Biochemical Pharmacology 83 (2012) 524–530

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Bismuth ions inhibit the biological activity of non-amidated gastrins *in vivo*

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ARTICLE INFO

Article history:
Received 26 October 2011
Accepted 30 November 2011
Available online 8 December 2011

Keywords:
Bismuth ions
Colorectal mucosa
Ferric ions
Glycine-extended gastrin
Proliferation
Progastrin

ABSTRACT

The peptide hormone gastrin binds two ferric ions with high affinity, and iron binding is essential for the biological activity of non-amidated gastrins *in vitro* and *in vivo*. Bi^{3+} ions also bind to glycine-extended gastrin17 (Ggly), but inhibit Ggly-induced cell proliferation and migration in gastrointestinal cell lines *in vitro*. The aims of the present study were firstly, to establish the mechanism by which Bi^{3+} ions inhibit the binding of Fe^{3+} ions to Ggly, and secondly, to test the effect of Bi^{3+} ions on the activity of non-amidated gastrins *in vivo*. The interaction between Bi^{3+} ions, Fe^{3+} ions and Ggly was investigated by ultraviolet spectroscopy. The effect of Bi^{3+} ions on colorectal mucosal proliferation was measured in three animal models. *In vitro* in the presence of Bi^{3+} ions the affinity of Fe^{3+} ions for Ggly was substantially reduced; the data was better fitted by a mixed, rather than a competitive, inhibition model. In rats treated with Ggly alone proliferation in the rectal mucosa was increased by 318%, but was reduced to control values ($p < 0.001$) in animals receiving oral bismuth plus Ggly. Proliferation in the colonic mucosa of mice overexpressing Ggly or progastrin was significantly greater than in wild-type mice, but was no greater than control ($p < 0.01$) in animals receiving oral bismuth. Thus a reduction in the binding of Fe^{3+} ions to Ggly and progastrin in the presence of Bi^{3+} ions is a likely explanation for the ability of oral bismuth to block the biological activity of non-amidated gastrins *in vivo*.

1. Introduction

Bismuth compounds historically have been used to treat gastrointestinal disorders including gastritis, duodenal ulcers, colitis and acute diarrhoea [1]. Bismuth is effective in healing experimental ulcers in animals, and both gastric and duodenal ulcers in humans [2], possibly via inhibition of the growth of gastrointestinal pathogens such as *Helicobacter pylori* [3]. Protection against gastric mucosal damage induced by non-steroidal anti-inflammatory drugs, aspirin or alcohol has also been noted with bismuth salts. In the colon treatment with bismuth reduced acid-induced colitis in rats [4,5], and microscopic [6] and ulcerative [7] colitis in humans. The bismuth salt most commonly used for treatment of gastrointestinal conditions in medical practice in Australia is currently colloidal bismuth subcitrate. Pharmacological studies have demonstrated that following absorption bismuth binds to plasma proteins [8] and is distributed through most tissues [9].

Gastrin is a gastrointestinal peptide hormone that was originally identified as a stimulant of acid secretion. Gastrin is synthesized as a 101-residue precursor (preprogastrin) which, on removal of a 21-residue signal peptide, yields progastrin (80 residues). Proteolytic processing in the secretory vesicles of the antral G cell generates a number of intermediate, non-amidated progastrin-derived peptides, including glycine-extended gastrin17 (Ggly), which has the sequence ZGPWLEEEEEAYGWMDFG [10]. Removal of the C-terminal glycine and amidation of the penultimate phenylalanine yields amidated gastrin (Gamide). Gamide, acting through the cholecystokinin-2 receptor (CCK2R), is the major hormonal regulator of gastric acid secretion [11], and is a mitogen for normal gastric epithelium and some gastric cancers *in vitro* and *in vivo* [10,12,13]. In contrast, progastrin and Ggly have little direct effect on gastric acidity [14], but potentiate the effects of Gamide on acid secretion [15]. The major physiological role of progastrin and Ggly is in the colon, as progastrin and Ggly stimulate proliferation of a colonic cell line *in vitro* [16] and of the normal mucosa *in vivo* [17,18]. Such non-amidated gastrins may also act as growth factors in colorectal cancer [19].

