

## The protective effect of endothelin receptor antagonists against surgically induced impairment of gastrointestinal motility in rats

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Submitted May 11, 2019; accepted in final form August 12, 2019

### Abstract

Endothelin (ET) receptor antagonists: BQ-123 (ET<sub>A</sub>), BQ-788 (ET<sub>B</sub>), tezosentan (dual ET receptor antagonist) protect against the development of postoperative ileus (POI) evoked by ischemia-reperfusion (I/R). The current experiments explored whether ET antagonists prevent the occurrence of POI evoked by surgical gut manipulation. Intestinal transit was assessed by measuring the rate of dye migration subsequent to skin incision (SI), laparotomy (L), or laparotomy and surgical gut handling (L+M) in diethyl ether anaesthetized rats (E). Experimental animals were randomly sub-divided into two groups depending on the time of recovery following surgery: viz. either 2 or 24 h (early or late phase POI). E and SI did not affect the gastrointestinal (GI) transit. In contrast, L and L+M significantly reduced GI motility in comparison to untreated group (UN). Tezosentan (10 mg/kg), BQ-123 and BQ-788 (1 mg/kg) protected against development of L+M evoked inhibition of intestinal motility in the course of late phase, but not early phase POI. Furthermore, tezosentan alleviated the decrease in the contractile response of the longitudinal jejunal smooth muscle strips to carbachol *in vitro* induced by L+M. The serum ET(1–21) concentration was not increased in either the early or the late phase POI groups after surgery compared to control animals. This study indicates that delay in the intestinal transit in late phase of surgically induced POI involves an ET-dependent mechanism.

**Key words:** endothelins, gut manipulation, gastrointestinal motility rats, postoperative ileus

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## Introduction

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Postoperative ileus (POI), is an iatrogenic complication characterized by a transient cessation of coordinated bowel motility preventing the effective intestinal contents transit and the tolerance of food intake. Its reported incidence rate varies from 10 to 40% leading to increased morbidity, mortality and higher hospitalization costs (1–4). The pathophysiology of POI is complex and incompletely understood involving pharmacological, neural and immune-mediated mechanisms. The first, a neurogenic phase, represents a reaction to a surgical disruption of the peritoneum and bowel manipulations, which is mediated by adrenergic and non-adrenergic inhibitory reflexes. The second phase represents a time- and procedure-dependent inflammatory response to intestinal handling. Finally, a third phase mediated by increased vagal tone involving the activation of nicotinic receptors (specifically the  $\alpha 7$  receptor subunit) in the cell membranes of macrophages plays a considerable role in POI resolution. Due to the multifactorial aetiology of POI, the multimodal enhanced recovery after surgery (ERAS) programs involving several interventional modalities proved to be most successful (5–8).

The endothelins (ETs) are a family of 21 amino acid peptides with three distinct isoforms: ET-1, ET-2 and ET-3. ETs bind in mammals to G protein-coupled ET<sub>A</sub> and ET<sub>B</sub> cell surface receptors. Binding of ETs to their receptors mediates several functions including vasoconstriction, pain, inflammation and carcinogenesis (9). The ET-like immunoreactivity and specific binding sites are widely distributed in the GI tract (10). Although most of the ETs actions in the GI tract are contractile and occur via its direct action on smooth muscle cells (11), the net effect of ETs on GI motility depends on the animal species, the gut segment, the profile of stimulated ET receptors and interactions with other mediators acting at target sites (12). ET<sub>A</sub> and ET<sub>B</sub> are involved in the pathogenesis of intestinal dysmotility caused by ischemia-reperfusion (I/R), severe burns or acute pancreatitis (13–16).

The objectives of the current experiments were to investigate whether ET<sub>A</sub> and/or ET<sub>B</sub> receptor antagonists attenuate the development of surgically induced GI motility impairment *in vivo* and *in vitro*. Additionally, we investigated, whether the potential salutary effects of tezosentan correlate with plasma ET(1–21) concentration.

