1997).

We recently showed that gastrin is able to significantly modulate growth and migration levels in various human glioblastoma cells (Camby et al., 1996; De Hauwer et al., 1998). Using conventional CCK-1 and CCK-2 receptors antagonists, we observed that the gastrin-mediated response in the case of human glioblastoma cell growth relates to CCK-2 rather than to CCK-1 receptors, whereas complete inhibition by specific gastrin antagonists of gastrin-induced modifications growth level was not achieved (Camby et al., 1996). These features suggested the action of another type of gastrin receptor and/or binding protein. Our findings (Camby et al., 1996) were later confirmed by the fact that astrocytoma cells...
associated with the highest level of malignancy, e.g., glioblastomas, do not express CCK-2 receptors, whereas astrocytes and astrocytoma cells associated with low levels of malignancy do exhibit CCK-2 receptors (Reubi et al., 1997).

The aim of the present work was to identify the type of receptor able to mediate gastrin-induced biological effects in the human glioblastoma cell line U373, which do not express CCK-2 or CCK-1 receptor. We succeeded in identifying a specific binding site for the C-terminal heptapeptide of gastrin (G-7) implicated in the inhibition of U373 cell migration.

Materials and Methods

**Drugs.** Tris, MgCl₂, sucrose, KCl, KH₂PO₄, NaCl, NaHPO₄, NaOH, bacitracine, NaF, AlCl₃, LiCl, guanosine-5'-O-(3-thiotriphosphate), HEPES, phosphor-12-mristate-13-acetate (PMA), CaCl₂, MgSO₄, EGTA, EDTA, glucose, 3-isobutyl-1-methylxanthine, trichloroacetic acid (TCA), CaCO₃, ammonium formate, and DNA ladder products were provided by SDS (Peypin, France). Columns and Dowex UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Ethanol and HCl scintillation counting were purchased from Amersham Biosciences (Souffleweyersheim, France).

**Radioiodination of Peptides.** Bolton-Hunter reagent as described previously (Galleyrand et al., 1994). G-7 was radioiodinated by the chloramine T method. Radioiodination was performed at room temperature for a period of 1 to 1.5 min. G-7 was dissolved in 0.5 M phosphate-buffered saline, pH 7.5, at a concentration of 10 µg/20 µl. Ten microliters of ¹²⁵I-Nal (1 mCi) was added to the peptide solution (20 µl) and mixed. The reaction was initiated by adding 10 µl of a freshly prepared chloramine T solution at a concentration of 10 mg/ml (in 0.5 M phosphate buffer, pH 7.5). After 1 to 1.5 min, the radioiodination was stopped by adding 100 µl of a freshly prepared Na₂S₂O₅ solution at a concentration of 2 mg/ml in 0.5 M phosphate buffer, pH 7.5. Unreacted radioiodine and unlabeled peptide were separated from labeled peptide by reverse phase chromatography on a C₁₈ column. The fraction (1 ml) containing the radiolabeled peptide was divided into aliquots of 5 to 50 µl (about 1–3 × 10⁶ cpm) and stored at −20°C. The efficiency of the radioiodination reached 90% for iodine incorporation.

**Analysis of CCK Receptors Expression by RT-PCR Experiments.** Total RNA was extracted from each of the five tested human astrocytic tumor cell lines (U373, U87, SW 1088, HS683, and A172), from human Jurkat T cells, and from rat pancreatic acini by using the SV Total RNA isolation System. The Access RT-PCR System was designed for the reverse transcription, and the PCR amplification of a specific target RNA from total RNA was performed as described by the manufacturer. Total RNA (500 ng) was used for each RT-PCR experiment. Ten picomoles of upstream and downstream primer was added to the reaction mixture for the PCR amplification of the cDNA. The reaction mixture was subjected to 35 cycles of DNA polymerization (68°C, 2 min), denaturation (94°C, 30 s), and primer annealing (58°C, 1 min) in a thermal cycler (MJ Research, Watertown, MA).

