Peptide-Linked 1,3-Dialkyl-3-acyltriazenes: Gastrin Receptor Directed Antineoplastic Alkylating Agents

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The gastrin receptor is expressed in various human cancers, such as the adenocarcinoma of the colon. The peptide hormone gastrin and the C-terminal peptides derived from it act as growth factors for these cancers. The hypothesis for the present work was to use the gastrin receptor as a target for appropriately constructed cytotoxic agents. We developed methods to link tetragastrin and pentagastrin by their N-termini to cytotoxic 1-(2-chloroethyl)-3-benzyl-3-succinoyltriazene. These compounds, CBS-4 and CBS-5, respectively, whose complete structures were determined by multinuclear NMR and mass spectrometry, competed effectively with gastrin in an assay using either guinea pig stomach fundus or the rat acinar tumor cell line AR42J as the source of the receptor. CBS-5 was cytotoxic to AR42J cells but was not toxic to A549 human lung cancer cells, which do not express the receptor.

Introduction

Colon cancer has one of the highest incidence rates for all cancers in Western countries, especially in the U.S.[1] It has been a very difficult cancer to treat by chemotherapeutic methods, and no clearly effective drugs for adenocarcinoma of the colon are available. Several reports have been published discussing the role of gastrointestinal hormones in the etiology and prognosis of gastrointestinal carcinomas.[2a–d] It was shown that normal human gastric and colonic mucosa and adenocarcinomas of the human stomach and colon contain specific gastrin-binding receptors.[3a,b] In a clinical study,[4] 38 of 67 patients suffering from primary colon cancers exhibited significant levels of the gastrin binding receptor. Gastrin has been reported to promote the growth of colonic tumors in vivo[5] and in some colon tumor cell lines.[6a–c] Further evidence for its function as a growth factor is the in vitro antiproliferative effect of gastrin antagonists, such as proglumide and benzotript, on colon carcinoma cells.[7a,b]

Other tumors arising from hormone-responsive tissues, notably breast cancer, have been treated successfully by endocrine-related procedures,[8a,b] when the tumor had been shown to have specific receptors for the hormone(s) that stimulate growth of the tissue.

One of the major problems with chemotherapeutic drugs in general are their severe side effects due to unspecific interactions with naturally highly proliferative tissue. Some attempts have been made to enhance the specificity of drugs by using hormones or antihormones as carriers of cytotoxic agents to target specific receptors which may be present in tumor tissue.[9a–c] These studies were largely focused on steroid receptors, such as estrogen or progesterone receptor. We chose to examine the potential of the gastrin receptor (GR) as a target for chemotherapeutic drugs in the treatment of gastrointestinal tumors.

Gastrin, cholecystokinin (CCK), and the amphibian skin peptide, caerulein, are members of a family of gastrointestinal peptides, which share a common carboxyl terminal pentapeptide amide and exert their effects on a particular target tissue by interacting with the same class of receptors.[10] Structure–activity studies with varying peptide sequences have been performed.[11a–d] The results indicate that the smallest peptides which exhibit GR binding affinity are the tetragastrin and pentagastrin amide. Thus, the GR-binding ability of these small peptides could be used, in principle, to target cytotoxic agents attached to the N-terminus of the peptide.

We focused our attention on 1,3-dialkyltriazenes as the cytotoxic moiety. Triazenes in general show mutagenic[12a,b] and carcinogenic[13a,b] properties. Antitumor activity of various triazenes has also been demonstrated.[14a,b] 1,3-Dialkyl- and 1,3,3-trialkyltriazenes are very sensitive to proteolytic decomposition and hydrolyze under physiological conditions with half-lives measured in seconds.[13b] However, their stability in buffer was dramatically improved by substituting 1,3-dialkyltriazenes with acyl groups;[15] half-life times in the range of 2–1000 min were observed, depending on the acyl group. Some of these acyl triazenes have been found to possess significant chemotherapeutic properties.[14a] For the design of a carrier-linked 1,3-dialkyltriazene conjugate it was therefore appropriate to use an acyl-functionalized linker between the peptide and the triazene, which would yield a drug with a reasonable stability under physiological conditions. This paper discusses our attempts to prepare peptide-linked acyltriazenes which would exhibit strong binding to GR.

Results and Discussion

We chose tetragastrin (Trp-Met-Asp-Phe-NH_2) and β-alanine-modified pentagastrin (β-Ala-Trp-Met-Asp-Phe-NH_2) for our initial studies in targeting the triazene. Since the crucial part for the recognition of gastrointestinal peptides by the receptor is the carboxyl terminal sequence Trp-Met-Asp-Phe-amide, there is a logical requirement to attach these peptides to the cytotoxic moiety by their N-terminal amino function. We

