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Bioactivity of Analogs of the N-Terminal Region of Gastrin-17

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Abstract

Gastrin-17-Gly (G17-Gly) has been shown to bind to non-CCK nanomolar and micromolar affinity sites on DLD-1 and HT-29 human colonic carcinoma cells and to stimulate cellular proliferation. However, in previous studies, we showed that C-terminal truncation of the gastrin-17 (G17) to the G17 analog G17(1–12) and then to G17(1–6)-NH_2 did not remove the ability to bind to DLD-1 cells or to activate proliferation. This implies that residues and/or structural motifs required for bioactivity at these receptors rest in the N-terminal region of G17. In this work, radioligand binding studies conducted with further C-terminally truncated analogs revealed that sequences as short as G17(1–4) still bind to a single receptor with micromolar affinity. Additionally, cell proliferation assays showed that G17(1–12) stimulates proliferation of DLD-1 cells, as of HT-29 cells, but the sequences shorter than G17(1–6)-NH_2, including nonamidated G17(1–6), were incapable of stimulating proliferation. These observations indicate that the tetrapeptide pGlu-Gly-Pro-Trp is the minimum N-terminal sequence for binding to the probable growth-promoting site on DLD-1 cells. Since analogs shorter than G17(1–6) are able to bind the receptor, these peptides may be of use for developing selective antagonists.

1. Introduction

While it is accepted that gastrin-17 (G17) (pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2) has a role in the development and growth of gastric cancers, consensus has not been reached regarding its effect on colon cancers. Recent research has suggested that elevated gastrin levels in the circulation do not play a role in cancer promotion in the colon [8,15–16,28,30,35], and that CCK_2 receptors, responsible for the mediation of gastric acid secretion and epithelial growth promoting effects of G17, are largely absent from the colon and thus do mediate such effects [5,12–14,31–32]. Additionally, it has been found that processing intermediates in the formation of G17 including progastrin and G17-Gly are secreted by colon cancer cells and are more frequently present in the colon than is G17 [9,20, 27]. These intermediates have been shown to stimulate the growth of the colonic mucosa *in vitro* and in rats [4,10,21,29,34–35], and to stimulate the proliferation of normal and neoplastic colon cells not expressing CCK_2 receptors [6,18,22,33,36].

The growth-promoting effects of G17-Gly have been shown to be mediated by a putative, non-CCK receptor by several groups, through a mechanism not involving the C-terminal tetrapeptide sequence Trp-Met-Asp-Phe-NH_2 of G17, which is essential for binding and activation of the CCK_2 receptor [3,7,23–24,26,37,39–40]. Nanomolar and micromolar affinity

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receptors for G17-Gly on primary tissues and on cultured cell lines have been detected [17, 19,25,33,36,38,41]. In our previous study we demonstrated the simultaneous presence of G17-Gly and G17 nanomolar and micromolar binding sites on the DLD-1 and HT-29 human colon cancer lines by performing radioligand binding assays employing a wide concentration range of unlabeled G17-Gly [1]. Subsequently it was shown that these two sites are responsible for a biphasic growth effect when DLD-1 cells are treated with G17-Gly. Further studies revealed that both C-terminal analogs [Leu^{15}]G17(6–17)-Gly and [Leu^{15}]G17(11–17)-Gly bind to a single site on DLD-1 cells with close to micromolar affinity, while the N-terminal analog G17 (1–12) stimulates nonbiphasic proliferation of HT-29 cells [2]. These results suggest that the N-terminal region of G17 is essential for binding and activation of a nanomolar affinity receptor that mediates the growth-promoting effects of the peptide.

In our previous study, we showed that G17(1–12) binds to two sites on DLD-1 cells with similar affinities to that of G17-Gly[11]. We further truncated G17 to produce G17(1–6)-NH_2 to find that it binds to DLD-1 cells at a single site with micromolar affinity and it also can stimulate cell proliferation. Thus, neither the C-terminal tetrapeptide of G17 which is essential for binding the CCK_2 receptor nor even the full pentaglutamyl sequence of the central portion of the peptide is necessary for binding or activation of the putative receptor on the cancer cells. In this work, we examined the growth effect of G17(1–12) on DLD-1 cells, as well as further truncated G17 (Table 1.) to determine the minimal N-terminal sequence required for binding and activation of the putative growth-promoting receptor. We also examined the effect of C-terminal capping with an amide group on the ability of these analogs to bind and activate the receptor.