Gamide, Ggly and progastrin all bind two ferric ions with high affinity [20,21]. The carboxylate groups in the side chains of glutamates 7, 8 and 9 were identified as the binding sites [22]. Mutation of glutamate 7 of Ggly to alanine reduced the stoichiometry of ferric ion binding from 2 to 1, and simultaneously completely abolished biological activity in cell proliferation and cell migration assays [22]. The observation that the iron chelator desferrioxamine (DFO) also completely blocked Ggly activity in cell proliferation and migration assays indicated that glutamate 7 was important as a ferric ion ligand rather than as a site of interaction

Abbreviations: CCK2R, cholecystokinin-2 receptor; CRC, colorectal carcinoma; Ggly, glycine-extended gastrin; PBS, phosphate-buffered saline.

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doi:10.1016/j.bcp.2011.11.030

with the Ggly receptor [22]. Treatment of rats and mice with DFO also blocked the effects of Ggly and progastrin on proliferation of the normal rectal mucosa *in vivo* [23]. In contrast mutation of glutamate 7 of Gamide to alanine had no effect on biological activity, even though the stoichiometry of ferric ion binding was again reduced from 2 to 1 [24]. This observation was consistent with previous reports that the minimum active fragment of Gamide was the C-terminal tetrapeptide amide. The receptors for Ggly and progastrin have not been definitively identified, although several candidates have been proposed. For example a gastrin-binding protein first identified in porcine gastric mucosal membranes binds both Ggly and Gamide with similar low affinity [25,26]. In the case of progastrin, biological activity may be mediated via membrane-bound annexin II in both normal and cancerous gastrointestinal cell lines [27,28].

Studies by fluorescence and NMR spectroscopy have shown that Bi^{3+}ions also bind to glutamates 7, 8 and 9 of Ggly [29]. However no direct evidence has yet been presented to indicate whether bismuth ions compete for the same Ggly binding site as ferric ions, or whether their binding at a different site alters the Ggly structure with consequent reduction in ferric ion binding and in the affinity of Ggly for its receptor. To investigate the mechanism by which Bi^{3+} ions bind to Ggly the interaction between Ggly, Bi^{3+} ions and Fe^{3+} ions was investigated by ultraviolet absorption spectroscopy. The observation that Bi^{3+} ions inhibit Ggly-induced inositol phosphate production, and proliferation and migration of gastrointestinal cell lines *in vitro* [29], suggested that Bi^{3+} ions might also be able to interfere with the stimulatory effects of non-amidated gastrins on normal and neoplastic colonic tissue *in vivo*. To determine whether Bi^{3+} ions are able to block the effects of Ggly and progastrin in the normal colorectal mucosa *in vivo*, three animal models were used. In the first model, proliferation in the hypoplastic rectal mucosa of rats that had undergone colostomy was stimulated by short-term infusion of Ggly via Alzet mini-osmotic pumps [30]. In the second and third model, FVB/N mice were engineered to overexpress progastrin (hGAS mice [17]) or Ggly (MTI/Ggly mice [18]), respectively. In all three models Bi^{3+} ions were administered by oral gavage of bismuth citrate.

2. Materials and methods

2.1. Peptides and chemicals

Ggly (93% pure) was from Auspep (Tullamarine, Australia). Bismuth nitrate and bismuth citrate were from Sigma–Aldrich (Castle Hill, Australia).

2.2. Animals

Male Sprague-Dawley rats weighing between 250–300 g were used for the colostomy model [30]. Twelve-week-old male and female transgenic FVB/N mice overexpressing progastrin (hGAS mice [17]) or Ggly (MTI/Ggly mice [18]) were utilized as models of high circulating non-amidated gastrins. All animals were maintained in standard laboratory conditions on a 12-h:12-h light/dark cycle with free access to water and pellets of sterilized standard rat chow or mouse feed (Ridley AgriProducts, Pakenham, Australia) as appropriate, and mortality during the experiments was nil. All experiments were conducted at the Austin Health Department of Surgery (Melbourne, Australia) with ethics approval from Austin Health Animal Ethics Committee.

2.3. Absorption spectroscopy

Absorption of Ggly (10 μM in 10 mM Na acetate, pH 4.0, containing 100 mM NaCl and 0.005% Tween 20) in the presence of increasing concentrations of bismuth and/or ferric ions was measured at 280 nm against a buffer blank, in 1 ml quartz cuvettes thermostatted at 298 K, with a Cary 5 spectrophotometer (Varian, Mulgrave, Australia).