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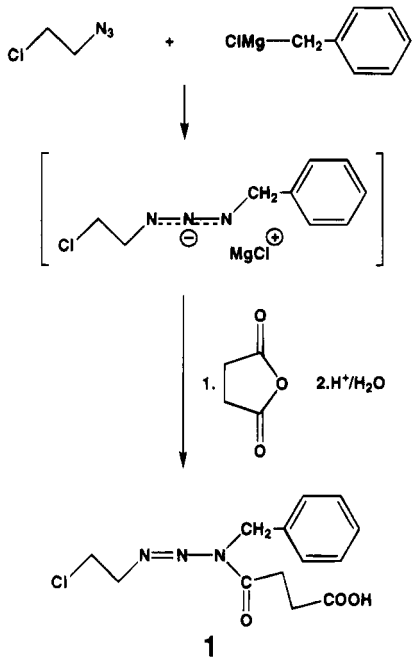
Scheme 1



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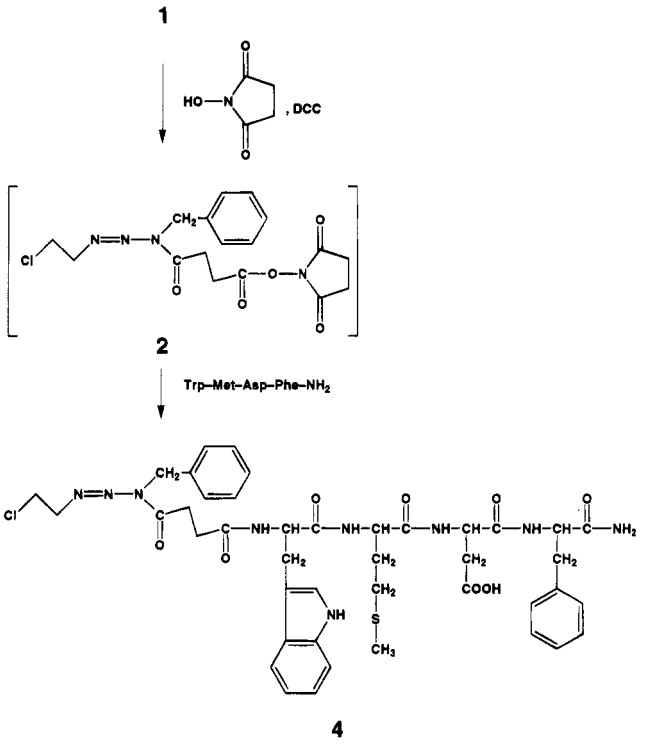
used succinic acid to link the 1,3-dialkyltriazene to the peptide. Here we describe the syntheses of *N*-[1,3-dialkyltriazene-3-ylsuccinoyl]peptides, their activity in binding to the gastrin receptor, and preliminary cytotoxicity studies in AR42J cells.

Synthesis of CBS-4 and CBS-5. The succinoyl linker was attached to the 1-(2-chloroethyl)-3-benzyltriazene by the nucleophilic attack of the 1,3-dialkyltriazene anion on succinic anhydride. Our previous work has established the triazene anion as a strong nucleophile.[15] This resulted in the formation of 1-(2-chloroethyl)-3-benzyl-3-succinoyltriazene (**1**, CBS) and simultaneously generated a new carboxyl functional group. The IUPAC nomenclature for this compound is 3-benzyl-3-(3-carboxypropanoyl)-1-(2-chloroethyl)triazene. The CBS acronym was derived from the common name which highlights the 2-chloroethyl group. The product which is isolated contains the succinoyl group on the benzyl-bearing nitrogen. The other isomer has never been isolated. We suspect that if it is formed, it decomposes rapidly in an autocatalytic reaction. The identity of the correct isomer was proved by mass spectrometry.

The EI + mass spectrum gave a prominent peak corresponding to MNa^+ at *m/z* 320. The exact mass measurement of this peak was consistent with the empirical formula. The MH^+ peak at *m/z* 298 was also observed. The base peak was *m/z* 230 ($MNa^+ - 91 + H^+$), which could have arisen from the loss of a benzyl group or the (chloroethyl)diazonium group. The absence of a peak at *m/z* 232, however, indicates that the base peak must have been produced by the loss of the chloroethyldiazonium ion (i.e., no peak for ^{37}Cl). It follows, therefore, that the structure of **1** must have the chloroethyl group at N-1 and the benzyl and succinoyl groups at N-3. The isomeric structure with both the 2-chloroethyl group and the succinoyl group on the same nitrogen is excluded by this observation. Since all of the described compounds were derived from **1**, they must all have the isomeric structures as indicated.