2. Materials and methods

2.1. Solid phase peptide synthesis resins and amino acids

Rink Amide AM, Fmoc-Trp(Boc)-Wang, and Fmoc-Tyr(OtBu)-Wang resins were from NovaBioChem (San Diego, CA, USA). Fmoc-Glu(OtBu)-Wang and Fmoc-Leu-Wang resins were from Advanced ChemTech (Louisville, KY, USA). H-Tyr(OtBu)-HMPB-ChemMatrix resin was from Matrix Innovations (Montreal, Quebec, Canada). Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Trp(Boc)-OH, Fmoc-Pro-OH, Fmoc-Ala-OH, and pGlu-OH were from Advanced ChemTech. Fmoc-Glu(OtBu)-OH and Fmoc-Tyr(OtBu)-OH were from NovaBioChem.

2.2. Peptide synthesis, cleavage, and purification reagents

DMF, DMSO, NMP, DCM, acetone, methanol, ammonium bicarbonate, acetonitrile, sodium hydroxide, and sodium chloride were from Fisher (Fair Lawn, NJ, USA). DIEA and HBTU were from Chem-Impex (Wood Dale, IL). TIS, thioanisole, EDT, piperidine, and diethyl ether were from Sigma-Aldrich (Milwaukee, WI, USA). TFA was from Sigma-Aldrich (peptide synthesis grade), Chem-Impex (peptide synthesis grade), and Pierce (Rockford, IL) (HPLC grade).

2.3. Cell culture and radioligand binding materials and reagents

DLD-1 human colon carcinoma cells were from American Type Tissue Collection (ATCC) (Manassas, VA, USA). RPMI-1640 (with L-glutamine) growth media, Cellstripper non-enzymatic cell dissocation solution and antibiotic/antimycotic solution (10,000 I.U. penicillin, 10,000 μg/mL streptomycin, and 25 mg/mL amphotericin B) were from Mediatech Inc. (Herndon, VA, USA). Trypsin-EDTA (0.25% trypsin and 1.0 M EDTA) and fetal bovine serum were from Atlanta Biologicals (Lawrenceville, GA, USA). HEPES, Tris-HCl, potassium chloride, potassium phosphate (monobasic), and sodium phosphate (dibasic) were from Fisher. Tris, magnesium chloride, sodium chloride, potassium phosphate (dibasic), calcium chloride,

D-(+)-glucose, and trypan blue were from Sigma Aldrich. [Leu^{15}]G17-Gly was prepared as previously reported [1].

2.4. Radiolabeling of G17(1–12)

Custom iodination of G17(1–12) and subsequent HPLC purification was carried out by Peninsula Laboratories using the chloramine T method (Peninsula Laboratories, San Carlos, CA, USA). The specific activity of [^{125}I-Tyr^{12}]G17(1–12) was 782–1832 Ci/mmol.

2.5. Peptide synthesis and cleavage

Peptides (Table 1.) were synthesized, as previously described [11], on an Advanced ChemTech "Apogee" synthesizer in 0.25 mmol scale using *N*-α-Fmoc protected amino acids and the appropriate polystyrene resins: a Rink Amide AM resin for all amidated, and the appropriate Wang linker resins for all non-amidated peptides. G17(1–12) was additionally synthesized using a H-Tyr(OtBu)-HMPB-ChemMatrix resin. The side chains of Tyr and Glu were *t*-Bu protected, while the side chain of Trp was *t*-Boc protected.

Peptides were cleaved from their respective resins using thioanisole / EDT / water / phenol / TIS / TFA (5:2.5:5:5:1:81.5, v/v/v/v/v) cleavage cocktail. This mixture was prepared and chilled to −10 °C before use. The resin was agitated in the mixture with a magnetic stirrer for 15 minutes at 0 °C, and then for 105 minutes at room temperature. After cleavage, the peptide was precipitated by adding ice cold diethyl ether. The peptide and resin were collected by filtration using a medium porosity sintered glass filter, washed several times with ice-cold diethyl ether, and dried under vacuum. The peptide was then dissolved in neat TFA. The solution was filtered and the volume was reducted to 1–2 mL using a Buchi rotary evaporator (Buchi, Flawil, Switzerland). The peptide was again precipitated by adding 100-fold excess (by volume) of ice-cold diethyl ether followed by incubation at 4 °C for 30 minutes. The peptide was collected by filtration using a fine porosity sintered glass filter.

2.6. Peptide purification and characterization

Peptides were analyzed and purified on a dual pump HPLC apparatus (Gilson, Middleton, WI, USA) as previously described [2,11]. For analytical HPLC either a Vydac 218TP54 column (C_{18}, 5 μm particle size, 4.6 mm × 250 mm) (Grace Vydac, Hesperia, CA, USA) or a Phenomenex 00G-4252-Y0 "Luna" column (C_{18}, 5 μm particle size, 3 mm × 250 mm) (Phenomenex, Torrance, CA, USA) were used at flow rates of 1 mL/min and 0.5 mL/min, respectively, with an initial injection concentration of 1 mg/mL. For purification, a semi-preparative Phenomenex 00G-4252-N0 "Luna" column (C18, 5 μm particle size, 10 mm × 250 mm) was used at a flow rate of 5 mL/min, with an initial injection concentration of 4 mg/mL. The molecular weights of peptides were confirmed by ES-MS using a SCIEX API150EX mass spectrometer (Perkin-Elmer Life Sciences, Boston, MA, USA). Peptides were obtained at greater than 95% purity.