2.4. Rat defunctioned colon model

Alzet minipumps (reservoir volume of 2 ml, pumping rate of 2.5 μl/h for 28 days; Alza, Mountain View, CA) were filled with Ggly (0.53 mg/ml in 0.1% bovine serum albumin in PBS) and primed in 0.9% saline overnight at 37 °C. Rats were anesthetized with inhaled Isoflurane (Abbott, North Ryde, Australia) throughout the operation. The rectum was defunctioned, and the pump was inserted in the peritoneal cavity in the same operation, as described previously [30]. Bismuth ions were administered by oral gavage with bismuth citrate. There were four treatment groups, with sizes as indicated in parentheses: group 1, Control, 0.1% bovine serum albumin in PBS ($n = 8$); group 2, Ggly only, 0.53 mg/ml in 0.1% bovine serum albumin in PBS ($n = 7$); group 3, Bismuth only, bismuth citrate, 135 mg/kg/12 h three times a week ($n = 8$); group 4, Ggly plus Bismuth, Ggly 0.53 mg/ml in 0.1% bovine serum albumin in PBS, bismuth citrate, 135 mg/kg/12 h three times a week ($n = 7$).

2.5. Measurement of proliferation

Proliferation of the hypoplastic rectal mucosa in defunctioned rats was determined by counting vincristine-arrested metaphase nuclei as described previously [30]. In brief rats were injected with vincristine (100 mg/kg, Oncotain; Mayne Pharma, Mulgrave, Australia) to arrest proliferating cells in metaphase and were euthanized 3 h later. All culling was done between 1200 and 1300 h to avoid diurnal variation in mucosal proliferation. Descending colon and rectum were collected, fixed in 10% formalin overnight, and embedded in paraffin (Department of Anatomical Pathology, Austin Health, Victoria, Australia). The nuclei arrested in metaphase were counted in 20 crypts for each haematoxylin and eosin-stained section using an Olympus BH-2 light microscope.

Twelve week-old hGAS, MTI/Ggly, and FVB/N mice were treated with bismuth citrate 270 mg/kg/day in PBS or PBS alone by oral gavage five times a week for 4 weeks. Mice were weighed before euthanasia and anesthetized by Forthane (isoflurane) inhalation before blood was collected by cardiac puncture. Colons were harvested, fixed, and paraffin embedded according to standard techniques. Sections of colon tissue were dewaxed and stained for Ki67 with a monoclonal anti-mouse primary antibody (Dakopatts, Copenhagen, Denmark) and a goat anti-mouse IgG-horseradish peroxidase secondary antibody (Dakopatts) with diaminobenzidine as substrate. The labelling index was calculated as the number of immunopositive cells multiplied by 100 and divided by the total number of cells per colonic crypt. The heights of 20 colonic crypts per specimen were measured in pixels using Image Proplus software (MediaCybernetics, Silver Spring, MD).

2.6. Radioimmunoassay

Serum concentrations of progastrin and Ggly were measured by radioimmunoassay as described previously with antibodies 1137 and 7270, respectively [31].

2.7. Curve fitting and statistical analysis

Data (expressed as means ± S.E.M.) for the binding of ferric ions to Ggly were fitted to a two-site ordered model with the program BioEqs [32–34]. Data for the binding of ferric ions to Ggly in the presence of various concentrations of bismuth ions were fitted to

competitive and mixed models with the program BioEqs. In the competitive model bismuth ions bind to the same site as ferric ions so that the species $GglyBiFe_2$ cannot form, while in the mixed model bismuth ions bind to a different site from ferric ions and the species $GglyBiFe_2$ can form. Statistical significance was determined by t-test with Bonferroni's correction using the program SigmaStat (Jandel Scientific, San Rafael, CA).

3. Results

3.1. Bismuth ions competitively inhibit the binding of ferric ions to Ggly

To determine whether or not bismuth ions competed with ferric ions for binding to Ggly, the effect of bismuth ions on the Fe^{3+}-induced enhancement of the Ggly absorption spectrum was investigated. Although addition of bismuth nitrate up to a molar ratio of 5 had relatively little effect on Ggly absorbance at 280 nm, the presence of bismuth ions significantly reduced the enhancement of Ggly absorbance on subsequent addition of ferric ions (Fig. 1A and B). Simultaneous fitting of the ferric ion titration curves obtained at different $[Bi^{3+}]$ with the program Bioeqs gave a better fit with a mixed model (Fig. 1A) than with a competitive model (Fig. 1B). The best fit affinity constants for the mixed model, which are presented in Table 1, indicate that the presence of bismuth ions results in a nearly 50-fold reduction in the affinity of Ggly for ferric ions.