Primers were designed on the basis of published cDNA sequences of rat CCK-1 and human CCK-2 receptor genes (Lee et al., 1993; Takata et al., 1995). Rat CCK-1 and human CCK-2 receptor fragments were generated using two different sets of primers: for the CCK-1 receptor, 5'-CTGCTACGCTGTCGGAAC-3' (sense) and 5'-GGAGACTCTAAGGCTTGGCAAAAT-3' (antisense); and for the CCK-2 receptor, 5'-CTACCTAGGGTCTCTTGGACG-3' (sense) and 5'-TCAGCCAGGCCCAGTGTGCT-3'(antisense). The PCR products were analyzed by electrophoresis on 2% agarose gels.

**Binding Studies.** The day before the experiment, cells were seeded in 24-multiwell culture plates (200,000 cells/ml) and incubated in MEM supplemented with 5% FCS, 0.6 mg/ml glutamine, 200 IU/ml penicillin, and 200 mg/ml streptomycin. Before the binding experiment, cells were washed twice with PBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 142 mM NaCl, and 10 mM NaH₂PO₄, pH 6.95) containing BSA (1 g/l). The binding experiments were performed at a final volume of 500 µl of MEM containing BSA (1 g/l). Kinetics experiments were performed at the indicated temperatures in presence of 140 pM ¹²⁵I-G-7. For displacement experiments, cells were incubated at 10°C for 40 min in the presence of ¹²⁵I-G-7 (140 pM) with or without various concentrations of unlabeled peptides or antagonists. Saturation experiments were performed in the same conditions in the presence of various concentrations of ¹²⁵I-G-7. Nonspecific binding was determined in the presence of 10 μM G-7. Incubation was terminated by washing the cells twice with PBS supplemented with BSA (20 g/l). Cells were then solubilized in 1 ml of 1 M NaOH. Samples were removed from the plates, placed in tubes, and the associated radioactivity was determined. Incubations were performed in duplicate, and the mean values were used for calculations.

For binding experiments performed in the presence of aluminum tetrafluoride (AlF₄⁻), the cells were preincubated with or without a combination of NaF (30 mM) and AlCl₃ (10 µM) for 30 min at 37°C. Negative control was carried out using a combination of 30 mM NaCl and 10 µM AlCl₃ in the same conditions. Cells were then incubated at 10°C for 40 min in the presence of ¹²⁵I-G-7 (140 pM) with or without 10 µM G-7 for nonspecific binding.

The binding of other radiolabeled ligands [¹²⁵I-BH-CCK-4 (70 pM), ¹²⁵I-BH-CCK-8 (35 pM), and ¹²⁵I-BH-[Leu]₁[gly]₃ (5–17) (50 pM)] has been tested under the same experimental conditions (40 min, 10°C). Nonspecific binding was determined by using 10 µM of each corresponding unlabeled peptide.

**Measurement of Inositol Phosphates Production.** Intracellular inositol phosphates production was determined as described by Qian et al. (1993). U373 cells (100,000 cells/ml) were plated in 24-multiwell culture plates in MEM maintenance medium supplemented with 5% FCS (v/v) and allowed to attach overnight. Then cells were washed with 1 ml of the same medium containing antibiotics (200 IU/ml penicillin and 200 µg/ml streptomycin) and cultured for 16 h in 1 ml of the same medium containing 2.5 µCi of myo-[²⁵]H]inositol (16–20 Ci/mmol). The cells were washed in 199 medium + antibiotics and incubated (20–30 min, 37°C) in the same medium containing 20 mM LiCl. Loaded cells were then washed with 1 ml of IP buffer (135 mM NaCl, 20 mM HEPES, 2 mM CaCl₂, 1.2 mM MgSO₄, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose, and 0.5% BSA, pH 7.45) and incubated with or without agonist in a final volume of 500 µl of IP buffer. After 1-h incubation at 37°C, the reaction was stopped by removing the incubation medium and adding 1 ml of a mixture of ethanol/HCl (2000:1, v/v). One milliliter of each aliquot was applied to a column containing 1 ml of a 1:2 (v/v) Dowex AG-1-X8
anion exchange resin in distilled water. The columns were washed with 2 × 3 ml of distilled water and 2 × 2 ml of 40 mM ammonium formate. Inositol phosphates were eluted with 2.5 ml of 1 M ammonium formate. The radioactivity of each eluate was counted after addition of 10 ml of Complete Phase Combining System solution. For the positive control, U373 cells were incubated with a combination of 30 mM NaF and 10 μM AlCl₃ under the same experimental conditions.

