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ARTICLE

Lubeluzole: from anti-ischemic drug to preclinical antidiarrheal studies

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Abstract

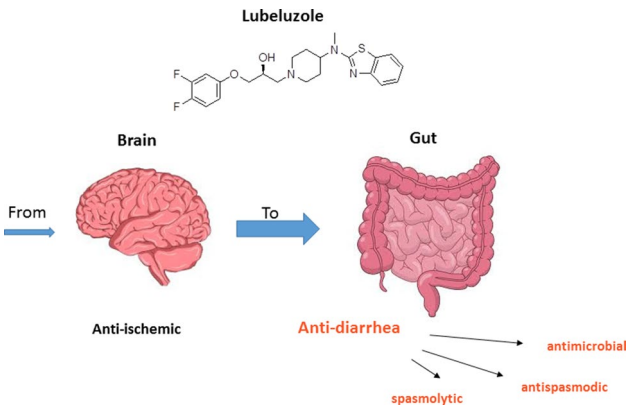
Background Lubeluzole, a neuroprotective anti-ischemic drug, was tested for its ability to act as both antibiotic chemosensitizing and antipropulsive agent for the treatment of infectious diarrhea.

Methods In the present report, the effect of lubeluzole against antidiarrheal target was tested. The antimicrobial activity towards Gram-positive and Gram-negative bacteria was investigated together with its ability to affect ileum and colon contractility.

Results Concerning the antimicrobial activity, lubeluzole showed synergistic effects when used in combination with minocycline against four common Gram-positive and Gram-negative bacteria (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922), although relatively high doses of lubeluzole were required. In ex vivo experiments on sections of gut smooth muscles, lubeluzole reduced the intestinal contractility in a dose-dependent manner, with greater effects observed on colon than on ileum, and being more potent than reference compounds otilonium bromide and loperamide.

Conclusion All above results identify lubeluzole as a possible starting compound for the development of a novel class of antibacterial adjuvants endowed with spasmolytic activity.

Graphic abstract



Keywords Lubeluzole · Diarrhoea · Ion channels · Antibiotics · Synergism · Gut contractility

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Extended author information available on the last page of the article

Abbreviations

Ach	Acetylcholine
ATCC	American Type Culture Collection
BSCA	Basal spontaneous contraction activity
CaM	Calmodulin

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CLSI	Clinical and Laboratory Standard Institute
CFU	Colony forming units
DMSO	Dimethyl sulfoxide
EFS	Electrical field stimulation
FIC	Fractional inhibitory concentration
FICI	Fractional inhibitory concentration index
FFT	Fast Fourier transform
GI	Gastrointestinal
hERG	Human ether-à-go-go related gene
MCA	Mean contraction amplitude
MDR	Molecular drug resistance
MH	Muller Hinton
MHB	Mueller Hinton broth
MICs	Minimal inhibitory concentrations
OB	Otilonium bromide
OD	Optical density
PSS	Physiological salt solution
SCV	Spontaneous contraction variability
SEM	Standard error of the mean
SC	Spontaneous contractions
SCV	Spontaneous contraction variability
UTHSCSA	University of Texas Health Science Center at San Antonio

Introduction

Food borne diseases are illnesses that result from the consumption of food contaminated with pathogenic bacteria, viruses, or parasites. They cover either clinically mild gastroenteritis that requires only outpatient cares (e.g., traveller's diarrhoea) [1] or severe illness that can culminate in a prolonged hospitalization. The symptoms generally include fever, abdominal cramping, dehydration, nausea, and diarrhoea, with the latter representing one of the main causes of morbidity and mortality mostly in infants and immunocompromised population, especially in developing regions [2, 3]. The management of foodborne illness includes symptomatic treatment with antidiarrheal medications, such as anticholinergics and antimotility agents, as well as antiemetics in patients with clinically significant vomiting. In case of acute infectious diarrhoea, antibiotic therapy may be necessary to prevent bacteremia, especially in the elderly, infants, and immunocompromised patients [2]. Unfortunately, bacterial agents associated with gastroenteritis have become increasingly resistant to various conventional antimicrobial therapies [4–6], indicating the need to search for new effective molecules. Multi-target non-antibiotic drugs that would allow the management of diarrhoea inhibiting the intestinal motility while potentiating conventional antibiotics might represent a valuable therapeutic option.

Regarding the electrical regulation of the gastrointestinal (GI) motility, it is well known that many of the voltage-sensitive ion channels are involved [7–9]. Voltage-gated Ca^{2+} channels are abundantly expressed in GI tissues [8, 10] and calcium channel blocking agents notoriously inhibit smooth muscle contraction [10–12]. On the other hand, voltage-gated Na^{+} channels also contribute to the GI motility as further demonstrated when the constipation effect of ranolazine has been recently attributed to the Na^{+} current inhibition [13]. Furthermore, human ether-à-go-go-related gene (hERG) K^{+} channel is involved in the formation of slow waves in the smooth muscle cells thus contributing to gastrointestinal motility [14].

Since 2006, we have been involved in the study of lubeluzole (Fig. 1), a clinically relevant anti-ischemic drug which gave constipation in 20% of the treated subjects in the course of its clinical trials [15] and proved to be a Na^{+}, Ca^{2+}, and hERG channel blocker [16–18]. These evidences prompted us to investigate the possible spasmolytic effects of lubeluzole on the GI by inhibiting the intestinal mechanical activity.

On the other hand, we have previously demonstrated that lubeluzole also acts as a calmodulin (CaM) antagonist [19]. Possible antibacterial properties against both Gram-positive and Gram-negative bacteria have been reported for CaM antagonists [20, 21]. Na^{+} and Ca^{2+} channel blocking activity might result in inhibition of bacterial chemotaxis [22], and lubeluzole contains in its structure the benzothiazole privileged structure found in numerous antibacterial agents [23, 24]. For all above, we also evaluated the possibility that lubeluzole was active against foodborne pathogenic bacteria associated with acute gastroenteritis. Furthermore, we also explored the possibility that lubeluzole displayed synergy with currently used antibacterial agents, since it is known that some calcium channel modulators, such as verapamil [25, 26], nifedipine, or diltiazem analogues, possess MDR reverting ability [27, 28].

Thus, the possible role of lubeluzole as anti-infective chemosensitizer agent endowed with antidiarrheal potential has been explored in this study, with the aim of identifying a new entry to compounds for a novel class of antidiarrheal agents.

