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Gastric Enterochromaffin-Like Cell Hyperplasia and Neoplasia in the Rat: An Indirect Effect of the Histamine H_2-Receptor Antagonist, BL-6341*

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ABSTRACT

Oral administration of BL-6341 hydrochloride, a long-acting histamine H_2-receptor antagonist, to rats for 2 years at doses of 10, 55 or 300 mg/kg/day resulted in several changes in the fundic (oxyntic) mucosa of the glandular stomach. The most significant alteration was a proliferation of argyrophil endocrine cells that was demonstrated to be enterochromaffin-like (ECL) cells. The ECL cell proliferation consisted of a continuum of changes involving diffuse hyperplasia, focal adenomatous hyperplasia, and carcinoid tumor formation at the highest dose level of 300 mg/kg. At 55 mg/kg only ECL cell hyperplasia occurred, and at the low dose of 10 mg/kg there were no remarkable proliferative changes. The reference compound, cimetidine (950 mg/kg), produced a degree of ECL cell proliferation that was slightly less, but not significantly different than, that observed with 55 mg/kg of BL-6341. Dose-related elevations of serum gastrin were observed with BL-6341, while cimetidine produced hypergastrinemia that was generally intermediate between that produced by the middle and low doses of BL-6341. The hypergastrinemia resulted from the pharmacologic inhibition of acid secretion, which is the negative feedback mechanism controlling the production of gastrin. Only the 300 mg/kg dose of BL-6341 produced a significant, sustained (24 hours) hypergastrinemia and carcinoid tumors. The chronic, sustained hypergastrinemia was considered to be the primary cause of the ECL cell carcinoid neoplasia. All genetic toxicology tests performed with BL-6341 were negative. It was concluded that the demonstrated hypergastrinemia represents an indirect, hormonal, epigenetic mechanism of tumorigenesis.

INTRODUCTION

The toxicologic testing of new, potent, long-acting histamine H_2-receptor antagonists and H^+, K^+-ATPase inhibitors has resulted in several types of proliferative gastric changes in chronic rat studies. The most noteworthy of these is the occurrence of gastric carcinoid tumors in the oxyntic mucosa (2, 18, 34). These proliferative endocrine changes are distinct from those produced by the gastric carcinogen, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), that produces adenocarcinomas primarily in the pyloric region of the stomach (28). The histamine H_2-blocker, tiotidine, has produced a dysplasia/carcinoma syndrome in rats that resembles the lesions associated with MNNG (38).

This report describes the occurrence of proliferative, gastric, endocrine cell changes in the fundic stomach of rats administered the long-acting H_2-antagonist, BL-6341 hydrochloride. It discusses the significance of these lesions, proposes a mechanism of tumorigenesis, and compares these observations with similar findings associated with other anti-secretagogues and those reported in man.

METHODS

Two-Year Rat Study. The carcinogenicity study was composed of 60 male and 60 female Charles River, Sprague-Dawley CD rats per dose group, and 110 male and 110 female negative controls. Rats were 8 weeks of age when the study was initiated. They were administered either BL-6341 hydrochloride by daily gavage at either 10, 55 or 300 mg/kg, cimetidine at 950 mg/kg, or water (control). BL-6341 is a long-acting histamine H_2-receptor antagonist (11, 15) with the chemical name 3-amino-4-[2-[(2-guanidinothiazol-4-yl)methylthio], ethylamino]-1,2,5-thiadiazole 1-oxide (11). At 1 year 10 male and 10 female rats in each group were nec-

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ropsied, except for cimetidine-treated rats. No cimetidine rats were necropsied at this time due to the large number of gavage-related deaths in this group. At approximately 2 years (104–106 weeks) all surviving rats were necropsied. All rats dying during the study or sacrificed in moribund condition were also necropsied. Necropsies consisted of a complete post-mortem examination with special emphasis on the stomach.

The stomachs were removed, opened along the greater curvature, washed in saline, examined closely, weighed, and pinned to a corkboard. Each was subsequently placed in a separate jar of neutral, buffered, 10% formalin. Any abnormal findings were recorded, and sections of all gross lesions, in addition to routine stomach samples from each rat, were prepared for histologic examination. Samples were taken from 1 area of the forestomach, 3 areas of the fundic (oxyntic) stomach, and 3 areas of the pyloric stomach.

Stomach sections were embedded in paraffin and all sections were stained with hematoxylin and eosin (H&E). In addition, the following special staining techniques were performed on selected samples: Grimelius (19) and Sevier-Munger (36) argyrophil silver stains, Fontana-Masson (31) argentaffin silver stain, periodic acid-Schiff (PAS) and alcian blue at pH 2.5.

As part of the stomach examination, the thickness of the fundic mucosa was measured microscopically with an ocular micrometer. The density of argyrophil endocrine cells in the inner portion of the fundic mucosa was evaluated by counting the number of cells with argyrophil granules that was observed in the field of view of a Zeiss Photomicroscope I, using a 40× objective and a 12.5× ocular. The base of the visual field was aligned with the base of the fundic mucosa prior to the determination of argyrophil cell densities. Measurements and density determinations were conducted in a blinded manner. The peroxidase-antiperoxidase (PAP) technique was used to examine gastric endocrine cells for the presence of histamine. The gastric specimens examined in this manner were from high dose and control rats in the 2-year study. Samples were fresh frozen in isopentane/acetone (1:1) at −70°C and transported in dry ice to R. Håkanson, in Lund, Sweden, for performance of the PAP stain. This examination utilized antibodies raised against a conjugate of histamine and bovine serum albumin.

The electron microscopic examination was conducted on formalin-fixed gastric tissue from Rat #106 that contained a carcinoid tumor. The tissue was post-fixed in osmic acid and embedded in epoxy resin, sectioned, and stained with uranyl acetate and lead citrate.

At the end of the 2-year carcinogenicity study, serum gastrin concentrations were determined on serum samples collected at time of necropsy, 23–30 hours after drug administration. Samples were examined from 15 male and 15 female rats from each group unless the number of rats surviving was less than this.

One-Month Gastrin Studies. In the 1-month gastrin studies that followed the carcinogenicity study, male Sprague-Dawley CD rats were administered, by daily gavage, either BL-6341 at 300, 55 or 10 mg/kg, cimetidine at 950 mg/kg, or water (control). This testing comprised 2 separate studies. Rats were approximately 15 weeks of age at initiation of studies. Serum gastrin concentrations were determined by collecting 1 ml of blood from 10 different rats in each group at 4, 12, and 24 hours after gavage dosing. These determinations were performed on day 1 and at approximately 2 and 4 weeks of study. At termination of the study, 10 rats in each group were necropsied and stomachs were examined histopathologically as previously described. (Serum gastrin analyses from the 1-month studies were performed by Hazleton Laboratories in Vienna, Virginia.)

Statistical analyses of stomach and body weights, serum gastrin concentrations, and gastric anatomical observations were carried out separately by sex in each study, using 1-way analysis of variance (ANOVA) procedures. For those studies evaluating several doses of the same drug, a linear contrast was set up in the ANOVA model to test for the significance of a dose-related trend. Comparisons between drug-treated and control groups were carried out by computing Dunnett t-statistics when group sizes were equal, or by computing t-statistics using the Bonferroni adjustment when group sizes were not equal. Comparisons among the drug-treated groups were carried out by computing Tukey or Tukey-Kramer t-statistics. Each set of (two-tailed) comparisons was performed at the 5% significance level.

Genetic Toxicology Studies. BL-6341 was tested for mutagenic and genotoxic potential in a series of *in vitro* and *in vivo* short-term genetic toxicology studies (Bristol-Myers Company, unpublished reports). Included in this battery of tests were an Ames/*Salmonella* microbial mutagenicity assay, DNA repair assays in human diploid fibroblasts and primary rat hepatocytes, a mammalian cell gene mutation assay in human lymphoblasts at TK and HGPRT loci, and an *in vivo* cytogenetics study in Chinese hamster bone marrow cells. In addition, an *in vivo* DNA binding study was performed to determine the potential of BL-6341 to interact with or bind to DNA from rat stomach epithelial cells following oral administration.