Measurement of Cyclic AMP. U373 cells (500,000 cells/dish) were cultured in MEM supplemented with 1% FCS, 0.6 mg/ml glutamine, 200 IU/ml penicillin, and 200 mg/ml streptomycin for 72 h in 10-cm tissue culture dishes (5 × 10⁶ cells/dish). The day before the experiment, cells were seeded in six-multiwell culture plates (100,000 cells/ml) and incubated in MEM containing 0.5 mM 3-isobutyl-1-methylxanthine, but without FCS and antibiotics. For the test, agonist was incubated for 30 min at 37°C. The reaction was stopped by adding 1 ml of 10% TCA, and neutralization was obtained by adding CaCO₃. Cells were scrapped and removed from the plates. Samples were centrifuged at 3000 rpm for 10 min, supernatants were acetylated with triethylamine/acetic anhydride (2:1, v/v), and cyclic AMP production was determined by means of a radioimmunoassay kit.

U373 Cells Proliferation Studies by [³H]Thymidine Incorporation. U373 cells (100,000 cells/ml) were plated in 24-multiwell culture plates in MEM maintenance medium supplemented with 5% FCS (v/v) and allowed to attach overnight. Then cells were cultured for 24 h in a serum-free medium supplemented with 0.2% BSA (w/v). They were also treated for 24 h with the various compounds. DNA synthesis was estimated by the measurement of [³H]thymidine incorporation into precipitable TCA material. The [³H]thymidine (0.5 μCi/well) was added during the last hour of the 24-h treatment period for a 4-h duration. To remove unincorporated [³H]thymidine, the cells were then washed twice with PBS supplemented with BSA (2 g/l). DNA was precipitated with 5% TCA (w/v) at 4°C for 30 min. The precipitates were washed twice with 95% ethanol, dissolved in 1 ml of NaOH, and analyzed in a liquid scintillation counter after neutralization with 1 ml of 1 M HCl. The incubations were performed in duplicate and the mean values were used for the calculations.

Transient Transfection. The plasmid pFos-Luc used for transfection experiments was purified according to alkaline lysis method by using the plasmid maxi kit. For reporter gene analysis, U373 cells were trypsinized and plated in 24-multiwell culture plates (200,000 cells/ml) in MEM maintenance medium supplemented with 5% FCS (v/v) and then cultured in the same medium but supplemented with 0.3% FCS. Twenty-four hours later, cells were incubated with the various tested compounds. After 8-h incubation, cells were treated as described previously for assaying the luciferase activity (Astruc et al., 1995). PMA was used as positive control. Luciferase activity was measured using a 1450 microbeta luminescence counter (PerkinElmer Wallac, Gaithersburg, MD). Incubations were performed in triplicate, and the mean values were used for the calculations. Results are expressed in arbitrary units.

