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Full paper

## RQ-00201894: A motilin receptor agonist causing long-lasting facilitation of human gastric cholinergically-mediated contractions

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## ABSTRACT

The aim was to characterise RQ-00201894, a novel non-macrolide motilin agonist, using human recombinant receptors and then investigate its ability to facilitate cholinergic activity in human stomach. A reporter gene assay assessed motilin receptor function. Selectivity of action was determined using a panel of different receptors, ion channels, transporters and enzymes. Cholinergically-mediated muscle contractions were evoked by electrical field stimulation (EFS) of human gastric antrum. The results showed that RQ-00201894, motilin and erythromycin acted as full motilin receptor agonists ( $EC_{50}$ : 0.20, 0.11, 69 nM, respectively). In this function, RQ-00201894 had >90-fold selectivity of action over its ability to activate the human ghrelin receptor ( $EC_{50}$  19 nM) and greater selectivity over all other receptors/mechanisms tested. In human stomach RQ-00201894 0.1–30  $\mu$ M concentration-dependently increased EFS-evoked contractions (up to 1209%;  $pEC_{50}$  6.0). At 0.1–10  $\mu$ M this activity was usually prolonged. At higher concentrations (3–30  $\mu$ M) RQ-00201894 also caused a short-lasting muscle contraction, temporally disconnected from the increase in EFS-evoked contractions. RQ-00201894 10  $\mu$ M did not consistently affect submaximal contractions evoked by carbachol. In conclusion, RQ-00201894 potently and selectively activates the motilin receptor and causes long-lasting facilitation of cholinergic activity in human stomach, an activity thought to correlate with an ability to increase gastric emptying.

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## 1. Introduction

The gastrointestinal (GI) hormone motilin is released from the upper gastrointestinal tract during hunger to evoke large, migrating contractions of the stomach which represent phase III of a pattern of GI movements during hunger, known as the migrating motor complex (MMC) (1). Further, this release of motilin may contribute to the mechanisms by which the sensation of hunger is initiated (2). Interestingly, however, motilin receptor agonists are also the target for developing new drugs which cause prolonged stimulation of gastric emptying of meals. This follows the discovery, in 1989, that the anti-biotic drug erythromycin is a non-selective

motilin receptor agonist (3), followed by its subsequent 'off label' use as a gastric prokinetic agent (e.g. treating patients with gastroparesis, helping to control blood glucose levels in diabetic patients and increasing gastric emptying in patients receiving enteral feeding or requiring clearance of gastric contents before endoscopy or emergency surgery and its use in critically ill patients needing rapid intubation) (1). In addition, azithromycin, another antibiotic drug which activates the motilin receptor, is used to treat certain patients with gastro-oesophageal reflux, where increasing gastric emptying facilitates clearance of acid from the oesophagus (4). Nevertheless, there is concern that antibiotic drugs should not be used when there is no infection to treat, since this may contribute to growing anti-bacterial drug resistance (5). Erythromycin and azithromycin also have additional actions (e.g. inhibition of purinergic P2X channels and cytochrome 3A4 by erythromycin, and in rare cases, the potential for cardiac QT prolongation, arrhythmia and sudden cardiac death by erythromycin and azithromycin) (1, 4,

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6, 7). Finally, the optimal doses required to treat patients with gastroparesis are not rigorously established, with most investigators now using doses which are lower than those required to treat infections, an approach which appears to retain gastric prokinetic activity with minimal adverse events such as the induction of nausea (1).

Progress in identifying a non-antibiotic drug which acts selectively at the motilin receptor has been slow; as yet, no selective motilin receptor agonist has entered clinical practice. In part, the slow progress can be attributed to the complex macrolide structures of erythromycin, azithromycin and related molecules. This complexity greatly impedes the ability to undertake the precise structure-activity studies needed to optimise the selectivity of action, efficacy and duration of action of a motilin receptor agonist suitable for clinical use, in addition to the pharmacokinetic characteristics which enable such a molecule to become a successful drug (8). Thus, the identification of motilin receptor agonists with simpler structures, including those derived from dihydro-triazolopyridazine-1,3-dione-based amino acids (9) and more recently, the low molecular weight receptor agonist, camicinal (GSK962040 (10)) are now providing improved opportunities to develop effective motilin receptor agonists and successful drugs.