TABLE I.—Characteristics of carcinoid tumors in the fundic mucosa of rats in the carcinogenicity study.

Compound (mg/kg)	Rat no.	Sex	Fate[a]	Lesion size (mm)	Locally invasive
BL-6341	8	M	ST	1.0	No
(300)[b]	19	M	ST	0.7	No
	36	M	DW	2.0[c]	Yes
	85	F	ST	1.1	No
	106	F	ST	2.0[c]	Yes

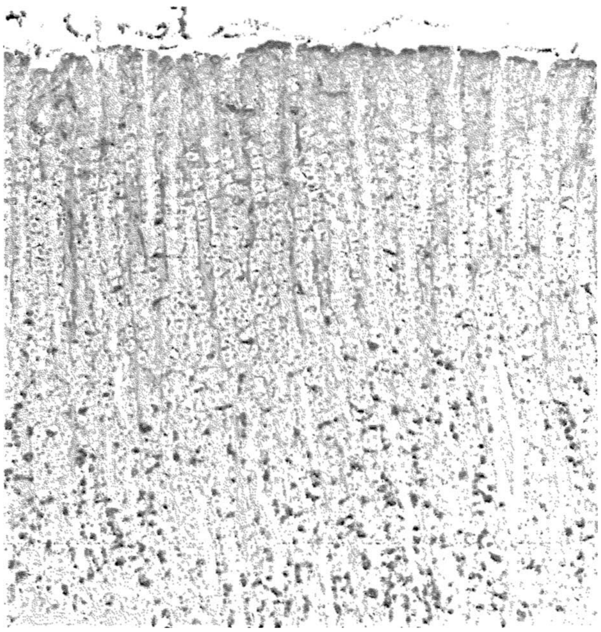
[a] ST: sacrificed at termination (weeks 104–105); DW: died on week 96.
[b] This dose represents a 420 multiple of the clinical dose.
[c] Lesion observed at necropsy.

RESULTS

Two-Year Rat Study. The treated rats remained in good general health throughout the 2-year test period. Lower mean body weight in the BL-6341 high dose rats and the cimetidine-treated rats was the only drug-associated clinical observation. Deaths occurred in 47/100, 52/100 and 55/100 high, intermediate, and low dose rats, respectively, that received BL-6341; and in 99/120 cimetidine-dosed and 111/200 of the negative control rats. Many of these deaths were considered to be due to pulmonary irritation, which was gavage-related, especially in the cimetidine-treated rats. The marked pulmonary irritation liability of cimetidine, when administered at high doses to rats by gavage, is well-documented (30).

Gross examination of stomachs at necropsy revealed 2 focal lesions in high dose rats that were subsequently demonstrated to be drug-related changes by histopathologic examination. These consisted of a 2-mm ulcer in the fundic mucosa of Rat #36 and a 2-mm focal thickening in the fundic mucosa of Rat #106.

Microscopic examination of the stomachs revealed several findings considered to be drug-related, along with a number of spontaneous changes. The most significant drug-related observations in the stomachs of rats administered BL-6341 were the prominent endocrine cell proliferations and the carcinoid tumors in the fundic portion of the gastric mucosa. Carcinoids were observed in 5/100 rats in the high dose (300 mg/kg) group. No gastric carcinoids were seen in rats at either lower dose (55 or 10 mg/kg) of BL-6341, or with cimetidine or the negative controls (Table I). Four of the 5 rats with carcinoids were sacrificed at the end of the study at



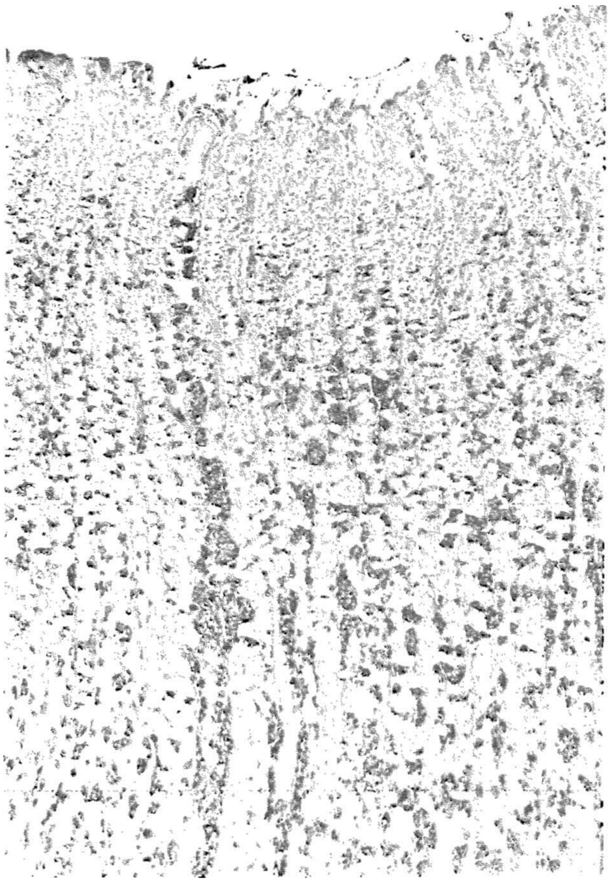
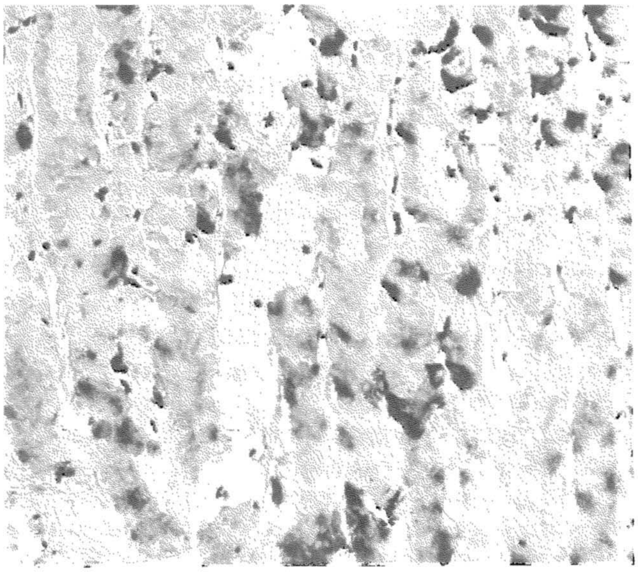
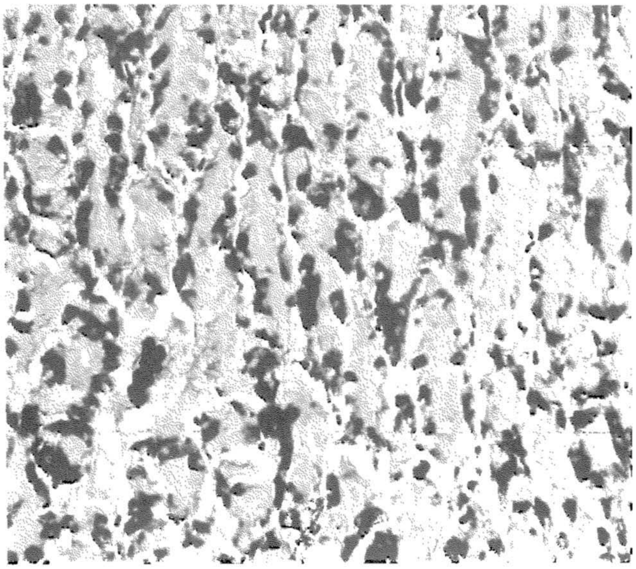


FIG. 1.—(Left) Dark-staining argyrophil cells diffusely scattered throughout basal half of fundic mucosa of a control rat at 2 years. Grimelius stain. ×125.