Quantitative Determination of U373 Cell Migration. The motility levels of the living U373 cells were quantitatively determined by means of a computer-assisted phase-contrast microscope (Olympus, Brussels, Belgium) equipped with a charge-coupled device camera (Hitachi Densihi, Tokyo, Japan). The technical procedure has been detailed previously (De Hauwer et al., 1997, 1998). Briefly, our software enabled each U373 cell in the culture under study to be isolated automatically on the basis of specific morphological characteristics. The automatic extraction of a cell from its background was followed by a computer-assisted process that enabled the coordinates of its center of gravity (centroid) to be mathematically determined. Because this operation was performed every 4 min, the trajectory of each cell centroid could be computed by interpolation. From these trajectories was calculated the maximum relative distance to the origin (the quantitative MRDO variable) of each cell. This variable constitutes the greatest linear distance between the original and subsequent positions of a cell divided by the period of observation during tracking cell, which was at most 48 h in the present experiments. At the beginning of the experiments (t = 0 h) between 20 and 40 malignant tumor cells were present in the field of the phase-contrast microscope (100×) computed by the charge-coupled device camera. At the end of the experiments (t = 48 h), the trajectories of between 120 and 400 tumors cells have been analyzed in each experimental condition. Each experiment was carried out in triplicate, and the data obtained were pooled for the sake of clarity in Fig. 7. The influence of G-7 was assayed at three distinct concentrations, e.g., 10⁻⁹, 10⁻⁷, and 10⁻⁵ M.

Results

Analysis of CCK-1 and CCK-2 Receptor Expression on Various Human Glioma Cell Lines

We used RT-PCR experiments to investigate whether various human tumoral glioma cell lines (U373, U87, SW 1088, Hs683, and A172) expressed the classical CCK-1 or CCK-2 receptor (Fig. 1, A and B). Expression of CCK-1 receptor in rat pancreatic acini and CCK-2 receptor in human Jurkat T cell line were used as positive RT-PCR controls. The integrity of each total RNA was tested by localizing the two rRNA subunits after electrophoresis on a denaturing agarose gel before each RT-PCR experiment (data not shown). Moreover, for each total RNA preparation, we checked by RT-PCR the correct amplification of the ubiquitary GAPDH gene (Fig. 1C).

As shown in Fig. 1, we didn’t detect any amplification of CCK-1 and/or CCK-2 receptor in the different astrocytoma cell lines. The positive controls yielded the expected results for the CCK-1 and CCK-2 receptors because a 293 and 610 bp were amplified, respectively.

To confirm the absence of the classical CCK-1 or CCK-2 receptors, we chose the direct approach of measuring the specific binding of various CCK/gastrin agonists radiolabeled at the N-terminal part with a Bolton-Hunter reagent [¹²⁵I-BH-CCK-4, ¹²⁵I-BH-CCK-8, and ¹²⁵I-BH-[Leu₆₁]gastrin(5-17)] on the five cell lines described previously. These compounds are usually used to pharmacologically characterize the two classical CCK receptor subtypes (Noble et al., 1999). Our results showed that none of these cell lines bind any labeled compound (data not shown). These data confirmed those obtained by means of RT-PCR, e.g., that none of the five glioma cell lines expressed any detectable levels of CCK-1 or CCK-2 receptor subtypes.

Evidence of a New Gastrin Binding Site in U373 Cell Line

Although we didn’t detect any classical CCK/gastrin receptor in the various human astrocytoma cell lines described previously and to understand by which mechanism gastrin-17 can inhibit the motility of U373 cells (De Hauwer et al., 1997, 1998), we decided to test the binding of various modified gastrin analogs in this cell line. When [¹²⁵I]-labeled C-terminal heptapeptide of gastrin H-Ala-
Tyr(\(^{125}\text{I})\)-Gly-Trp-Met-Asp-Phe-NH\(_2\)] was used, a specific binding was detected. Nonspecific binding tested in the presence of 10 \(/H_9\) gastrin-7 represented about 20\% of the total binding.