2.7. Cell culture

DLD-1 human colon carcinoma cells were grown in monolayer culture in RPMI 1640 media containing 10% fetal bovine serum and 1% (v/v) of antibiotic/mycotic solution (Gibco, Invitrogen Corporation, Carlsbad, CA, USA). This medium was changed every 2–3 days. Cells were incubated at 37 °C in a 5% CO_2 atmosphere and maintained at subconfluent levels. Cultures were passaged by detachment of cells with trypsin-EDTA and reseeding cells 1:4 to 1:10. Cells after passage 25–30 were harvested at 70–90% confluency.

2.8. Radioligand binding assays with intact cells

The binding of peptides in competition with [^{125}I-Tyr^{12}]G17(1–12) to receptors on DLD-1 cells was determined. Cells were detached from culture flasks using a non-enzymatic cell dissociation solution (Cellstripper). Cells (3–4 million cells/well) were incubated with shaking for 5 h at 28 °C in a buffer solution (pH 7.4; 300 μL/) containing 20 mM HEPES, 1 mM calcium chloride, 5 mM potassium chloride, 2.2 mM magnesium chloride, 120 mM sodium chloride and 6 mg/mL glucose with 50 pM [^{125}I-Tyr^{12}]G17(1–12) and 10^{-12} M to 10^{-4} M test peptide. Suspensions were rapidly filtered through glass fiber filters (pore size 3.1 mm; Pall Life Sciences, East Hills, NY, USA) that had been presoaked at 4 °C in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% bovine serum albumin using a Brandel cell harvester (Biomedical R&D Laboratories, Gaithersburg, MD, USA). Filters were washed twice with ice-cold 50 mM Tris-HCl buffer, air-dried and were transferred to scintillation tubes. Bound radioactivity was counted using a Wallac model 1277 Gammamaster automated gamma counter (EG&G, Inc., Wellesley, MA, USA) with a counting efficiency of 50%. Non-specific binding was determined in the presence of 10^{-4} M G17(1–12).

2.9. Cell proliferation assays

Cells (7–10 × 10^3) were seeded in culture medium (400 μL) containing 10% fetal calf serum in 48-well plates and allowed to attach and reach 40–50% confluence. The medium was then replaced with serum-free growth medium and cells were starved for 24 h. 10^{-12} M to 10^{-5} M test peptide was added to the inner 24 wells of the 48-well plates, and cells were allowed to grow for 72 h. Medium was removed and the cells were detached using trypsin-EDTA and counted visually using a hemocytometer under a brightfield microscope.

2.10. Statistical analysis

All data were analyzed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Comparison of fit for one and two site binding was determined using the F-test. The statistical significance of cell proliferation data was determined using one-way ANOVA with the Dunnett post-test.

3. Results

3.1. Competition binding to intact DLD-1 cells

[^{125}I-Tyr^{12}]G17(1–12) bound to DLD-1 cells in a saturable and displaceable manner. Total binding represented less than 1% of the total added radioligand in all assays.

3.1.1. G17(1–6)—G17(1–6) displaced [^{125}I-Tyr^{12}]G17(1–12) in a dose dependent manner (Figure 1). Specific binding was 44–52% of total binding. The peptide failed to displace all radioligand. Nonlinear regression of the binding results fit the data to a single-site model. The IC_{50} value for binding was 5.9×10^{-6} M.

3.1.2 G17(1–5)-NH_2 and G17(1–5)—G17(1–5)-NH_2 displaced [^{125}I-Tyr^{12}]G17(1–12) in a dose dependent manner (Figure 2). Specific binding was 47–67% of total binding. The peptide failed to displace all radioligand. Nonlinear regression of the binding results fit the data to a single-site model. The IC_{50} value for binding was 1.7×10^{-5} M.

The nonamidated peptide again displaced [^{125}I-Tyr^{12}]G17(1–12) in a dose dependent manner, but once again, not all radioligand was displaced. Specific binding was 39–64% of total binding. Nonlinear regression of the binding results fit the data to a single-site model. The IC_{50} value for binding was 8.1×10^{-6} M.

3.1.3 G17(1–4)-NH_2 and G17(1–4)—G17(1–4)-NH_2 displaced [^{125}I-Tyr^{12}]G17(1–12) in a dose dependent manner (Figure 3). Not all radioligand was displaced. Nonlinear regression of the binding results fit the data to a single-site model. The IC_{50} value for binding was 3.9×10^{-5} M.

