

**The Role of Endogenous Gastrin in the  
Development of Enterochromaffin-like Cell  
Carcinoid Tumors in *Mastomys natalensis*:  
A Study with the Specific Gastrin Receptor Antagonist AG-041R**

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We examined the effects of a newly synthesized gastrin receptor antagonist, AG-041R, on the growth of enterochromaffin-like (ECL) carcinoid tumors in *Mastomys natalensis* both *in vitro* and *in vivo*. AG-041R was as potent as the well known gastrin antagonist L365,260 in inhibiting not only the gastrin-induced release of histamine from but also histidine decarboxylase (HDC) gene expression in the ECL carcinoid tumor cells. AG-041R also inhibited gastrin-induced DNA synthesis and c-fos gene expression in the tumor cells. Furthermore, AG-041R significantly inhibited the growth of the transplanted *Mastomys* ECL carcinoid tumors *in vivo*. From these data, it is concluded that endogenous gastrin is involved in the growth of ECL carcinoid tumors in *Mastomys natalensis*. Moreover, AG-041R is shown to have a potential as an anti-neoplastic agent for ECL carcinoid tumor of the stomach.

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## INTRODUCTION

In addition to stimulating histamine release along with the resulting acid secretion from parietal cells, gastrin has a trophic effects on enterochromaffin-like (ECL)<sup>e</sup> cells in the fundic mucosa. Indeed, it is well known that patients with hypergastrinemia such as type A gastritis or Zollinger-Ellison syndrome with multiple endocrine neoplasia (MEN) type I have a tendency to develop ECL carcinoid tumor of the stomach [1-5]. For example, in our series of patients, 22 out of 25 patients with gastric carcinoid tumors had hypergastrinemia, whereas only one patient with colon carcinoid tumor had hypergastrinemia (Table 1). Moreover, we have identified significant expression of gastrin receptor mRNAs in all of the five gastric carcinoid tumors with hypergastrinemia tested to date (Figure 1). Although most carcinoid tumors of the stomach are quite indolent and tend to remain localized to their site of origin for extremely long periods [4, 5], several cases have been reported to have metastasized to local lymph nodes [4, 5]. Thus, it is possible that gastrin receptor antagonists may be more important as anti-neoplastic agents for gastric carcinoid tumors than as anti-acid agents.

In the present study, therefore, we examined the effects of a newly developed gastrin receptor antagonist, AG-041R, on the growth of ECL carcinoid tumors in *Mastomys natalensis* both *in vitro* and *in vivo*.

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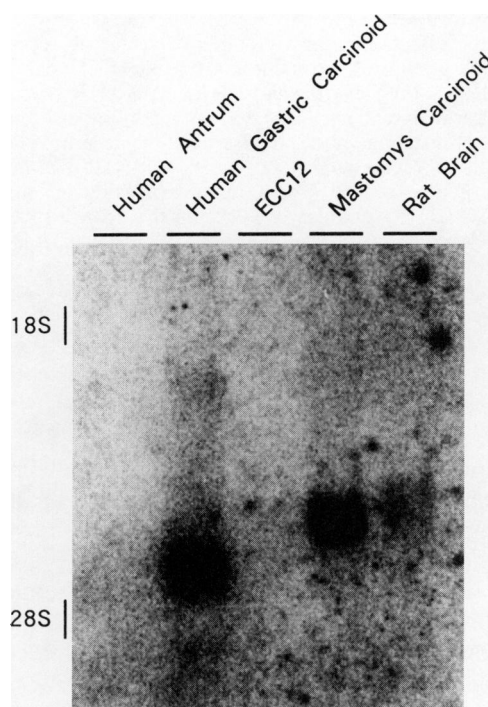
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<sup>e</sup>*Abbreviations:* ECL, enterochromaffin-like; HDC, histidine decarboxylase; MEN, multiple endocrine neoplasia; DMSO, dimethylsulfoxide; CMC, carboxymethylcellulose.