RQ-00201894 is a new non-macrolide, small molecule motilin receptor agonist (11). The pharmacology of RQ-00201894 is now defined in terms of its potency, efficacy and selectivity at the human motilin receptor. In addition, the ability of RQ-00201894 to facilitate gastric cholinergic motor transmission has been characterised using human isolated stomach. This is an important assay not just because it reflects the functions of the main excitatory motor nerves within the stomach, but because it also identifies motilin receptor agonists which induce either a long-lasting facilitation of cholinergic activity, more suitable for a gastric prokinetic drug or, like motilin itself, induce a shorter-lasting facilitation consistent with its role in mediating phase III MMC activity (12). Some of the data have previously been presented as meeting abstracts (13, 14).

## 2. Materials and methods

### 2.1. Activity of RQ-00201894, motilin and erythromycin at the human recombinant motilin receptor

CHO cells stably expressing both NFAT/ $\beta$ -lactamase reporter gene and human motilin receptor were seeded into 384-well black/clear-bottom plates. After overnight incubation, the culture medium was replaced by serum free medium at 37 °C for 1.5 h. The cells were then treated with the motilin receptor agonists at 37 °C for 4.5 h. Afterwards, CCF4/AM, a fluorogenic substrate (Invitrogen) was added and the cells incubated for 2 h. The fluorescence intensities were measured (Functional Drug Screening System; Hamamatsu Photonics) with excitation at 405 nm and emission at 465 and 540 nm. The concentration-response curves for RQ-00201894, motilin, and erythromycin were expressed as a percentage of the maximal control activity (50  $\mu$ M erythromycin), and analyzed using GraphPad Prism (GraphPad Software) to obtain EC<sub>50</sub> values. Data represent mean  $\pm$  S.E.M. of three independent experiments performed in duplicate.

### 2.2. Evaluation of selectivity of action

The activity of a single concentration of RQ-00201894 (10  $\mu$ M) was examined using a range of 64 different GPCRs, transporters, enzymes and ion channels expressed in native tissues and cell lines expressing human or rat recombinant proteins, provided by Cerep (Cerep, Paris, France), using standard radioligand binding, functional and enzyme assays. Specifically for the human ghrelin

receptor, at which the effects of a range of concentrations of RQ-00201894 were studied, the receptors were transiently expressed in HEK293 cells and after seeding into 96 well plates (20,000 cells/100  $\mu$ l/well) their function assessed using a Ca<sup>2+</sup> influx assay with fluorescence intensity measured as above, as a ratio of emission intensities (510 nm from 340 nm excitation/510 from 380 nm). The concentration-response curves of RQ-00201894 and human ghrelin were expressed as percentage in proportion with maximal control activity (1  $\mu$ M human ghrelin).

### 2.3. Human stomach

The method followed that which we have previously described (12). In brief, segments of stomach were obtained from patients undergoing surgery for obesity. The study was approved by the East London Research Ethics Committee 1, (REC reference number 10/H0703/71, SSA reference number 10/H0703/76), and written informed consent was obtained from all patients. Tissues were transferred to the laboratory within 2 h after resection in Krebs' solution (mM: NaCl 121.5, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, KCl 4.7, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, glucose 5.6) equilibrated with 5% CO<sub>2</sub> and 95% O<sub>2</sub> and were used immediately or after overnight storage at 4 °C in fresh, oxygenated Krebs' solution.