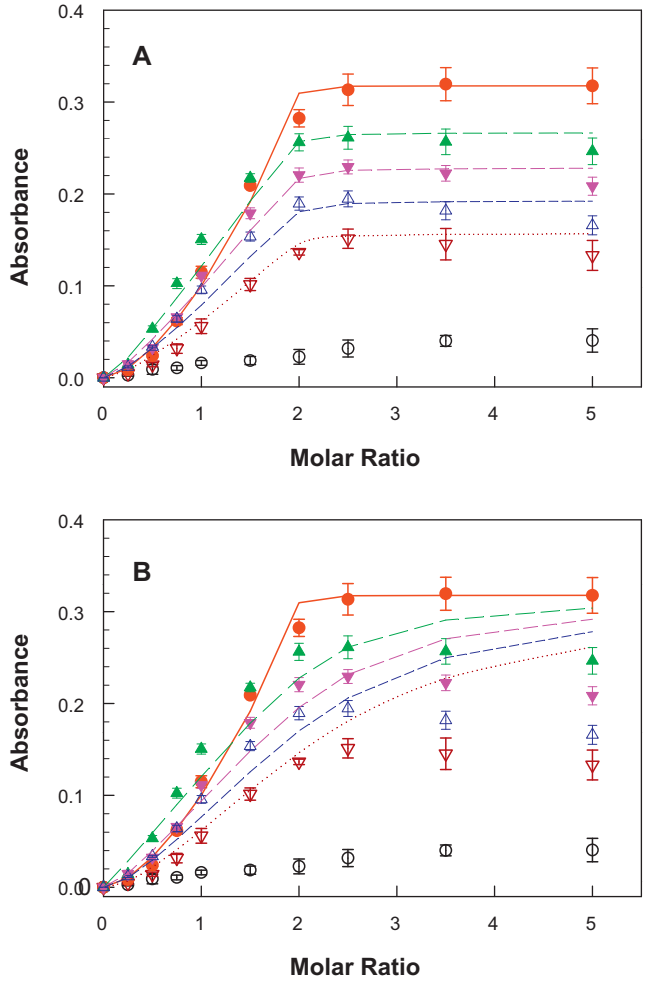

Fig. 1. Bismuth ions inhibit the binding of ferric ions to Ggly. (A) and (B) Addition of ferric ions to 10 μM Ggly (●) in 10 mM Na^+ acetate, pH 4.0, 100 mM NaCl, 0.005% Tween 20, 298 K resulted in an increase in the absorption at 280 nm. Addition of ferric ions to 10 μM Ggly containing 10 μM (▲), 20 μM (▼), 32.5 μM (△), or 50 μM bismuth ions (▽) caused a progressively smaller change in absorption. Addition of bismuth ions to 10 μM Ggly up to a molar ratio of 5 did not cause a substantial change in absorbance (○). Points are means of at least three separate experiments; bars represent the SEM. Lines represent the best fit to either a mixed (A) or competitive (B) two site model with the program BioEqs; the appropriate dissociation constants are given in Table 1. The better fit was obtained with the mixed model.

3.2. Bismuth inhibits Ggly-stimulated proliferation in the rat colorectal mucosa

The effect of bismuth ions on Ggly-stimulated proliferation in the colorectal mucosa of defunctioned rats was assessed by counting metaphase-arrested nuclei. The metaphase index was significantly increased by 3.2-fold in the rats treated with Ggly compared to control rats (Fig. 2). The stimulation of proliferation by Ggly was reversed by bismuth treatment, while bismuth alone did not affect the proliferative index in the rectally defunctioned rats.

3.3. Bismuth inhibits biological activity of PG and Ggly in the colon of hGas and MTIGgly mice

The effect of bismuth on the colorectal mucosa of mice overexpressing non-amidated gastrins was assessed by counting cells stained for the proliferative marker Ki67. The percentage of cells stained for Ki67 was significantly greater in both hGas mice and MTI-Ggly mice compared to wild-type FVBN mice. Following bismuth treatment the percentage of cells stained for Ki67 was significantly decreased in both hGas and MTI-Ggly mice (Fig. 3A and B). No significant changes were observed in crypt height (data not shown).

3.4. Serum progastrin concentrations are significantly increased in mice treated with bismuth

As reported previously [17], hGas mice have high concentrations of circulating progastrin as a result of hepatic expression of a human gastrin minigene. Following bismuth treatment the concentration of circulating progastrin increased a further 5-fold (Table 2). In contrast bismuth treatment had no significant effect on circulating Ggly in the hGAS or MTIGgly mice, and slightly decreased Ggly in the FVBN mice.

4. Discussion

In the current study we have investigated the interaction between trivalent bismuth ions (Bi^{3+}), trivalent ferric (Fe^{3+}) ions

Table 1
Affinities of Ggly for bismuth and ferric ions.