FIG. 2.—(Right) Prominent increase in argyrophil cells throughout all portions of thickened mucosa, except foveolae, of a rat receiving BL-6341 at 300 mg/kg for 2 years. (Compare to Fig. 1.) Grimelius stain. ×125.





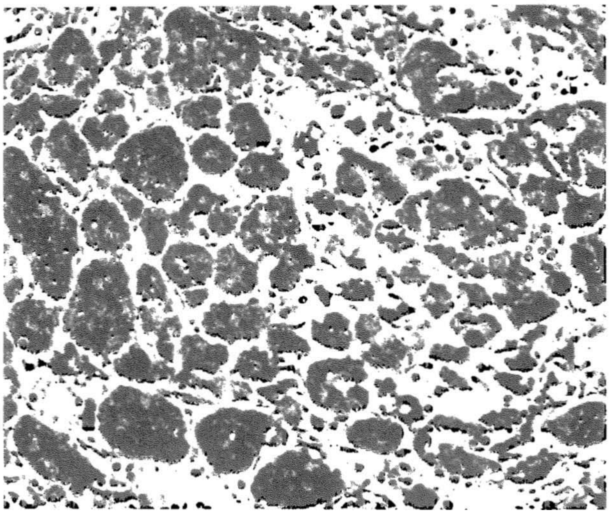


FIG. 3.—(Left) Dark-staining, histamine immunoreactive ECL cells in basal half of fundic mucosa of control rat at 2 years. PAP stain. ×250.

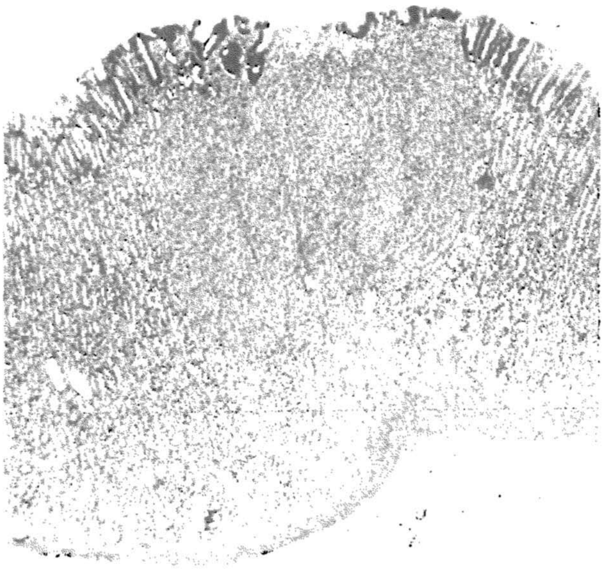
FIG. 4.—(Right) Marked increase in number of histamine immunoreactive ECL cells in fundic mucosa of a rat receiving BL-6341 at 300 mg/kg for 2 years. (Compare to Fig. 3.) PAP stain. ×250.

FIG. 5.—(Bottom) Focal, adenomatous hyperplasia of dark-staining ECL cells in fundic mucosa of high dose (300 mg/kg) rat at 2 years. Sevier-Munger stain. ×250.

week 104 or 105. One of the 5 rats died at week 96 of causes unrelated to the small gastric carcinoid that was observed at necropsy (Table I).

The hyperplastic and neoplastic cells were composed of true argyrophil endocrine cells since their secretory granules stained positively with the Grimelius and Sevier-Munger argyrophil stains and negatively with the Fontana-Masson argentaffin stain. The results of these silver stains, especially the Sevier-Munger technique, suggested that the carcinoid tumors were composed of enterochromaffin-like (ECL) cells (20, 22, 23). The immunoperoxidase technique demonstrated the presence of histamine in the proliferated endocrine cells in the fundic mucosa. The ECL cell is the only gastric endocrine cell in the rat stomach that contains histamine (22, 23), and these cells are found exclusively in the fundic (oxyntic) mucosa.

The carcinoid tumors in this study were part of a larger spectrum of endocrine cell alterations in the fundic mucosa. This spectrum consisted of a continuum of proliferative ECL cell changes, ranging from diffuse hyperplasia to focal, adenomatous hyperplasia, to carcinoid tumor formation. The diffuse hyperplasia was demonstrated by uniform proliferations of argyrophil cells (Figs. 1, 2) and cells immunoreactive for histamine (Figs. 3, 4). In a number of high dose rats, these ECL cell proliferations took



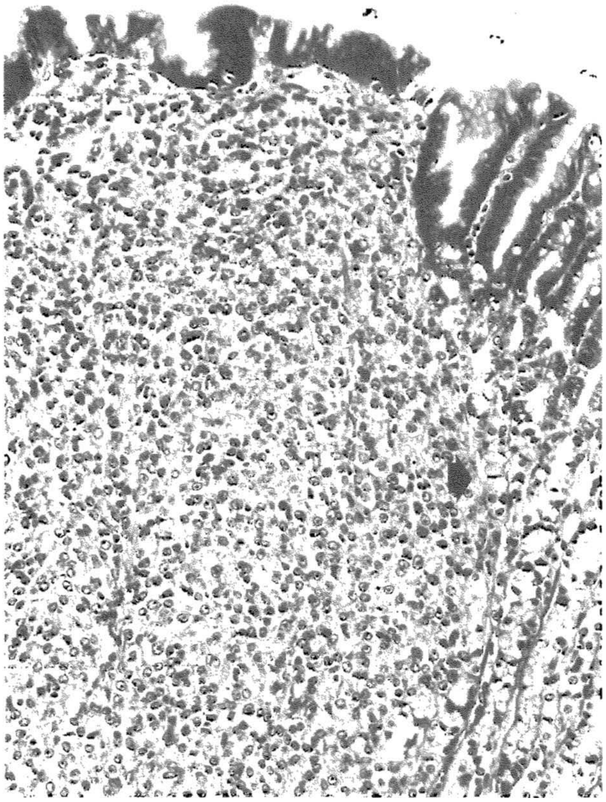


FIG. 6.—(Top) Intramucosal carcinoid extending from base to surface of the mucosa in a high dose (300 mg/kg) rat. H&E stain. ×30.

FIG. 7.—(Bottom) Lateral border (arrow) of intramucosal carcinoid in Fig. 6 and compression of overlying foveolae. H&E stain. ×250.

the form of focal or adenomatous hyperplasia (Fig. 5). This type of hyperplasia occurred in rats with and without carcinoids. Some degree of ECL cell proliferation was observed in all high dose rats sacrificed at the termination of the 2-year study. In many of the high dose rats, the ECL cell hyperplasia was prominent enough that it could be recognized with routine hematoxylin and eosin (H&E) staining, wherein the ECL cells had pale-staining cytoplasm.

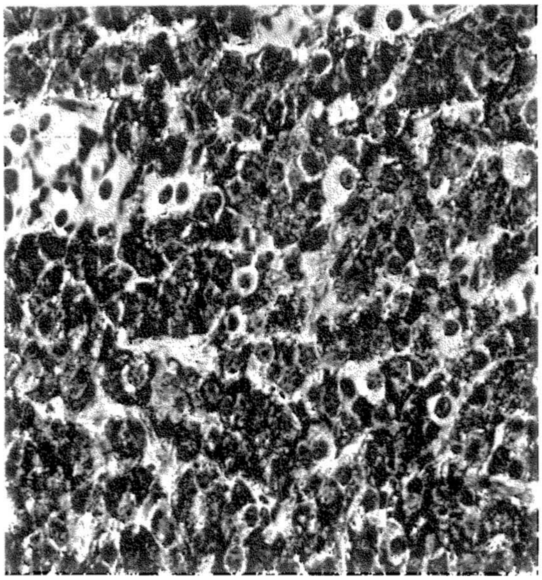
In 2 high dose rats, foci of argyrophil endocrine cells, that had no apparent association with intramucosal carcinoids, had penetrated beneath the muscularis mucosae. One of these rats did have an intramucosal carcinoid in an unrelated, distant area of the fundic mucosa, while only prominent, focal ECL cell hyperplasia was detected in the mucosa of the second rat.