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## Materials and Methods

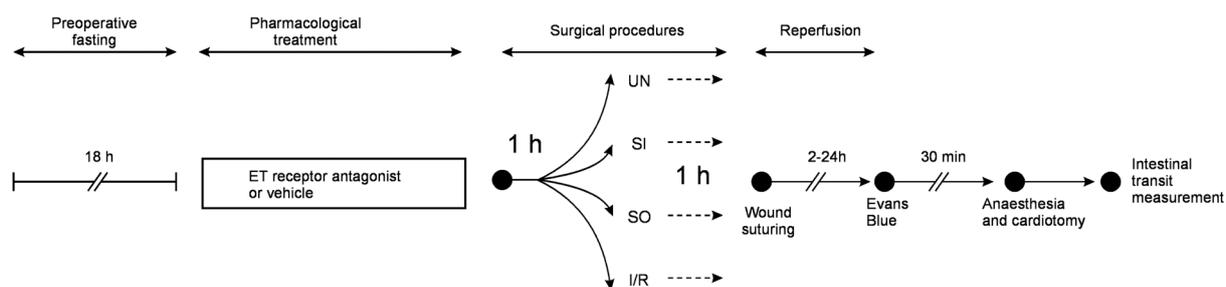
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### *Experimental protocol*

All experimental procedures were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and had been approved by the Bioethics Committee for Studies on Animals, Medical University of Gdańsk. Male Albino-Wistar rats (200–250 g, 8 h fasting with free access to tap water) were allocated randomly to one of the five experimental groups:

- 1) control, untreated animals subjected neither to anaesthesia nor to surgery (UN,  $n=5$ )
- 2) ether anesthetized animals (E,  $n=7$ )
- 3) ether anesthetized animals subjected to skin incision (SI,  $n=10$ )
- 4) ether anesthetized animals subjected to laparotomy (L,  $n=10$ )
- 5) ether anesthetized animals subjected to laparotomy with subsequent surgical gut manipulation (L+M,  $n=12$ ).

Surgical gut manipulation is defined as gut evisceration followed by mechanical stimulation of both the cecum and small intestine using aseptic procedures (17). Rats, which belonged to L and L+M cohorts were randomly sub-divided into two groups that recovered after either 2 or 24 h respectively, which correspond to the early and late phases of POI respectively.



**Fig. 1.** Experimental flowchart depicting the experiment investigating the effects of ET antagonists: tezosentan (10 mg/kg), BQ-123, BQ-788 (1 mg/kg) on the intestinal transit of Evans blue in untreated, conscious rats (UN) or animals subjected to ether anaesthesia (E), skin incision (SI), laparotomy (L) or laparotomy with subsequent surgical gut manipulation (L+M). Respective controls in each experimental group received an equal volume of vehicle instead of test article. All tested agents or vehicle were administered intraperitoneally (i.p.) 1 h prior to surgery.

Animals in each group received 0.15 ml of Evans blue at the designated time point (2 or 24 h) *via* an oro-gastric tube and 30 min later animals were sacrificed by cardiotomy under deep E. The small intestines were excised and, to avoid tissue stretching, gently laid on corkboard for measurements. An observer, who was unaware of the treatment the animals were receiving, measured the most distal point of dye migration from the pylorus (Fig. 1).

### *The effects of ET antagonists on the intestinal transit*

The effects of the intraperitoneally (i.p.) injected tezosentan (10 mg/kg), BQ-123 or BQ-788 (1 mg/kg) were investigated in UN, SI, L or L+M. Controls in each experimental group received an equal volume of the respective vehicle instead of the test agent. All agents were administered one hour before surgery. The time of ET antagonists administration and their doses were chosen based on the results of the previous experiments (15).

The number of animals contained in the experimental groups investigating the early POI equalled: UN ( $n=6$ ), L ( $n=15$ ) or L+M ( $n=25$ ). The cohorts used to investigate the late POI included: UN ( $n=7$ ), L ( $n=14$ ) or L+M ( $n=20$ ).