## MATERIALS AND METHODS

### Drugs

AG-041R is a recently discovered potent nonpeptide and nonbenzodiazepine derivative CCK<sub>B</sub>/gastrin receptor antagonist with low affinity for CCK<sub>A</sub> receptors (Table 2). AG-041R [3R-1(2,2-Diethoxyethyl)-3-((4-methylphenyl)aminocarbonylmethyl)-3-((4-methylphenyl)ureido)-indoline-2-one] was synthesized by Chugai Pharmaceutical Co. (Tokyo, Japan) (Figure 2). AG-041R was dissolved with dimethylsulfoxide (DMSO) for the *in vitro* study and was suspended in 0.3 percent carboxymethylcellulose (CMC) for the *in vivo* study.



**Figure 1. Gastrin receptor gene expression in human and *Mastomys* ECL carcinoid tumor of the stomach.** ECC12 is a gastric endocrine cell line [10]. The size of human gastrin receptors is slightly larger than those of *Mastomys* and of rats.

**Table 1. Carcinoid tumor in the gastrointestinal tract.**

	Hyper-gastrinemia		
	(+)	(-)	Unknown
Colon carcinoid	1	28	10
Gastric carcinoid	22*	3	4
Endocrine Cell	2	1	1
Cancer of the stomach			

\*One case is ZE syndrome with MEN type-I, and the other 21 cases are type A gastritis. The data were obtained in Kobe, Japan between 1989 and 1996.

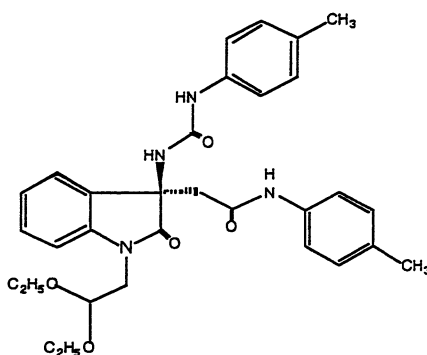


Figure 2. Chemical structure of AG-041R.

L-365,260 (provided by Merck & Sharp, Philadelphia, PA) was dissolved with DMSO, and Lansoprazole (LPZ; Takeda Pharmaceutical CO., Tokyo) was suspended in 0.3 percent CMC.

#### *Preparation of the ECL carcinoid tumor cells and its cell membranes in *Mastomys natalensis**

A primary ECL carcinoid tumor arose in the glandular stomach of a two-year-old *Mastomys natalensis* female. The tumor was serially transplanted into the thigh muscles of one- to two-month-old male *Mastomys* [6] and was then used in this experiment. The transplanted tumors from *Mastomys* were minced and then dispersed with 0.4 mg/ml collagenase (type I) (Sigma St. Louis, MO) and 4 mg/ml dispase (Sanko Chemicals, Tokyo, Japan). After washing twice, the dispersed cells were suspended in Krebs-Ringer Bicarbonate buffer (pH 7.4) with 10 mM HEPES, 1 mg/ml BSA and 2.5 mM D-glucose.