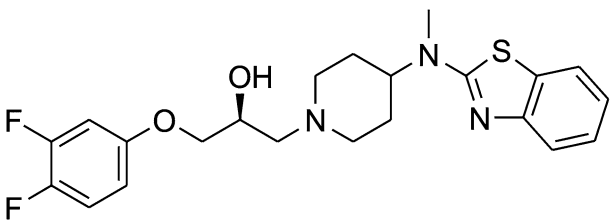

Fig. 1 Chemical structure of lubeluzole

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich or Alfa Aesar at the highest quality commercially available. Details about preparation of Lubeluzole are previously reported [19, 29, 30].

Antimicrobial activity

Combination lubeluzole/ciprofloxacin

Lubeluzole was stocked at 1220 µg/ml in 100% DMSO and diluted in sterile distilled water containing 1% DMSO (final concentrations: 512–4 µg/ml). Ciprofloxacin [≥ 98.0% (HPLC) Merck Life Science S.r.l., Milan, Italy] was dissolved in distilled water at 1.700 µg/ml and filter-sterilized (0.22 µm). This solution was further diluted with sterile saline solution (0.85% NaCl) at the final concentrations of 1–0.05 µg/ml.

Possible synergistic effects of lubeluzole with ciprofloxacin were studied for its antimicrobial activity against the foodborne pathogens *Yersinia enterocolitica* DSM 4780 and *Bacillus cereus* DSM 4313 stored in ISPA-CNR Microbial Collection of Bari and generally associated with acute gastroenteritis [31, 32]. The strains were routinely cultured in their appropriate media before being screened using agar disk diffusion assay method according to EUCAST guidelines (EUCAST, 2017) [33]. Briefly, a sterile cotton swab soaked with 0.5 McFarland solution of each strain was spread on Petri dishes with Muller Hinton agar (MH, Tryptone, 17.5 g/l; Beef extract, 2 g/l; Soluble starch, 1.5 g/L and Agar, 17 g/l). Then, cellulose disks (Oxoid), soaked with 20 µl of the different lubeluzole and ciprofloxacin solutions and their combinations, were applied to the surface of seeded agar plates. Plates were incubated at 37 °C for 16–20 h. Diameters (nearest millimetre) of inhibition zones around the assayed disks were measured with a calliper from the back of plate held above a dark background. Alternatively, diameters of inhibition halos were digitally measured on high-resolution images of inoculated plates using University of Texas Health Science Center at San Antonio (UTHSCSA) ImageTool 3.0 software (https://ddsdx.uthscsa.edu/dig/itdesc.html).

Subsequently, antibacterial combination screening of the most sensitive strains was carried out in broth against lubeluzole in the presence of ciprofloxacin following the methodology reported in Caputo et al. [34]. Screening was carried out in 200 µl in 96-well flat-bottom MIC plates, in duplicate, using Mueller Hinton Broth (MHB) with 1% DMSO lubeluzole and ciprofloxacin concentrations nearest their minimal inhibitory concentrations (MICs). The inoculum (1%, v/v, corresponding to *ca.* 5×10^5 CFU/ml) of each assayed strain was prepared from the overnight culture. Test volume was 200 µl per well. Background controls (8 wells per plate) contained only growth medium with DMSO (these were also the sterility controls). Growth controls, also eight wells per plate, contained growth medium with DMSO and inoculum. Plates were incubated at 37 °C for 24 h and growth kinetics were monitored every 10 min at 600 nm using an automated Microplate reader (Varioskan Flash, Thermo Fisher, Milan, Italy). The percentage growth for each test well was calculated as:

$$\left[\text{Optical density (OD)} - \text{mean background}\right] / \left[\text{mean growth} - \text{mean background}\right] \times 100,$$

as reported by Ejim et al. [35]. Hits were considered to be active compound concentrations where the growth average of the three replicates was < 55% of the mean growth control displaying mean difference greater than three standard deviations.

Combination lubeluzole/minocycline

Four bacterial strains from American Type Culture Collection (ATCC, Rockville, MD, USA) were used to test the antibacterial properties of lubeluzole and minocycline, alone and in combination. In particular, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used. The bacterial species were cultured on Mueller Hinton agar (MHA, Oxoid) and each bacterial suspension was composed of 2–3 colonies of each strain taken from an MHA plate and dissolved in 2 ml of MHB. The resulting suspensions were diluted with 0.85% NaCl solution and then adjusted to 0.5×10^{-3} to 2.5×10^{-3} CFU/ml (0.5 Mc Farland) [36]. The starting inocula were confirmed by plating serial dilutions and determining the colony counts. A total of 0.1 ml of each bacterial suspension were dispensed into serially diluted wells containing the drugs, reaching the final drug concentration. The MICs of lubeluzole and minocycline were determined by the broth microdilution method, according to the CLSI (Clinical and Laboratory standard Institute, 2018) protocol M7A10 guidelines. After the addition of 0.1 ml of inoculum, the trays were incubated at 35 °C for 48 h. MIC was defined as the lowest concentration that did not result in any visible growth of the bacterial strains compared to their growth in the control well. The MIC values are given in µg/ml. Four independent MIC determinations were performed.

Microdilution checkerboard method

In the combination assays, the checkerboard procedure described by White et al. [37] was followed to evaluate the synergistic action of the minocycline with lubeluzole. Twelve double serial dilutions of the two drugs were prepared following the same method used to evaluate the MIC values. In the first step of our experiments, the serial dilutions ranged from 40 to 5% of lubeluzole MIC and from 25 to 3% of minocycline MIC, in combination each other; subsequently, the percentage of the two drugs have been reversed and 40–5% of minocycline MIC and 25–3% of lubeluzole MIC have been used in combination. MIC data of minocycline and lubeluzole were converted into Fractional Inhibitory Concentration (FIC) determined using the formula:

$$\mathrm{FIC} = \left(\mathrm{MIC}^{\mathrm{combination}}/\mathrm{MIC}^{\mathrm{alone}}\right).$$

FIC index (FICI) for each combination has been then calculated by adding the minocycline FIC value and the lubeluzole FIC value at that combination. Generally, FICI value was interpreted as follows: (i) a synergistic effect when value ≤ 0.5; (ii) an additive or indifferent effect when value > 0.5 and < 1; (iii) an antagonistic effect when value > 1.