### *In vitro experiments*

Rats were randomly divided into three groups: UN, L+M and animals pre-treated with 10 mg/kg tezosentan 1 h prior to L+M. Subsequent to L+M animals were sub-divided into early- and late-phase POI groups, depending on their post-surgical recovery time, i.e. 2 vs. 24 h respectively.

Full-thickness longitudinal smooth muscle strips were isolated as described previously (18) and mounted vertically at 2.0 g of resting tension in water jacketed glass chambers to equilibrate at 37° C for 90 min before the beginning of experiment. The buffer was changed every 5 min except during the contact time of tissues with carbachol (parasympathetic agent). The activity of each longitudinal smooth muscle strip was recorded isotonicly with a PIT 212 force displacement transducer (COTM, Białystok, Poland) connected to TZ-4100 line recorders (Laboratori Pristroje, Prague, Czech Republic). Carbachol (1 nM–30 μM) was applied at increasing concentrations at 15 min intervals and the buffer changed every 5 min. As soon as the peak contraction had developed, the tissues were washed out until the length of the strip returned to basal levels. The maximum myogenic response was defined as the contraction that could not be increased further by a higher carbachol concentration. The viability and reproducible contractility of each strip was examined at the end of each experimental session by a submaximal contractile response to carbachol, at the same concentration as at the start. Experiments were performed using at least 8–15 different tissue strips.

### ***Biochemical measurements of ET(1–21) in blood plasma***

Measurements were performed using a conventional, 96-well, sandwich enzyme immunoassay (ELISA No. BI-20052, Biomedica GmbH, Vienna, Austria). Blood samples were collected from rat aortae and processed according to the manufacturer's instructions. The following groups of animals were included in the measurements: UN ( $n=15$ ), L+M ( $n=10$  and 8 respectively) and rats pre-treated with tezosentan prior to L+M ( $n=9$ ). Rats from the latter groups were left to recover for 24 h subsequent to surgery.

### ***Drugs***

Tezosentan was a generous gift of Drs. M. Clozel and M. Iglarz (at the time of donation at Actelion Pharmaceuticals Ltd., Allschwil, Switzerland). Tezosentan was dissolved in saline whereas BQ-123 and BQ-788 were dissolved in a few drops of DMSO and the sample volume was adjusted to the desired concentration using normal saline (Fresenius Kabi, Kutno, Poland). Diethyl ether was purchased from Polskie Odczynniki Chemiczne SA (Gliwice, Poland). BQ-788, BQ-123, Evans blue, DMSO, carbachol and all other chemicals were obtained from Sigma-Aldrich (Poznań, Poland).

### ***Statistical analysis***

The length of small intestine between animals in all experimental groups was not statistically different in the course of this study (data not shown). Therefore the distance covered by Evans blue was expressed as centimetres of dye transit and the results were demonstrated as a mean value  $\pm$  S.E.M. for the number of rats included in each group. ET(1–21) concentrations were expressed as a mean concentration and  $EC_{50}$  results were presented as a mean concentration values  $\pm$  S.E.M. for the number of samples/number of muscle strips included in each group. Taking into account the degree of the invasiveness of the surgical procedures (UN<E<SI<L<LM) and to compare the small intestinal Evans blue transit in those animals a sequentially applied unpaired *t*-test has been used (UN vs. E; UN vs. SI; UN vs. L; UN vs. LM). In order to investigate the effects of ET antagonists on the intestinal motility in the early and late phase of POI *in vivo* and *in vitro*, the transit comparisons have been performed using one-way analysis of variance (ANOVA) followed in case of statistical significance by Bonferroni multiple comparisons correction. Several experimental cohorts have been studied: (UN vs. UN+T); (UN vs. UN+BQ-123); (UN vs. UN+BQ-788); (L vs. L+T); (L vs. L+BQ-123); (L vs. L+BQ-788); (L+M vs. L+M+T); (L+M vs. L+M+BQ-123); (L+M vs. L+M+BQ-788). Two-tailed *P* values of less than 0.05 were taken to indicate significant difference.