Species	Mixed		Competitive	
	K (nM)	Abs (%)	K (nM)	Abs (%)
GasFe	5.5	100	5.5	100
GasFe2	12	317.7	12	317.7
GasBi	53	100	0.09	100
GasBiFe	244	100	6120	100
GasBiFe2	229	100	NA	NA
χ^2	19.6		29.2	

The data presented in Fig. 1 for the change in absorbance on addition of ferric ions to Ggly (10 μM in 10 mM Na^+ acetate, pH 4.0, 100 mM NaCl, 0.005% Tween 20, 298 K) in the presence of various concentrations of bismuth ions were fitted to competitive and mixed models with the program BioEqs [32–34]. In the competitive model bismuth ions bind to the same site as ferric ions so that the species $GglyBiFe_2$ cannot form, while in the mixed model bismuth ions bind to a different site from ferric ions and the species GglyBiFe can form. The better fit was obtained with the mixed model. NA, not applicable.

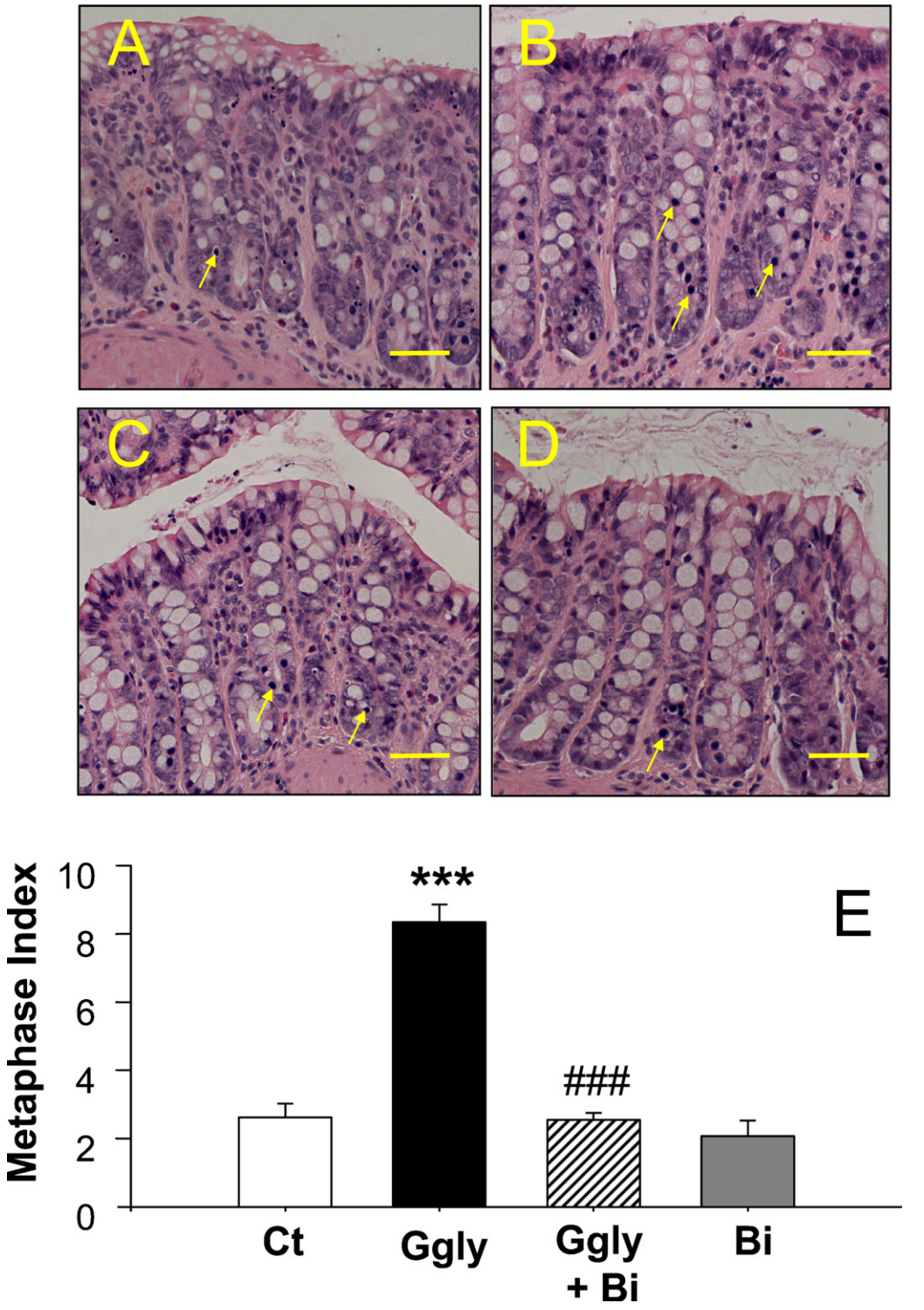

Fig. 2. Bismuth ions inhibit Ggly-stimulated proliferation in rat colorectal mucosa. Rats that had been subjected to a colostomy to induce hypoplasia of the defunctioned rectal mucosa were infused with Ggly (0.53 mg/ml in 0.1% bovine serum albumin in PBS) or with 0.1% bovine serum albumin in PBS alone via Alzet miniosmotic pumps. Bismuth ions were administered by oral gavage with bismuth citrate (135 mg/kg/12 h three times a week). After 4 weeks animals were injected with vincristine and sacrificed 3 h later. Sections from the colorectal mucosa of control (A), Ggly-treated (B), bismuth- and Ggly-treated (C), or bismuth-treated (D) rats were stained with haematoxylin and eosin and examined microscopically to determine the metaphase index (mean number of metaphase-arrested nuclei per crypt; examples indicated by arrows) in the defunctioned rectum (E). Images were taken at 20× magnification, and the scale bars represent 50 μm. Data are means ± SEM, where $N = 7$. The significance of differences from the control ($^{***}p < 0.001$) or between the bismuth- and Ggly-treated and corresponding Ggly-treated samples ($^{###}p < 0.001$) was determined by *t*-test with Bonferroni's correction.

and glycine-extended gastrin (Ggly) *in vitro*. We have also examined the ability of bismuth to inhibit the proliferative activity of non-amidated gastrins *in vivo* in three different animal models.

Binding of bismuth to Ggly has previously been demonstrated by fluorescence quenching and by NMR spectroscopy [29]. Broadening of the resonances of glutamates 7, 8 and 9 suggested that, as with ferric ions, these three residues formed part of the bismuth binding site. However the better fit of the binding data presented in Fig. 1 to a mixed rather than a competitive inhibition model (as defined in the legend to Table 1) clearly indicates that