Gut motility ex vivo studies

Animals

All procedures in animals were performed in accordance with Guidelines and Authorization for the Use of Laboratory Animals (Italian Government, Ministry of Health). All procedures followed the guidelines of animal care and were approved by the Ethics Committee of the University of Bologna (Bologna, Italy) (Protocol 14/72/12).

Male Sprague–Dawley rats weighing 220–250 g at arrival (Harlan, San Pietro al Natisone, Udine, Italy) and male guinea pigs 300–350 g (Charles Rivers Laboratories, Calco LC, Italy) were used. Animals were housed in an animal facility with monitored temperature and light (12 h cycle and 21 ± 2 °C). The animals were allowed to acclimate to the environment for at least 7 days. All animals had free access to water and food, but they were deprived of food overnight before sacrifice.

Rat proximal colon

After general anesthesia (pentobarbital 80 mg/kg *ip*), rats were killed by cervical dislocation. A 3-cm section of proximal colon (taken at 1 cm from ileocecal sphinter) was obtained through a midline incision of the abdomen and was immediately placed in a cooled modified Krebs' solution (pH 7.4) of the following composition (mM): NaCl 113, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ (H_2O) 2.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.5, and ascorbic acid 5.5. It was then cleaned and rinsed, and a circular ring 0.5 cm long was cut and mounted in an organ bath (20 ml) filled with modified Krebs' solution, maintained at 37 °C and aerated with a mixture of 95% O_2 and 5% CO_2. One end of the circular ring was connected to a metal rod, while the other end was attached to a strain gauge transducer (FORT 25, WPI, Sarasota, FL, USA). Isometric tension was measured by the PowerLab data acquisition system and recorded using Chart 5.5.5 (ADInstruments, Castle Hill, Australia). The tissue was allowed to equilibrate for at least 30 min prior to the start of the experiment. An initial load of 0.5 g tension was applied to the preparation. Concentration–response curves to ACh (0.1–100 μM) were constructed on rat colon before and after 30-min lubeluzole (0.001–1 μM) or 20-min loperamide (0.1 and 1 μM) incubation. The data are expressed as percentage of tension values reached with the highest dose of acetylcholine used (100 μM).

Moreover, the specimens were subjected to transmural stimulation (Electrical Field Stimulation, EFS) at frequencies of 1, 3, 5, and 10 Hz (14 V, 1 ms, pulse trains lasting 10 s) through two parallel platinum electrodes connected to a stimulator (Digital Stimulator, LE 12106, Letica, Ugo Basile, Italy). The EFS induces an immediate relaxation of specimens followed, at the end of EFS, by a contraction called off-contraction. This contractile response is the result of nervous reflex as it is abolished by tetrodotoxin, and reduced by atropine and by tachykinin antagonist [38].

Guinea pig gut segments

The animals were sacrificed by cervical dislocation, and the organ required was set up rapidly under a suitable resting tension in 15 ml organ bath containing appropriate physiological salt solution (PSS) consistent warmed (see below) and buffered to pH 7.4 by saturation with 95% O_2–5% CO_2 gas mixture. After the equilibration period (see specific method), for all the tissue cumulative-concentration curves of lubeluzole (0.1, 0.5, 1, 5, 10, 50, and 100 μM) first dissolved in DMSO and then diluted with PSS. According to this procedure, the concentration of DMSO in the bath solution never exceeded 0.3%, a concentration which did not produce appreciable effects. During the generation of cumulative concentration–response curves for ileum and proximal colon, the next higher concentration of the compounds was added only after the preparation reached a steady state.

In all experiments, otilonium bromide (OB) was used as reference compound.

Ileum The terminal portion of ileum (2–3 cm near the ileo-caecal junction) was cleaned, and segments 1 cm long of ileum were set up under 1 g tension at 37 °C in organ baths containing Tyrode solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.20, $KH_2PO_4 \cdot 2H_2O$ 1.19, $NaHCO_3$ 25 and 11 glucose equilibrated with 95% O_2–5% CO_2 gas at pH 7.4. The intestine was removed above the ileo-caecal junction. Segments of 2 cm length were mounted under a resting tension of 300–400 mg. Strips were secured at one end to a force–displacement transducer (FT 0.3, Grass Instruments Corporation) for monitoring changes in isometric contraction.

Proximal colon Starting approximately 1 cm distal from the cecocolonic junction, two segments of about 1 cm of the guinea pig proximal colon were cut. The proximal colon was cleaned by rinsing it with De Jalon solution of the following composition (mM): NaCl 155, KC1 5.6, $CaCl_2$ 0.5, $NaHCO_3$ 6.0, and 2.8 glucose. Then the mesenteric tissue was removed. The two segments were suspended in organ baths containing gassed, warm de Jalon solution under a load of 1 g maintained at 37 °C. Tension changes in longitudinal muscle length were recorded.

Spontaneous contraction

The tracing graphs of spontaneous contractions (SC) of ileum and colon were continuously recorded with the Lab-Chart Software (AD Instruments, Bella Vista, New South Wales, Australia). After the equilibration period (about 30–45 min according to each tissue) cumulative-concentration–response curves of drug were constructed. At the end of each single concentration, the following parameters were evaluated considering a 5 min stationary period:

- Mean contraction amplitude (MCA), calculated as the mean force value;
- Spontaneous contraction variability (SCV), measured as the standard deviation (g) of the force contraction values;
- Basal spontaneous contraction activity (BSCA), calculated as the percentage (%) variation of each mean force value (g) with respect to the control;
- Spontaneous contractions rates through a standard FFT (fast Fourier transform) analysis and the evaluation of the absolute powers of the following frequency bands of interest (expressed in Hz):][0.0,0.1];][0.1,0.2];][0.2,0.3];][0.3,0.4];][0.4,0.5];][0.5,0.6];][0.6,0.7];][0.7,0.8];][0.8,0.9];][0.9,1.0].

All the calculations were performed in a post-processing phase with the Lab Chart Software. To avoid errors due to the presence of artefacts, the period of analysis was chosen by a skilled operator.

Induced contraction

One set of experiments was carried out using heighty K^+ concentration for ileum and colon [39]. Briefly, after the equilibration period, gut strips were contracted by washing in specific PSS (ileum and colon) containing 80 mM KCl (equimolar substitution of K^+ for Na^+). When the contraction reached a plateau (about 30–45 min) cumulative-concentration curves of lubeluzole were constructed. Concentrations of lubeluzole were added cumulatively to the bath allowing for any relaxation to obtain an equilibrated level of force. Tension changes were recorded isometrically.