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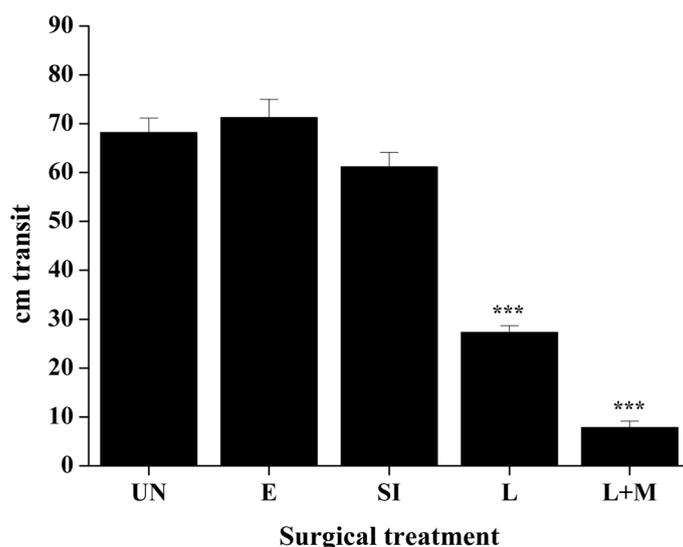
## **Results**

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### ***Effects of E and surgery on the intestinal transit***

In the course of pilot experiments Evans blue migrated over a distance of  $68.17 \pm 2.98$  cm of a total length of  $102 \pm 3.18$  cm of the small intestine in the conscious UN rats. E and SI did not affect the intestinal transit of Evans blue  $71.25 \pm 3.75$  cm of  $109 \pm 8.88$  cm and  $61.17 \pm 2.94$  cm of  $105 \pm 2.87$  cm, respectively.

On the other hand, both L and L+M significantly reduced intestinal motility, the dye migrating only  $27.33 \pm 1.38$  cm out of  $99.99 \pm 3.62$  cm in the former group and only  $7.83 \pm 1.3$  cm out of  $112 \pm 7.28$  cm in the latter group (Fig. 2). The length of small intestine between experimental groups was not statistically different in any experiment.



**Fig. 2.** Small intestinal Evans blue transit in conscious UN rats, animals undergoing E, SI, L, and LM. Results are shown as cm migration of the dye and are presented as a mean  $\pm$  S.E.M. ( $n=5-12$ ). Statistical significance was calculated using sequentially applied unpaired  $t$ -test: \*\*\*UN vs. L or UN vs. L+M ( $P<0.0001$ ).

### *Protective effects of tezosentan, BQ-123 and BQ-788 against surgically-induced inhibition of GI motility*

Tezosentan, BQ-123 or BQ-788 did not affect the intestinal motility of U or SI animals. Similarly, they did not prevent the development of the early phases of POI induced by L or L+M (Fig. 3).