The mucosa, muscularis mucosa and submucosal plexus were removed by blunt dissection. Strips of gastric antrum (3–5  $\times$  15 mm) were cut approximately parallel to the circular muscle fibres and mounted in tissue baths (containing Krebs solution at 35 °C, gassed with 5% CO<sub>2</sub> in O<sub>2</sub>) for measurement of changes in muscle tension using pre-calibrated isometric force transducers (AD Instruments, Chalgrove, UK) linked to a data acquisition system (Biopac Inc., CA, USA). After an initial application of 2 g tension the strips were allowed to recover for 60 min, during which the bath solutions were changed every 15 min. The strips were then stimulated via two parallel platinum ring electrodes connected to a stimulator (STG2008, Scientifica, Uckfield, UK). Electrical field stimulation (EFS) was applied at 5 Hz for 10 s using 50 V (c.200 mA) and 0.5 m bipolar pulse duration, repeated every 1 min. These parameters evoked monophasic, cholinergically-mediated contractions, attenuated by simultaneous activation of nitrgergic inhibitory neurons (12), and were applied continuously until consistent responses were obtained (bath solution changed every 15 min).

In the experiments to examine the effects of RQ-00201894 on EFS-evoked contractions, each strip of stomach muscle was exposed to only a single concentration of RQ-00201894, which was then left in contact with the tissue for at least 60 min. The following measurements were taken:

- 1 Change in baseline muscle tension (expressed as a % of at least three pre-treatment EFS-induced contractions) and duration of any change,
- 2 Maximum change in EFS-evoked contractions (determined by measuring at least three EFS-induced responses at a given time-point, expressed as a percentage of the mean of at least three pre-treatment EFS-induced responses (100%)) and the time taken to achieve maximum response,
- 3 The length of time over which the maximum increase in EFS-evoked contraction was maintained during the continuous presence of the compound, measuring the time of fade of response if appropriate,
- 4 The occurrence of irregular contractions in response to EFS after application of RQ-00201894, recorded as a simple presence or absence.

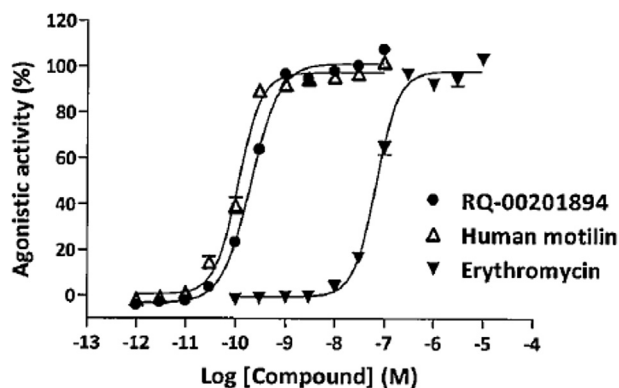


Fig. 1. Concentration-response curves of RQ-00201894, human motilin, and erythromycin at human motilin receptors expressed in CHO cells, obtained using a  $\beta$ -lactamase reporter gene assay. Data represent percentage relative to the maximal control activity at 50  $\mu$ M erythromycin and show arithmetic mean  $\pm$  SEM of three independent variables.

In separate experiments to investigate the effects of RQ-00201894 on contractions evoked by carbachol, a concentration of 1  $\mu$ M carbachol was used (representing the concentration causing ~50% of maximum contraction; data not shown) and consistent responses were obtained (5 min contact, repeated at 15 min intervals) before RQ-00201894 was added.

#### 2.4. Statistical analysis

Data obtained from experiments using the recombinant receptors are expressed as geometric means with 95% confidence ranges. Curves were fitted using a 4 parameter (log) agonist response curve. Data obtained from the human stomach experiments are expressed as medians and ranges or as the mean  $\pm$  standard error of the mean; n values are the numbers of patients. Curves were fitted using a 3 parameter (log) agonist response curve. Changes of baseline tension, EFS magnitude and responses to carbachol were compared using Wilcoxon signed rank tests.  $P < 0.05$  is considered as statistically significant. All data were analysed using GraphPad Prism 5.