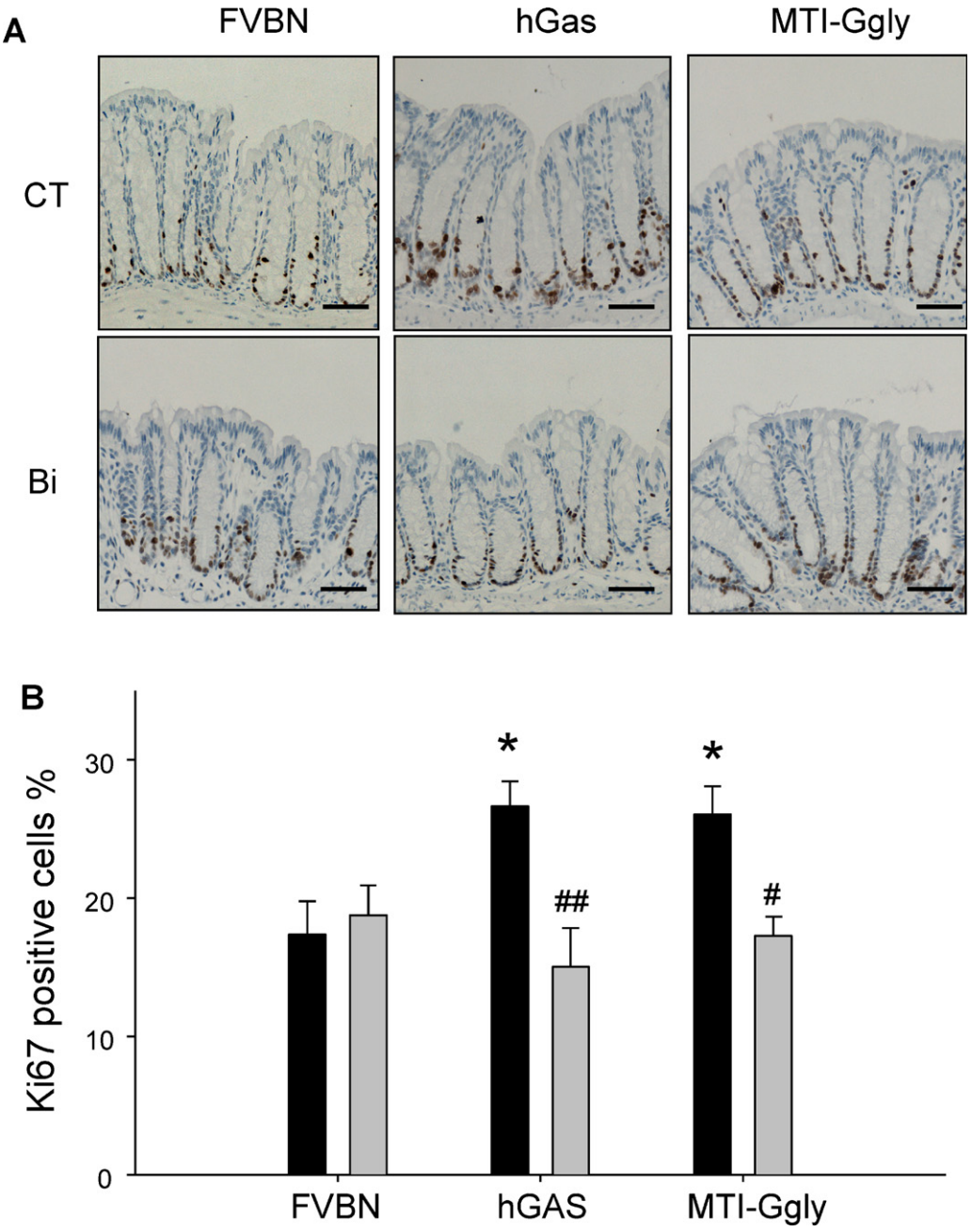

Fig. 3. Bismuth ions inhibit proliferation in the colorectal mucosa of mice over-expressing non-amidated gastrins. (A) Representative sections from the colons of control FVB/N mice, hGAS mice over-expressing progastrin, or MTI/Ggly mice over-expressing Ggly which had been treated with PBS or bismuth ions were stained with an antibody against the proliferation marker, Ki67. Images were taken at 20× magnification, and the scale bars represent 50 μm. (B) The proliferation index in hGAS mice overexpressing progastrin was significantly greater than in FVB/N mice ($^{*}p < 0.05$). The proliferation index in hGAS and MTI-Ggly mice was significantly reduced following treatment with bismuth ions (gray bars) compared with treatment with PBS (black bars) ($\#\#p < 0.01$). Bismuth ions had no significant effect on the proliferation index in wild-type FVB/N mice. The proliferation index represents the number of Ki67-positive cells expressed as a percentage of the total number of cells per crypt for a total of 20 crypts per section. Data are means ± SEM, where $N = 8$. Statistical significance was determined by *t*-test with Bonferroni's correction.

the Ggly-bismuth complex can still bind two ferric ions, and hence implies that the bismuth and ferric ion binding sites are not identical. Such a difference in binding site is not unexpected given the large difference in ionic radius between the bismuth (117 pm) and ferric (69 pm) ions. One possible explanation for the involvement of glutamates 7, 8 and 9 in both binding sites, as observed by NMR spectroscopy [29], is that at least one of the glutamates may simultaneously ligate both a bismuth and a ferric ion, in the same way that glutamate 7 has been proposed to simultaneously ligate both of the two bound ferric ions in the GglyFe2 complex [29]. However, further structural work will be required to establish the exact ligation of the bismuth ion in the GglyBi complex.