A slow autocatalytic decomposition of **1** was observed,

Scheme 2



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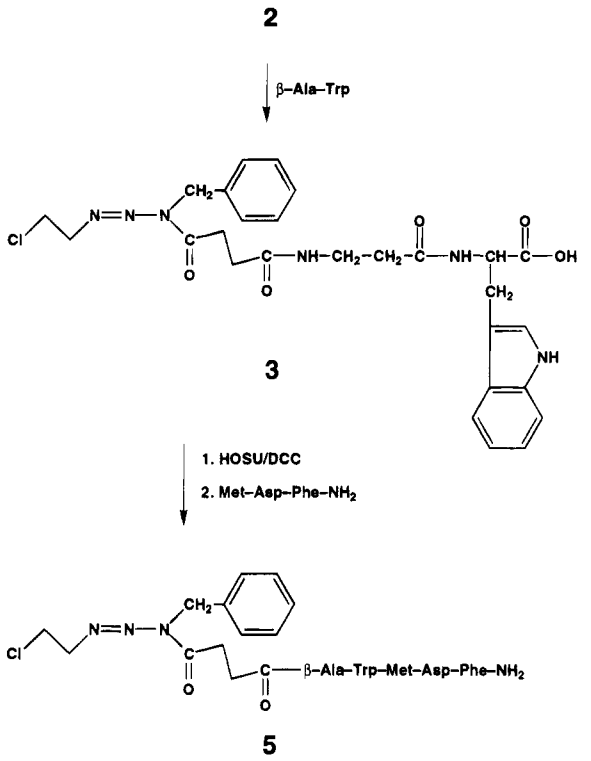
presumably due to the presence of the free carboxyl group. We have previously noted that acyltriazenes are subject to acid-catalyzed decomposition.[16] To circumvent this problem, this crucial intermediate was always freshly prepared before the next synthetic step.

In choosing the appropriate coupling method to link the sensitive triazene moiety to an amino acid or peptide, we had to focus on compatible solvent systems and techniques which required minimal side chain protection. The best results for activating **1** were obtained by forming the hydroxysuccinimide ester **2** in tetrahydrofuran (THF). Since the condensing agent, dicyclohexylcarbodiimide, partially forms the unreactive isourea derivative of **1**, **1** was used in excess relative to the more expensive peptide or amino acid. The coupling reaction was performed in acetonitrile/water, a suitable solvent system for both components, the triazene and the peptide. The tetrapeptide was directly reacted with the succinimide ester of **1** in the synthesis of CBS-Trp-Met-Asp-Phe-NH_2, (**4**, CBS-4), as shown in Scheme 2.

Due to the difficulties expected to be encountered in the removal of the carboxylic protecting group in the presence of the triazene moiety, the aspartic acid side chain on the peptide was left unprotected. In their effort toward the total synthesis of CCK, Bodanszky et al.[17] reported succinimide derivatives from aspartyl residues as major side products when unbranched amino acids such as BOC-Gly were used to acylate the tetrapeptide Trp-Met-Asp-Phe amide. To avoid this complication, the pentapeptide part was attached to **1** in a 2 + 3 addition. β-Ala-Trp was reacted with **1** to give CBS-β-Ala-Trp (**3**, CBS-2), which was linked to Met-Asp-Phe amide in the last step to produce CBS-(β-Ala-Trp-Met-Asp-Phe-NH_2) (**5**, CBS-5). The conversion of protected **1** (CBS) to **5** (CBS-5) is shown in Scheme 3.

Structural Elucidation. The proton assignments for CBS-4 and CBS-5 are listed in Table 1. The data

Scheme 3



were derived from the analysis of COSY,[18a,b] TOCSY,[19a,b] and HMQC[20] 2D NMR spectra. Higher order systems and signal overlapping complicated the determination of coupling constants. To separate these out, the spin systems were calculated and spin simulated and compared with the experimental spectra.

Significant differences between CBS-5 and CBS-4 were observed in the methionine region. Since there are no differences between the two structures in the neighboring amino acids, conformational changes seem to be the obvious cause. Pincus et al.[11] have performed conformational computational analyses on the CCK peptides active on the gastrin receptor (GR). CCK-4 (tetragastrin) tends to adopt predominantly α-helical structures, whereas the corresponding residue of CCK-7 and caerulein (CER-7) are distinctly nonhelical. CCK-7 and CER-7 are not able to inhibit the binding of CCK-4 to GR. On the other hand, little gastrin, a 17-amino acid peptide, which contains the closely related Tyr-Gly sequence, binds avidly to GR, but not to the peripheral nervous system CCK receptor. The preferred conformations of the active hexapeptide fragment from little gastrin, unlike CCK-7 and CER-7, are predominantly α-helical in the COOH-terminal tetrapeptide fragment. On the basis of these studies, it is likely that some conformational differences exist between CBS-4 and CBS-5. Unfortunately NOESY and ROESY analysis of CBS-4 revealed that the molecule was highly mobile conformationally, and it was impossible to assign a sufficient number of crosspeaks to make it possible to deduce a unique conformational orientation for the molecule in solution. This apparent conformation flexibility suggests that both CBS-4 and CBS-5 ought to be able to adopt a conformation which would allow binding to GR.