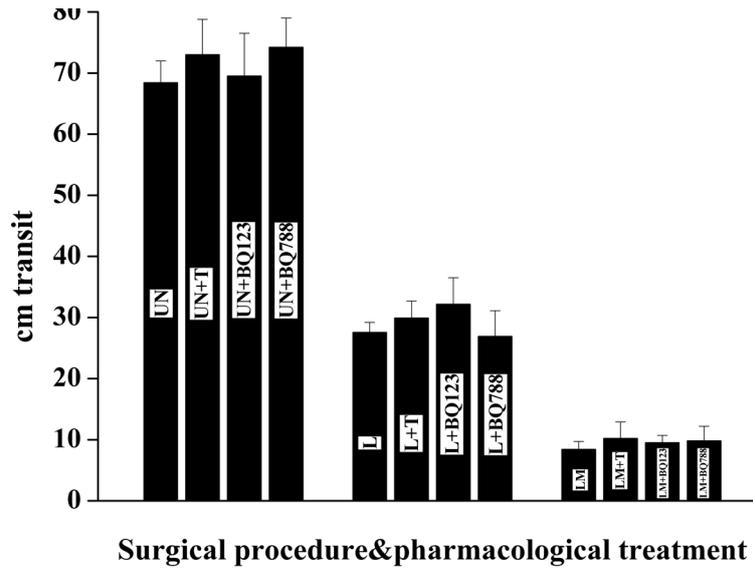
No effect of ET blockers has been observed on the GI motility inhibition evoked by L in the late phase of POI. Contrastingly, all ET blockers attenuated the development of additional inhibitory effects of surgical gut manipulation following L (L+M) during the late phase of POI (Fig. 4).

### *Results of in vitro experiments*

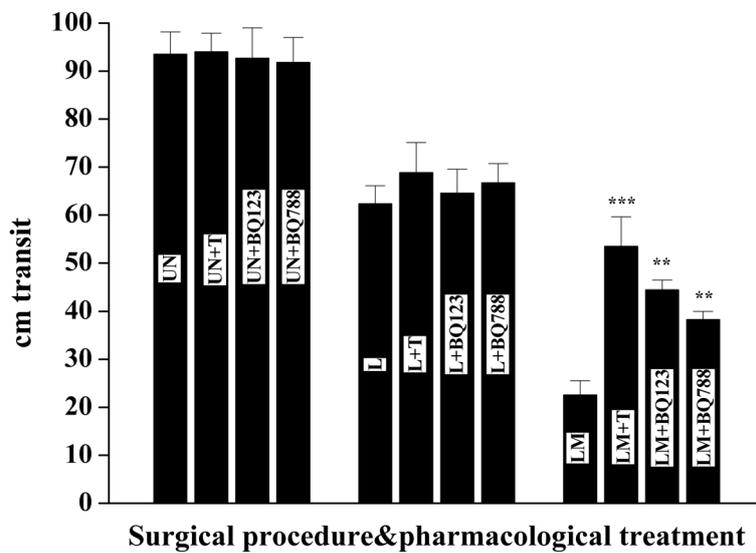
Carbachol evoked concentration-dependent contractions of ileal strips yielding typical response curves in the range from 1 nM–3  $\mu$ M in U, effective concentration 50% ( $EC_{50}$ ) reaching  $34.90 \pm 7.86$  nM. L+M caused a considerable inhibition of GI motility moving the respective concentration-contraction curves to the right, increasing the  $EC_{50}$  of carbachol to:  $776 \pm 31.26$  nM and  $299 \pm 14.92$  nM at 2 and 24 h respectively post-surgery ( $P<0.01$ ). Tezosentan pre-treatment prior to L+M markedly decreased the  $EC_{50}$ s of carbachol in strips isolated from animals in the late phase of POI. Their  $EC_{50}$ s value reached:  $77.15 \pm 10.62$  ( $P<0.05$ ; Fig. 5).