Statistical analysis

Data obtained from guinea pig were analyzed by the Student's *t* test and presented as means ± S.E.M. [40] and *P* value less than 0.05 has been considered significant. The potency of lubeluzole defined as IC_{50} was calculated from concentration–response curves (Probit analysis using Litchfield and Wilcoxon [40] or GraphPad Prism® software [41, 42].

Data from rats were analyzed by two-way ANOVA for repeated measure followed by *t* test for paired data. Results are presented as mean ± SEM. The level of significance was set at $p < 0.05$. For each experiment, 5–6 preparations were used.

Results

Antimicrobial activity

As the antimicrobial activity concerns, we first evaluated lubeluzole against foodborne pathogenic bacteria involved in acute gastroenteritis infections (*Yersinia enterocolitica* and diarrheal *Bacillus cereus* group) [43–48], but no assayed strains were inhibited at all the concentrations of lubeluzole (4–512 µg/ml) tested by disk diffusion assay. Then, we investigated lubeluzole possible synergistic effects when used in combination with an antibiotic; we choose ciprofloxacin, given the clinical relevance of the latter as an antibiotic of choice in the treatment of infectious diarrhea (500 mg/twice daily) [31, 32].

MIC values of the chosen antibiotic against *Y. enterocolitica* DSM 4780 and *B. cereus* DSM 4313 were 0.025 and 0.05 µg/ml, respectively. When lubeluzole was tested in combination with ciprofloxacin on Petri dishes seeded with DSM 4313, appreciable increases in inhibitory halo diameters were registered with 0.5 µg/ml of ciprofloxacin and 64 or 128 µg/ml of lubeluzole. By contrast, lubeluzole did not modify the activity of ciprofloxacin against DSM 4780. Based on these results, the antibacterial activity of

Table 1 Growth percentages of *B. cereus* DSM 4313 at 16 h of incubation in presence of different concentrations of ciprofloxacin and lubeluzole, assayed alone or in combination

	Lubeluzole (μg/ml)		
	0	64	128
Ciprofloxacin (μg/ml)			
0	100 ± 7	53.2 ± 2.0	1.0 ± 0.2
0.025	98.6 ± 1.7	51 ± 3	0.7 ± 0.5
0.05	82 ± 11	44.5 ± 2.8	1.3 ± 0.8
0.5	0.40 ± 0.01	0.6 ± 0.1	0.2 ± 0.1

The values represent mean ± standard deviation ($n = 3$) comparing OD600 nm of each culture to the mean OD of the control growth (no compounds added)

Table 2 Growth percentages of *Y. enterocolitica* DSM 4780 at 16 h of incubation in presence of different concentrations of ciprofloxacin and lubeluzole, assayed alone or in combination

	Lubeluzole (μg/ml)		
	0	64	128
Ciprofloxacin (μg/ml)			
0	100 ± 6	97 ± 6	3.1 ± 0.4
0.025	99.4 ± 1.8	21 ± 6	3.1 ± 1.5
0.05	8 ± 3	35.4 ± 2.0	2.7 ± 0.5
0.5	5.2 ± 2.8	2.8 ± 1.1	2.2 ± 0.2

The values represent mean ± standard deviation ($n = 3$) comparing OD600 nm of each culture to the mean OD of the control growth (no compounds added)

ciprofloxacin (0.025, 0.05, and 0.5 μg/ml) and lubeluzole (64 and 128 μg/ml) was then evaluated, both alone and in combination, in broth cultures of DSM 4313 and DSM 4780. After 16 h of incubation, the growth control cultures of DSM 4313 and DSM 4780 strains reached growth plateau (1.227 OD and 0.353 OD on average, respectively). Surprisingly, both bacterial cultures were inhibited in the presence of high concentration of lubeluzole (128 μg/ml); only DSM 4313 strain showed a 53.2% reduction in maximum growth at 64 μg/ml concentration of lubeluzole (Tables 1, 2). Conversely, the inhibitory activity of ciprofloxacin was confirmed on both tested strains (Tables 1, 2), although at higher concentrations than those observed in the disk diffusion assay. No synergy was observed on DSM 4313, since the strong or moderate inhibition of bacterial growth obtained combining sub-lethal concentration of ciprofloxacin (0.05 and 0.025 μg/ml) with 128 and 64 μg/ml of lubeluzole, respectively, could rather be attributed to the lubeluzole effect alone. Only a moderate decrease in DSM 4313 growth was found when the sub-lethal concentration of ciprofloxacin (0.05 μg/ml) was combined with 64 μg/ml of lubeluzole (Table 1). Conversely, lubeluzole at 64 μg/ml concentration reduced the DSM 4780 growth percentage to one-fifth when used in combination with ciprofloxacin (0.025 μg/ml). Seemingly, an antagonistic effect was obtained combining 0.05 μg/ml of ciprofloxacin and 64 μg/ml of lubeluzole, with the growth percentage being higher than that observed when lubeluzole was used in combination with 0.025 μg/ml ciprofloxacin (35.4% and 21.1% of growth percentage, respectively). Therefore, among all the combinations tested, only the association 0.025 μg/ml ciprofloxacin–64 μg/ml lubeluzole gave an interesting effect, since the MIC of the two drugs in combination is halved compared to their individual MIC values.

On the contrary, the inhibition observed with the associations 0.05 or 0.5 μg/ml ciprofloxacin–64 μg/ml lubeluzole could be conceivably due to ciprofloxacin alone, with its MIC value being 0.05 μg/ml. Likewise, when lubeluzole was tested at its MIC value (128 μg/ml) in association with ciprofloxacin, the antimicrobial effect observed could be derived from lubeluzole, regardless of a possible synergistic effect with the antibiotic.

To explore the wideness of the antibacterial spectrum of lubeluzole, its effect on two common strains of Gram-positive (*Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213) and Gram-negative (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) which are etiologic agents causing diarrhea has also been evaluated as well as the effects of its combination with antibiotics.