**Kinetic Experiments**

The association of \(^{125}\text{I}-\text{G}-7\) was first tested at 37 °C, the temperature used by De Hauwer et al. (1997, 1998) for migration experiments. Our results showed a transient binding with a maximal binding around 15 min. At 60 min, no more specific binding was detected (Fig. 2A). We further explained this result by a degradation of the radioligand at 37 °C in the presence of the cells (Fig. 2B). The association of \(^{125}\text{I}-\text{G}-7\) was also tested at lower temperatures. At 10 °C, we found that binding was time-dependent and reached a steady state with an apparent pseudo first order rate constant \(K_{\text{app}}\) of 2.5 \times 10^{-5} \text{ s}^{-1} \text{ mol}^{-1} \text{ (Fig. 2C). In these conditions, no degradation of the radioligand (Fig. 2D) was detected. To avoid a degradation of the radioligand, the subsequent binding experiments were performed at 10 °C. From the dissociation experiments, we found a \(K_{-1}\) value of 2.8 \times 10^{-4} \text{ s}^{-1} \text{ (Fig. 2E). From the \(K_{+1}\) and \(K_{-1}\) values, the \(K_d\) of \(^{125}\text{I}-\text{G}-7\) has been evaluated at 2 \times 10^{-9} \text{ M}.**

**Scatchard Analysis**

Saturation experiments showed that specific binding was saturable, whereas nonspecific binding was a linear function of the radiolabeled peptide concentration. \(^{125}\text{I}-\text{G}-7\) apparently interacted with a single class of sites (Fig. 3) characterized by the following parameters (mean ± S.D.): \(K_D = 2.5 \times 10^{-9} \text{ M and } B_{\text{max}} = 790,000 \text{ sites/cell (n = 3 independent experiments).}\)

**Pharmacological Profile of Gastrin-7 Binding Site**

The effects of several CCK/gastrin receptor agonists and antagonists on \(^{125}\text{I}-\text{G}-7\) binding were investigated. G-7, sCCK-8, G-13, G-17, the C-terminal glycine-extended G-17 (G-17-Gly-OH), L-365,260 (CCK-2 receptor antagonist), and L-364,718 (CCK-1 receptor antagonist) were tested for their
ability to inhibit specific binding of the $^{125}\text{I-G-7}$ on U373 cells. As shown in Fig. 4A, G-7 and sCCK-8 induced a dose-dependent inhibition of $^{125}\text{I-G-7}$ binding with $K_i$ values, respectively, of 0.5 ± 0.2 and 2 ± 1.3 μM (mean ± S.D. of three separate experiments, each performed in duplicate). On the other hand, our results showed that G-13, G-17, G-17-GlyOH, L-365,260, and L-364,718 were unable to inhibit $^{125}\text{I-G-7}$ binding. These results showed that $^{125}\text{I-G-7}$ seemed to interact with a specific target, which was different from the classical CCK-1 or CCK-2 receptor.

Structure-activity relationship studies were carried out. Longer (Fig. 4B) and shorter (Fig. 4C) gastrin fragments were tested for their ability to inhibit $^{125}\text{I-G-7}$ binding on U373 cells (Table 1). Our results showed that the most potent analog was the C-terminal heptapeptide of gastrin (G-7). The C-terminal octa- and nonapeptide (G-8 and G-9) still retained some affinity, as did the C-terminal hexapeptide (G-6). Moreover, neither the C-terminal decapeptide (G-10), the C-terminal pentapeptide (G-5), nor the tetrapeptide (G-4) was able to displace $^{125}\text{I-G-7}$ from its binding sites. Our results showed that G-7 corresponds to the more potent structure derived from gastrin able to bind the new target on U373 cells.