Three of the 5 carcinoids in the high dose rats were limited to the fundic mucosa (Table I). They consisted of small foci (<2 mm) of neoplastic ECL cells which extended from the base of the mucosa to the foveolae (Fig. 6). The borders of the carcinoids were well-demarcated from the more normal glandular tissue (Fig. 7), and the cytoplasm of neoplastic cells was pale-staining with H&E stain. Grimelius or Sevier-Munger silver stains demonstrated each tumor to be composed of argyrophil endocrine cells (Fig. 8). The 3 intramucosal carcinoids appeared to be well-differentiated, and prominent argyrophil granules were demonstrated within the cytoplasm. As a group, these carcinoids presented somewhat different morphologic patterns, including solid (Fig. 9), acinar (Fig. 10), and mixed solid and trabecular.

In 2 rats, the carcinoid tumors penetrated the muscularis mucosae, resulting in local invasion of the submucosa (Table I). In one, the carcinoid appeared well-differentiated, with the cellular pattern consisting mostly of nests of plump, oval cells located on prominent basement membranes (Fig. 11). The cytoplasm of cells in this carcinoid stained very lightly with H&E, but stained intensely with argyrophil silver stains (Fig. 12).

Only 1 carcinoid caused ulceration of the overlying mucosa (Fig. 13) and invasion of the muscularis mucosae (Fig. 14). This carcinoid had a trabecular pattern. The cytoplasmic granules within the ECL cells were smaller, less numerous, and stained less intensely with argyrophil stains than did those in the other carcinoids. This difference was considered to be due to the lesser degree of differentiation of this neoplasm. Similar observations in regard to degree of differentiation and granule size and prominence have been made on malignant gastric neuroendocrine tumors in man (37). Even though some carcinoids penetrated the muscularis mucosae and invaded the submucosa, no distant metastases were observed.

Electron microscopic examination demonstrated that the proliferated and neoplastic cells were endocrine cells which contained prominent, vesicular,



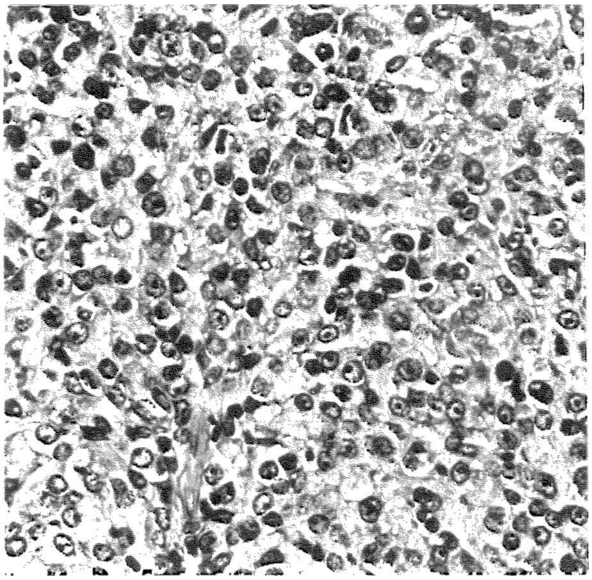


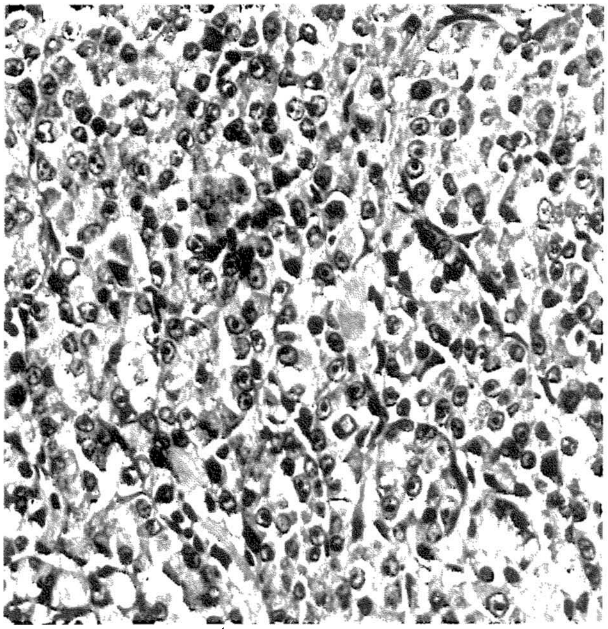
FIG. 8.—(Left) Intramucosal carcinoid demonstrating strong argyrophil reaction (dark stain) of cytoplasmic granules. Sevier-Munger stain. ×500.

FIG. 9.—(Right) Solid pattern of pale-staining, neoplastic ECL cells in an intramucosal carcinoid. H&E stain. ×500.

secretory granules (Fig. 15). The granules had a wide, clear halo around an electron-dense core, that was variable in shape and often eccentrically located. Nuclei contained prominent nucleoli and well-stained chromatin, but were otherwise unremarkable. These findings are consistent with those described for ECL cells in rats (20, 22, 26) and in man (5, 12, 27, 37).

Although there were no carcinoid tumors at 55 mg/kg of BL-6341 or in the rats receiving the reference compound (cimetidine at 950 mg/kg), proliferations of argyrophil endocrine cells could be detected in these groups at both 1 and 2 years by use of appropriate silver stains. At 1 year, the argyrophil endocrine cell increase was significantly greater than in controls at both 300 and 55 mg/kg of BL-6341 (Table II). The argyrophil cell density for the 10 mg/kg group was slightly higher than, but not significantly different from, controls. No cimetidine-treated rats were examined at 1 year, due to the higher mortality rate for this group.

Evaluation of argyrophil cell density at 2 years



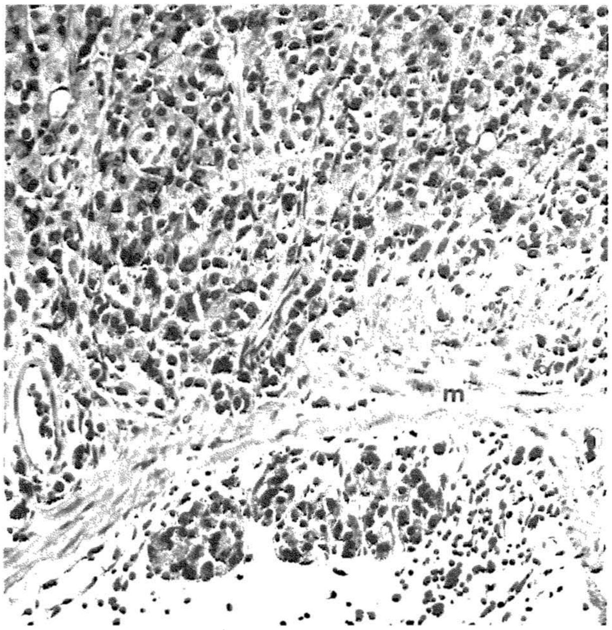
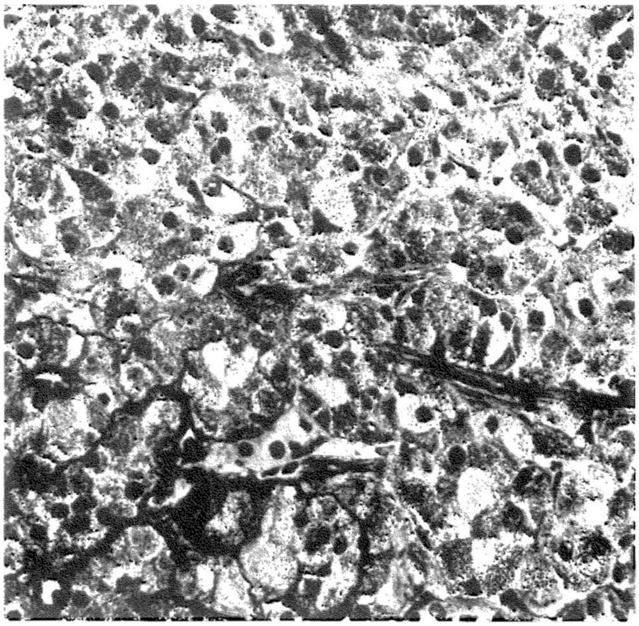


FIG. 10.—(Left) Intramucosal carcinoid demonstrating an acinar pattern of neoplastic ECL cells. H&E stain. ×500.