Some experiments are carried out in combination with minocycline [35]. Fractional inhibitory concentration (FIC) index (FICI) has been used as a measure of synergy, with FICI value ≤ 0.5 indicating synergy. At first, experiments with minocycline concentrations ranging from 40 to 5% and lubeluzole concentrations ranging from 25 to 3% of their corresponding MIC values were performed. In the second set of experiments, minocycline and lubeluzole concentrations have been inverted (lubeluzole: 40–5%; minocycline: 25–3%) and the inhibiting results, in agreement with those obtained with the reciprocal percentages, are depicted in Fig. 2. The FICI values corresponding to the lowest concentration of minocycline in combination with lubeluzole are reported in Table 3. As can be seen in Fig. 2 and Table 3, in all the tested bacterial strains, the minocycline MICs determined in the presence of lubeluzole were significantly reduced. FICI values indicated synergistic behaviour for numerous combinations thus indicating lubeluzole as an efficient chemosensitizer of minocycline antibiotic activity.

Possible use of lower amounts of the antibiotic in a combination therapy with the non-antibiotic lubeluzole is suggested. It is worth noting that concentrations as low as 3% of the antibiotic MIC values could give 100% inhibition of bacteria growth in the four strains, when in combination with lubeluzole concentrations ranging from 20 to 40% of its corresponding MIC depending on the strain. The possibility to reverse minocycline resistance through the combination therapy of the antibiotic with lubeluzole should be taken into account.

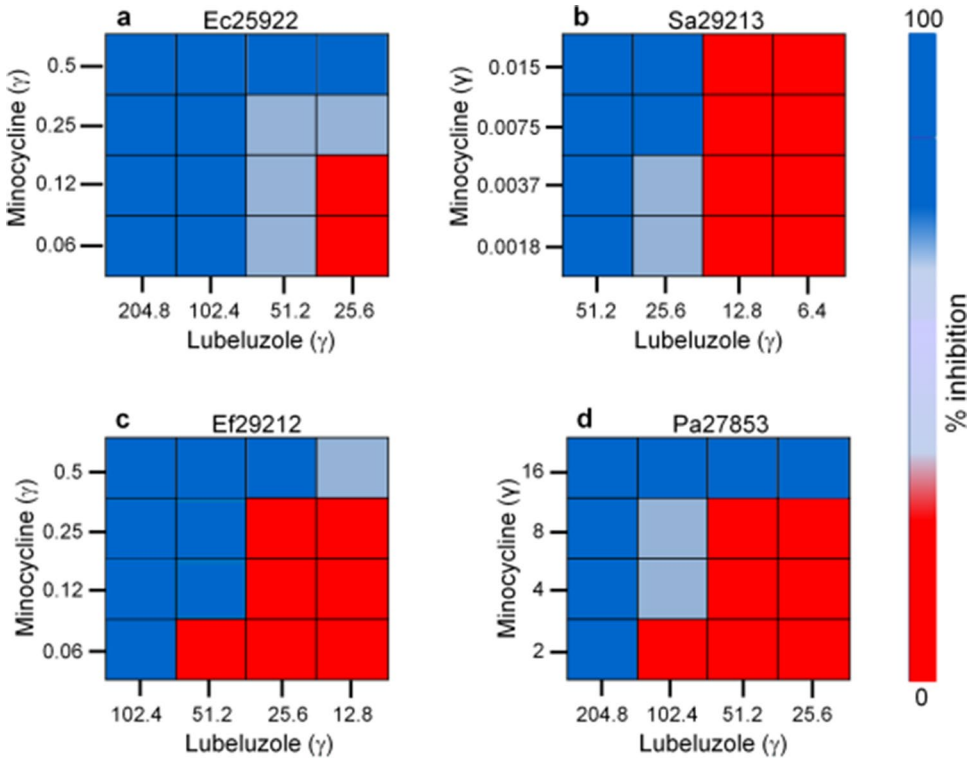

Fig. 2 Antimicrobial effect of lubeluzole in combination with minocycline. Inhibitory effect of the MIC dilutions of lubeluzole/minocycline against *Staphylococcus aureus* ATCC 29213 (**a**), *Escherichia coli* ATCC 25922 (**b**), *Enterococcus faecalis* ATCC 29212 (**c**), and *Pseudomonas aeruginosa* ATCC 27853 (**d**)

Table 3 Lubeluzole-minocycline fractional inhibitory concentration (FIC) and FIC indices (FICI)

	MICo[a] (μg/mL)	MICc[b] (μg/mL)	FIC[c]	FICI[d]
Escherichia coli ATCC 25922				
Minocycline	2	0.06	0.03	0.23
Lubeluzole	512	102.4	0.2	
Staphylococcus aureus ATCC 29213				
Minocycline	0.06	0.0018	0.03	0.43
Lubeluzole	128	51.2	0.4	
Enterococcus faecalis ATCC 29212				
Minocycline	4	0.06	0.03	0.43
Lubeluzole	256	102.4	0.4	
Pseudomonas aeruginosa ATCC 27853				
Minocycline	64	2	0.03	0.43
Lubeluzole	512	204.8	0.4	

[a]MIC alone

[b]MIC in combination

[c]$FIC = MIC^{combination}/MIC^{alone}$

[d]FICI = FIC of minocycline + FIC of lubeluzole

Spasmolytic effect on rat colon

Lubeluzole was tested for its spasmolytic effect through ex vivo experiments on sections of rat proximal colon with loperamide, a well-known antidiarrheal agent, being used as a reference compound. At first, the effects of two different concentrations (0.1 and 1 μM) of lubeluzole and loperamide on colonic contractile response to acetylcholine (ACh, 0.1–100 μM) were evaluated (Fig. 3a, b, respectively).

Although the two compounds showed a similar behaviour at 1 μM, a slight difference was observed at lower concentration (0.1 μM), since lubeluzole reduced the colonic contractile response to 100 μM acetylcholine to less extent than loperamide (tension was reduced to about 80% and 90%, respectively). Moreover, a statistically significant effect was observed at 10 μM acetylcholine only in the presence of lubeluzole (For all statements concerning statistical significance, see supplementary materials). Hence, even lower concentrations of lubeluzole were investigated. Interestingly, at 100 μM acetylcholine, lubeluzole 0.01 μM reduced the colonic contraction to the same extent of loperamide 1 μM, with tension being of about 70% for both drugs. No antipropulsive effect was observed at 0.001 μM lubeluzole (data not shown). Based on these findings, it may be said that lubeluzole was about two orders of magnitude more potent than loperamide. A similar behaviour was observed when the reduction of the contractile response that follows the inhibitory response to electrical field stimulation (off-contraction) was evaluated. In fact, lubeluzole reduced the off-contraction at concentrations one order of magnitude lower than loperamide (0.1 and 1 μM, respectively), at all frequencies of transmural stimulation (Fig. 4a, b, respectively).