Gastrin Receptor Binding Assay. In our assay, specific binding to GR was determined by subtracting from total radioiodinated gastrin bound, the amount

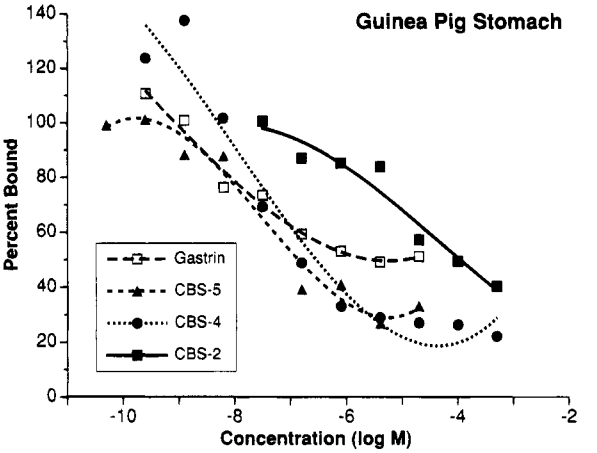


Figure 1. Competition binding of gastrin and various CBS derivatives to the gastrin receptor in the guinea pig gastric mucosa. The lines are a computer-generated best fit through the experimental points. The points are an average of three separate samples. The standard error of each point was smaller than 5%.

bound in the presence of 1000-fold excess nonradioactive gastrin. These experiments (data not shown) established that our assay conditions measured 70–80% specific gastrin binding to GR and yielded an experimental K_d of 3.2 nM for gastrin in the guinea pig gastric glands and 1.4 nM in AR42J cells. Nonradioactive gastrin caused a detectable inhibition of [^{125}I]gastrin binding to guinea pig gastric glands at the level of 1.2 nM and progressed to total inhibition at approximately 10 μM. The 50% inhibition level (EC_{50}) was about 0.6 μM. CBS-4 had an EC_{50} of 0.13 μM, and consistent with the foregoing conformational argument, CBS-5 was also a good specific inhibitor with an EC_{50} of 0.08 μM. On the other hand, CBS-β-Ala-Trp (CBS-2), the dipeptide-linked triazene precursor to CBS-5, was a much poorer inhibitor, inhibiting specific gastrin binding within the range of 1–500 μM with an experimental EC_{50} of 63 μM (see Figure 1).

Nonradioactive gastrin caused a detectable inhibition of [^{125}I]gastrin binding to AR42J cells between 1.2 nM and 100 μM, with an EC_{50} of about 1 μM. CBS-2, in turn had a much higher EC_{50} of about 200 μM. These results were consistent with the pattern we obtained using guinea pig gastric glands (Figure 2).

Colony Survival Assays. We attempted to assess the cytotoxicity of the peptide-linked triazenes in view of their ability to preferentially bind to GR on the surface of receptor-bearing cells in culture. All human colon tumor cell lines used showed undetectable levels of GR by our assay (data not shown). Although some papers[23a,b] report detectable GR in some colon or gastric tumor lines, there is considerable discrepancy in the literature as to their presence in most commercially available lines[22] as measured by competition assays. Recently, evidence for autocrine gastrin production in some colon carcinomas has been reported,[23a,b] which suggests that GR could be present in cultured cells, however saturated by gastrin and thus undetectable in competition assays.

Our results on the effects of added gastrin and CBS compounds on the AR42J line, an azaserine-induced rat acinar cell carcinoma line,[24] are described in Figure 3. Gastrin and pentagastrin enhanced colony growth in these cells, and CBS-4 and CBS-2 were not toxic even

Table 1. ^{1}H-NMR Assignments of CBS-4 and CBS-5 in MeOD (500 MHz)[a]

assignm	CBS-4, **4** δ (ppm)	mult (J, Hz)	CBS-5, **5** δ (ppm)	mult (J, Hz)
Trp-HD	7.55	t (1.0), d (7.9)	7.54	t (1.0), d (7.9)
Trp-HG	7.36	t (0.9), d (8.2)	7.31	t (0.9), d (8.1)
Trp-HA	7.23	nd	7.09	nd
Trp-HF	7.05	d (1.0), d (8.2), d (7.0)	7.07	d (1.0), d (8.2), d (7.0)
Trp-HE	7.00	d (0.9), d (7.0), d (7.9)	6.98	d (1.2), d (8.0), d (7.0)
Trp-CH	4.52	d (8.0), d (4.5)	4.56	d (5.9), d (7.5)
Trp-CH_2	3.32	d (4.5), d (14.9)	3.25	d (0.9), d (5.9), ab (14.8)
	3.26	d (8.0), d (14.9)	3.18	d (0.7), d (7.5), ab (14.8)
Phe	7.26, 7.22, 7.16	nd[b]	7.26, 7.21	nd
Bz	7.19, 7.14, 7.09	nd	7.26, 7.20	nd
			7.16	
Bzl-CH_2-N	5.11, 5.05	d (15.3)	5.09	s
=N-CH_2	4.09	d (13.8), d (5.7)	4.06	ab (5.4)
	4.04	d (13.8), d (6.0)	4.05	ab (4.9)
CH_2-Cl	3.85	d (5.7), d (6.0)	3.86	ab (4.9)
			3.85	ab (5.4)
Phe-CH	4.54	d (11.1), d (6.7)	4.52	d (4.6), d (9.8)
Phe-CH_2	3.29	d (6.7), d (13.8)	3.26	d (14.1), d (4.6)
	3.00	d (14.1), d (11.1)	2.96	d (14.1), d (9.8)
β-Ala			3.43	d (5.6), d (6.6), ab (13.7)
			3.37	d (7.8), d (5.5), ab (13.7)
			2.44	d (5.6), d (7.8), ab (14.8)
			2.38	d (6.6), d (5.5), ab (14.8)
linker	3.29	d (17.8), d (4.9), d (5.6)	3.20	d (7.8), d (6.4), ab (17.7)
	3.21	d (17.8), d (9.9), d (5.2)	3.11	d (6.2), d (6.8), ab (17.7)
	2.64	d (15.4), d (9.9), d (4.9)	2.56	d (6.2), d (7.8), ab (15.5)
	2.54	d (15.4), d (5.6), d (5.2)	2.50	d (6.8), d (6.4), ab (15.5)
Asp-CH	4.53	d (8.6), d (5.2)	4.53	d (6.8), d (7.4)
Asp-CH_2	2.65	d (5.2), d (17.0)	2.65	d (16.1), d (6.8)
	2.54	d (8.6), d (17.0)	2.55	d (16.1), d (7.4)
	4.10	d (9.6), d (2.8)	4.26	
Met-CH	2.08	d (13.5)	2.23	d (5.4), d (9.0), ab (13.3)
CH_2-S-Met	2.03	nd	2.18	d (7.0), d (8.7), ab (13.3)
Met-CH_3	1.89	s	1.96	s
Met-CH_2	1.76, 1.73	nd	1.89, 1.77	nd

