

Dual α_{2C} /5HT_{1A} Receptor Agonist Allyphenyline Induces Gastroprotection and Inhibits Fundic and Colonic Contractility

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Abstract

Background Allyphenyline, a novel α_2 -adrenoceptor (AR) ligand, has been shown to selectively activate α_{2C} -adrenoceptors (AR) and 5HT_{1A} receptors, but also to behave as a neutral antagonist of α_{2A} -ARs. We exploited this unique pharmacological profile to analyze the role of α_{2C} -ARs and 5HT_{1A} receptors in the regulation of gastric mucosal integrity and gastrointestinal motility.

Methods Gastric injury was induced by acidified ethanol in Wistar rats. Mucosal catalase and superoxide dismutase levels were measured by assay kits. The effect of allyphenyline on electrical field stimulation (EFS)-induced fundic and colonic contractions was determined in C57BL/6 mice.

Results Intracerebroventricularly injected allyphenyline (3 and 15 nmol/rat) dose dependently inhibited the development of mucosal damage, which was antagonized by ARC 239 ($\alpha_{2B/C}$ -AR and 5HT_{1A} receptor antagonist), (S)-WAY 100135 (selective 5HT_{1A} receptor antagonist), and JP-1302 (selective α_{2C} -AR antagonist). This protection was accompanied by significant elevation of mucosal catalase and superoxide dismutase levels. Allyphenyline (10^{-9} – 10^{-5} M) also inhibited EFS-induced fundic contractions, which was antagonized by ARC 239 and (S)-WAY 100135, but not by JP-1302. Similar inhibition was

observed in the colon; however, in this case only ARC 239 reduced this effect, while neither selective inhibition of α_{2C} -ARs and 5HT_{1A} receptors nor genetic deletion of α_{2A} - and α_{2B} -ARs influenced it.

Conclusions Activation of both central α_{2C} -ARs and 5HT_{1A} receptors contributes to the gastroprotective action of allyphenyline in rats. Its inhibitory effect on fundic contractions is mediated by 5HT_{1A} receptors, but neither α_2 -ARs nor 5HT_{1A} receptors take part in its inhibitory effect on colonic contractility in mice.

Keywords Allyphenyline · Gastroprotection · Fundic relaxation · Colonic motility · Alpha_{2C} adrenergic receptor · 5HT_{1A} receptor

Introduction

α_2 -adrenoceptors (α_2 -ARs) belong to the superfamily of G-protein-coupled receptors and consist of three different molecular subtypes (α_{2A} , α_{2B} , and α_{2C}) [1], which are widely distributed in the central nervous system (CNS) and in peripheral tissues and possess distinct functional and pharmacological properties (for reviews see [2–4]). The synthesis of subtype-selective ligands and generation of genetically engineered mice have allowed significant advances in our understanding of the role of each subtype. Several studies demonstrated that most of the classical effects of α_2 -AR agonists, such as hypotension, bradycardia, or sedation are mediated entirely or predominantly by the α_{2A} -subtype [2, 4].

Since the pioneering work of Paton and Vizi [5], who reported the localization of presynaptic α_2 -ARs on cholinergic myenteric nerves and their inhibitory action on the release of acetylcholine, a vast amount of data have

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been accumulated on the role of these receptors in the gastrointestinal (GI) tract as well. Besides cholinergic neurons, α_2 -ARs are also localized on extrinsic noradrenergic neurones (autoreceptors), as well as on enterocytes and smooth muscles, and their activation results in various effects, such as inhibition of GI motility, secretion and visceral sensation, and increased intestinal absorption of water and electrolytes [6, 7]. Moreover, there is evidence that α_2 -ARs localized in the CNS are also involved in the inhibition of GI motility and secretion [8–10]. Due to all these actions, α_2 -ARs are considered attractive therapeutic targets for the treatment of a wide range of GI disorders, including functional dyspepsia, irritable bowel syndrome, and inflammatory bowel diseases [7, 11]. However, most GI effects are mediated mainly by the α_{2A} -AR subtype [4, 7, 12], and pharmacologic modulation of this subtype in the above-mentioned diseases by non-selective or subtype-selective α_2 -AR ligands may result in numerous undesirable side effects outside the GI tract (see above).

In the last two decades, our research group demonstrated that α_2 -AR agonists induce a potent defensive effect in the stomach and reduce the extent of mucosal injury in both acid-dependent (indomethacin) and acid-independent (ethanol) ulcer models [12–17]. More importantly, our results clearly showed that this protective effect is mediated by α_{2B} - and α_{2C} -ARs localized in the brain, most probably in the dorsal vagal complex, and α_{2A} -ARs do not have any significant role in it [12, 14, 17]. Accordingly, we proposed that selective $\alpha_{2B/C}$ -AR agonists may represent a novel group of gastroprotective agents, which are devoid of several side effects mediated by the α_{2A} -AR subtype, such as hypotension, bradycardia, or sedation [17]. However, although the site of gastroprotective action is likely to be central, it should be considered that α_{2B} -ARs are located also postsynaptically on vessels [18, 19] and appear to be involved in the vasoconstrictor response to α_2 -AR agonists [20] and in salt-induced hypertension [21]. Consequently, selective α_{2C} -AR stimulants are likely to be even more favorable and safer potential therapeutic agents against gastric mucosal injury than the mixed $\alpha_{2B/C}$ -AR agonists, since they are devoid of adverse effects due to activation of both α_{2A} - and α_{2B} -ARs.

In the recent years, allyphenyline, a novel α_2 -AR ligand, endowed with peculiar pharmacological profile has been synthesized and characterized [22–24]. Among the three α_2 -AR subtypes, allyphenyline behaves as an agonist only at the α_{2C} -AR subtype, while it does not bind to α_{2B} -ARs and behaves as a neutral antagonist at the α_{2A} -AR subtype in vitro [22]. In line with it, allyphenyline enhanced morphine analgesia and attenuated morphine withdrawal symptoms in vivo (due to its α_{2C} -AR agonist property), but it was devoid of any sedative effect (due to its α_{2A} -AR-antagonism) [23, 24].