FIG. 11.—(Right) Invasive mucosal carcinoid demonstrating foci of neoplastic cells that have penetrated through the muscularis mucosae (M) and incited a mild inflammatory cell response. Grimelius stain. ×250.



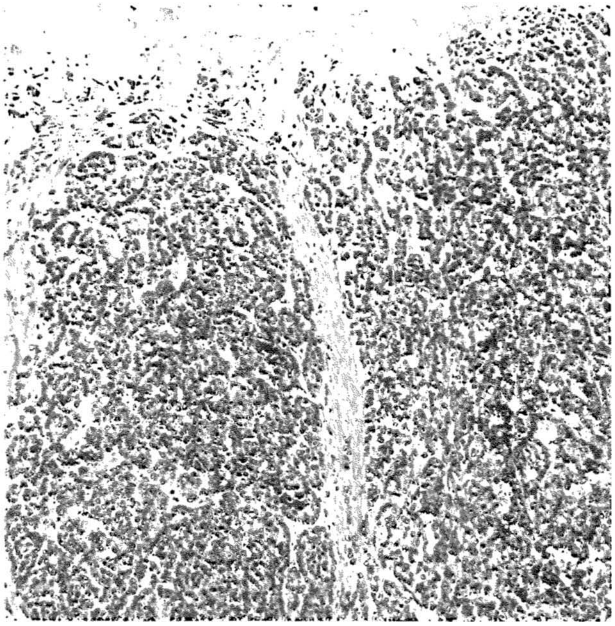


FIG. 12.—(Left) Prominent staining of argyrophil cells in an invasive carcinoid. Cells are arranged in a nest-like pattern with prominent basement membranes. Sevier-Munger stain. ×500.

FIG. 13.—(Right) Ulceration of fundic mucosa by a more undifferentiated carcinoid. Trabecular pattern and collagenous intracarcinoid septae are recognizable. H&E stain. ×125.

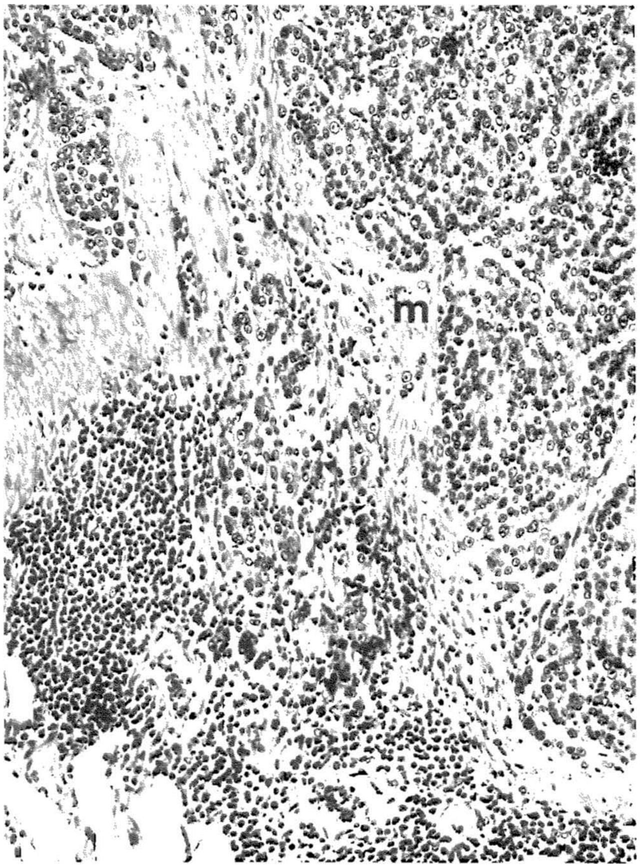


FIG. 14.—Invasion through muscularis mucosae (M) by carcinoid in Fig. 13. Neoplastic foci in submucosa have elicited a prominent, mononuclear cell inflammatory response. H&E stain. ×250.

showed similar findings to those at 1 year at 300 and 55 mg/kg of BL-6341 (Table II, Fig. 16). Cimetidine-treated rats also had a significant increase in argyrophil cell density at 2 years compared to controls. This increase was slightly less than, but not significantly different from, that associated with 55 mg/kg of BL-6341. The argyrophil cell density for cimetidine was significantly less than that produced by 300 mg/kg of BL-6341. At both 1 and 2 years there was also a dose-related trend ($p < 0.01$) for

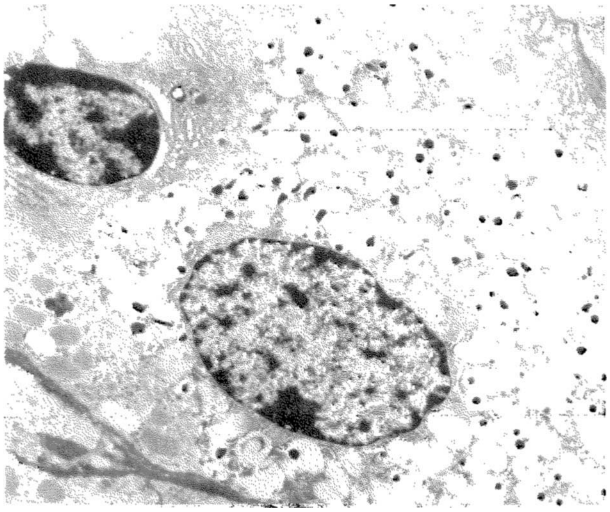


FIG. 15.—Ultrastructural appearance of a carcinoid cell showing many vesicular, secretory granules with wide, clear halos and electron dense cores. Uranyl acetate and lead citrate stain. ×14,400.

TABLE II.—Effect of BL-6341 or cimetidine on argyrophil cell density in fundic mucosa from rats in a carcinogenicity study.

		Argyrophil cell density[a]				
Time	Sex	Vehicle control	BL-6341 (10 mg/kg)	BL-6341 (55 mg/kg)	BL-6341 (300 mg/kg)	Cimetidine (950 mg/kg)
1 Year	M	98 ± 20	114 ± 17[b]	134 ± 18[b,c]	184 ± 25[c,d,e]	—
	F	111 ± 19	133 ± 20[b,d]	169 ± 12[b,c,e]	211 ± 11[c,d,e]	—
2 Years	M	126 ± 24	130 ± 19[b,d,f]	186 ± 18[b,c,e]	217 ± 13[c,d,e,f]	163 ± 24[b,c,e]
	F	152 ± 16	150 ± 20[b,d,f]	202 ± 21[b,c,e]	254 ± 37[c,d,e,f]	203 ± 28[b,c,e]

[a] Values are group means ± SD of the number of argyrophil cells observed in the field of view using a 40× objective and 12.5× ocular; n: 5 rats (1 year) or 10 rats (2 years), except cimetidine males (2 years) n:4. No cimetidine-treated rats were sacrificed at 1 year. Three fields per rat were examined and the mean value was used for each individual rat to generate the group means.
[b] Significantly different from BL-6341 at 300 mg/kg ($p < 0.05$).
[c] Significantly different from vehicle control ($p < 0.05$).
[d] Significantly different from BL-6341 at 55 mg/kg ($p < 0.05$).
[e] Significantly different from BL-6341 at 10 mg/kg ($p < 0.05$).
[f] Significantly different from cimetidine at 950 mg/kg ($p < 0.05$).

the ECL cell density increases associated with BL-6341.