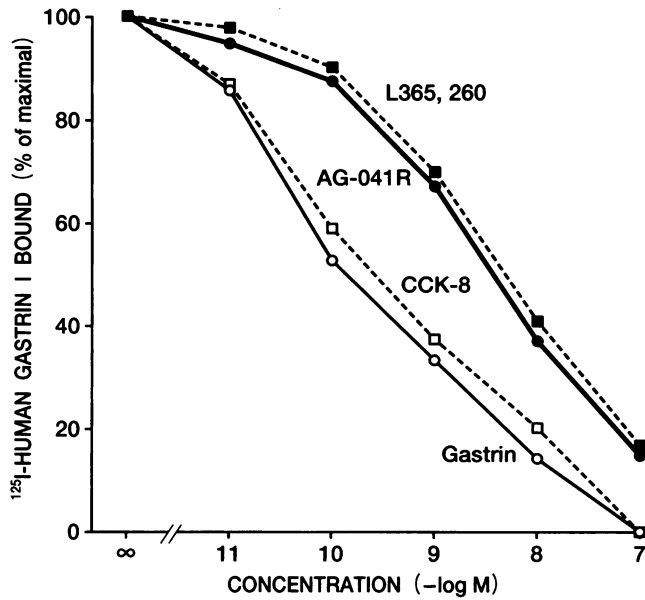
A crude membrane preparation of the ECL tumor was obtained as described previously [7]. The tumor tissue was minced and homogenized with a glass homogenizer in HEPES buffer (10 mM, pH 7.4) containing 1 mg/ml bacitracin. After filtration through surgical gauze, the homogenate was centrifuged at 1,000 x g for 2 min. The supernatant was then recentrifuged at 20,000 x g for 15 min. After washing twice, the resulting pellet was resuspended in 10 mM HEPES buffer (pH 5.5) containing 120 mM NaCl, 4.7 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM ethylene glycol-bis ( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mg/ml bacitracin, and 5 mg/ml BSA.

#### *<sup>125</sup>I-human gastrin I binding experiment*

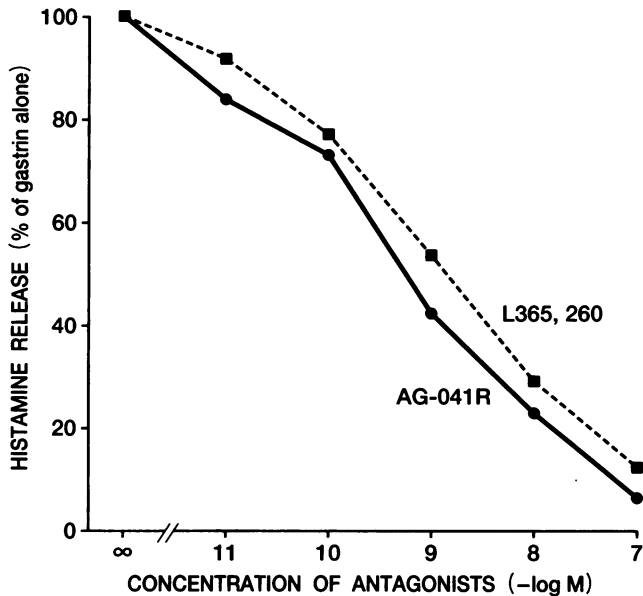
Binding studies were carried out in a volume of 500  $\mu$ l 10 mM HEPES buffer (pH 7.4) containing 25 pM <sup>125</sup>I-gastrin and 300  $\mu$ g crude membrane protein with or without various unlabeled agents. Following incubation at 24°C for 60 min, bound and free hormone fractions were separated by centrifugation, and radioactivity remaining bound to the pellets was counted [7].

#### *Measurement of histamine release*

After 10 min of preincubation at 37°C in a shaking bath, the dispersed cells were incubated for 30 min with or without various test agents in Krebs'-Ringer bicarbonate buffer (pH 7.4) with 10 mM HEPES, 1 mg/ml BSA, 2.5 mM D-glucose and 0.1 mM IBMX (10<sup>6</sup> cells/ml) [8]. The reactions were then stopped by adding 4 ml ice-cold incubation medium; after centrifugation, the supernatant was stored at -30°C until assay. The histamine concentration in each sample was directly measured by an RIA kit (Immunotech S.A., Marseilles, France).



**Figure 3.** Inhibition of <sup>125</sup>I-human gastrin I binding to the ECL carcinoid tumor membrane of *Mastomys* by gastrin receptor antagonists. The data are means from triplicate samples, and are expressed as percentages of maximal specific binding in the presence of  $10^{-7}$  M CCK8. Representative data from three separate experiments are shown.