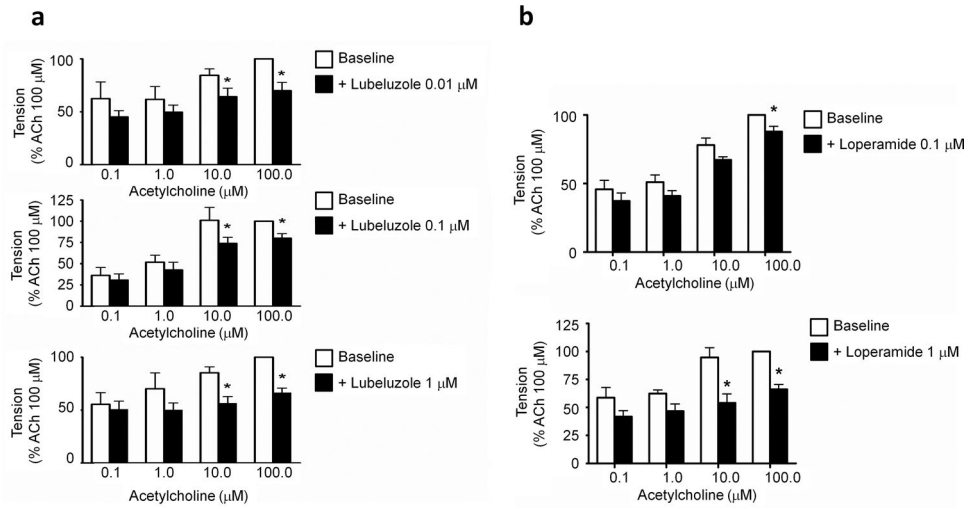

Fig. 3 Effect of lubeluzole on acetylcholine rat proximal colon induced contractility. The rat contractile responses to in vitro administration of acetylcholine (ACh, 0.1–100 μM) recorded in the absence of the drugs (baseline) as well as after the 30-min incubation with lubeluzole (0.01–1 μM) (**a**) or the 20-min incubation with loperamide (0.1 and 1 μM) (**b**). Each point represents the mean ± SEM of the tension expressed as percentage of contractile response to ACh 100 μM obtained in 5–6 preparations. At each lubeluzole or loperamide concentration used, the differences between the corresponding means were verified using two-way analysis of variance for repeated measures followed by *t* test for paired samples as post hoc test (*$p < 0.05$)

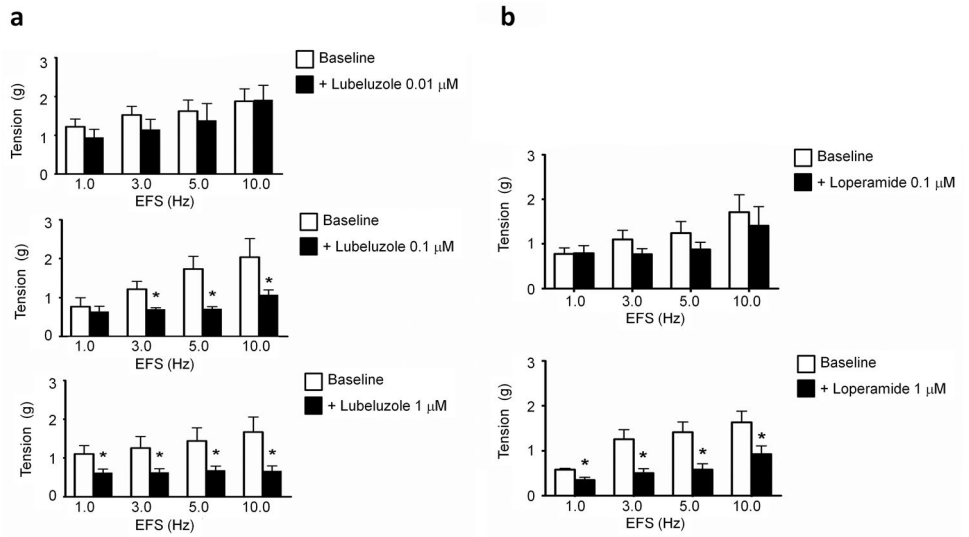

Fig. 4 Effect of lubeluzole on rat colon electric field stimulation (EFS). The off-contraction recorded in the absence of the drugs (baseline) as well as after the 30-min incubation with lubeluzole (0.01–1 μM) (**a**) or the 20-min incubation with loperamide (0.1 and 1 μM) (**b**). Each point represents the mean ± SEM of the tension expressed in grams (g) obtained in 5–6 preparations. At each lubeluzole or loperamide concentration used, the differences between the corresponding means were verified using two-way analysis of variance for repeated measures followed by *t* test for paired samples as post hoc test (*$p < 0.05$).

Relaxant activity on guinea pig gut tissues

Lubeluzole effect on the spontaneous contractility of isolated guinea pig ileum and colon has been evaluated in terms of reduction of tone (basal spontaneous contraction activity, BSCA), spontaneous contraction variability (SCV), mean contraction amplitude (MCA), and spontaneous contraction rates (FFT). On ileum, lubeluzole reduced the tone with a similar efficacy but lower potency than the reference drug OB (Fig. 5a). The spontaneous ileum basal contractility (SC, MCA, SCV, FFT) was also reduced in a dose-dependent manner, although at higher doses than OB (Fig. 5b,