The nonamidated peptide again displaced [^{125}I-Tyr^{12}]G17(1–12) in a dose dependent manner, but all radioligand was displaced. Specific binding was 59–71% of total binding. Nonlinear regression of the binding results fit the data to a single-site model. The IC_{50} value for binding was 3.9×10^{-5} M.

3.1.4 G17(1–3)-NH_2—G17(1–3)-NH_2 did not displace [^{125}I-Tyr^{12}]G17(1–12) at concentrations as high as 10^{-3} M.

3.2 Cell proliferation assays

G17(1–12) stimulated significant proliferation of DLD-1 cells in a non-biphasic, dose-dependent manner. (Figure 4). N-terminal G17 analogs shorter than G17(1–6) did not stimulate proliferation of DLD-1 cells at concentrations as high as 10^{-5} M in 8 experiments with 4 replicates for each concentration of peptides.

4. Discussion

4.1 Radioligand binding assays with while DLD-1 cells

G17(1–6) bound to and displaced [^{125}I-Tyr^{12}]G17(1–12) from a single site on DLD-1 cells. The analog bound a receptor with low (micromolar) affinity, and failed to displace all radioligand, resembling G17(1–6)-NH_2 but with slightly lower affinity [11]. The low binding might be either to the G17-Gly low affinity site, or to the G17-Gly high affinity site. [Leu^{15}]G17(6–17)-Gly and [Leu^{15}]G17(11–17)-Gly also bound one site with similarly low affinity and displacement of receptors [2].

Sequential C-terminal truncation of G17(1–6) resulted in analogs with decreasing affinities for the receptor. G17(1–5) and G17(1–5)-NH_2 have slightly lower binding affinity than the G17 (1–6) analogs, and similarly fail to displace all radioligand.

G17(1–4) and G17(1–4)-NH_2 had identical IC_{50} values for the receptor, at 3.9×10^{-5} M. Another drop in affinity was seen, for a total of about an order of magnitude drop from the G17 (1–6) set of analogs. The concentration range was extended to 10^{-3} M because of the extremely low affinity exhibited by the analogs. Strangely, here G17(1–4)-NH_2 shows similar behavior to the longer analogs in displacing about ¾th of the specifically bound radioligand, but G17 (1–4) displaces all specifically bound radioligand. It is not clear what this means, given the incomplete displacement of radioligand by all of the other unlabeled analogs, especially G17 (1–4)-NH_2 at concentrations as high as 10^{-3} M. It is presumably possible that the analogs still bind two sites similar to G17(1–12), though this seems highly unlikely given the binding of [Leu^{15}]G17(6–17) and [Leu^{15}]G17(11–17). Additionally, the short length of the analogs seems to belie the possibility that they would be able to bind to two separate sites, except perhaps in the case that high and low affinity sites are simply conformational states of the same receptor, such as with GPCRs bound and unbound to their accompanying G-proteins.

The failure of G17(1–3)-NH_2 to displace specifically bound radioligand at concentrations as high as 10^{-3} M indicates that the Trp residue is essential for binding to the receptor. Thus, the N-terminal region contains a four residue sequence which is essential for binding to the putative G17-Gly receptor, much like the C-terminal tetragastrin sequence is essential for binding CCK_2-R. This finding is in agreement with our previous results [1], which showed that [Leu^{15}]G17-Gly and [Leu^{15}]G17 displace tritiated [Leu^{15}]G17-Gly with similar affinities at

both binding sites on DLD-1 membranes. Clearly, unlike CCK_2-R, the putative G17-Gly receptor or receptors do not rely on tetragastrin and the amidated C-terminus for binding, which explains the much greater ability of G17-Gly to bind and activate the receptor(s), as opposed to CCK_2-R, upon which G17-Gly has virtually no effect.

4.2 Proliferation effect of analogs on DLD-1 cells

The stimulation of proliferation of DLD-1 cells by G17(1–12) is less than the maximum stimulation effected by [Leu^{15}]G17-Gly or [Leu^{15}]G17 on DLD-1 cells seen previously [1]. Therefore, it is possible that the C-terminal region of both G17 and G17-Gly acts to stabilize any structures important for binding that exist in the N-terminal region of G17(1–12).

The non-biphasic stimulation of proliferation by G17(1–12) is resembles that that of the effect of the peptide on HT-29 cells [2]. The revelation that G17(1–12) binds both high and low affinity sites on DLD-1 cells indicates that this sequence is an antagonist to the low affinity (proliferation retarding) receptor site. It appears that he two receptor sites work in tandem to promote and retard proliferation, and that the residues responsible for binding the low affinity site rest in the C-terminal portion of the peptide.