Regarding other receptors, allyphenyline also binds to and activate 5HT_{1A} receptors [25], and dual activation of α_{2C} -ARs and 5HT_{1A} receptors by this compound elicited antidepressant- and anxiolytic-like effects in rodents [25, 26]. 5HT_{1A} receptors have been implicated in numerous GI processes, such as gastric mucosal protection [27, 28], inhibition of cholinergic gastric contractions [29, 30], as well as modulation of colonic motility [31]. It should be emphasized that ulcer disease is often accompanied by increased gastric motility [32], consequently inhibition of GI motility may contribute to the therapeutic effect of an anti-ulcer agent.

Based on the receptorial actions, we hypothesized that allyphenyline may induce gastroprotective action (due to dual activation of α_{2C} -ARs and 5HT_{1A} receptors) and may also inhibit GI motility, in spite of the lack of α_{2A} -AR activation (due to 5HT_{1A} receptor agonism).

Our results confirmed our expectations and demonstrated that (1) allyphenyline potently inhibits the development of ethanol-induced mucosal damage, and central α_{2C} -ARs and 5HT_{1A} receptors are both involved in this action, (2) allyphenyline concentration dependently suppresses cholinergic contractions in the stomach, which effect is—at least partly—mediated by 5HT_{1A} receptors, but not by α_{2C} -ARs, and (3) allyphenyline also suppresses cholinergic contractions in the colon, but this effect does not depend on the activation of 5HT_{1A} receptors and α_2 -ARs. Hence, the unique receptorial profile makes allyphenyline particularly attractive for the treatment of various GI disorders, such as peptic ulcers, functional dyspepsia (FD), or irritable bowel syndrome (IBS), because both mucosal protection, fundic relaxation, and inhibition of colonic motility can be achieved without inducing α_{2A} - and α_{2B} -AR-mediated unwanted side effects.

Methods

Synthesis of Allyphenyline

Allyphenyline (2-(1-(2-allylphenoxy)ethyl)-4,5-dihydro-1H-imidazole) [22] was obtained from 2-(2-allylphenoxy)propanenitrile [33] by treatment with sodium methoxide in methanol and subsequent condensation with ethylenediamine, as reported in Fig. 1.

Animals

For in vivo analysis of gastric mucosal defensive processes, male Wistar rats weighing 150–170 g (Simmelweis University) were used [15], while for ex vivo analysis of gastric and colonic contractility C57BL/6 mice from both sexes (20–25 g, National Institute of Oncology) were used

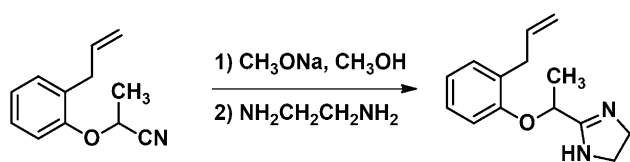


Fig. 1 Synthesis of allylphenylene (50 % yield). The free base was transformed into the oxalate salt; which was recrystallized from 2-PrOH: mp 154–155 °C. $^1\text{H NMR}$ (DMSO): δ 1.53 (d, 3, CH_3), 3.42 (m, 2, CH_2CH), 3.88 (s, 4, $\text{NCH}_2\text{CH}_2\text{N}$), 5.06 (dd, 2, $\text{CH}=\text{CH}_2$), 5.40 (q, 1, OCH), 5.95 (m, 1, $\text{CH}=\text{CH}_2$), 6.92–7.26 (m, 4, ArH), 7.81 (br s, 1, NH , exchangeable with D_2O). Anal. Calcd for $\text{C}_{14}\text{H}_{18}\text{N}_2\text{O}$ $\text{H}_2\text{C}_2\text{O}_4$: C, 56.80; H, 6.55; N, 8.28. Found: C, 56.69; H, 6.71; N, 8.13

[34]. α_{2A} - and α_{2B} -AR knockout mice (with the same C57BL/6 genetic background) were the generous gift of Prof. L. Hein (University of Freiburg, Germany). The generation of these mouse lines has been described previously in detail [20, 35]. Genotypes were confirmed by subtype-specific polymerase chain reactions (with a TProfessional Basic Thermocycler, Biometra) performed with genomic DNA isolated from tail biopsies as described previously [36]. Animals were housed in a temperature- and humidity-controlled room at a 12-h light/dark cycle under conditions of animal housing and experimentation according to ethical guidelines issued by the Ethical Board of Semmelweis University, based on EC Directive 86/609/EEC.

All procedures conformed to the European convention for the protection of vertebrate animals used for experimental and other scientific purposes, and all efforts were made to minimize the suffering of animals. The experiments were approved by the National Scientific Ethical Committee on Animal Experimentation and permitted by the government (Food Chain Safety and Animal Health Directorate of the Central Agricultural Office (PEI/001/1493-4/2015)).

In Vivo Analysis of Gastric Mucosal Protective Processes

Before the experiments, rats were deprived of food for 24 h with free access to tap water. Gastric mucosal lesions were induced by acidified ethanol (98 ml absolute ethanol + 2 ml concentrated HCl), which was given intragastrically in a volume of 0.5 ml/rat by an oral gavage using a stainless steel cannula. All test compounds were injected into the lateral brain ventricle (intracerebroventricularly, i.c.v.) 10 min before the ethanol challenge in a volume of 10 μl , as described previously [15]. 60 min after the injection of ethanol, the animals were killed, and the stomachs were excised and opened along the greater curvature. The flattened stomachs were photographed and

pixel numbers of the damaged and total mucosal areas were determined using a free photo-editing software. The percentage of the damaged mucosal area in each stomach was calculated as follows: [(pixel number of ulcerated mucosa/pixel number of total mucosal area) \times 100]. After the stomachs were photographed, gastric mucosa was separated on a cooled plate, shock-frozen in liquid nitrogen, and stored at -80 °C until further assayed.