Two other types of gastric epithelial cells appeared to be slightly increased in number, although they were not directly quantitated. The number of parietal and chief cells within the oxyntic mucosa of rats in the carcinogenicity study was presumed to be increased at the two highest dose levels, since their densities appeared relatively unchanged while the mucosal area they occupied was significantly greater than normal due to an increase in mucosal thickness. Therefore, the increases in parietal and chief cells constituted part of the increased thickness of the oxyntic mucosa observed at 1 month and 2 years (Table III), along with the quantitated increase in argyrophil endocrine cells (Table II). Unlike the ECL cells, the parietal and chief cells did not extend into regions of the mucosa where they are not normally found.

Another drug-related change in the fundic stomach was the occurrence of altered chief cells. These consisted of markedly hypereosinophilic, slightly hypertrophic, chief cells in generally normal locations within the fundic mucosa. These cells tended to line the base of the gastric glands, but also were occasionally found in the central portion of the mucosa. The incidence of this change at the 300 mg/kg dose of BL-6341 was 30% at 3 months, 90% at 1 year, and 41% at 2 years. The change, therefore, was not progressive with continued dosing and was also demonstrated to be reversible in the 3-month study. No drug-related association with gastric ulceration, hyperkeratosis, intestinal metaplasia, cystic glandular dilatation or mucosal, mononuclear cell infiltrations could be ascertained, since these findings were noted with approximately equal frequency in treated and control rats.

The serum gastrin concentrations, which were determined at 2 years, at 23–30 hours after drug administration, demonstrated an increase of 116% above controls in the female rats administered 300 mg/kg of BL-6341. Other groups did not show significant increases above controls. Since these samples had not originally been intended for serum gastrin evaluation, they were not handled in an optimum manner for this analysis. Therefore, special 1-month serum gastrin studies were subsequently conducted to further investigate this question.

One-Month Rat Studies. Evaluation of serum gastrin concentrations at 4, 12, and 24 hours postdose in the first rat gastrin study, which compared 300 mg/kg of BL-6341 to 950 mg/kg of cimetidine, showed that BL-6341 at 300 mg/kg consistently produced significant increases in gastrin concentrations when compared to controls (Table IV, Fig. 16). These differences remained significant for all 3 test periods, resulting in a consistently sustained (24 hours) hypergastrinemia. Cimetidine, at 950 mg/kg, produced 4-hour serum gastrin values that were sig-

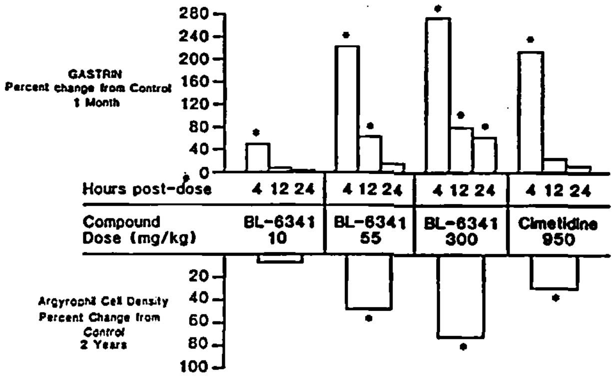


FIG. 16.—Comparison of 1-month serum gastrin concentrations to 2-year argyrophil cell densities in basal fundic mucosa of male rats. Values based on means of 10 rats. (*) Significant differences from controls $p < 0.05$.

TABLE III.—Fundic mucosal thickness in rats administered BL-6341 or cimetidine for 1 month or 2 years.

Time	Sex	Mucosal thickness in micrometers[a] Vehicle control	BL-6341 (10 mg/kg)	BL-6341 (55 mg/kg)	BL-6341 (300 mg/kg)	Cimetidine (950 mg/kg)
1 Month	M	556 ± 21	559 ± 15[b]	579 ± 15	591 ± 31[c,d,e]	567 ± 20[b]
2 Years	M	638 ± 36	652 ± 62[b,d,f]	739 ± 59[b,c,d,e]	1,008 ± 82[c,d,e,f]	653 ± 33[b,c,f]
	F	634 ± 61	624 ± 36[b,f]	736 ± 93[b,c,e]	976 ± 112[c,d,e,f]	660 ± 73[b]

[a] Values are group means ± SD of 10 rats (BL-6341) or 20 rats (cimetidine, vehicle control), since 1-month study I and II data were combined.
[b] Significantly different from BL-6341 at 300 mg/kg ($p < 0.05$).
[c] Significantly different from vehicle control ($p < 0.05$).
[d] Significantly different from cimetidine at 950 mg/kg ($p < 0.05$).
[e] Significantly different from BL-6341 at 10 mg/kg ($p < 0.05$).
[f] Significantly different from BL-6341 at 55 mg/kg ($p < 0.05$).

nificantly greater than controls, but the 12-hour and 24-hour post-dose gastrin concentrations were comparable to controls after study day 14–15. The 4-hour serum gastrin concentrations for cimetidine were significantly lower than those for 300 mg/kg of BL-6341 on all test days. The 12- and 24-hour gastrin concentrations for cimetidine were significantly lower than for 300 mg/kg of BL-6341 at both 2 and 4 weeks.

In the second 1-month gastrin study, which compared the lower doses of BL-6341 to cimetidine, serum concentrations were highest and significantly increased over controls 4 hours after dosing in all treated groups (Table IV). Gastrin concentrations decreased proportionally with additional time after dosing (Fig. 16). BL-6341 at 55 mg/kg and cimetidine at 950 mg/kg produced generally similar gastrin concentrations at 4 hours post-dose; the concentrations for cimetidine at 12 or 24 hours post-dose were intermediate between the levels for 55 and 10 mg/kg of BL-6341. A sustained (24 hours) hypergastrinemia was not produced with either 55 mg/kg of BL-6341 or with cimetidine following multiple daily dosing. The lowest dose of 10 mg/kg of BL-6341 consistently elevated serum gastrin at only 4 hours post-dose, and these concentrations were significantly lower than for the 55 mg/kg dose of BL-6341 or cimetidine.

Argyrophil cell density and mucosal thickness were evaluted in the gastric oxyntic mucosa of rats used in both of the 1-month gastrin studies, and results were correlated with serum gastrin concentrations. At doses of 300 and 55 mg/kg of BL-6341 there were significant increases in the density of argyrophil endocrine cells, considered to be ECL cells (Table V). No increase in ECL cell density was observed at 10 mg/kg of BL-6341. Cimetidine, in each of the 1-month studies, produced an increase in argyrophil cell density which was significantly different from that of controls, but not significantly different from that associated with BL-6341 at 55 or 300 mg/kg.

Evaluation of fundic mucosal thickness in the 1-month studies showed significant increases over controls with BL-6341 at 300 mg/kg, and a marginal

TABLE IV.—Effect of BL-6341 or cimetidine on serum gastrin in rats.

Study	Compound	Gastrin concentrations (pg/ml)[a] 4 hours	12 hours	24 hours
I	BL-6341 (300 mg/kg)	781 ± 130[b,c]	359 ± 180[b,c]	286 ± 109[b,c]
	Cimetidine (950 mg/kg)	549 ± 80[b,d]	224 ± 68[d]	191 ± 34[d]
	Vehicle control	207 ± 24	198 ± 38	176 ± 19
II	BL-6341 (10 mg/kg)	290 ± 64[b,c,e]	172 ± 16[e]	157 ± 21
	BL-6341 (55 mg/kg)	620 ± 196[b,f]	274 ± 178[b,f]	168 ± 13
	Cimetidine (950 mg/kg)	695 ± 96[b,f]	202 ± 30[b]	158 ± 13
	Vehicle control	192 ± 23	167 ± 15	155 ± 11

[a] Values are means ± SD of 10 rats on study day 28 or 29. Since control values were significantly different between the 2 studies, data were not combined.
[b] Significantly different from vehicle control ($p < 0.05$).
[c] Significantly different from cimetidine at 950 mg/kg ($p < 0.05$).
[d] Significantly different from BL-6341 at 300 mg/kg ($p < 0.05$).
[e] Significantly different from BL-6341 at 55 mg/kg ($p < 0.05$).
[f] Significantly different from BL-6341 at 10 mg/kg ($p < 0.05$).