Contrary to the results seen with its amidated form [11], G17(1–6) did not stimulate proliferation of DLD-1 cells at concentrations as high as 10^{-5} M. This seems at variance with the close binding affinities. However, as C-terminal amidation has been shown to be essential to binding of CCK_2-R, C-terminal capping by an amide group may also be necessary to stabilize this short analog to permit activation of the putative receptor, in lieu of the longer sequence of G17(1–12) and the full G17.

All analogs shorter than G17(1–6) were unable to stimulate proliferation of DLD-1 cells. These results show that a peptide length of at least six residues from the N-terminus, along with C-terminal amidation, is required to form structures important to activation of the receptor. Since analogs shorter than G17(1–6) are able to bind to the receptor, these peptides may be of use for developing selective antagonists.

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Abbreviations

Boc	butyloxycarbonyl
CCK	cholecystokinin
DCM	dichloromethane
DIEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
EDT	1,2-ethanedithiol
EDTA	ethylenediaminetetraacetic acid
ESI-MS	electrospray ionization mass spectrometry

Fmoc	9-fluorenylmetoxycarbonyl
G17	gastrin-17
G17-Gly	gastrin-17-Gly
HBTU	O-benzotriazolyl-N,N,N′,N′-tetramethyluronium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
NMP	*N*-methylpyrrolidinone
OtBu	O-*t*-butyl
pGlu	pyroglutamic acid
TFA	trifluoroacetic acid
TIS	triisopropylsilane
Tris	tris(hydroxymethyl)aminomethane

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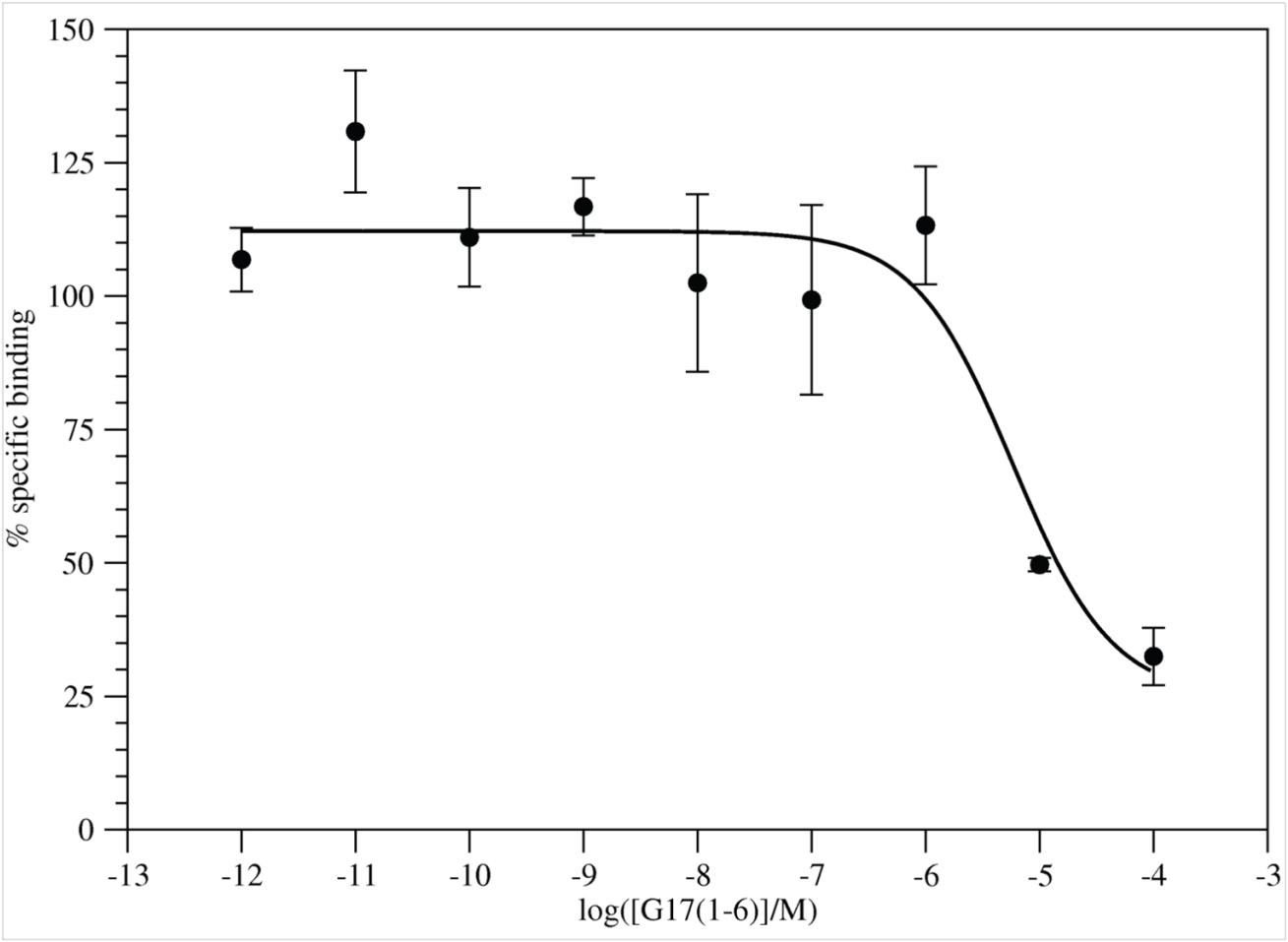