[a] For designations refer to structure. [b] nd, complex multiplet, not determined.

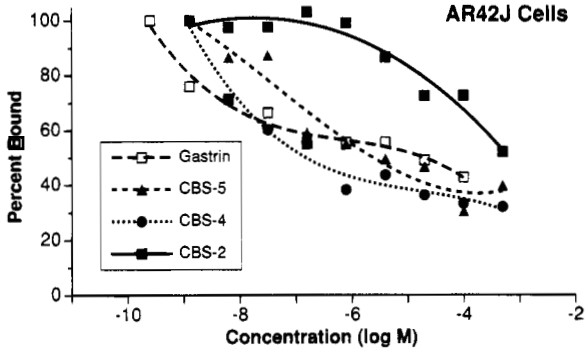


Figure 2. Competition binding of the gastrin and the CBS derivatives to the gastrin receptor on AR42J rat pancreatic acinar cell carcinoma cells in culture. The lines are a computer-generated best fit through the experimental points. The points are an average of three separate determinations, and the standard error for each point was less than 5%.

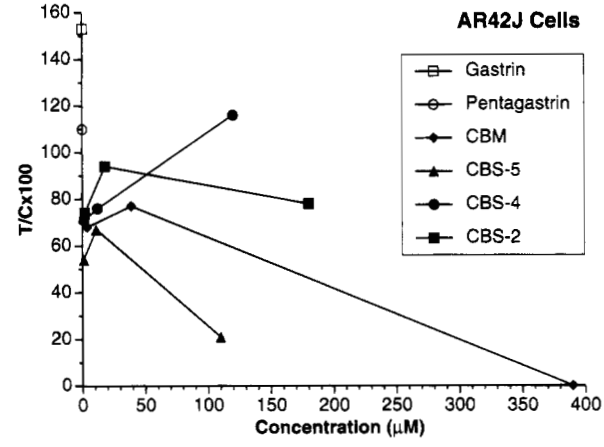


Figure 3. Colony survival assay of AR42J cells treated with gastrin, pentagastrin, the CBS drugs, and CBM. The cells were treated with 0.01 μM gastrin and pentagastrin every 4 days for the duration of the experiment. The CBS derivatives and CBM were applied once at the doses indicated. The points are an average of colony counts from three wells.

at the highest concentrations used (150–200 μM). However, CBS-5 exhibited good toxicity. The model compound, 1-(2-chloroethyl)-3-benzyl-3-(*N*-methylcarbamoyl)triazene (CBM),[31] whose structure is similar to that of CBS, was 3-fold less toxic than CBS-5. Experiments on the chemical stability of CBS-4 and CBS-5 showed that both chemicals were stable under the conditions of the assay. Figure 4 compares, in a simultaneous experiment, the relative toxicities of CBS-4, CBS-5, and CBM in AR42J cells and in a nonreceptor bearing cell line, A549. Clearly the presence of the GR in AR42J cells allows preferential uptake of CBS-5, thus increasing the toxicity of this compound roughly 3-fold when compared to CBM, which exhibited similar toxicity in both cell lines. The lack of cytotoxicity of CBS-4 in this experiment is difficult to explain. We noted, however, that CBS-4 is significantly less soluble then