TABLE V.—Effect of BL-6341 or cimetidine on argyrophil cell density in fundic mucosa in 2 rat 1-month studies.

Study	Argyrophil cell density[a] Vehicle control	BL-6341 (10 mg/kg)	BL-6341 (55 mg/kg)	BL-6341 (300 mg/kg)	Cimetidine (950 mg/kg)
I	73 ± 8	—	—	103 ± 18[b]	99 ± 11[b]
II	89 ± 8	90 ± 6[c,d]	130 ± 8[b,e]	—	123 ± 12[b,e]

[a] Values are group means ± SD of the number of argyrophil cells observed in the field of view using a 40× objective and a 12.5× ocular; n = 10 male rats. Three fields per rat were examined and the mean value used for each individual rat to generate the group means. Since control values were significantly different between the two studies, data from groups common to both studies were not combined.
[b] Significantly different from vehicle control ($p < 0.05$).
[c] Significantly different from cimetidine at 950 mg/kg ($p = 0.05$).
[d] Significantly different from BL-6341 at 55 mg/kg ($p = 0.05$).
[e] Significantly different from BL-6341 at 10 mg/kg ($p = 0.05$).

difference at 55 mg/kg. The mucosal thickness in cimetidine-treated rats in both studies was not significantly different from that in controls (Table III).

Related Studies. Gastric secretory studies with BL-6341 hydrochloride and cimetidine in chronic gastric fistula rats have demonstrated significant differences in acid inhibition at 24 hours after drug administration. BL-6341, at 300 mg/kg, produced a statistically significant reduction in acid concentration and acid output at this time point, while doses of 55 or 10 mg/kg of BL-6341 or 950 mg/kg of cimetidine did not (R. L. Cavanagh, Bristol-Myers Co., unpublished data). Therefore, only the 300 mg/kg dose of BL-6341 was associated with a significant, 24-hour gastric hypochlorhydria, which in turn was considered to be the cause of the sustained hypergastrinemia observed in rats administered this same dose (Table IV).

Genetic Toxicology Studies. The results of the genetic toxicology studies are summarized in Table VI. BL-6341 hydrochloride was negative in all of the studies performed. The results indicate that BL-6341 did not exhibit mutagenic, clastogenic or genotoxic potential in any of these test systems. These negative results support the lack of *in vivo* DNA binding observed in rat stomach epithelial cells, and indicate that carcinoid tumors observed in the stomach of rats in the 2-year carcinogenicity study most likely were not induced by a direct genotoxic mechanism.

DISCUSSION

Hypergastrinemia. The gastric proliferation of argyrophil endocrine (ECL) cells was the pathologic change of greatest importance in the 2-year carcinogenicity study with BL-6341, since it resulted in ECL cell neoplasia in the fundic mucosa. It has been well-established that hypergastrinemia will produce a generalized trophic effect on the gastric mucosa, and an increase in the activity, size and number of ECL cells in the oxyntic mucosa (13, 21–23, 29). Hypergastrinemia occurs when gastric pH is elevated, since

TABLE VI.—Genetic toxicology results with BL-6341.

Endpoint	Assay	Doses/concentrations	Results
Gene mutation	Ames/*Salmonella* microbial mutation (42)	375–3,000 μg/0.1 ml/well (±S9)[a]	negative (2 studies)
Gene mutation	Human lymphoblasts at TK and HGPRT loci (43)	800–2,400 μg/ml (−S9)[b] 1,250–5,000 μg/ml (+S9)[b]	negative negative
DNA damage/repair	DNA repair in human diploid fibroblasts (46)	375–1,500 μg/ml (±S9)[b]	negative
DNA damage/repair	DNA repair in primary rat hepatocyte cultures (47)	0.001–200 μg/ml	negative
DNA damage	DNA binding *in vivo* in rat stomach epithelium (44, 48)[c]	300 mg/kg	negative
Chromosomal aberration	*In vivo* cytogenetics in Chinese hamster bone marrow cells (45)[d]	300 mg/kg	negative

[a] With and without metabolic activation.
[b] +S9: with metabolic activation; −S9: without.
[c] ~400 μCi ^{14}C-BL-6341 per rat.
[d] The LD_{50} of BL-6341 is 561 mg/kg.

gastric (antral) acidity is the negative feedback mechanism for inhibition of gastrin release from G-cells in the pyloric antrum. Since the desired pharmacologic activity of histamine H_2-receptor antagonists is suppression of gastric acid production, these compounds produce significant hypergastrinemia, as a secondary effect, when given at very high doses in animal toxicologic studies. The duration of the elevated gastrin levels is in direct proportion to the duration of activity of the antisecretagogue administered.

At the end of the 2-year carcinogenicity study with BL-6341, serum gastrin levels collected at necropsy, 23–30 hours after drug administration, were significantly increased in the high dose BL-6341 female rats when compared to controls. This was in contrast to cimetidine-treated rats, that had serum gastrin concentrations lower than controls at the same time period after dosing. The 2 female high dose BL-6341-treated rats that had gastric ECL cell carcinoid tumors both had sustained increases of serum gastrin at the termination of the 2-year study. The 3 male high dose BL-6341 rats with carcinoids were not among those 15 male rats that had previously been randomly selected for blood chemical evaluation at termination of the study.

In a single-dose rat antisecretory study, the gastric antisecretory activity of BL-6341 at 24 hours after administration was considerably greater than for cimetidine when both were administered at the highest doses used in the rat carcinogenicity study. At this 24-hour time point there still was significant inhibition of acid secretion by 300 mg/kg of BL-6341, but no inhibition by cimetidine at 950 mg/kg. These findings are consistent with the demonstrated prolonged duration of activity for BL-6341 (15) and its classification as a long-acting H_2-receptor antagonist that produces tighter binding to H_2-receptors than does cimetidine (11). These secretory observations correlated very well with serum gastrin concentrations observed in the 1-month rat gastrin studies. In the study utilizing the highest dose of BL-6341 (300 mg/kg), serum gastrin levels were significantly greater than for cimetidine on all test days after day 1. This was true even at 4 hours after dosing, when serum gastrin concentrations for cimetidine were also increased (Table IV). Of even greater importance is the observation that only the highest dose of BL-6341 produced a consistent hypergastrinemia at 12 and 24 hours after drug administration.

At 55 mg/kg of BL-6341, serum gastrin concentrations were slightly greater, but not significantly different from, those associated with cimetidine at 950 mg/kg. Neither produced a sustained (24 hours) hypergastrinemia following multiple daily administrations; 10 mg/kg of BL-6341 produced the lowest serum gastrin levels at each test period.