**Action of AlF$_4^-$ on $^{125}\text{I-G-7}$ Binding**

To test the putative coupling of the new target expressed in the U373 cells with G proteins, we studied $^{125}\text{I-G-7}$ binding in the presence of 30 mM NaF + 10 μM AlCl$_3$, as described by Lallement et al. (1995). As shown in Fig. 5, the cell treatment induced a 93 ± 2% inhibition of $^{125}\text{I-G-7}$ binding (mean ± S.D. of three experiments each performed in duplicate). As a control, we showed that $^{125}\text{I-G-7}$ binding was not affected when U373 cells were treated with 30 mM NaCl + 10 μM AlCl$_3$ (data not shown). We can hypothesize that the new gastrin binding site expressed in U373 cells is coupled to G proteins. Another explanation could be that the signing ini-
tiated by AlF₄⁻ acts upon the G-7 receptor and decreases its affinity for the new target.

**Binding of ¹²⁵I-G-7 on Human Astrocytes**

We have tested the ability of ¹²⁵I-G-7 to bind to two astrocytic cell lines to determine whether this new binding site was specific of tumoral model. In our experimental conditions, we showed that the two nontumoral astrocytic cell lines tested (HFA 2 and HFA 2.1) were able to bind the labeled C-terminal heptapeptide of gastrin with the same pharmacological profile than on U373 cells (data not shown).

**Role of New Gastrin Binding Site on U373 Cells**

**Effect of G-7 on Second Messengers Production.** By using sensitive techniques used previously by our group on various cellular models (Oiry et al., 1999; Poosti et al., 2000), we have tested the effect of G-7 on intracellular inositol phosphates accumulation (Fig. 6A) and cAMP production (Fig. 6B). Our results showed that 10⁻⁷ M G-7 did not induce any accumulation of inositol phosphates or cAMP. As controls, we found that NaF + AlCl₃ and forskolin, respectively, induced an accumulation of inositol phosphates and a cAMP production. Moreover, in accordance with the literature, we found that substance P was able to induce a dose-dependent liberation of IPs (8 times the basal) by interacting with the neurokinin-1 receptor, a Gq-coupled receptor endogenously expressed on U373 cells (Takeda et al., 1991; Raddatz et al., 1995; data not shown).

**Effect of G-7 on Cell Growth.** We tested the effect of various concentrations of G-7 on [³H]thymidine accumulation in U373 cells. Our results did not evidence any stimulation of growth over the basal value in the presence of G-7, whereas PMA used as a positive control was able to stimulate U373 cells growth (data not shown).

**Effect of G-7 on c-Fos Expression.** We investigated whether G-7 could influence c-Fos gene transcription. To this end, transient expression experiments were performed by transfecting the plasmid in which the c-Fos promoter drives the luciferase reporter gene. As shown in Fig. 6C, G-7 induced c-Fos gene transcription with about a 5-fold increase compared with the control. Moreover, to confirm that these G-7 effects were not mediated by the classical CCK/gastrin receptors, we have tested the effects of L-364,718 and L-365,260 on G-7 induction. These two CCK/gastrin receptor antagonists were without effect on c-Fos gene transcription induced by G-7. These results suggested that G-7 was able to induce c-Fos gene transcription and that this induction was not mediated by the classical CCK/gastrin receptors.

**Effect of G-7 on Cell Migration.** The cell migration was quantitatively determined by means of a computer-assisted phase-contrast microscope, a new technique developed by De Hauwer et al. (1997). The data illustrated in Fig. 7 clearly indicated that G-7 induced a dose-dependent decrease of U373 cell migration, with a significant response being obtained at 10⁻⁹ M.

**Discussion**

About one-third of brain tumors are diffuse astrocytic tumors associated with dismal prognoses because of their marked capacity to diffusely invade the normal brain parenchyma. Their level of malignancy increases from astrocytomas (World Health Organization/WHO grade II) to anaplastic astrocytomas (WHO grade III) and glioblastoma (WHO grade IV). The human U373 cellular model used in our experiments exhibited biological and histopathological proper-