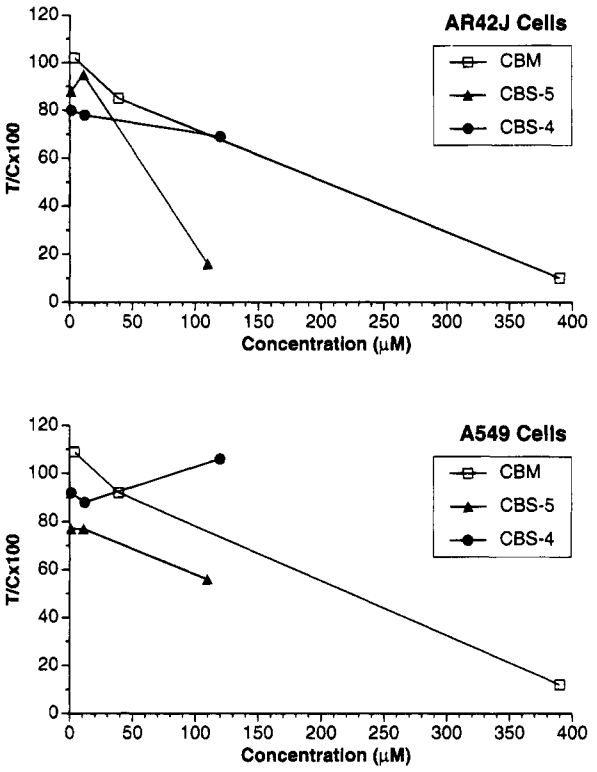


Figure 4. Colony survival assay of AR42J cells (top) and A549 human lung adenocarcinoma cells (bottom), treated with CBM, CBS-4, and CBS-5. The assays were performed simultaneously. Each point is an average of colony counts from three wells.

CBS-5 in the assay medium since it precipitated from solution at concentrations required for cytotoxicity. Alternatively, the tetrapeptide conjugated cytotoxic agent may be processed differently after binding to the receptor than CBS-5. Experiments to clarify this issue are underway. We were forced to use AR42J cells in our assays rather than a colon carcinoma line because the latter do not express GR in culture, even though most colon cancers appear to express the receptor. Other workers have also noted this phenomenon.[22]

Conclusion

The synthesis of gastrin peptide-linked alkylating agents was accomplished. NMR investigations showed that the molecules were very flexible, with no specific conformations being evident from NOESY and ROESY experiments. The compounds CBS-4 and CBS-5 bound to the gastrin receptor in guinea pig stomach and in AR42J cells. CBS-5 was found to be more cytotoxic to AR42J cells than CBM, the model unconjugated drug, but CBS-4 was not cytotoxic. Pentagastrin, the parent peptide of CBS-5, as well as gastrin itself were found to stimulate AR42J cell growth in culture. Thus, the activity of CBS-5 in this assay strongly suggests that the hypothesis for receptor-mediated cytotoxicity has been demonstrated. The lack of cytotoxicity for CBS-4 may be due to a simple solubility problem. This compound is poorly soluble in the medium and may be coming out of solution during the period of the assay. Experiments to determine this or some other possible explanations are underway in our laboratory.

Experimental Section

Materials. The preparation of chloroethyl azide was described earlier.[25] All chemicals used in the synthesis were reagent grade (Bachem, Philadelphia, PA; Aldrich, Milwaukee, WI) and were used without additional purification. NMR spectroscopy was performed on a Varian VXR-500 spectrometer (500 MHz for ^{1}H). Chemical shifts are reported relative to TMS in CD_3OD. Spin calculations and simulations were carried out using LAACON 3 program[26] on a VAX 8650 at the Biomedical Supercomputing Center at FCRDC. Nuclear Overhauser effects in the proton NMR spectrum were measured using the two-dimensional pulse sequence NOESY[27] and ROESY.[28] Mass spectra, including exact mass measurements, were carried out on a VG-Micromass ZAB-2F spectrometer equipped with a VG data system, Model 2035, or a VG-Micromass Model 7070 spectrometer.

3-Benzyl-3-(3-carboxypropanoyl)-1-(2-chloroethyl)triazene (1, CBS). Chloroethyl azide (1.72 mL, 20 mmol) was dissolved in 80 mL of dry THF and cooled to 55 °C. A 2 M solution of benzylmagnesium chloride in THF (10 mL) was added dropwise under nitrogen. The mixture was stirred for 1 h at room temperature. After cooling to −55 °C a solution of 2 g (20 mmol) of succinic anhydride in 20 mL of dry THF was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred for 1.5 h. Then, 50 mL of water and 150 mL of diethyl ether were added. The reaction mixture was cooled to 0 °C and acidified to pH 4 (1 N HCl). The phases were separated. The water phase was extracted with diethyl ether (2 × 20 mL). The combined organic phases were washed with brine and dried over the Na_2SO_4. A slightly yellow oil was obtained after evaporation of the solvent. The product was purified by column chromatography on silica gel; pentane:diethyl ether = 1/1. Yield: 4.35 g of a crystalline product. Mp 89−90 °C. MS calcd *m/z* for MNa^+ $C_{13}H_{16}ClN_3NaO_3$ 320.07784, found 320.080. ^{1}H-NMR Data ($CDCl_3$): δ 7.22 (s, 5 H), 5.14 (s, 2 H), 4.07 (t, J = 6.1 Hz), 3.81 (t, J = 6.1 Hz), 3.18 (t, J = 6.8 Hz), 2.80 (t, 6.8 Hz).