#### 2.5. Drugs

In the experiments using the recombinant motilin and ghrelin receptors, RQ-00201894 and erythromycin were dissolved and diluted with 100% DMSO before final addition to the appropriate assay buffer Motilin receptor assay: 100 ml of HBSS (1000 ml + 1 M HEPES 20 ml), 5 ml BSA (20 mg/ml) and 1 ml DMSO; Ghrelin receptor assay: 100 ml of HBSS (1000 ml + 1 M HEPES 20 ml) and adding to the cells. Human motilin was dissolved with distilled water before serial dilution using 100% DMSO and assay buffer, as above. Human ghrelin was dissolved with distilled water. For all other experiments with recombinant receptors, RQ-00201894 was

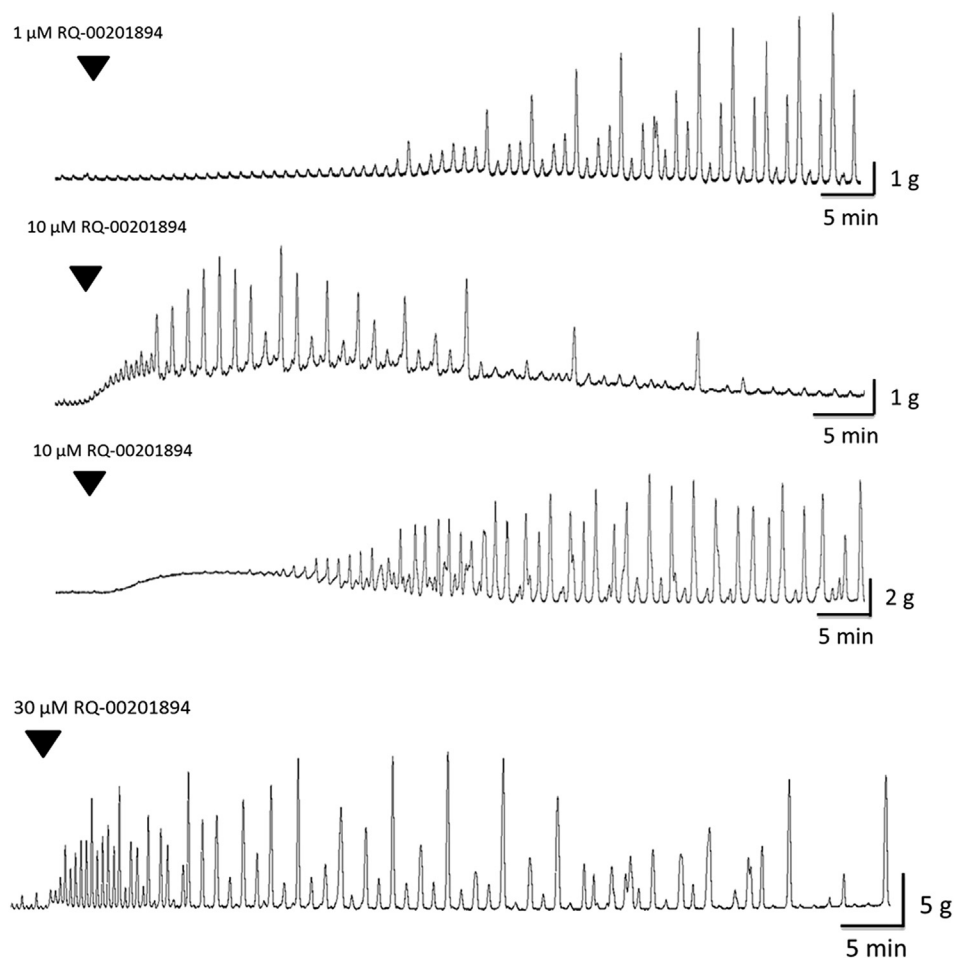


Fig. 2. Representative experimental records showing the responses of circular muscle strips of human antrum to the continuous presence of 1–30  $\mu$ M RQ-00201894. The contractions are shown in response to EFS (50 V, 0.5 m bipolar pulse duration, 5 Hz) given for 10 s, every 1 min. Note the different response kinetics after application of RQ-00201894.

dissolved in 100% DMSO at 10 mM. In the experiments using human stomach, all drugs were freshly prepared prior to use. Carbachol and RQ-00201894 were dissolved in dH<sub>2</sub>O.