Endocrine (ECL) Cell Proliferation. Since gastrin has trophic effects on rat ECL cells, the greater degree and duration of hypergastrinemia with BL-6341 explain the greater ECL proliferation (hyperplasia and neoplasia) observed in the carcinogenicity study with 300 mg/kg of this long-acting H_2-antagonist (Fig. 16). When the gastrin concentrations at lower doses of BL-6341 and for cimetidine are compared to the histologic gastric findings at these doses in the carcinogenicity study, it appears that both the degree and duration of the hypergastrinemia were important factors for argyrophil endocrine cell proliferation. Even though there were no carcinoid tumors at 55 mg/kg of BL-6341 or 950 mg/kg of cimetidine, significant increases in argyrophil cell density were observed with these doses of both compounds in the carcinogenicity study (Table II). These results indicate that the reference compound, cimetidine, when administered at approximately 56 times the human clinical dose for 2 years, elicited a trophic effect on argyrophil endocrine cells that was slightly less than, but not significantly different from, that observed with 55 mg/kg of BL-6341, which represents a dose 77 times the anticipated clinical dose. These endocrine cell proliferations were associated with transient hypergastrinemias of less than 24 hours duration, as demonstrated in the 1-month rat gastrin studies (Fig. 16). Also to be noted is the correlation between the lowest, most transient, hypergastrinemias occurring at 10 mg/kg of BL-6341, and the absence of any significant increase of ECL cells at this dose in the carcinogenicity study. The results suggest that transient hypergastrinemias of sufficient magnitude can produce some argyrophil endocrine cell proliferation but not carcinoids.

This explanation is supported by the findings in carcinogenicity studies with omeprazole (18, 26) and SKF-93479 (2) in which doses that produced sustained hypergastrinemia were associated with carcinoids. Although no serum gastrin data were reported for loxtidine (34), it is probable that a similar situation may have occurred with this long-acting H_2-antagonist.

ECL cell density in control rats in the 1-month, 1-year and 2-year studies increased with age (Tables II, V). This observation has also been reported by other investigators, and could be a factor in the delayed development of ECL cell carcinoids in rats observed in this and other studies (18, 34).

Mechanism of Tumorigenesis. In subchronic rat studies with omeprazole and ranitidine, the degree of ECL cell proliferation that was observed reflected the increases in plasma gastrin levels produced by these antisecretagogues (29, 39). If rats were antrectomized, hypergastrinemia and ECL cell increases did not occur.

In these studies antrectomy should have decreased gastric acidity, while at the same time preventing hypergastrinemia and ECL cell proliferation. These findings, therefore, indicate that the ECL cell proliferations associated with omeprazole were not a direct result of a decrease in gastric acidity, but rather were due to hypergastrinemia. This concept is further supported by gastric changes that occur in Zollinger-Ellison syndrome, in which argyrophil and ECL cell hyperplasia and carcinoid tumor formation have been documented, under conditions of gastric acid hypersecretion and concurrent hypergastrinemia (6, 9).

Further evidence for the primary role of hypergastrinemia in this proliferative process is the observation by Blom (3) that subcutaneous administration of pentagastrin to rats for 3 months at 250 μg/kg produces an increase in endocrine cell density similar to that observed after oral treatment with omeprazole at 40 μmol/kg. In light of these studies and the previously mentioned reports of carcinoid tumor association with BL-6341, SKF-93479, and omeprazole, we conclude that hypergastrinemia, rather than gastric hypochlorhydria, is the primary cause of both gastric endocrine cell proliferation and carcinoid tumor formation in rats administered very high doses of long-acting, acid antisecretagogues for 2 or more years.

Cimetidine did not produce any gastric tumors, either in this study or in a previously conducted carcinogenicity study in rats (30). The absence of a sustained elevation of serum gastrin is probably the reason why short-acting H_2-antagonists have not produced carcinoids in rat carcinogenicity studies.

The negative results of six different genetic toxicology tests conducted with BL-6341 indicate that the compound is not genotoxic, and suggest an epigenetic mechanism of carcinoid tumor formation. This is consistent with the proposed indirect, hormonal mechanism of tumorigenesis.

The carcinoid tumors associated with the administration of BL-6341 were quite different from the gastric tumors produced in rats by the H_2-antagonist, tiotidine. Tiotidine-related gastric lesions consisted of a dysplasia/carcinoma complex. They occurred as early as 6 months, were not carcinoids, and were located primarily in the pyloric (antral) region of the stomach (38). These changes are similar to those produced by the potent gastric carcinogen MNNG (28).

Other Gastric Changes. Tinctorial changes in chief cells similar to those observed with BL-6341 have been reported following the administration of the food additive sodium metabisulfite (1). These changes were reversible, and the additive proved negative in a carcinogenicity study (40). The same chief cell alteration observed with BL-6341 has been associated with the H_2-receptor antagonist famotidine. It is considered to be reversible and of no toxicologic significance and has also been observed in rats administered omeprazole (10, 18). An apparently similar gastric alteration, but one not considered to involve chief cells, was observed in a rat carcinogenicity study with loxtidine (34). This chief cell alteration has, therefore, been reported for the more potent and/or longer acting H_2-receptor antagonists, but not for cimetidine or ranitidine. However, if ranitidine is administered to rats twice a day by gavage at 750 mg/kg for 3 months, the eosinophilic chief cell alteration can be observed (R. S. Hirth, unpublished data). It would appear that this alteration is pharmacologically mediated and related to the degree and duration of gastric hypoacidity that is produced (3).

In the rat carcinogenicity study the numbers of parietal and chief cells in the fundic mucosa appeared to be increased at the 2 higher doses of BL-6341. These increases were considered to be a part of the generalized thickening of the oxyntic mucosa, due to the trophic effect of gastrin. This type of generalized gastric trophism of gastrin has been well-substantiated (22, 23).

Increases in parietal cell volume, density, or numbers have been reported following the administration of the H_2-antagonists cimetidine (17) and metiamide (41), and the antacid Maalox (32), due to resultant hypergastrinemia. They have also been observed following the administration of pentagastrin (16), and as a result of the increased serum concentrations of endogenous gastrin that are associated with the Zollinger-Ellison syndrome in man (33) and dogs (24). The proliferative effect of gastrin on chief cells has been reported less often; however, this alteration does not appear to have been investigated as thoroughly (33). The non-endocrine cells of the fundic mucosa of rats have been recently shown to increase proportionally as a result of prolonged administration of pentagastrin (3). In all of the preceding examples, there was also a generalized thickening of the oxyntic mucosa. This suggests that hypergastrinemia was the primary factor for the trophic effects on both the endocrine and non-endocrine cells in the gastric mucosa.

Risk Assessment. The assessment of human risk associated with the administration of BL-6341 essentially involves an evaluation of the trophic effects of hypergastrinemia. The trophic effects on the rat ECL cell are well-established; the carcinogenicity study with BL-6341 extends the range of this trophism to include carcinoid tumors. Nothing, however, is known about the function of the human ECL cell. The stimuli they functionally respond to and the peptide hormone they produce are unknown (23). They do not contain histamine, as does the rat ECL cell (21, 35). Nevertheless, it is believed that the same trophic effects that operate in the rat also apply to man (23).

Gastric argyrophil and ECL cell hyperplasia and neoplasia are reported to have occurred in man under very specific conditions. These conditions include non-antral (Type A) atrophic gastritis (8, 9, 12, 14, 37), Zollinger-Ellison syndrome (6, 9, 14), pernicious anemia (4, 12, 14, 25, 27), and surgical gastrojejunostomy without antral resection (7). The single factor common to all of these conditions is the potential for severe, chronic, hypergastrinemia. Therefore, in man, as in the rat, there does appear to be an association between gastric endocrine cell proliferation and chronic, sustained hypergastrinemia.

This response, however, most likely represents a threshold effect; doses below those causing severe, sustained hypergastrinemia would not be expected to result in ECL cell carcinoid tumors. The absence of genotoxic potential of BL-6341 would support a threshold phenomenon, as would the dose-response relationship and demonstrated no-effect dose in the rat-carcinogenicity study.

The clinical dose of BL-6341 in man would not be expected to produce sustained hypergastrinemia, since 24-hour anacidity has not been observed at clinical doses (Bristol-Myers Company, unpublished data). It is therefore proposed that the gastric carcinoid tumors associated with the administration of only the 300 mg/kg dose of BL-6341 (420 times proposed clinical dose) to rats for 2 years poses no human risk, if the administration of this drug to man avoids sustained hypergastrinemia and produces no greater serum gastrin concentrations than those associated with the clinical use of short-acting H_2 antagonists.

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