**TABLE 1**

<table>
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<tr>
<th>Gastrin Fragments</th>
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![Fig. 5. Effect of NaF/AlCl₃ on ¹²⁵I-G-7 specific binding on the U373 astrocytic cell line. Cells were preincubated at 37°C for 30 min with or without 30 mM of NaF + 10 μM of AlCl₃, then the binding experiment was performed for 40 min at 10°C. Non-specific binding was evaluated in the presence of 10 μM G-7. Results are expressed as percentages of the specific binding and are means ± S.D. of three separate experiments, each performed in duplicate.](image-url)
ties characteristic of a malignant astroglioma. The exceptional capacity of isolated astrocytic tumor cells to diffuse from the tumor body into the normal surrounding brain parenchyma is due to two major events, namely, invasion and motility (De Hauwer et al., 1998).

Although no effective treatment exists today against malignant astrocytomas, and because we observed that gastrin is able to significantly modulate the cell growth and motility of human glioblastoma cells (Camby et al., 1996; De Hauwer et al., 1998), we believe that gastrin-based therapy could represent an interesting approach. Because we did not evidence any classical CCK/gastrin receptor type on various astrocytic tumoral cell lines, we investigated whether another gastrin-binding protein could be expressed and could explain some gastrin-induced biological activities on the human astroglioma cell line U373. In recent years, similar strategies have enabled new receptors or binding sites to be discovered. For example, Seva et al. (1994) showed that glycine-extended progastrin exerts its biological activity on the AR4-2J pancreatic tumor cell line by interacting with specific receptors differing from the classical CCK-1 or CCK-2 receptor. On the other hand, Singh et al. (1995) described on Swiss 3T3 fibroblasts the possible presence of a novel class of gastrin-prefering receptors that bind amidated and glycine-extended forms of gastrin with almost equal affinity. More recently, Litvak et al. (1999) observed that the JMV 1155 compound H-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Gly-OH, which is a C-terminal modified gastrin analog, inhibited the G-17-Gly-OH-induced growth of an in vivo transplanted human colon cancer by interacting with a specific receptor different from CCK-1 or CCK-2 receptor.

The aim of this study was to pharmacologically characterize the target that mediates the biological effects induced by gastrin on U373 astroglioma cells. We found that these cells did not bind usual CCK/gastrin agonists radiolabeled by a Bolton-Hunter reagent but were able to bind the radiolabeled C-terminal heptapeptide of gastrin (\(^{125}\text{I}-\text{gastrin-7}\)). We showed that G-7 was able to induce a dose-dependent displacement of \(^{125}\text{I}-\text{G-7}\) binding, whereas none of the CCK/gastrin receptor agonists and antagonists tested were able to inhibit \(^{125}\text{I}-\text{G-7}\) binding with affinities corresponding to a classical CCK-1 or CCK-2 pharmacological profile. These results strongly suggested that on the U373 cell line, \(^{125}\text{I}-\text{G-7}\)

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**Fig. 6.** Effect of G-7 on \(^{3}\text{H}\)inositol phosphates production (A), cAMP accumulation (B), and c-Fos gene expression (C) on the U373 astrocytic cell line. For the quantitation of IPs and cAMP, U373 cells were incubated at 37°C in the presence of \(10^{-7}\) M G-7, \(30\) mM NaF + \(10\) \(\mu\)M AlCl\(_3\) (A, IPs positive control), and \(10^{-7}\) M forskolin (B, cAMP positive control). \(^{3}\text{H}\)IPs and cAMP accumulations were determined, and results (mean ± S.D. of three separate experiments) were, respectively expressed as disintegrations per minute or picomoles per milliliter. The effect of G-7 was tested in the absence and in the presence of CCK/gastrin antagonists (L-364,718 and L-365,260) on luciferase activity resulting from the human c-Fos promoter transiently expressed in the astrocytic tumor cell line U373 (C). PMA was used as a positive control. Results (expressed in arbitrary units) are means ± S.D. of three separate experiments, each performed in triplicate. CT, control.