**Figure 4.** Effect of gastrin receptor antagonists on the gastrin ( $10^{-8}$  M)-induced increase of histamine release from the ECL carcinoid tumor cells of *Mastomys*. The values are means from triplicate samples and are expressed as percentages of histamine release induced by gastrin ( $10^{-8}$  M) alone.

### Cell incubation and RNA blot analysis

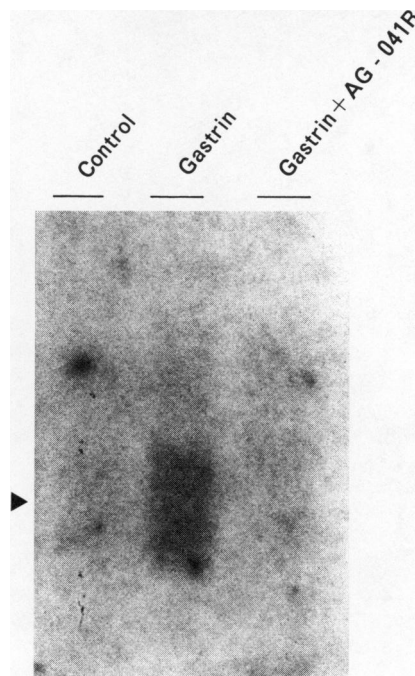
Dispersed cells were incubated with or without gastrin ( $10^{-8}$  M) in the presence or absence of AG-041R ( $10^{-7}$  M) for 1h. After washing, total cellular RNA was extracted as described [9]. Samples were electrophoresed on a 1 percent agarose/formaldehyde gel, and transferred to nitrocellulose filters. Filters were hybridized at  $42^{\circ}\text{C}$  with  $^{32}\text{P}$ -labeled HDC or c-fos cDNA probes. After 16h hybridization, filters were washed twice for 20 min in  $2 \times \text{SSC}$ , 0.1 percent SDS at  $50^{\circ}\text{C}$ , and autoradiographed.

### $^3\text{H}$ -thymidine incorporation

Dispersed cells were incubated under serum starved conditions for 5h. The cells were then incubated in 24-well plates in the presence or absence of gastrin ( $10^{-8}$  M) with or without various concentrations of AG-041R or L365,260 for 10h followed by a further 5h incubation with methyl- $^3\text{H}$ -thymidine (1 mci/ml) (Amersham, Buckinghamshire, UK). The radioactivity of the incorporated  $^3\text{H}$ -thymidine in 6 percent trichloroacetic acid-insoluble precipitates was counted after lysing in 1 percent SDS/10 mM NaOH.

### In vivo growth experiments

Dispersed tumor cells ( $10^7$  each) were transplanted into the thigh muscles of a one month-old male *Mastomys*. The *Mastomys* animals were then divided into four groups (8 animals per group). One group was used as control. The second group received daily non-parenteral administration of LPZ at a dose of 10 mg/kg for one month. The third group was given AG-041R (30 mg/kg) orally for one month. The fourth group received concomitant administration of AG-041R (30 mg/kg) and LPZ (10 mg/kg). One month after the start of the administration of the drugs, the animals were anesthetized, the tumors in the thigh muscles were taken out, and their diameters measured.



**Figure 5.** Effect of AG-041R ( $10^{-7}$  M) on the increase of histidine decarboxylase (HDC) gene expression induced by gastrin ( $10^{-8}$  M).

## RESULTS AND DISCUSSION

AG-041R displaced the specific binding of  $^{125}\text{I}$ -human gastrin I to the ECL carcinoid tumor cell membranes in *Mastomys* in a dose-dependent fashion with a potency similar to L365,260 (Figure 3). This binding inhibition by AG-041R was associated with a dose-dependent inhibition of the histamine release from these carcinoid tumor cells induced by gastrin, again with a similar potency to L365,260 (Figure 4). In agreement with these data, AG-041R inhibited the gastrin-induced increase of HDC gene expression in the carcinoid tumor cells (Figure 5).