Figure 1.
Binding of G17(1–6) (●) to DLD-1 cells. Results are from 3 experiments with 6 replicates for each concentration. Error bars are ± SEM.

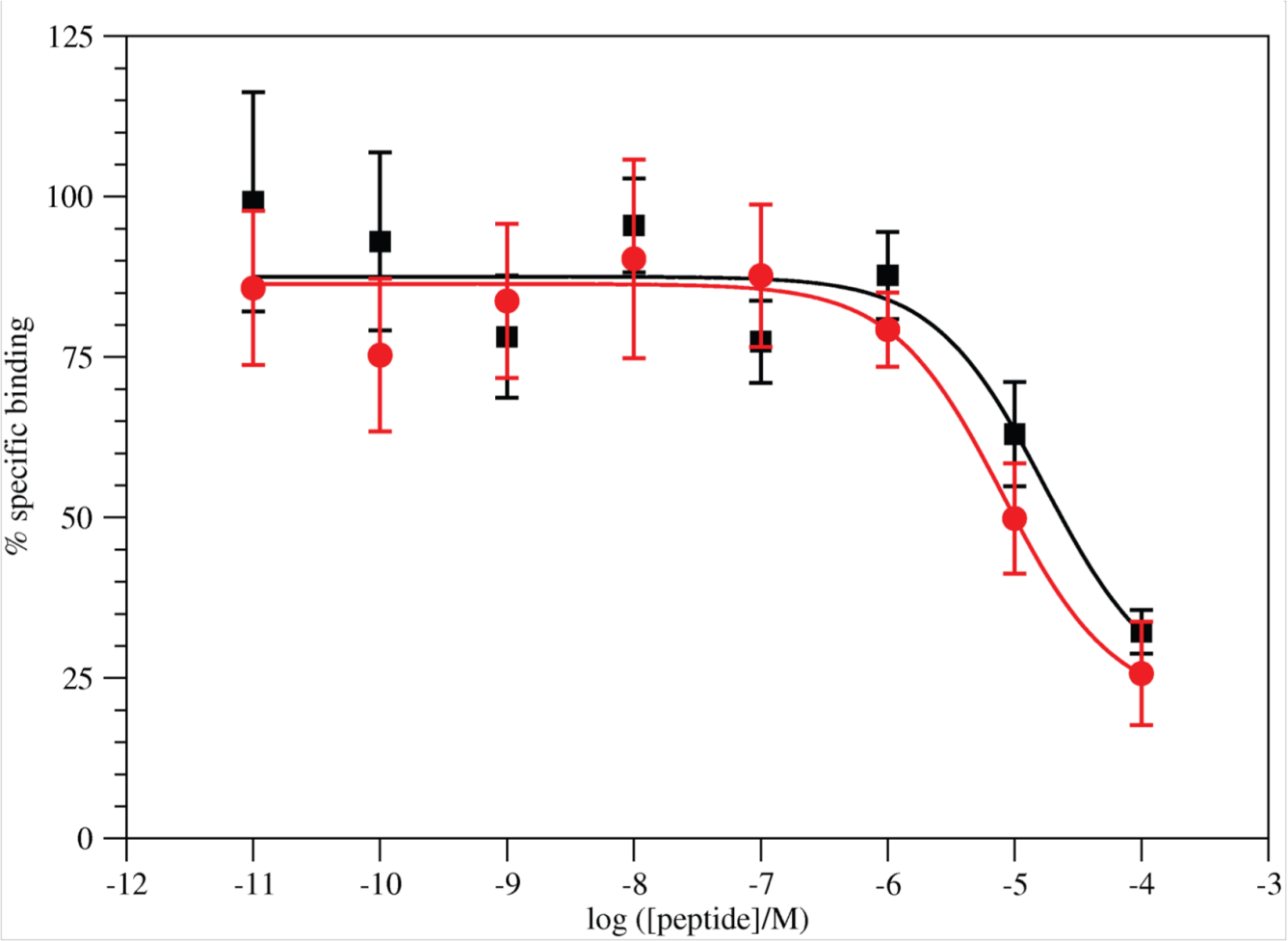

Figure 2.