Study Design

In four consecutive experiments, a total of 80 rats were randomly divided into 16 groups (four groups/experiment, five rats/group). In the first experiment, rats received water per os and vehicle i.c.v. (absolute control group), acidified ethanol per os and vehicle i.c.v. (ethanol control group), or acidified ethanol per os and allylphenylene i.c.v. at 2 different doses (3 and 15 nmol). In the three other experiments, rats received acidified ethanol per os and vehicle, allylphenylene (15 nmol), an α_2 -AR and/or 5HT $_{1A}$ receptor antagonist, or the combination of allylphenylene and the respective antagonist i.c.v. The applied doses of drugs were selected based partly on our preliminary results, partly on the literature data [16, 17, 25, 26].

Catalase Assay

Catalase (CAT) enzyme activity was measured by using an assay kit (Cayman Europe, Tallinn, Estonia) according to the manufacturer's instructions. This kit utilizes the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2 . The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen. One unit of CAT was defined as the amount of enzyme that causes the formation of 1 nmol of formaldehyde per minute at 25 °C. CAT activity was expressed in nmol/min/mg tissue.

Superoxide Dismutase Assay

Superoxide dismutase (SOD) activity was measured by using an assay kit (Cayman Europe, Tallinn, Estonia) according to the manufacturer's instructions. This kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine, and measures the activity of all three types of SOD (Cu/Zn, Mn, and Fe-SOD). One unit of SOD was defined as the amount of enzyme needed to exhibit 50 % dismutation of the superoxide radical (O_2^-). SOD activity was expressed in unit/mg tissue.

Ex Vivo Analysis of Fundic Contractility

The preparation of gastric fundus strips was performed as described previously [34]. Briefly, mice were killed by cervical dislocation, and the entire stomach was removed and placed in Petri dish containing Krebs solution and aerated with carbogen (mixture of 95 % O₂ and 5 % CO₂). The fundal part was separated from the pyloric part of each stomach, and 2- to 2.5-cm-long fundus strips were prepared by transverse cuts on each side of the strip parallel to the longitudinal muscle. From each stomach, only one strip was prepared.

The isolated gastric fundus strips were suspended between upper (ring) and lower (straight) electrodes in organ baths of 5 ml volume containing Krebs solution aerated with carbogen at 37 °C. Krebs solution has the following composition (mM/L): NaCl, 118.0; NaHCO₃, 25.0; KCl, 4.7; KH₂PO₄, 1.2; glucose, 11.0; CaCl₂, 2.5; MgSO₄, 1.2. The upper end of the strip was attached by thread to a transducer, connected to a computer through an amplifier. The resting tension was adjusted to 0.5 g. To induce contraction of gastric fundus strip, electrical field stimulation (EFS) was used with the following stimulation parameters: pairs (200 ms pulse distance) of rectangular impulses (1 ms pulse width, 25 shocks, 9 V/cm, i.e., supramaximal intensity of 5 Hz) were repeated in 60 s. Each impulse induced a fast contractile response, followed by a slower relaxation response, whose amplitudes were constant over a period of 60 min (pilot experiments). The contractions were abolished by atropine (1 μM), indicating the predominant role of the cholinergic system in the contractile response, while the relaxations were inhibited by N^ω-Nitro-L-arginine (L-NNA, 10 μM), indicating the activation of nitrergic pathways.

After setting up, strips were allowed to equilibrate for 30–45 min before adding the first concentration of allyphenyline or clonidine (non-selective α₂-AR agonist, used as a reference compound). A period of 5 min was allowed to elapse between concentration increment. Cumulative concentration–response curves were constructed for both agonists. When the effects of the antagonists (ARC 239, (S)-WAY 100135, and JP-1302) on the inhibitory effect of allyphenyline were assessed, the antagonists (1 μM) were added to the organ bath 20 min prior the first concentration of allyphenyline. The antagonists in this concentration were effective and selective [34, 37–39]. Drugs were given in volumes <1 % of total bath volume.

The 50 % effective concentrations (EC₅₀) and maximal inhibitory effect (E_{\max}) of agonists were calculated from the nonlinear regression of individual sigmoid concentration response curves. The average of five peaks of contractions before adding the agonists was taken as 100 %

(control). The inhibitory effect of the agonists (the mean of five peaks) was expressed as a percentage to the control contractions.

Ex Vivo Analysis of Colonic Contractility

Besides the stomach, the most distal (~2 cm) segment of the colon was also removed from the same mouse. The same steps were followed as in the preceding case, but based on preliminary studies stimulation parameters were modified, and single rectangular impulses (1 ms pulse width, 1 shock, 9 V/cm, i.e., supramaximal intensity of 5 Hz) were repeated in 30 s. These impulses induced a fast, slight relaxation response followed by a contractile response, with constant amplitudes over a period of 60 min, and were abolished by L-NNA (10 μM) and atropine (1 μM), respectively.

Chemical Compounds

Clonidine hydrochloride (non-selective α₂-AR agonist, PubChem CID 2803) and JP-1302 trihydrochloride hydrate (selective α_{2C}-AR antagonist, PubChem CID 540335) were purchased from Sigma-Aldrich (St. Louis, USA), while (S)-WAY 100135 dihydrochloride (selective 5HT_{1A} receptor antagonist, PubChem CID 6604840) and ARC 239 dihydrochloride (α_{2B/C}-AR and 5HT_{1A} receptor antagonist, PubChem CID 609483) were obtained from Tocris Bioscience (Bristol, UK). Allyphenyline (PubChem CID 24906198) was synthesized as described above. All drugs were dissolved in saline (in vivo) or in distilled water (ex vivo). For controls the respective vehicles were used.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis of the data was performed either with Student's *t* test (two treatment groups), or with one-way ANOVA followed by Newman–Keuls post hoc test, or (in the case of comparing concentration–response curves) by two-way repeated measures ANOVA followed by Holm–Sidak post hoc test. A probability of $p < 0.05$ was considered statistically significant.

Results

The Effect of Allyphenyline on Ethanol-Induced Mucosal Damage

Oral administration of acidified ethanol induced multiple longitudinal hemorrhagic lesions on the gastric mucosa (damaged mucosal area: 9.3 ± 1.9 %, $n = 20$, Fig. 2).