### 3. Results

#### 3.1. Activity of RQ-00201894 at the recombinant human motilin receptor

RQ-00201894, motilin and erythromycin were equi-effective as motilin receptor agonists, the  $E_{max}$  values for RQ-00201894 and motilin being similar to the activity evoked by the maximum concentration of erythromycin (50  $\mu$ M) (Fig. 1). In this assay, the  $EC_{50}$  values for RQ-00201894, motilin and erythromycin were, respectively, 0.20 (geometric mean with 95% confidence limits: 0.19–0.22), 0.11 (0.07–0.19) and 69 (51–94) nM; the  $E_{max}$  values were 101 (99–103), 97 (95–100%) and 98 (95–101%); and the Hill slopes were 1.5 (1.3–1.7); 1.9 (1.5–2.3) and 1.9 (1.6–2.3) all from 3 independent experiments performed in triplicate.

#### 3.2. Selectivity data

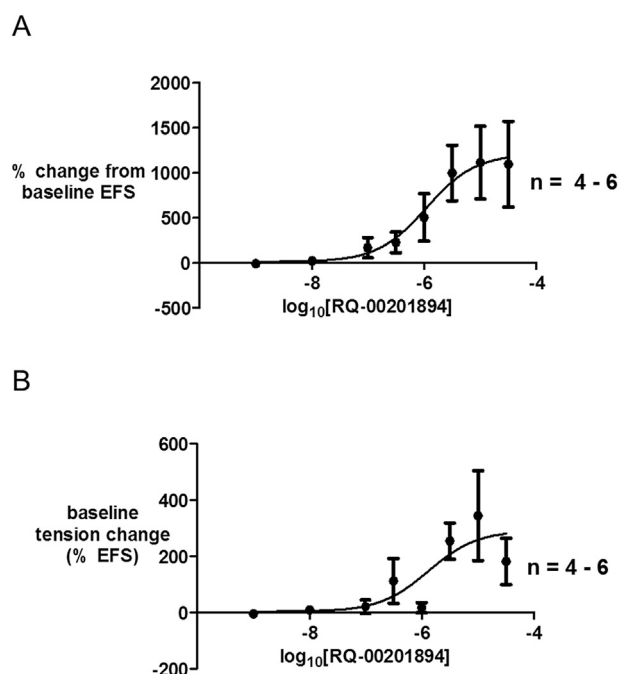
RQ-00201894 10  $\mu$ M had little or no activity when tested against a Cerep panel of 64 receptors, ion channels and enzymes. In the binding studies the exception was the Na<sup>+</sup> channel ( $IC_{50}$  1.5  $\mu$ M). In the functional assays the exception was the 5-HT<sub>1B</sub> receptor ( $EC_{50}$  8.6  $\mu$ M). Further, RQ-00201894 acted as an agonist at the human ghrelin receptor but at concentrations approximately 95 times higher than those required to activate the motilin receptor ( $EC_{50}$  19 (geometric mean with 95% confidence limits: 11–34) nM at the ghrelin receptor, compared with 4.9 (2.0–12) nM for ghrelin itself;  $n = 3$  independent experiments).

#### 3.3. Effect in human stomach

52 strips of human isolated antrum muscle were obtained from 8 patients undergoing sleeve gastrectomies for obesity. The male to female ratio was 1:1; median age (range) = 51 (25–60). 3 tissues were used on the same day as the operation, and 5 were used following overnight storage in fresh oxygenated Krebs solution at 4 °C, following removal of the mucosa.

After achieving consistent responses to EFS (obtained after 208 (189–256) minutes), the application of RQ-00201894 0.1–30  $\mu$ M concentration-dependently increased the amplitude of the EFS-evoked contractions (Figs. 2 and 3; Table 1), with an  $E_{max}$  of  $1209 \pm 183\%$  and a  $pEC_{50} = 6.0 \pm 0.4$ ,  $n = 4–6$  patients for each concentration;  $P = 0.03$  at 10  $\mu$ M; lower concentrations (0.001, 0.01  $\mu$ M) had no consistent activity. At low concentrations (0.1–10  $\mu$ M) this excitatory action of RQ-00201894 was slow in onset and after reaching maximum, was usually maintained for the remainder of the experiment; at the highest concentration (30  $\mu$ M) the response faded during the continued presence of the compound (Figs. 2 and 4; Table 1). Notably, the facilitation of the EFS-evoked responses did not always occur in a regular manner, with large and small EFS-evoked contractions appearing at irregular intervals especially at the higher concentrations (Figs. 2 and 4).