Binding of G17(1–5)-NH_2 (■; Results are from 7 experiments with 18 replicates for each concentration.) and G17(1–5) (● Results are from 5 experiments with 16 replicates for each concentration.) to DLD-1 cells. Error bars are ± SEM.

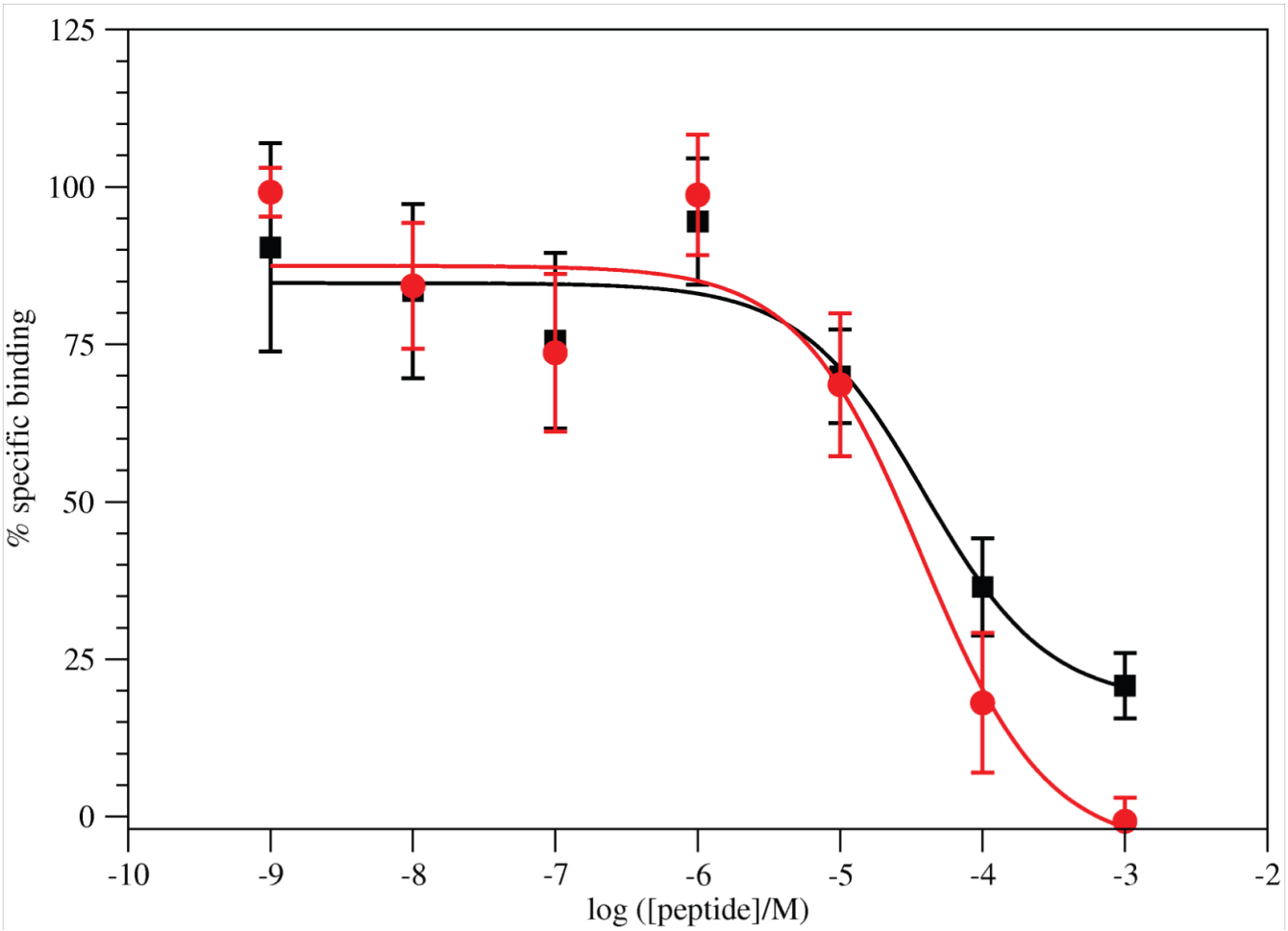

Figure 3.
Binding of G17(1–4)-NH_2 (■; Results are from 7 experiments with 15 replicates for each concentration.) and G17(1–4) (●; Results are from 4 experiments with 10 replicates for each concentration.) to DLD-1 cells. Error bars are ± SEM.