AG-041R clearly suppressed the gastrin-induced enhancement of c-fos gene expression (Figure 6). Moreover, AG-041R dose-dependently inhibited the gastrin-induced

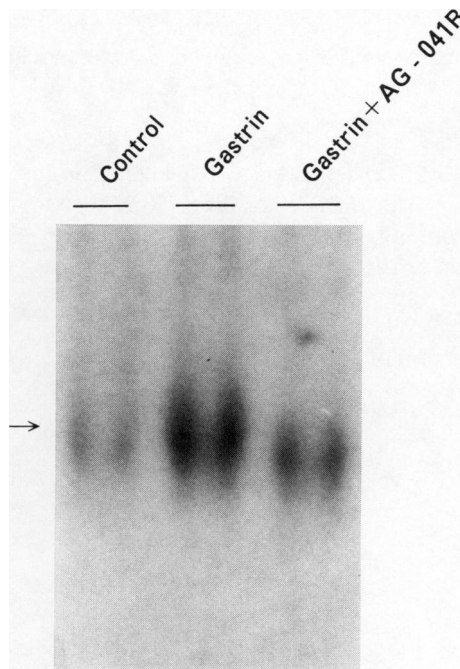


Figure 6. Effect of AG-041R ( $10^{-7}$  M) on the increase of c-fos gene expression induced by gastrin ( $10^{-8}$  M).

Table 2.  $\text{IC}_{50}$  of AG-041R on the bindings of  $^{125}\text{I}$ -Gastrin to guinea pig isolated gastric glands and  $^{125}\text{I}$ -CCK8 to rat pancreatic acinar membranes.

Compound	CCK-B receptors	CCK-A receptors	$\text{IC}_{50}$ Ratio (CCKA/CCKB)
AG-041R	1.11	620.0	558.6
Y M022	1.02	19.7	19.3
L 365,260	2.19	949.0	433.3
L 364,718	338.0	0.45	0.001
Human gastrin	9.61	356.0	37.0
CCK-8	1.29	0.21	0.16

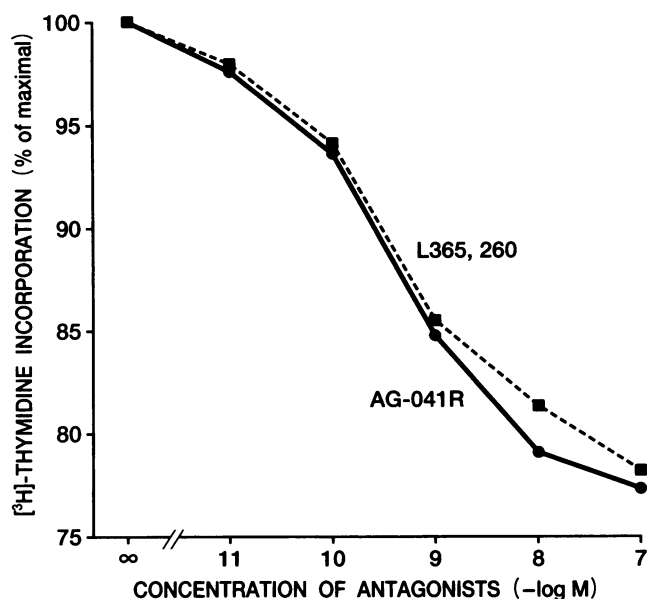


Figure 7. Effects of gastrin antagonists on the gastrin ( $10^{-8}$  M)-induced increase of  $^3\text{H}$ -thymidine incorporation into the ECL carcinoid tumor cells of *Mastomys*. The data are means of quadruplicate samples, and are expressed as percentages of maximal incorporation induced by gastrin ( $10^{-8}$  M).