### *Serum ET(1–21) concentration*

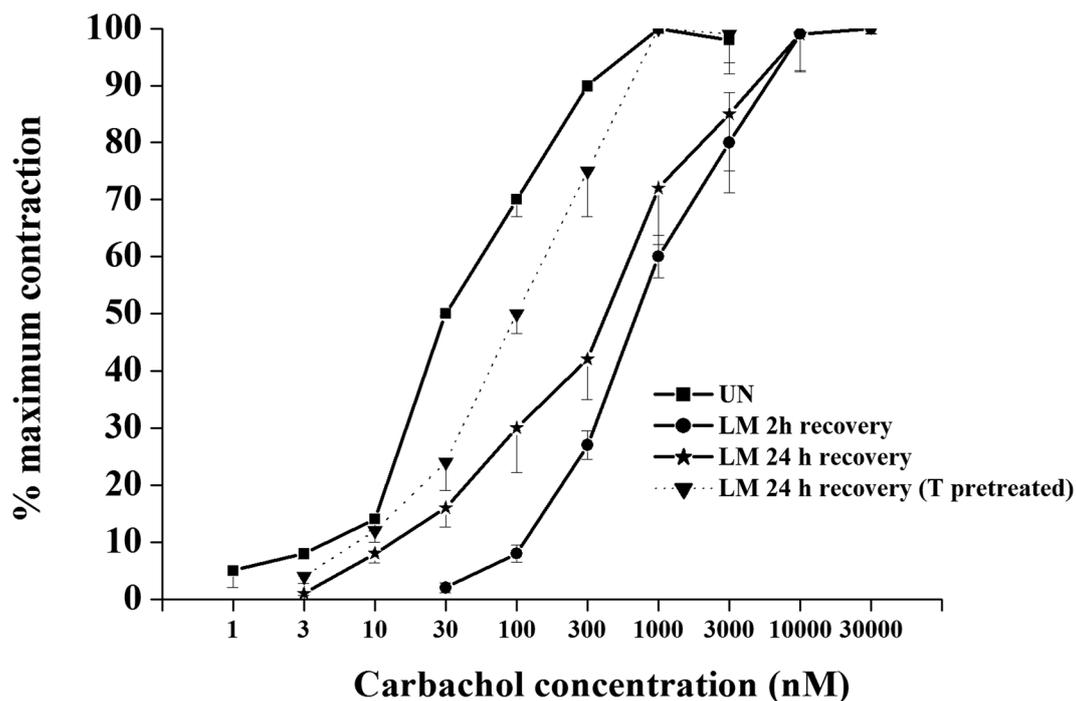
The ET(1–21) levels in plasma of control rats were  $11.35 \pm 1.93$  pg/ml. They were not different from the concentrations observed 2 or 24 h in L+M animals:  $9.42 \pm 2.5$  pg/ml or  $10.12 \pm 1.92$  pg/ml ( $n=8$ ). In the latter group tezosentan pre-treatment did not significantly affect the ET(1–21) concentration:  $7.56 \pm 3.86$  pg/ml.



**Fig. 3.** The effect of tezosentan (T-10 mg/kg, i.p.), BQ-123 or BQ-788 (1 mg/kg, i.p.) pre-treatment on the small intestinal transit of Evans blue in conscious UN animals or rats subjected to SI, L and LM. Experiments were performed 2 h post-surgery. Results are shown as cm migration of the dye and are represented as mean  $\pm$  S.E.M. ( $n=6-25$ ). Results were compared using one-way ANOVA finding no significant difference among each four columns within UN, L and L+M animals.



**Fig. 4.** The effect of tezosentan (T-10 mg/kg, intraperitoneally), BQ-123 or BQ-788 (1 mg/kg, intraperitoneally) pre-treatment on the small intestinal transit of Evans blue in untreated animals (UN) or rats subjected to skin incision (SI), laparotomy (L) and laparotomy followed by surgical gut manipulation (LM). Experiments were performed 24 h post-surgery. Results are shown as cm migration of the dye and are represented as mean  $\pm$  S.E.M. ( $n=7-20$ ). Results were compared and statistical significance was calculated using one-way ANOVA followed in case of statistical significance by Bonferroni t-test. Following comparisons have been made: UN vs. UN+T or UN+BQ-123 or UN+BQ-788; L vs. L+T or L+BQ-123 or L+BQ-788; L+M vs. L+M+T or L+M+BQ-123 or L+M+BQ-788). Statistical significances have been observed for: L+M vs. L+M+T\*\*\* ( $P<0.001$ ); L+M vs. L+M+BQ-123\*\* ( $P<0.01$ ); L+M vs. L+M+BQ-788\* ( $P<0.05$ ).