I.c.v. injection of allyphenyline (3 and 15 nmol/rat) 10 min before the ethanol challenge dose dependently reduced the development of mucosal lesions, and at the higher dose, it induced almost complete inhibition (damaged areas: 1.4 ± 0.5 and 0.2 ± 0.1 %, respectively, $n = 5$, $p < 0.01$, which correspond to 21.7 and 4 % of the damaged area in the respective ethanol control group, Fig. 2).

As Fig. 3 shows, the formation of mucosal lesions in the ethanol-treated animals was associated with significantly reduced SOD activity in the gastric mucosa (0.47 ± 0.04 vs 0.32 ± 0.02 unit/mg tissue, $n = 5$, $p < 0.001$), while this reduction was completely prevented by allyphenyline (0.53 ± 0.03 unit/mg tissue, $n = 5$, $p < 0.001$), indicating that it counteracted the pro-oxidant effect of ethanol. Similarly, the mucosal CAT activity was significantly higher in the allyphenyline + ethanol-treated group than in the ethanol control group (vehicle + ethanol: 0.27 ± 0.03 , allyphenyline + ethanol: 0.56 ± 0.15 nmol/min/mg tissue, $n = 5-10$, $p < 0.05$).

Analyzing the Involvement of α_2 C-ARs and 5HT_{1A} Receptors in the Gastroprotective Effect of Allyphenyline

The mucosal protective action of i.c.v.-injected allyphenyline was completely inhibited by ARC 239

(50 nmol/rat i.c.v., Fig. 2). Although this compound is widely used as an $\alpha_{2B/C}$ -AR antagonist, there is evidence that it also binds to 5HT_{1A} receptors with nanomolar affinity [37]. Hence, the inhibitory action of ARC 239 indicates only that at least one of these receptors is involved in mediating the effect of allyphenyline. For further analysis, we used (S)-WAY 100135 (50 nmol/rat) and JP-1302 (56 nmol/rat), selective antagonists of 5HT_{1A} receptors and α_2 C-ARs, respectively [38, 39]. As Fig. 2 shows, both compounds inhibited the effect of allyphenyline, suggesting that both α_2 C-ARs and 5HT_{1A} receptors contribute to the gastroprotection. When given alone, none of the antagonists influenced significantly the development of ethanol-induced lesions.

The Effect of Allyphenyline on EFS-Induced Fundic and Colonic Contractions

As Fig. 4 demonstrates, allyphenyline inhibited EFS-evoked cholinergic contractions in both fundus and colon in a concentration-dependent fashion. While in the stomach allyphenyline was one order of magnitude less potent than clonidine, a prototypical non-selective α_2 -AR agonist (EC₅₀ values are 240.8 ± 71.6 and 20.8 ± 6.2 nM, respectively), in the colon they had comparable potencies and efficacies (Table 1). Figure 4b, d shows representative

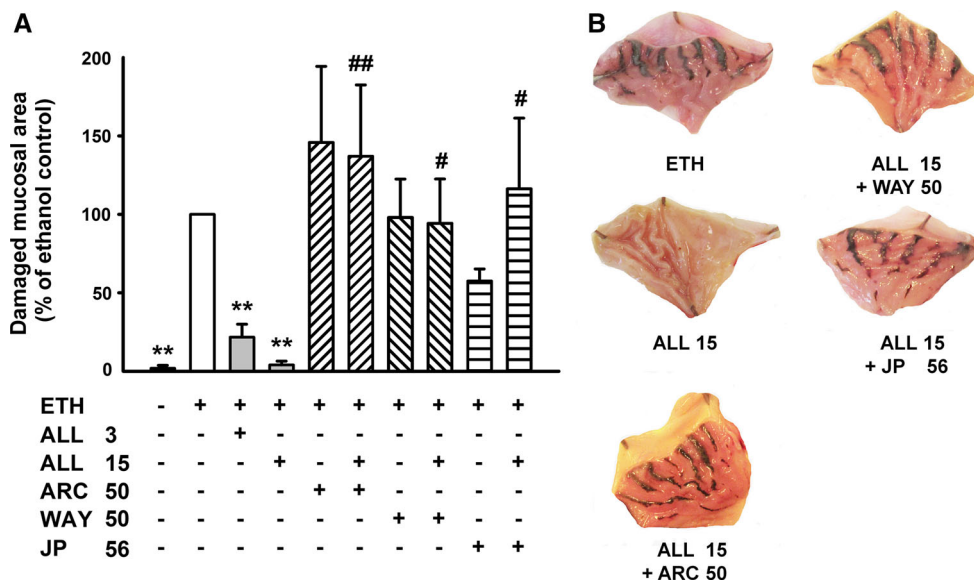


Fig. 2 a The effect of allyphenyline (ALL, 3 and 15 nmol/rat) on ethanol (ETH)-induced gastric mucosal injury in the absence and presence of ARC 239 (ARC, 50 nmol/rat), (S)-WAY 100135 (WAY, 50 nmol/rat), and JP-1302 (JP, 56 nmol/rat). Allyphenyline was injected intracerebroventricularly (i.c.v.) either alone or together with the respective antagonists 10 min before the ethanol challenge. In order to compare the inhibitory effects of the antagonists used in three different experiments, the damaged mucosal area of each stomach was normalized to the mean value of the respective ethanol control

group (100 %). Each column represents mean (\pm SEM) of 5 rats. $**p < 0.01$ compared with ethanol-treated group (column 2); $\#p < 0.05$, $##p < 0.01$ compared with allyphenyline 15 nmol-treated group (column 4) (ANOVA, Newman-Keuls post hoc test). **b** Representative macroscopic pictures demonstrating the mucosal protective effect of allyphenyline (ALL) and the inhibitory effect of ARC 239 (ARC), (S)-WAY 100135 (WAY), and JP-1302 (JP) on it. The dark, livid areas represent the hemorrhagic ulcerous part of the mucosa