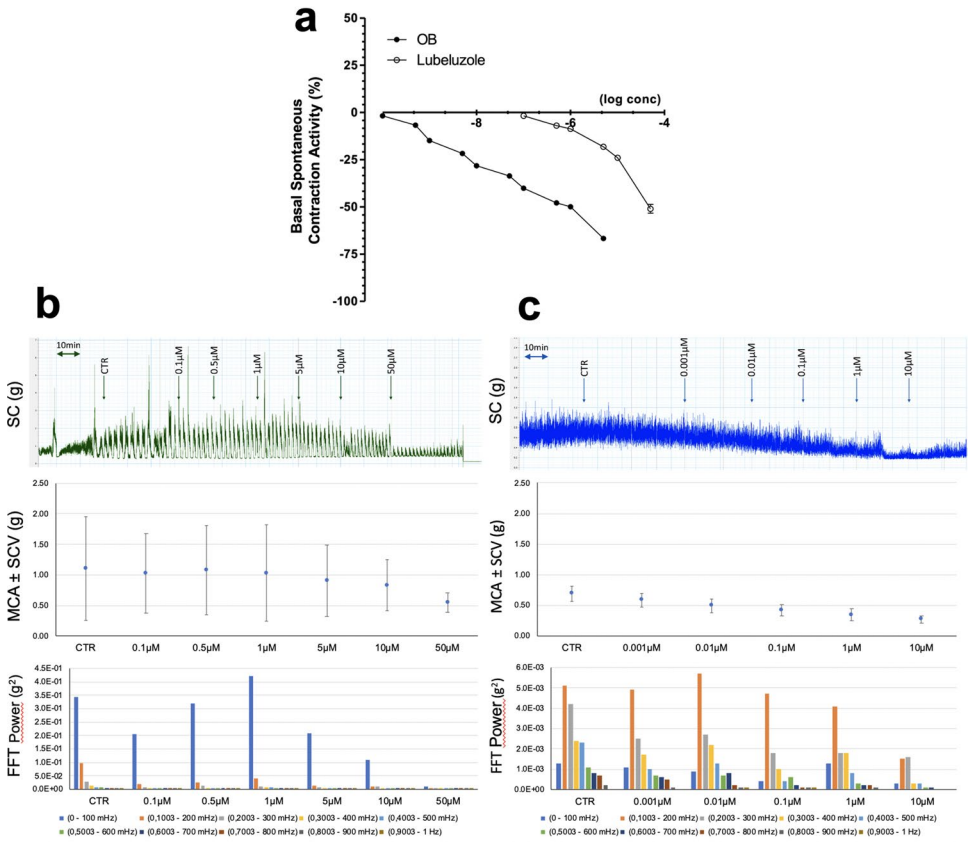

Fig. 5 Effect of lubeluzole on guinea pig spontaneous ileum contractility. Cumulative concentration–response curves for lubeluzole and OB on guinea–pig ileum basal spontaneous contraction activity (each point is the mean ± SEM of four-six experiments. Where error bars are not shown, these are covered by the point itself) (**a**); focus on experimental original recording of the concentration–response curve of lubeluzole (**b**) and OB (**c**) on ileum spontaneous contraction (SC), mean contraction amplitude ± spontaneous contraction variability (MCA ± SCV), percentage band powers of control, and after addition of each concentration observed in the same experiment (FFT)

c, respectively). In fact, a significant reduction of SC was observed at 50 μM and 10 μM concentrations of lubeluzole (Fig. 5b) and OB (Fig. 5c), respectively.

Furthermore, a gradual increasing reduction of MCA and SCV than control was observed in the OB concentration range 0.1–10 μM (Fig. 5c), with a similar result being obtained only in the presence of 50 μM lubeluzole (Fig. 5b). Finally, the lubeluzole concentration needed for observing a visible FFT reduction was five times higher than OB concentration (50 and 10 μM, respectively; Fig. 5b, c), thus confirming the lower potency of lubeluzole compared to the reference compound. Interestingly, lubeluzole was as potent as OB on colon tone reduction (Fig. 6a) and more potent than OB on both SC and MCA (Fig. 6b, c). Unlike OB which does not significantly modify the FFT, lubeluzole reduced all frequencies in a dose-dependent manner.

In addition, the effect of lubeluzole on L-type calcium channels, whose block usually induces constipation, has been investigated in K^+-depolarized (80 mM KCl) guinea pig intestinal smooth muscle. Under these conditions, all transmembrane mechanisms are blocked and the contraction is due to the movement of calcium through the slow channels. As reported in Table 4, lubeluzole was more potent than the reference compound OB on both the ileum and colon strips and, interestingly, performed more potently on colon than ileum with an IC_{50} value eightfold lower (0.18 and 1.4 μM, respectively). Figure 7 illustrates the above observations.

Discussion

In this paper, the evaluation of lubeluzole as adjuvant in antibacterial therapy as well as spasmolytic agent for the treatment of infectious diarrhea has been reported.

Lubeluzole showed an albeit mild antibacterial activity against two foodborne pathogenic bacterial strains (*Y. enterocolitica* DSM 4780 and *B. cereus* DSM 4313) as well as *Staphylococcus aureus*. When used in combination with known antibiotics, lubeluzole slightly reduced the MIC value of ciprofloxacin (at 64 μg/ml and 0.025 μg/ml, respectively) against *Y. enterocolitica* DSM 4780, while interesting synergistic effects were observed when used in combination with minocycline against four common Gram-positive and Gram-negative bacteria. This effect is in line with a recent study describing the well-known opioid loperamide as a chemosensitizer agent able to enhance the antimicrobial efficacy of minocycline against bacterial pathogens [35]. Lubeluzole concentrations ranging from 25.6 to 51.2 μg/ml synergize with minocycline (0.0018–0.015 μg/ml) in *Staphylococcus*