In our previous study of the inhibition of the biological activity of Ggly by bismuth ions *in vitro* the data was interpreted to favour competitive inhibition [29]. This interpretation was based on the fact that the straight lines on a secondary plot intercepted above the ordinate, rather than on the ordinate as would be expected for mixed inhibition (see Fig. 5C in [29]). Re-examination of the original data indicates that the intersection is only slightly above the ordinate and hence, after allowance for experimental error, is not inconsistent with mixed inhibition. Since the spectroscopic

Table 2
Bismuth ions increase circulating progastrin concentrations in hGas mice.

Strain	[Ggly] (pM)		[Human progastrin] (pM)	
	+PBS	$+Bi^{3+}$	+PBS	$+Bi^{3+}$
hGas	610 ± 360	910 ± 390	1140 ± 340	6020 ± 1800*
MTI/Ggly	34 ± 3	33 ± 3	NP	NP
FVBN	37 ± 3	21 ± 2**	NP	NP

Circulating gastrin concentrations in mice over-expressing non-amidated gastrins were measured by radioimmunoassay as described in Section 2. Ggly or human progastrin concentrations in bismuth-treated and PBS-treated mice were compared statistically by *t*-test with Bonferroni's correction. NP, not present, as human progastrin is only present in the hGAS mice.
* $p < 0.05$.
** $p < 0.01$.

data presented in Fig. 1 are clearly in favour of mixed inhibition, we conclude that the GglyBi complex adopts a different conformation from the GglyFe complex and is therefore not recognized by the Ggly receptor.