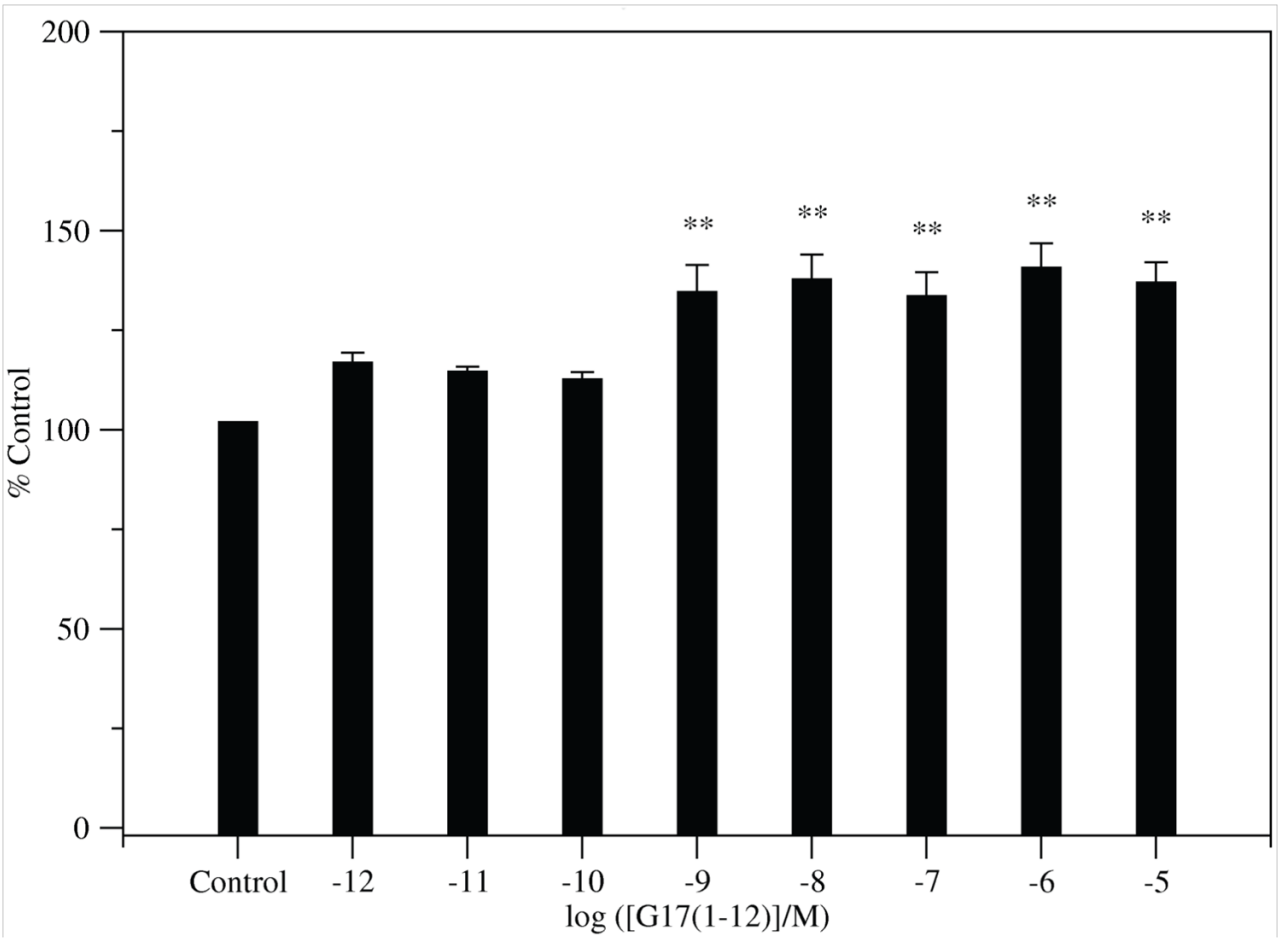

Figure 4.
Proliferation of DLD-1 cells treated with G17(1–12). Error bars are ± SEM. (ANOVA: Dunnett post-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Table 1

Synthetic *N*-terminal analogs of G17.

Peptide	Sequence
G17(1–12)	pGlu-Gly-Pro-Trp-Leu-(Glu)5-Ala-Tyr
G17(1–6)	pGlu-Gly-Pro-Trp-Leu-Glu
G17(1–5)	pGlu-Gly-Pro-Trp-Leu
G17(1–5)-NH_2	pGlu-Gly-Pro-Trp-Leu-NH_2
G17(1–4)	pGlu-Gly-Pro-Trp
G17(1–4)-NH_2	pGlu-Gly-Pro-Trp-NH_2
G17(1–3)-NH_2	pGlu-Gly-Pro-NH_2