**Fig. 7.** Influence of G-7 on the level of migration of living U373 cells. The trajectories of the cells were calculated by means of computer-based phase-contrast videomicroscopy over a 48-h period observation. The chart details the influence induced by \(10^{-9}\), \(10^{-7}\), and \(10^{-5}\) M G-7. Data presented in Fig. 7 resulted from three independent sets of results, which were pooled. Results corresponded to means ± S.D. (CT, control in absence of G-7).
seemed to interact with a target different from the classical CCK/gastrin receptors. Likewise, G-17-Gly-OH could not inhibit 125I-G-7 binding, suggesting that the new gastrin binding site is different from the glycine-extended gastrin receptor previously described by Seva et al. (1994). By using longer and shorter gastrin analogs, structure-activity relationships enabled us to show that G-7 corresponded to both the minimum and maximum gastrin-related structure able to bind the new target on U373 cells. Moreover, we showed that 125I-G-7 did bind two nontumoral astrocytic cell lines.

From the kinetics and Scatchard experiments, the deduced affinity of 125I-G-7 on U373 cells was about 2 × 10^{-9} M, although from competition experiments, we found an affinity of 2 × 10^{-7} M for the homologous unlabeled peptide. The behavior of the labeled and the unlabeled G-7 seemed to be different on the U373 cells. This difference may account on the iodinated tyrosine, which is used as a radioligand.

After showing that G-7 interacted with a new gastrin binding site on U373 cells, we further study the putative biological effect of this gastrin fragment. Although the new binding site seemed to be coupled to G proteins, G-7 did not induce any production of inositol phosphates or cAMP. We have tested the influence of this compound upstream in the intracellular events cascade. We have tested the G-7 effect on the c-Fos promoter regulation for two major reasons. First, although it has been described that gastrin modulates the rate of growth of various glioblastoma cell lines, it is well known that the c-Fos transcription factor plays a central role in the cell motility processes (Lauffenburger and Horwitz, 1996). Our results showed that G-7 was able to induce c-Fos gene expression. Because the CCK-1 or CCK-2 receptor antagonists were without any effect on the G-7 stimulation, we conclude that the CCK-Gene expression induced by G-7 is mediated by a new gastrin site, different from the classical CCK/gastrin receptors. On the basis of the results obtained by De Hauwer et al. (1998) showing that gastrin-17 inhibits a dose-dependent manner the motility of U373 cells, we have tested the effect of G-7 on the same cell line. As described for G-7, we found that G-7 was able to significantly inhibit U373 cell migration.

Our future goal will be now to study in detail the intracellular pathways leading to activation of c-Fos gene transcription and inhibition of cell motility by G-7 on U373 cells. As described recently for the CCK-2 receptor (Rozenzweig and Walsh, 2001), we can suggest that our new G-7 binding site might be coupled to Rho via Ga12 and/or Ga13 and thereby lead to cell migration, although phosphorylation of intermediate proteins specifically involved in cell migration (Rho kinase, myosin light chain, and actin).

Although our binding results showed that G-7 did not interact with the new gastrin binding site and that U373 cells did not express the classical CCK-1 or CCK-2 receptor, our future goal will be now to understand by which mechanism G-17 can exert its biological activity. It is a possibility that G-17 is hydrolyzed, producing G-7, which is responsible to the biological activity by interacting with the new target.

In conclusion, the data presented in this study suggest the existence of a new and specific gastrin binding site in the human astrocytic tumor cell line U373, a cell line that does not express the classical CCK-1 or CCK-2 receptor. In addition, although dramatic levels of astrocytoma cell migration in the brain parenchyma constitute the major feature of malignancy in high-grade astrocytomas, the new gastrin binding site exhibits a specific affinity for the C-terminal heptapeptide of gastrin that strongly inhibits astrocytoma cell migration. All of these data suggested that G-7 could constitute a new target for the development of new therapeutic strategies against malignant astroglia.

References


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