**Fig. 5.** Non-cumulative concentration-response curves of the smooth muscle strips exposed to carbachol. Data were normalized as percentages of the maximal response to carbachol and plotted against carbachol concentration. For the sake of the clarity of the picture (maximum value on the ordinate is 100%) data are presented as means – S.E.M. and not means  $\pm$  S.E.M. for at least 8–15 different tissue strips. Animals were allowed either 2 or 24 h recovery period subsequent to surgery. Prior to surgery rats from the latter group were randomly sub-divided into two experimental groups, the first one those pre-treated with tezosentan (T, 10 mg/kg, i.p.) and the second one those receiving tezosentan's vehicle prior to surgery.

## Discussion

The current experiments involved three types of nociceptive stimuli: SI, L, and L followed by a subsequent mechanical stimulation of both the cecum and the small intestine (L+M). The results were in accord with those of De Winter et al. (19), with SI exhibiting no marked effect on the GI transit, whereas L caused a significant delay, an effect additionally potentiated by gut manipulation (L+M).

Tezosentan, BQ-123 and BQ-788 have not shown any marked effects on the movement of Evans blue in UN rats or animals subjected to E, SI and L, which remains in concert with the data showing that  $ET_A$  and  $ET_B$  antagonists do not affect GI transit or GI smooth muscle contractions under basal conditions (15, 20–22). In contrast, other authors have demonstrated that  $ET_A$  and  $ET_B$  inhibit gastric and colonic motility in guinea pigs (23). These discrepancies can at least be partially ascribed to methodological differences.

As opposed to the early phase POI,  $ET_A$  and  $ET_B$  blockers reversed the additional inhibition of intestinal transit evoked by L+M during the late phase POI, with tezosentan being the most efficacious. However, the difference between tezosentan, BQ-123 and BQ-788 failed to reach statistical significance. The diminished GI transit observed *in vivo* was mirrored by the decreased contractile responses of the longitudinal jejunal smooth muscle strips to carbachol *in vitro*. The pre-operative administration of tezosentan alleviated this dysfunction.

It is not fully feasible to elucidate the molecular mechanisms of the salutary effects of ET antagonists based on the results of the current experiments alone. An attempt at explanation may involve the analgesic and

anti-inflammatory properties of ET blockers (9). ET-1 acts as an algogen in the peripheral nervous system and is involved in a variety of pain states, including inflammatory, neuropathic and cancer pain (24), for example ET-1 administered i.p. induces abdominal writhing in mice (25, 26). Moreover ET-1 plays a role in the pathogenesis of inflammation (24, 27), for instance ET-1 plasma and/or synovial fluid concentrations are higher in patients suffering from active rheumatoid arthritis, osteoarthritis or gout than their healthy counterparts (9). The dual ET-receptor blockers: bosentan and tezosentan demonstrate potent anti-inflammatory activities (28, 29).

BQ-123, BQ-788 and tezosentan counteracted in a targeted manner the additional inhibitory component caused by surgical gut manipulation (L+M) in the late phase POI, without affecting gut dysfunction after L alone. This demonstrates that the salutary effects of ET-receptor blockade in the late phase POI result from specific inhibition of ET effects rather than from its non-specific analgesic or anti-inflammatory properties. This conclusion is supported by the fact that BQ-123, BQ-788 and tezosentan were not effective in reversing GI motility inhibition caused by L alone in the early or late phase POI, where L is a potent nociceptive and inflammatory stimulus (30, 31). Similar observations concerning the lack of activity of ET-receptor antagonists subsequent to L were made in the I/R POI model (15).

It has been shown that open GI surgical procedures with extensive visceral manipulations led to a marked increase in plasma ET, whereas minor interventions such as SI do not increase ET-1 concentration (32–34). The magnitude of the systemic concentration of ET-1 was proportional to the length of the operation and the systemic levels of ETs continued to increase further 6–24 h postoperatively in comparison to the intraoperative period (33). The time-course of ETs release subsequent to tissue injury and the extent of the surgical insult may at least partly explain the ineffectiveness of ETs receptor blockade in the inhibition of GI motility caused by L and by L+M in early POI, which remains in concert with the observed lack of changes in ET (1–21) concentration 2 h post-surgery (35).