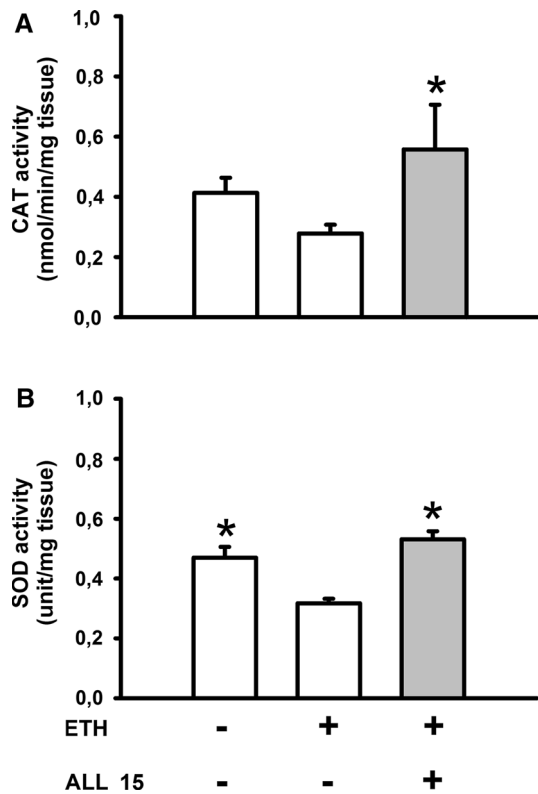
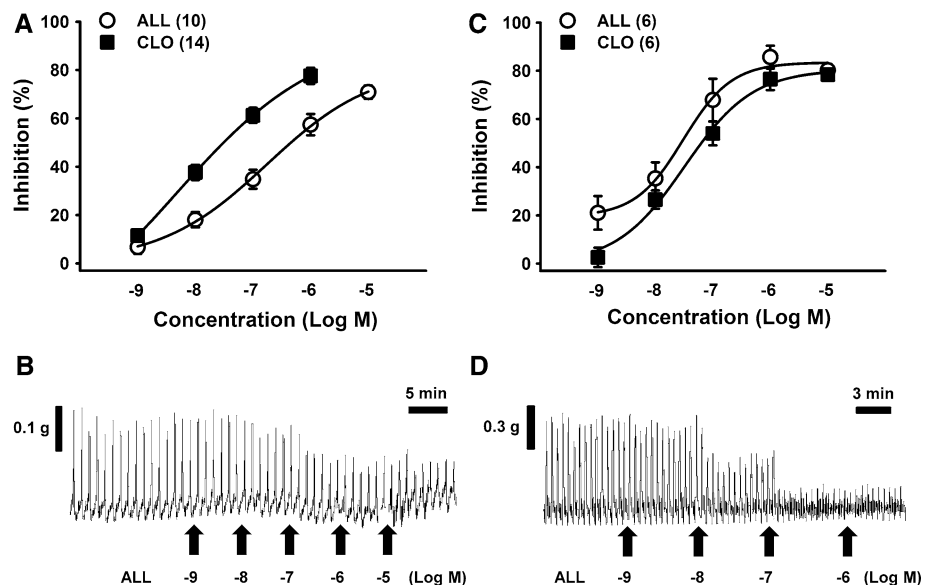


Fig. 3 The effect of ethanol (ETH) and allyphenyline (ALL) on the mucosal catalase (CAT, **a**) and superoxide dismutase (SOD, **b**) activity. Allyphenyline (15 nmol) was injected intracerebroventricularly (i.c.v.) 10 min before the ethanol challenge. Each column represents mean (\pm SEM) of 5–10 rats. * $p < 0.05$ compared with ethanol-treated group (column 2) (ANOVA, Newman-Keuls post hoc test)

tracings illustrating the effect of allyphenyline on EFS-induced fundic and colonic cholinergic contractions. In contrast to the EFS-evoked contractile response,

Fig. 4 The inhibitory effect of allyphenyline (ALL) and clonidine (CLO) on cholinergic fundic (**a**) and colonic (**c**) contractions induced by EFS in C57BL/6 mice. The numbers in brackets indicate the number of experiments. Both agonists were added cumulatively into the organ bath at increasing concentration every 5 min. Each concentration response curve represents the mean \pm SEM values of inhibition. **b**, **d** Representative tracings illustrating the inhibitory effect of allyphenyline (ALL, 10^{-9} – 10^{-5} M) on the EFS-induced fundic and colonic contractions in C57BL/6 mice



allyphenyline did not influence the nitregic relaxation (not shown).

Analyzing the Involvement of α_{2C} -ARs and $5HT_{1A}$ Receptors in the Allyphenyline-Evoked Inhibition of Fundic and Colonic Contractions

ARC 239 (1 μ M) did not influence the amplitude of EFS-induced twitch contractions in the stomach or in the colon per se, but significantly reduced the inhibitory effect of allyphenyline in both organs (Figs. 5a, 6a). However, in the stomach it shifted the concentration–response curve of allyphenyline rightward, and increased its EC_{50} value without significantly altering its E_{max} , while in the colon it reduced allyphenyline’s E_{max} , without significantly changing its EC_{50} value (Table 1). These results suggest that the interactions between allyphenyline and ARC 239 in the two organs might involve different mechanisms of action. (S)-WAY 100135 (1 μ M), similarly to ARC 239, did not alter EFS-induced fundic and colonic contractions alone, but significantly reduced the effect of allyphenyline in the stomach. On the other hand, it failed to modify that in the colon (Figs. 5b, 6b; Table 1). In contrast to the other two antagonists, JP-1302 (1 μ M) affected the effect of allyphenyline neither in the stomach nor in the colon (Figs. 5c, 6c; Table 1).