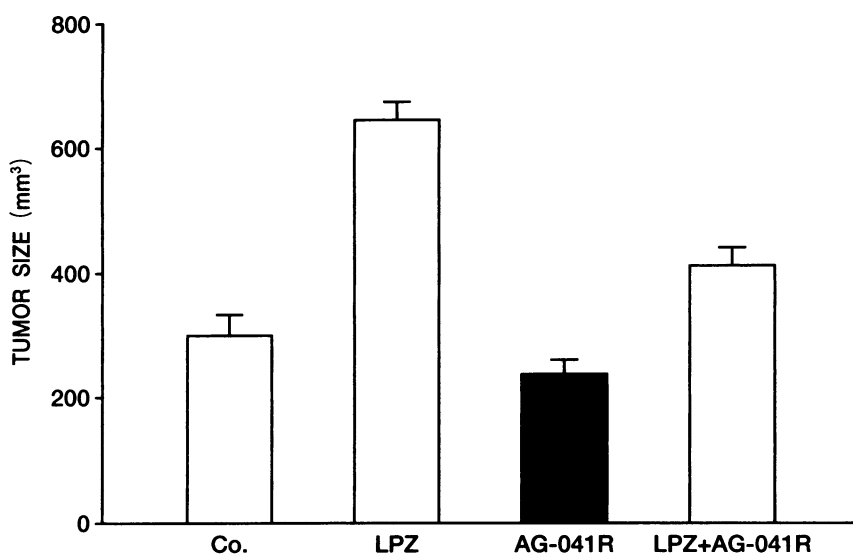


Figure 8. Effects of AG-041R on the growth of *Mastomys* ECL carcinoid tumors transplanted to the thigh muscles of male *Mastomys*. The data are the diameter of the transplanted tumors one month after the transplantation and are expressed as means  $\pm$  SE from eight animals.

increase of  $^3\text{H}$ -thymidine incorporation into the tumor cells (Figure 7). These data clearly indicate that AG-041R is a potent antagonist of gastrin receptors on *Mastomys* ECL carcinoid tumor cells, inhibiting not only histamine release but also cell growth.

Therefore, we next examined the effects of AG-041R on the growth of *Mastomys* ECL carcinoid tumors *in vivo*. As shown in Figure 8, LPZ significantly enhanced the growth of the transplanted ECL carcinoid tumors ( $p < .01$  vs control). AG-041R significantly reduced this LPZ-enhanced tumor growth. However, the most noteworthy finding was that AG-041R significantly inhibited not only the LPZ ( $p < .01$  vs LPZ alone)-induced but also the spontaneous growth of the transplanted ECL carcinoid tumors ( $p < .01$  vs control). Since *Mastomys* exhibits normal level of serum gastrin (data not shown), this finding strongly suggests that circulating gastrin, even at physiological concentrations, plays a role in the development of ECL carcinoid tumors in *Mastomys natalensis*.

It should be noted that some patients with gastric carcinoid tumors do not have hypergastrinemia [4, 5], and the growth of carcinoid tumors in those patients is believed to be independent of their serum gastrin levels. In case of *Mastomys*, it is considered that in addition to gastrin certain genetic factors play a role in the development of the ECL carcinoid tumors. Yet even under such conditions, serum gastrin at physiological concentrations appears to have a trophic action on the growth of the ECL carcinoid tumors. Thus, whether or not the growth of sporadic gastric carcinoid tumors without concomitant hypergastrinemia in humans is dependent on physiological levels of gastrin in the blood is an interesting question that remains to be elucidated. In this regard, it is worth noting that gastric carcinoid tumors without hypergastrinemia usually have a worse prognosis than those with hypergastrinemia [4, 5]. Thus, specific antagonists for gastrin receptors like AG-041R might also be useful for the treatment of the carcinoid tumors of the stomach with or without hypergastrinemia.

In conclusion, the present study clearly showed that gastrin is involved in the growth of the ECL carcinoid tumor of *Mastomys*. Moreover, AG-041R may be useful for the treatment of ECL carcinoid tumors of the stomach.

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