Relatively higher concentrations of RQ-00201894 (3–30  $\mu$ M) also consistently evoked a short-lasting muscle contraction (an  $E_{max}$  of  $292 \pm 76\%$  and a  $pEC_{50} = 5.9 \pm 0.6$ ,  $n = 4–6$  patients for each concentration;  $P = 0.03$  at 10  $\mu$ M;  $n = 6$ ) which appeared temporally disconnected from the increase in EFS-evoked contractions, reaching maximum more quickly and usually fading completely before the increase in EFS-evoked contraction had reached maximum (Figs. 2 and 3; Table 1); the lower concentrations of RQ-



**Fig. 3.** Effects of RQ-00201894 on contractions to EFS in circular muscle strips from human gastric antrum. Panel A displays the concentration relationship of the facilitation of EFS by RQ-00201894 0.001–30  $\mu$ M. Panel B displays the concentration relationship of the increase in baseline muscle tension induced by RQ-00201894. EFS (50 V, 0.5 m bipolar pulse duration, 5 Hz) was given for 10 s, every 1 min.  $n = 4–6$  patients for each concentration; drugs were applied non-cumulatively.

00201894 contracted the muscle in 2 of 5 tissues at 1  $\mu$ M, in 3 of 5 tissues at 0.3  $\mu$ M and in 2 of 6 tissues at 0.1  $\mu$ M.

In separate experiments, RQ-00201894 10  $\mu$ M had no consistent ability to affect the magnitude of contractions evoked by a submaximally-effective concentration of carbachol (1  $\mu$ M; contractions were  $83 \pm 13\%$  of the responses before RQ-00201894 addition;  $n = 4$ ,  $P > 0.05$ ).

## 4. Discussion

RQ-00201894 demonstrated good potency as an agonist at the human recombinant motilin receptor expressed in CHO cells, as well as selectivity of action when compared with the higher concentrations needed to activate the ghrelin receptor and more especially, interact with a range of other GPCRs, transporters, ion channels and enzymes. This selectivity of action as a motilin receptor agonist contrasts with the non-selective activity for erythromycin, azithromycin and other macrolide structures with antibiotic and motilin receptor agonist activity (1, 4, 6, 7; see Introduction for details). The potency of RQ-00201894 as a motilin receptor agonist in this assay was similar to that of motilin itself and >300 times greater than the potency demonstrated by erythromycin. It is likely that RQ-00201894 binds to the orthosteric binding site of the motilin receptor, as increasing concentrations of GM-109 (15) have been previously demonstrated to cause surmountable rightward shifts in the concentration-response curves to RQ-00201894 in the NFAT/ $\beta$ -lactamase assay (13).

In human isolated gastric antrum, RQ-00201894 0.1–30  $\mu$ M concentration-dependently facilitated cholinergically-mediated contractions. This activity was slow to reach maximum and once achieved, was usually prolonged (with the exception of 30  $\mu$ M, the highest concentration tested, where fade was clearly observed during the experiments). Notably, the potency of RQ-00201894 in

**Table 1**

Summary of effects of RQ-00201894 on baseline muscle tension and on neuronally-mediated contractions during electrical field stimulation in human isolated colon.