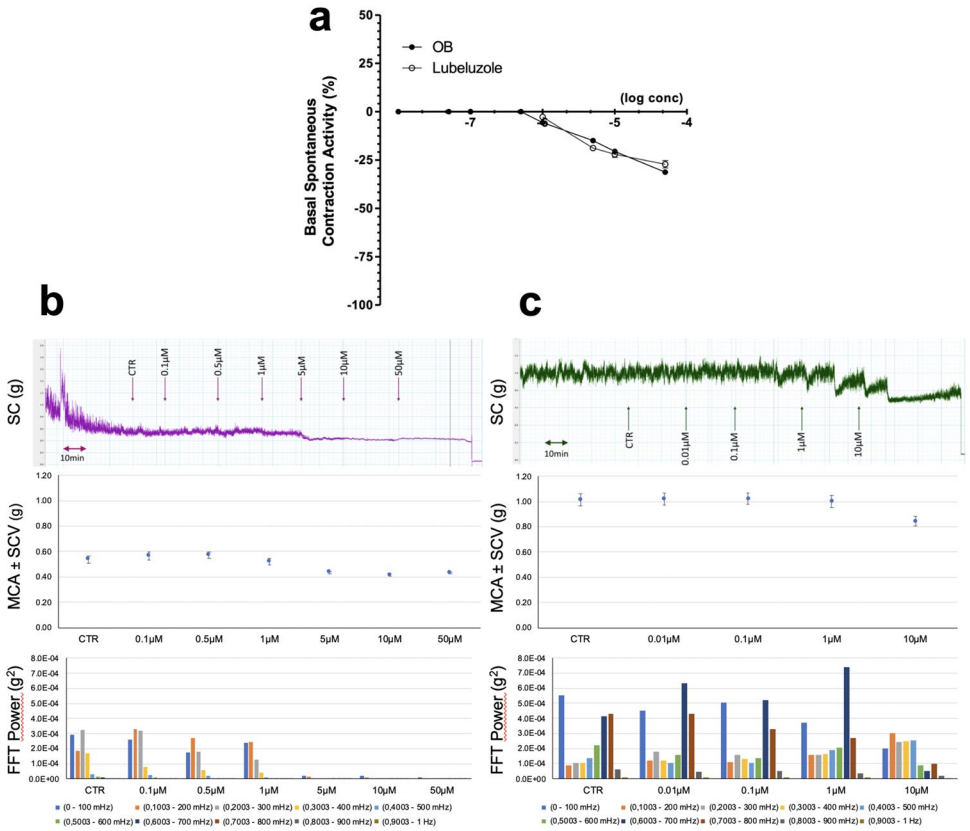

Fig. 6 Effect of lubeluzole on guinea pig spontaneous colon contractility. Cumulative concentration–response curves for lubeluzole and OB on guinea–pig colon basal spontaneous contraction activity (each point is the mean ± SEM of four-six experiments. Where error bars are not shown, these are covered by the point itself) (**a**); focus on experimental original recording of the concentration–response curve of lubeluzole (**b**) and OB (**c**) on colon spontaneous contraction (SC), mean contraction amplitude ± spontaneous contraction variability (MCA ± SCV), percentage band powers of control, and after addition of each concentration observed in the same experiment (FFT)

Table 4 Relaxant activity of lubeluzole and OB on K^+-depolarized guinea pig gut smooth muscle

Compd	Ileum			Colon		
	Activity[a] (M ± SEM)	IC_{50}[b] (μM)	95% Conf lim (μM)	Activity[a] (M ± SEM)	IC_{50}[b] (μM)	95% Conf lim (μM)
Lubeluzole	90.0 ± 1.6 (10 μM)	1.4	1.1–1.8	83 ± 2.4 (0.5 μM)	0.18	0.15–0.21
OB	52.0 ± 2.4 (10 μM)	8.6	7.1–10.1	90 ± 2.3 (50 μM)	3.4	2.6–4.4

[a]Percent inhibition of calcium-induced contraction on K^+-depolarized (80 mM) guinea pig ileum and colon smooth muscle (at indicated concentration)

[b]Calculated from log concentration–response curves (Probit analysis by Litchfield and Wilcoxon [40] with $n = 6–7$)

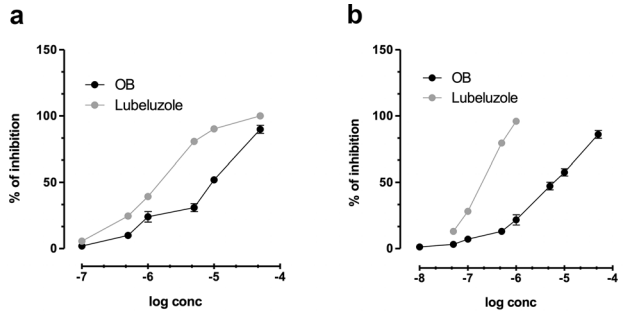

Fig. 7 Effect of lubeluzole on guinea pig induced ileum and colon contractility. Effect of Lubeluzole and Otilonium bromide (OB) on potassium chloride-induced contraction on isolated guinea pig ileum (**a**) and colon (**b**) strips. Each point is the mean ± SEM ($n = 5$ or 6). Where error bars are not shown; these are covered by the point itself

aureus ATCC 29213, with the MIC values being 128 μg/ml and 0.06 μg/ml, respectively.

In addition, we studied the relationship between loperamide and gut cholinergic receptors. Loperamide inhibits acetylcholine release on colon cholinergic receptors [49].

In ex vivo experiments, lubeluzole reduced the intestinal spontaneous contractility in a dose-dependent manner. When tested on rat proximal colon, lubeluzole inhibited the rat contractile response to in vitro administration of acetylcholine and was also able to reduce the so-called off-contraction, which is due to an interplay between several excitatory and inhibitory neurotransmitters, among which acetylcholine. On the other hand, lubeluzole was also able to reduce the tone and the rate of contractility at low and high stimulation pulse frequency of guinea pig ileum and colon segments. Many

neurotransmitters, when they bind to their respective receptors, activate the entry of calcium from the L-type channels as happens precisely for cholinergic receptors. For this reason, we evaluated the action of lubeluzole on potassium (80 mM) induced contractility. Under these experimental conditions, it is possible to see if the compound interferes with the movements of calcium through these channels [50]. Besides, it displayed a spasmolytic effect on K^+ (80 mM) induced contraction gut smooth muscles, thus interfering with the movements of calcium through the L-type calcium channels. In general, greater spasmolytic effects have been observed on colon than on ileum. It is noteworthy that lubeluzole was found to be much more potent than reference compounds (loperamide and OB), thus resulting in a promising spasmolytic agent.

Finally, it should be noted that the reduction of both low and high-frequency waves mostly in the colon not only would be responsible for the antipropulsive effect, but might also attenuate the painful spasm generally associated with infectious diarrhoea. If confirmed by future in vivo studies, the latter properties would indicate superiority versus loperamide which does not decrease the pain associated with diarrhoea and may cause cramps by itself [51].

All the above results highlight a possible role of lubeluzole as a non-antibiotic chemosensitizer in the antibacterial therapy aimed at gastroenteritis treatment. High concentrations of lubeluzole (64 µg/ml in combination with ciprofloxacin; 25.6–51.2 µg/ml in combination with minocycline) are required in vitro with respect to the in vitro active concentrations (often less than 2–4 µg/ml) of the commonly used antibiotics [52, 53]. Lubeluzole can be considered just as a candidate useful for the identification, through in-depth structure–activity relationship (SAR) studies, of a novel class of antibacterial adjuvants endowed with spasmolytic activity. The drug candidate optimization strategy should provide new lubeluzole analogues possibly endowed with higher chemosensitizing activity and lower systemic off-target effects. In vivo animal models should check cardiac liability, since QT interval prolongation was observed after lubeluzole parenteral administration [54].

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

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