***N*-[3-Benzyl-3-(carboxypropanoyl)-1-(2-chloroethyl)-triazene]-Trp-Met-Asp-Phe-amide (4, CBS-4).** Freshly prepared **1** (149 mg, 0.5 mmol) and hydroxysuccinimide (63 mg, 0.55 mmol) were dissolved in 5 mL of dry tetrahydrofuran. The solution was cooled to 0 °C, and dicyclohexylcarbodiimide (113.5 mg, 0.55 mmol) was added. After 1 h the ice bath was removed and the reaction mixture was stirred for 8 h at room temperature. The precipitated urea was filtered off, and the filtrate was concentrated and the residue dissolved in 10 mL of acetonitrile and filtered again. The peptide, Trp-Met-Asp-Phe-amide hydrochloride (Bachem) (238.3 mg, 0.4 mmol) was suspended in 5 mL of distilled water. Triethylamine (112 μL, 0.8 mmol) was added. The acetonitrile solution of the hydroxysuccinimide ester, **2**, was added dropwise with stirring. The reaction mixture was stirred at room temperature for 6 h. After dilution with 10 mL of water the reaction mixture was filtered and acidified to pH 4 (1 N HCl). The precipitate was collected and washed with water. The colorless solid was recrystallized from methanol/water (15 mL/15 mL). Further purification was carried out by HPLC under reverse-phase conditions using a preparative Waters μ-Bondapak RP-18 column (19 mm × 150 mm); eluent: acetonitrile/water/0.1%TFA gradient 40% to 60% acetonitrile (30 min). Anal. ($C_{42}H_{50}ClN_9O_8S·0.5H_2O$) C, H, N. ^{13}C-NMR spectrum: CO (176.96, 176.39, 176.10, 175.39, 174.32, 173.64, 172.87), Trp (137.10, 128.80, 124.88, 122.57, 119.98, 119.35, 112.46, 110.54, 56.98, 27.61), Phe (138.78, 130.34, 129.33, 128.00, 56.13, 38.18), Bz (138.10, 129.48, 128.53, 127.71, 44.08), Met (55.25, 30.80, 30.66, 15.01), Asp (51.98, 36.02), chloroethyl (63.97, 42.58), linker (30.91, 30.25).

***N*-[3-Benzyl-3-(carboxypropanoyl)-1-(2-chloroethyl)-triazene]-β-Ala-Trp, (3, CBS-2).** The hydroxysuccinimide ester of freshly prepared 3-benzyl-3-(3-carboxypropanoyl)-1-(2-chloroethyl)triazene (2.0 g, 5 mmol) was prepared in the manner described above. The dipeptide β-Ala-Trp (1 g, 3.63 mmol) was suspended in 20 mL of water/15 mL of acetonitrile. Triethylamine (700 μL, 3.63 mmol) was added. The hydroxysuccinimide ester dissolved in 25 mL of acetonitrile was added dropwise with stirring. The reaction mixture was stirred overnight. After dilution with 30 mL of water the pH was adjusted to 7.4 by adding 0.2 N sodium bicarbonate. The reaction mixture was washed twice with 15 mL of ethyl acetate and acidified to pH 3−4 (1 N HCl). The product was extracted

with ethyl acetate (3 × 30 mL). Yield: 1.44 g (71%) colorless amorphous solid. MS calcd *m/z* for MLi^+ $C_{27}H_{31}ClLiN_6O_5$ 561.2205, found 561.2262 (by FABMS). ^{1}H-NMR (500 MHz, CD_3OD): δ (assign., mult (*J*, Hz)) 7.55 (Trp_G, d(0.8), d(1.2), d(7.9)), 7.30 (Trp_D, t(0.9), d(8.1)), 7.22–7.08 (Bz, Trp_A, m), 7.06 (Trp_F, d(1.20), d(7.0), d(8.1)), 6.99 (Trp_E, d(1.0), d(7.0), d(8.1)), 5.09 (Bz, ab(14.9)), 5.08 (Bz, ab(15.0)), 4.75 ($TrpH_\alpha$, d(4.9), d(8.5)), 4.05 (NCH_2, d(5.2), d(6.3)), 3.85 ($ClCH_2$, d(5.1), d(6.3)), 3.39 (linker, t(7.5), ab(13.5)), 3.35 ($TrpH_\beta$, d(0.9), d(4.9), d(14.7)), 3.31 (linker, d(7.0), ab(13.5)), 3.16 ($TrpH_\beta$, d(0.8), d(8.5), d(14.8)), 3.12 (β-Ala, d(2.5), t(6.9)), 2.49, 2.45 (linker, t(6.9), ab(15.5)), 2.34 (β-Ala, d(1.6), d(6.7)).