These data indicate that $5HT_{1A}$ receptors are involved in mediating the inhibitory effect of allyphenyline on fundic motility, but neither these receptors nor α_{2C} -ARs take part in the inhibition of EFS-induced contractions in the colon. Though previous in vitro and in vivo studies did not indicate the involvement of α_{2A} - and α_{2B} -ARs in the action of allyphenyline [22–24], we also wanted to exclude the

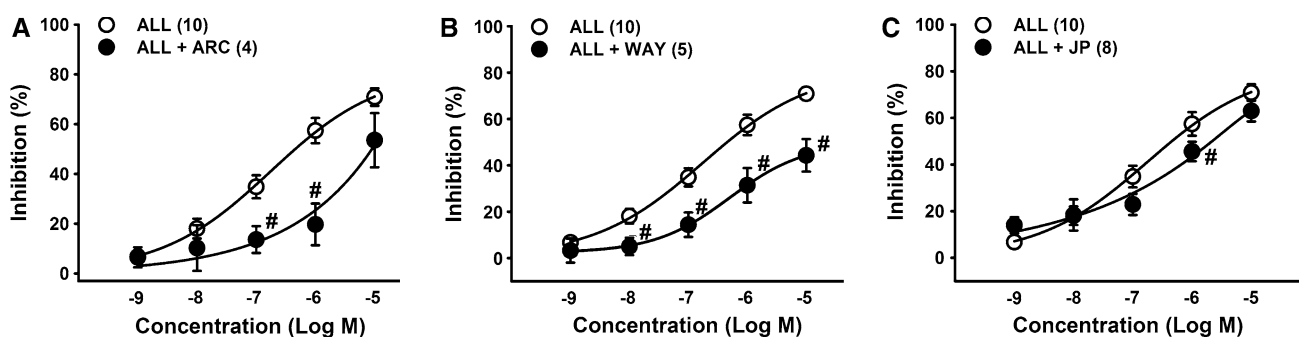
Table 1 EC₅₀ and E_{max} values of allyphenyline in the presence of α_{2C} -AR and 5HT_{1A} receptor antagonists

	Fundus			Colon		
	EC ₅₀ (nM)	E _{max} (%)	n	EC ₅₀ (nM)	E _{max} (%)	n
Clonidine	20.8 ± 6.2	83.2 ± 7.4	14	62.5 ± 43.5	78.8 ± 4.9	6
Allyphenyline	240.8 ± 71.6	74.7 ± 4.2	10	34.4 ± 15.8	92.3 ± 4.7	6
+ARC 239 1 μ M	4070 ± 1267 (<i>p</i> < 0.001)	68.4 ± 1.4 (ns)	4	115.5 ± 33.0 (ns)	59.8 ± 16.0 (<i>p</i> < 0.05)	6
+(S)-WAY 1 μ M	679.0 ± 197.6 (ns)	50.1 ± 9.1 (<i>p</i> < 0.05)	5	28.4 ± 9.2 (ns)	98.9 ± 0.6 (ns)	4
+JP-1302 1 μ M	514.0 ± 221.8 (ns)	78.4 ± 5.3 (ns)	8	26.2 ± 4.5 (ns)	84.9 ± 6.8 (ns)	8

Clonidine was used as a reference compound

n number of experiments, *ns* not significant

p < 0.05 was considered statistically significant (Student's *t* test)

**Fig. 5** The antagonistic effect of ARC 239 (ARC, 10⁻⁶ M, **a**), (S)-WAY 100135 (WAY, 10⁻⁶ M, **b**), and JP-1302 (JP, 10⁻⁶ M, **c**) on the inhibitory effect of allyphenyline (ALL, 10⁻⁹–10⁻⁵ M) on EFS-induced fundic contractions. Each circle represents mean ± SEM

values of inhibition (*empty circles* agonist alone, *filled circles*: agonist + antagonist), *numbers in brackets* indicate the number of experiments. #*p* < 0.05 (two-way repeated measures ANOVA, Holm-Sidak post hoc test)

participation of these receptors in the inhibitory effect of allyphenyline on colonic motility. Therefore, we compared its effect in wild-type, α_{2A} - and α_{2B} -AR KO mice. However, as Fig. 6d, e demonstrate, the concentration–response curves were the same in all lines, which rules out the involvement of these α_{2} -AR subtypes in the effect of allyphenyline.

Discussion

In the present study, we aimed to characterize the GI effects of a novel α_{2C} -AR agonist/ α_{2A} -AR antagonist/5HT_{1A} receptor agonist agent named allyphenyline, in terms of gastroprotection and regulation of GI motility. Our data demonstrate that allyphenyline possesses powerful gastroprotective action, which is mediated by central α_{2C} -ARs and 5HT_{1A} receptors, and inhibits both fundic and colonic contractions evoked by EFS, although probably via

different, 5HT_{1A} receptor-dependent and receptor-independent mechanisms, respectively.

It is well established that peptic ulcers develop as a result of impaired balance between the aggressive and protective factors of gastroduodenal mucosa [40]. Although introduction of antisecretory agents and antibiotic protocols against *H. pylori* resulted in a significant fall of the incidence of peptic ulcers, there is still a clinical need to find novel gastroprotective drugs which prevent the development and/or accelerate the healing of nonsteroidal anti-inflammatory drug (NSAID)-induced ulcers, *H. pylori* positive and negative gastroduodenal ulcers as well as stress-related ulcers in critically ill patients [41–43]. Since the historic article of Robert [44] on “gastric cytoprotection,” numerous endogenous and exogenous compounds and receptors have been shown to induce and mediate gastroprotection, i.e., prevention of ulcer formation without influencing gastric acid secretion (recent reviews: [42, 45–49]).