While interpreting ET(1–21) concentration alterations in current experiments in either 2 or 24 h post-surgical groups, it must be borne in mind that ETs act largely as autocrine or paracrine transmitters and therefore ET levels in peripheral blood are much lower than at the target site (12) meaning that the lack of the observed concentration changes in both early and late phases of surgically induced POI do not necessarily accurately represent changes at the organ level.

Our previous experiments provided evidence that ET receptor antagonists protected against I/R-induced intestinal dysmotility in a time- and dose-dependent manner at the early and late stages of reperfusion (15). This points out to the fact that I/R and surgical gut manipulation exert inhibitory effects on gut motility, which have different underlying pathophysiological mechanisms.

It is a well-known phenomenon that ET-1 released by stimulating endothelial cells contributes to the inflammatory process involving the activation of NF- $\kappa$ B and expression of pro-inflammatory cytokines including TNF- $\alpha$ , IL-1 and IL-6, which in turn stimulate ET-1 production. ET-1 increases the synthesis of TNF- $\alpha$  in macrophages and monocytes enhancing the inflammatory response by chemotaxis and phagocytosis of macrophages, monocytes and neutrophils. Increased production of reactive oxygen species in different types of cells occurs via the NF- $\kappa$ B, COX and NADPH oxidase-dependent pathways. ET-receptor blockers can inhibit some of the inflammatory process components and therefore it seems possible that the ongoing gut-wall located inflammatory process may contribute to the pathogenesis of POI (36).

ETs exert their cellular activities by acting on two cell-surface G-protein-coupled receptors. Type A (ET<sub>A</sub>) receptors are located primarily on vascular smooth muscle cells, whereas type B (ET<sub>B</sub>) receptors can be found amongst others on endothelial, vascular smooth muscle, and renal epithelial cells. Binding of ET-1

to ET<sub>A</sub> increases Ca<sup>2+</sup> influx and generates reactive oxygen species (ROS). In contrast, ET<sub>B</sub> receptors on the endothelium allow ET-1 to signal in an autocrine fashion and stimulate nitric oxide synthase (NOS) and NO production (9). Therefore, the interaction between ETs on ET<sub>A</sub> and ET<sub>B</sub> contributes to the pathogenesis of POI.

In a clinical context, the duration of POI in humans depends mainly on the recovery of colonic motility, whereas in this model both gastric and jejunal propulsion contribute to transit time and their individual effects cannot be separated. The exact origin of the released ETs cannot be determined from our experiments as several cells synthesize and release ETs (9) especially in response to traumatic and/or nociceptive stimuli. Additionally the interactions between ETs and other gut neurotransmitters, which affect gastrointestinal motility have not been studied in our experiments. Finally, quantifying the intestinal transit more precisely using radioisotopes might have provided more exact measurement results (37). On the other hand the employed model is simple, well established and the suppression of spike activity and the absence of migrating motor complex during small intestinal transit inhibition in rats make intestinal transit a reliable index of POI (38, 39).

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### Conflict of interest

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None of the manuscript authors have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within that could unduly influence their work, which need to be disclosed.

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### Acknowledgements

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This work was supported by G85 grant from the Ministry of Science and Higher Education and W184 grant from the Medical University of Gdańsk, Poland. Authors of the article are grateful to Drs. Clozel and Iglarz of Actelion Pharmaceuticals Ltd., Switzerland for their generous donation of tezosentan. Authors would like to thank Dr. Piotr Bandosz, MD, PhD of Medical University of Gdańsk for lending a helping hand with preparations of figures in the manuscript. Current affiliation of Roman Korolkiewicz is: Curevac AG, Frankfurt am Main, Germany.

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