***N*-[3-Benyl-3-(carboxypropanoyl)-1-(2-chloroethyl)triazene]-β-Ala-Trp-Met-Asp-Phe-amide, (5, CBS-5).** CBS-2 (**3**) (666 mg, 1.2 mmol) and hydroxysuccinimide (138 mg, 1.2 mmol) were dissolved in 30 mL of dry tetrahydrofuran. The reaction mixture was cooled to 0 °C. Dicyclohexylcarbodiimide (247 mg, 1.2 mmol) was added. The reaction mixture was stirred for 1 h at 0 °C followed by 8 h at room temperature. The precipitated urea was filtered off and the filtrate concentrated in vacuo. The residue was dissolved in 15 mL of acetonitrile and refiltered. The tripeptide Met-Asp-Phe amide hydrochloride (466 mg, 1 mmol), prepared according to the procedure of ref 29, was dissolved in 10 mL of water, and triethylamine (280 μL, 2 mmol) was added. The active ester was added dropwise, and the reaction mixture was stirred at room temperature for 6 h. Water (10 mL) was added, and 5% sodium bicarbonate was used to adjust the pH to 8. Diethyl ether (15 mL) was added, and the organic phase was separated. The water phase was acidified (1 N HCl) to pH 3–4 and extracted with ethyl acetate/methanol = 10/1 (3 × 40 mL). After evaporation of the solvent the colorless solid was recrystallized from methanol/water. Further purification was carried out by HPLC under reverse-phase conditions using a preparative Waters μ-Bondapak RP-18 column (19 mm × 150 mm); eluent: 70% methanol/30% water/0.05% TFA. Yield: 570 mg (60%). The NMR of this compound is presented in Table 1 and discussed above. The FAB mass spectrum gave MNa^+ at *m/z* 970 and MNa^+_2 at *m/z* 993. Anal. ($C_{45}H_{55}ClN_{10}O_9S\cdot H_2O$) C, H, N.

Biological Assays. Gastrin Receptor Binding Assay. The in vitro assay consisted of a competition binding with iodinated gastrin for the gastrin receptors from either guinea pig gastric glands or AR42J cells. All reagents were obtained from Sigma. The guinea pigs were male, strain 2NCR from the FCRDC animal facility (400–500 g). They were about 2 years old at the time of sacrifice and were maintained on a standard diet ad libitum until use. The AR42J cells were kindly provided by Dr. R. T. Jensen (NIDDK, NIH).

Typically, gastric glands were obtained from freshly isolated guinea pig stomachs by a modification of the procedure of ref 30. Gastric fundal mucosa was scraped off the supporting tissue in the cold and finely minced. It was then incubated with 0.1% collagenase in 0.1% BSA supplemented Hepes buffer (Hepes-1) at 37 °C for 45 min. The digest was passed three times through a 10 cm^3 syringe, filtered through a nylon mesh, and centrifuged at 250*g* for 5 min. After two consecutive washes in Hepes-1 the final gland pellet was resuspended in 12.5 mL of Hepes-1 containing 0.25 mg/mL of bacitracin. This preparation was added in 100 μL volumes to siliconized microfuge tubes containing the above buffer, 150 fmol (3-[^{125}I]-iodotyrosyl^{12})-gastrin-*1* (Amersham, 2000 μCi/mmol) at a specific activity of 0.37 μCi/pmol and varying amounts of each drug or nonradioactive gastrin-*1* (human) in a total volume of 200 μL. Compounds were added from concentrated stock solutions prepared in dimethyl sulfoxide (DMSO), and appropriate DMSO control samples were included in each assay. Samples were gently shaken at room temperature for 30 min. Twice the volume of cold Hepes-1 was added to samples followed by centrifugation at 4000*g* for 2 min. Gland pellets were washed once with the same buffer and the tips of the tubes containing the washed pellets were cut and placed directly into scintillation vials. Samples were counted in a γ counter.

Alternatively, AR42J cells were scraped and washed three times by centrifugation in cold Hepes-1 buffer at 1000 rpm in a tabletop centrifuge. The cells were then suspended in Hepes-1 with bacitracin as above to a concentration of approximately 10^7 cells/mL; 100 μL volumes of this preparation were then used in the assay as described above, in place of guinea pig glands, and processed similarly.

Colony Survival Assay. The rat pancreatic acinar carcinoma cell line AR42J[26] was provided as a courtesy of R. T. Jensen. The human non-small lung adenocarcinoma A549 cell line was obtained from American Type Culture Collection (Rockville, MD). Culture conditions consisted of Dulbecco's MEM with 10% Fetal Bovine Serum (all from Sigma) in a 5% CO_2 atmosphere at 37 °C with complete humidity. Assays involved seeding single cell suspensions at low densities in six-well culture dishes for 24–48 h and adding fresh medium containing compounds at varying concentrations from DMSO stocks as well as appropriate DMSO controls. Colonies were allowed to grow to 1–2 weeks with medium changes every 5 days. Wells were fixed in methanol and stained with 1% crystal violet (Sigma) in methanol. Colonies of greater than 30 cells were scored as survivors.

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