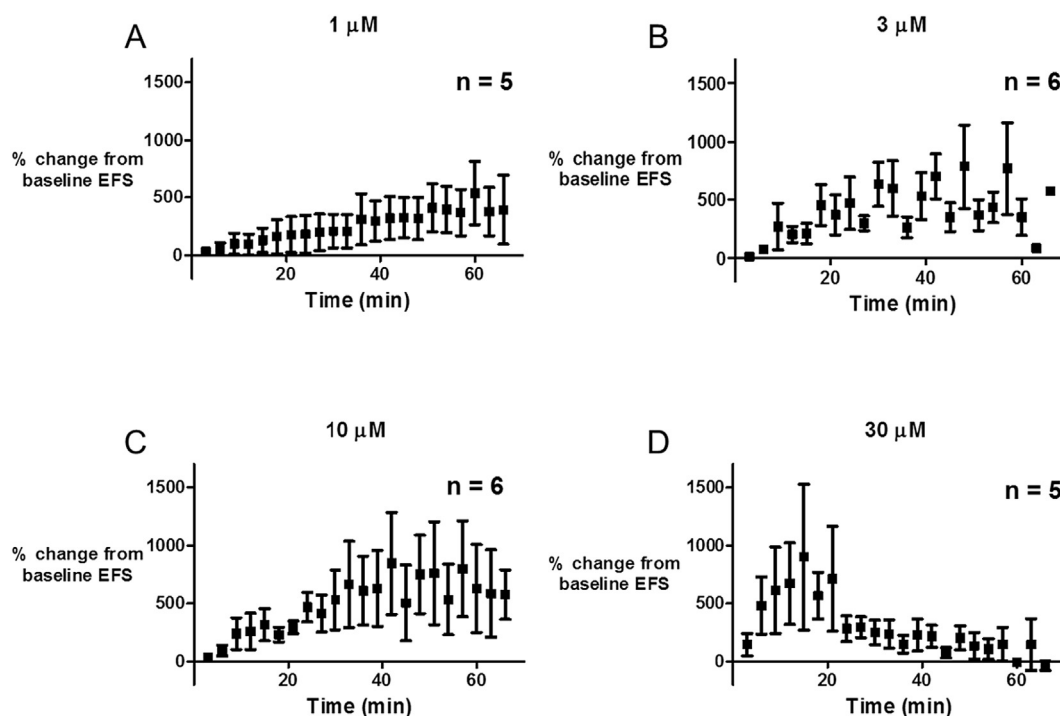
	Muscle [n]	Nerve [n]
Effective concentrations	0.3–30 $\mu$ M [5–6]	0.1–30 $\mu$ M [5–6]
$pEC_{50}$	$5.9 \pm 0.5$ [4–6]	$6.0 \pm 0.4$ [4–6]
$E_{max}$ (%) (mean $\pm$ standard error)	$292 \pm 76^*$ [4–6]	$1209 \pm 183$ [4–6]
$T_{max}$ at 10 $\mu$ M (min) (median, with range)	10 (5–16) [6]	44 (10–51) [6]
$T_{fade}$ at 10 $\mu$ M (min)** (median, with range)	29 (12–51) [6]	not observed [5] except in one tissue where the $T_{max}$ was short (10 min) and fade occurred within 17 min

The values shown are determined from a concentration–response curve for RQ-00201894 (0.001–30  $\mu$ M) constructed by adding single concentrations of the compound to separate tissues;  $n = 4–6$  patients for each concentration. \* % of contraction amplitude evoked by EFS prior to addition of RQ-00201894. \*\* the time at which the response faded is recorded as the point at which the increase in muscle tension had completely returned to baseline, or for EFS as the point at which the facilitation had declined by 50%.

the human gastric antrum was lower than that when tested at the human recombinant receptor (respectively,  $pEC_{50}$  values of 6.0 and 9.5). Similar differences in potencies have previously been reported for motilin, erythromycin, azithromycin and camicinal when comparing data obtained using recombinant receptors and human isolated stomach (4, 12), perhaps reflecting the additional need for these compounds to penetrate into the muscle and reach the motilin receptors on the cholinergic nerves. RQ-00201894 also evoked small increases in muscle tension. These contractions occurred consistently at concentrations (3–30  $\mu$ M) which were

higher than the threshold concentrations which increased EFS-evoked contractions, although at lower concentrations some—but not all—tissues contracted in response to RQ-00201894. Notably, the contractions were relatively rapid in onset and usually faded completely before RQ-00201894 had maximally increased EFS-evoked contractions. Such activity is consistent with the actions of other motilin receptor agonists in human stomach (4, 12). The different neuronal and muscle responses are thought to represent the main motor pathways by which low doses of motilin receptor agonists increase gastric emptying (by facilitation of cholinergic activity) or when given at higher doses, directly contract the stomach muscle to induce early satiety and nausea (exemplified by the use of erythromycin at the relatively high doses required for antibiotic treatment) (1).