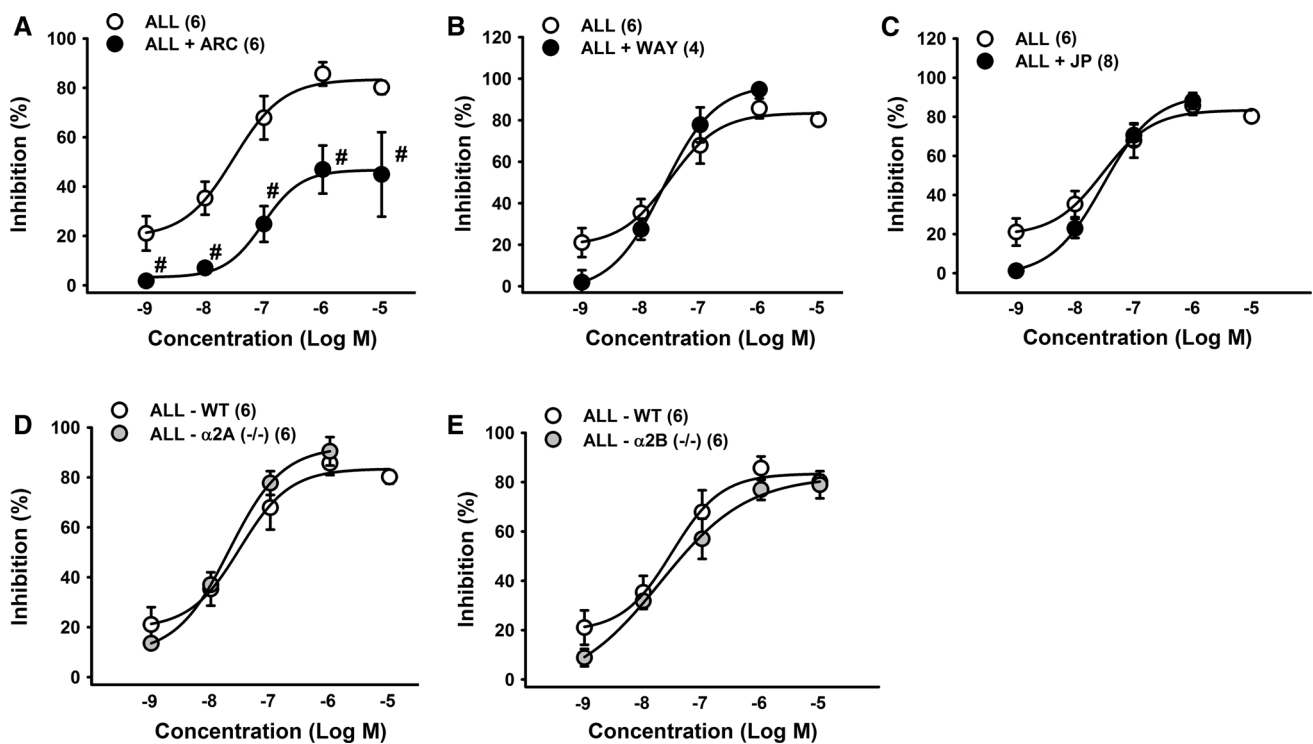


Fig. 6 a–c The antagonistic effect of ARC 239 (ARC, 10^{-6} M, a), (S)-WAY 100135 (WAY, 10^{-6} M, b), and JP-1302 (JP, 10^{-6} M, c) on the inhibitory effect of allyphenyline (ALL, 10^{-9} – 10^{-5} M) on EFS-induced colonic contractions. Each circle represents mean \pm SEM values of inhibition (empty circles agonist alone, filled circles agonist + antagonist), numbers in brackets indicate the number of experiments. # $p < 0.05$ (two-way repeated measures ANOVA, Holm-

Sidak post hoc test). d, e The inhibitory effect of allyphenyline (ALL) on EFS-induced colonic contractions in wild-type and α_{2A} - and α_{2B} -adrenoceptor-deficient C57BL/6 mice. Each circle represents mean \pm SEM values of inhibition (empty circles wild-type mice, filled circles respective knock out strain), numbers in brackets indicate the number of experiments

In the last two decades, our group provided mounting evidence for the gastroprotective effect of α_2 -AR agonists. We demonstrated that clonidine and rilmenidine, when given either peripherally (per os, subcutaneously) or centrally (i.c.v.), inhibited potently the direct necrotizing action of acidified ethanol on gastric mucosa in both rats and mice [12–17]. Our results also showed that the site of action is presumably within the CNS [16, 17], where the activation of α_{2B} - and α_{2C} -ARs initiates a chain of events resulting in vagally mediated release of mucosal protective factors, such as prostaglandins and nitric oxide [13, 16]. Because α_2 -AR agonists are widely used as antihypertensive drugs, as adjuncts to anesthetics and analgesics during the perioperative period, or as drugs to treat opiate withdrawal or attention-deficit/hyperactivity disorder [50], a potential anti-ulcer effect is of high clinical relevance. However, non-selective activation of all three α_2 -AR subtypes in the ulcer therapy would result in numerous undesired side effects, mainly mediated by the α_{2A} -AR subtype (hypotension, bradycardia, sedation), but partly also by the α_{2B} -AR subtype (vasoconstriction) [2, 4, 19]. Hence, selective stimulation of α_{2C} -ARs would provide a safer approach to treat upper GI mucosal lesions.

Besides α_2 -ARs also $5HT_{1A}$ receptors have been implicated in the regulation of gastric mucosal integrity. Various selective $5HT_{1A}$ receptor agonists, such as buspirone, 8-OH-DPAT, lesopitron (E-4424), E-4414, or E-4804, given either orally or i.p., were shown to inhibit gastric acid and pepsin secretion, to increase gastric adherent mucus levels and to reduce mucosal injury in both acid-dependent (cold-restraint stress, indomethacin, conditioned fear) and acid-independent (ethanol) ulcer models [27, 28, 51–53]. Whether the site of action is within the CNS or in the periphery has not been established, but Farre et al. [27] (based on unpublished observations) suggested that both central and peripheral effects may contribute to the mucosal protection. The possibility of a central component is indirectly supported by Hoshino and Sugizaki [54], who reported that destruction of the median raphe nucleus, a significant site of central 5-HT cell bodies, was associated with an increased incidence of gastric lesions.

Based on the data described above, we hypothesized that allyphenyline, a novel compound synthesized by Gentili et al. [22] possessing a particularly intriguing receptorial profile (α_{2C} -AR agonist/ α_{2A} -AR antagonist/ $5HT_{1A}$ receptor agonist), would exert potent gastroprotective action. Our

results confirmed this hypothesis and showed that centrally injected allyphenyline almost completely inhibited the development of gastric mucosal damage induced by ethanol, indicating its ability to recruit protective factors and enhance mucosal defense. This effect was inhibited by co-injection with ARC 239 ($\alpha_{2B/C}$ -AR and 5HT_{1A} receptor antagonist), (S)-WAY 100135 (selective 5HT_{1A} receptor antagonist), and JP-1302 (selective α_{2C} -AR antagonist), which suggests that central α_{2C} -ARs and 5HT_{1A} receptors contribute equally to the gastroprotective action. It is of note that similar dual receptorial action was observed by Del Bello et al. [25], who reported that yohimbine and (S)-WAY 100135 similarly contrasted the antidepressive-like effect of allyphenyline.