The ability of bismuth ions to block the proliferative effects of Ggly *in vivo* was first investigated in the rectal mucosa in rats with defunctioned recta. In this model the diversion of the faecal stream reliably induces an epithelial hypoplasia in the defunctioned rectal mucosa so that the trophic effects of administered growth factors are maximized. As observed previously [23,30], rats treated with Ggly alone showed a more than 3-fold increase (318% of control) in rectal mucosal proliferation. Treatment with bismuth ions was sufficient to inhibit completely the stimulatory effects of Ggly, as proliferation in those animals receiving oral bismuth together with Ggly was reduced to control values ($p < 0.001$).

Bismuth ions also blocked the proliferative effects of both progastrin and Ggly in the colorectal mucosa of transgenic mice overexpressing progastrin and Ggly. As observed previously [17,23], the percentage of proliferating cells was higher in the colorectal mucosa of transgenic mice overexpressing progastrin or Ggly than in wild-type controls. In both transgenic strains treatment with bismuth ions significantly inhibited the growth factor activities of the non-amidated gastrins, in support of the findings in the rat defunctioned rectum model. Our current results confirm our previous conclusion, based on the observation that the iron chelator DFO inhibited the biological activity of progastrin [23], that progastrin, like Ggly, requires ferric ions for biological activity. Thus, although different receptors have been proposed for Ggly [25,26] and progastrin [27,28], the proliferative effects of Ggly and progastrin in the colon appear to be mediated by a common receptor.

At first glance the ability of bismuth ions to block the proliferative effects of both progastrin and Ggly in the colorectal mucosa may seem to be inconsistent with the observations that treatment with bismuth reduced acid-induced colitis in rats [4,5]. However progastrin and Ggly, although they have little direct effect on gastric acidity [14], have both been shown to potentiate Gamide-induced acid secretion [15]. Presumably bismuth ions, which inhibit Ggly-induced proliferation and migration in cell lines *in vitro* [29], will also inhibit the potentiation of acid secretion by Ggly, and will thereby reduce the induction of ulcerative colitis by acid. Hence the inhibitory effect of bismuth ions on Ggly-induced proliferation in the colonic mucosa (Figs. 2 and 3) may not be apparent.

In the hGas mice overexpressing progastrin, bismuth treatment produced a further 5-fold increase in circulating concentrations of progastrin (Table 2). No significant change in circulating Ggly concentration was observed in the hGas mice, although in bismuth-treated wild-type mice the circulating Ggly concentration was significantly decreased. These observations suggest that, as previously reported for ferric ions [35], the binding of bismuth ions to progastrin inhibits processing during passage through the secretory pathway. Unfortunately due to the lack of a mouse-specific progastrin antibody we were unable to measure the circulating progastrin concentrations in wildtype or MTI-Ggly mice.

Bismuth ions are extremely effective as inhibitors of the biological activity of non-amidated gastrins. Thus in all three animal models bismuth ions are able to reverse completely the simulatory effects of Ggly and progastrin (Figs. 2 and 3). Interestingly in the hGas mice reversal was complete despite the significant increase in the circulating concentration of progastrin after bismuth treatment. This potency may at first sight appear inconsistent with the K_d of 53 nM for the GglyBi complex (Table 1), but it should be pointed out that the pH of the spectroscopic experiments was 4.0. The apparent K_i value determined in previous proliferation experiments at pH 7.6 *in vivo* was 0.29 nM [29].

Together with its safe dosing, antioxidant and mucosal healing qualities, bismuth is a good candidate for an all-round gastrointestinal protective agent. The present study further suggests that bismuth selectively inhibits the proliferative effects of non-amidated gastrins in the normal colorectal mucosa of both rats and mice. More work will be required to explore the possibility of using bismuth salts as therapeutic agents to block the proliferative effects of gastrins in colorectal cancer. At the very least, bismuth could slow the proliferation of established colorectal cancers and, at best, may inhibit progression through the adenoma-carcinoma sequence.

Acknowledgements

We thank Chelsea Dumesny and Mildred Yim for skilled assistance with the mice and radioimmunoassays, respectively. This work was supported by grant 5 RO1 GM065926-08 from the National Institutes of Health (to GB, AS), grants 400062 and 566555 (to GB, AS), 208926 (to GB) and 350235 (to AS) from the National Health and Medical Research Council of Australia.

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