In human stomach, RQ-00201894 is likely to increase cholinergic activity by facilitating the release of acetylcholine from the intrinsic cholinergic neurons of the stomach. Thus, in common with other motilin receptor agonists (4, 12) a high concentration of RQ-00201894 had no consistent ability to modulate the contractions evoked by a submaximally-effective concentration of carbachol. Notably, the increase in cholinergic activity by RQ-00201894 can occur in a prolonged manner. A broadly-similar long-lasting activity has previously been reported for erythromycin and azithromycin, drugs used to treat gastroparesis or increase gastric emptying, and also camicinal, under development for treatment of these disorders (4, 12; see Introduction for more detail). By contrast, a relatively short-lasting activity of motilin is consistent with its physiological role in inducing the migrating phase III contractions of the MMC (1, 4, 12, 16, 17). The explanation for these different profiles of activity for motilin and the synthetic motilin receptor agonists is unclear but ‘functional’ or ‘biased’ agonism has been hypothesised, with the molecules acting at one or more binding



**Fig. 4.** Duration of facilitation by RQ-00201894 of EFS-evoked contractions in circular muscle strips from human gastric antrum. For each tissue studied, the mean of three consecutive contractions were calculated consecutively throughout the experiment. Panels A–D shows the time course of the mean  $\pm$  S.E.M. response to application of RQ-00201894 1–30  $\mu$ M respectively. Note the different response kinetics and the irregularity of contractions during fade of response to RQ-00201894 (in which small contractions occurred in-between larger contractions), particularly at the higher concentrations. EFS (50 V, 0.5 m bipolar pulse duration, 5 Hz) was given for 10 s, every 1 min.  $n = 5–6$  patients for each concentration as displayed on panels.

sites on the motilin receptor to differentially influence different intracellular signalling pathways (18). Interestingly, the increase in EFS-evoked contractions caused by RQ-00201894 was not always consistent, especially at the higher concentrations tested, and the contraction amplitudes became irregular. The reason why irregular contractions should occur during EFS is unknown. One possibility, discussed by Broad *et al* (12), is that the activities of different groups of interstitial cells of Cajal (ICCs) within the intestinal wall become uncoordinated so that overall, the baseline muscle electrical activity rises and falls at a frequency which is markedly slower than the frequency of EFS, influencing the amplitudes of the contractions evoked by EFS. An ability of different groups of ICCs to move out of synchrony with each other has previously been observed in human isolated jejunum, a phenomenon linked to disordered GI movements (19).

In other studies with RQ-00201894 in conscious fasted dogs, the compound has been shown to induce high-amplitude contractions of gastric antrum and duodenum (similar to the phase III-like activity of the migrating motor complex (MMC) during hunger, mediated by endogenous motilin) without causing vomiting and even after suppression of spontaneous contractility by clonidine. Further, RQ-00201894 increased gastric emptying of meals containing acetaminophen in conscious dogs and cynomolgus monkeys (13, 20). Notably, the dog and human motilin receptors are phylogenetically distinct (21) with only 71% protein sequence identity between the two receptors and up to 2 log lower potencies for different motilin receptor agonists at the dog receptor (22, 23). Further, motilin receptor immunoreactivity has been detected within the enteric nervous system (ENS) but not in the muscle of dog stomach (21) whereas in humans motilin receptor immunoreactivity has been detected in both, with low concentrations of motilin facilitating cholinergic activity and higher concentrations directly contracting the muscle (12); these differences suggest significantly different dynamics of the response to motilin between the two species.

In summary, RQ-00201894 demonstrates good agonist potency and selectivity at the human motilin receptor and in human stomach, facilitates cholinergic activity in a manner that is broadly consistent with other non-peptide motilin receptor agonists. These data suggest that RQ-00201894 will promote human gastric emptying and should be evaluated as a potential new drug for treatment of disorders such as gastroparesis and in other groups of patients where there is a need to increase gastric emptying.

### Conflicts of interest

NT, MT, MS and TY are employees of RaQualia. GJS received funding from RaQualia for JB to conduct the human stomach studies. AG, UP and KM report no conflict of interest.

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