Although a detailed analysis of the peripheral factors involved in the mucosal protection was above the scope of the present study, we addressed the question of whether allyphenyline is able to counteract oxidative stress, an important factor in the pathomechanism of various experimental ulcers (e.g., NSAID, stress, ethanol) [55–58], as well as in *H. pylori*-associated ulcers [59]. It is well established that excessive production of reactive oxygen species (ROS) and/or their decreased elimination by antioxidant enzymes induce cell and tissue damage by promoting lipid peroxidation and increasing the production of inflammatory mediators and proinflammatory cytokines [60]. Reduced mucosal antioxidative capacity and increased level of lipid peroxidation products (e.g., malondialdehyde) correlate well with macroscopic and histological mucosal damage, and agents endowed with antioxidant property are able to prevent ROS-mediated cellular damage [55–58, 61]. Our results show that allyphenyline significantly increased the mucosal content of SOD [enzyme which catalyzes the dismutation of O₂^{•-} into less noxious hydrogen peroxide (H₂O₂)] and CAT (which accelerates the breakdown of H₂O₂ to water and oxygen) compared to the ethanol-treated group, suggesting that the gastroprotective action of allyphenyline—at least partly—relies on its ability to enhance the activity of mucosal antioxidant enzymes and reduce the damaging effect of ROS.

Besides inducing gastric mucosal protection, allyphenyline also inhibited EFS-induced cholinergic contractions in the murine fundus ex vivo. Compared to clonidine, a non-selective α_2 -AR agent, allyphenyline proved to be one order of magnitude less potent. Because among the three α_2 -AR subtypes allyphenyline activates only the α_{2C} -AR subtype [22, 23], which does not have any role in the modulation of gastric motility in mice [34], the inhibitory effect of allyphenyline may result from its serotonergic property, and not from an interaction with α_2 -ARs. Indeed, we found that (S)-WAY 100135 significantly reduced the inhibitory effect of allyphenyline in the

stomach, similarly to ARC 239, while JP-1302 had no relevant effect on it. These findings indicate the involvement of 5HT_{1A} receptors in the inhibitory response, which is in line with previous observations. Namely, presynaptic 5-HT_{1A} receptors were shown to mediate the inhibition of transmitter release from cholinergic nerve endings in the guinea pig antrum [29], and various 5HT_{1A} receptor agonists (buspirone, R137696 or flesinoxan) were shown to relax the proximal stomach in mice [30, 62], dogs [63], and also in humans [64, 65]. In accordance with the literature data [63], we found that nitrergic nerves are not involved in the 5-HT_{1A} receptor-mediated fundic relaxation, because allyphenyline did not influence the EFS-induced L-NNA-sensitive relaxation response.

Relaxation of the fundus, either by inhibiting the cholinergic drive or by stimulating the activity of inhibitory (nitrergic or VIP-ergic) neurons, may result in enhanced gastric accommodation [66]. Since impaired gastric accommodation is a hallmark of functional dyspepsia (FD) and contributes to a number of dyspeptic symptoms including nausea and early satiety, improving gastric accommodation by 5HT_{1A} receptor agonists is a promising approach to treat dyspeptic patients [66–68]. Supporting this concept, buspirone and tandospirone significantly improved the symptoms of FD patients [69, 70], although in the case of tandospirone the beneficial effect was attributed mainly to its anxiolytic property. Considering the potent gastroprotective, fundic relaxing and anxiolytic [26] properties of allyphenyline, we propose that this compound may be a useful tool in the therapy of both peptic ulcers and non-ulcer dyspepsia.

Our results demonstrate that in addition to the fundic relaxation, allyphenyline also potently inhibits cholinergic contractions in the colon, however, via an unknown mechanism. First, neither pharmacologic inhibition of α_{2C} -ARs by JP-1302 nor genetic deletion of α_{2A} - and α_{2B} -ARs altered the effect of allyphenyline. These results not only indicate that α_{2C} -ARs are not involved in the control of colonic motility, but also confirm the original findings of Gentili et al. [22] that allyphenyline does not activate the α_{2A} -AR subtype, which otherwise regulates colonic contractility [71]. Second, the effect was not affected by (S)-WAY 100135, which rules out the involvement of 5-HT_{1A} receptors. The exact role of these receptors in the control of colonic motility is still enigmatic [72], but recent findings suggest that these receptors take part in the modulation of colonic migrating motor complex by limiting the neurotransmitter output from intrinsic primary afferent neurons (IPANs) to ascending (S Type I) interneurons [31]. Our results suggest that 5-HT_{1A} receptors are not involved in the inhibition of cholinergic contractions in the murine colon. Interestingly, ARC 239 reduced the colonic effect of allyphenyline, which implies that it interferes with the

binding of allyphenyline to another, yet unidentified molecular target. However, even if the exact pharmacodynamic action remains to be established, the modulatory action of allyphenyline in the colon may be useful in various conditions with increased motility. It should be noted that there is a considerable overlap between FD and IBS patients [73, 74], and allyphenyline may have significant impact on most symptoms in these patients.

In summary, we demonstrated herein that allyphenyline possesses powerful gastroprotective action, which is mediated by central α_{2C} -ARs and 5HT_{1A} receptors, and inhibits both fundic and colonic contractions evoked by EFS, via 5HT_{1A} receptor-dependent and receptor-independent mechanisms, respectively. These results (1) confirm the role of central α_{2C} -ARs in the regulation of gastric mucosal integrity and provide the first evidence for the role of central 5HT_{1A} receptors in it, (2) confirm the presence and inhibitory effect of presynaptic 5HT_{1A} receptors on cholinergic motor neurons at the level of stomach, and (3) from a clinical aspect suggest that allyphenyline may be a particularly useful tool in the treatment of various GI disorders, such as peptic ulcers, functional dyspepsia, or irritable bowel syndrome.

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Compliance with ethical standards

Conflict of interest None.

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