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Muscarinic receptor 1 allosteric modulators stimulate colorectal emptying in dog, mouse and rat and resolve constipation

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Abstract

Background: Because M1 muscarinic receptors are expressed by enteric neurons, we investigated whether positive allosteric modulators of these receptors (M1PAMs) would enhance colorectal propulsion and defecation in dogs, mice and rats.

Methods: The potencies of the M1PAMs, T662 or T523, were investigated using M1 receptor expressing CHO cells. Effectiveness of M1PAMs on defecation was investigated by oral administration in mice and rats, by recording propulsive contractions in anaesthetized rats and by recording high amplitude propagating contractions in dogs.

Key Results: PAM EC₅₀ values in M1 receptor expressing CHO cells were 0.7 to 1.8 nM for T662 and 8 to 10 nM for T523. The compounds had 1000 fold lower potencies as agonists. In anesthetized rats, both compounds elicited propulsive colorectal contractions, and in dogs, mice and rats, oral administration increased fecal output. No adverse effects were observed in conscious animals. M1PAMs triggered propagated high amplitude contractions and caused defecation in dogs. Nerve-mediated contractions were enhanced in the isolated mouse colon. M1PAMs were equi-effective in rats with or without the pelvic nerves being severed. In two models of constipation in mice, opiate-induced constipation and constipation of aging, defecation was induced and constipation was reversed.

Conclusion and Inferences: M1PAMs act at targets sites in the colorectum to enhance colorectal propulsion. They are effective across species and they reverse experimentally-induced constipation. Previous studies have shown that they are safe in human. Because they provide an enhancement of physiological control rather than being direct agonists, they are predicted to provide effective treatment for constipation.

KEYWORDS: colokinetics, constipation, muscarinic receptors, defecation, enteric nervous system

Key Points

- Colorectal propulsion and defecation control involve enteric nervous system pathways, including cholinergic neurons that express M1 muscarinic receptors.
- We investigated two M1PAMs with nanomolar potency as PAMs, but 1000 fold less potency as agonists. The M1PAMs were orally active and caused defecation in dogs, mice and rats. They reversed defecation deficiency in opioid-induced constipation and constipation of aging.
- These M1PAMs have advantage as colokinetics because they enhance neurotransmission that normally occurs rather than being direct receptor stimulants.

1 INTRODUCTION

Early studies using non-subtype selective antagonists demonstrated an essential role of acetylcholine (ACh), acting through muscarinic receptors, in the control of gastrointestinal movements in animal models and in human.¹⁻⁴ More recently, subtypes of muscarinic receptors (M1r, M2r and M3r) have been identified on enteric neurons and muscle in human, and in the small intestine and colon of experimental animals.^{5,6} The M1 receptor is of particular interest as this receptor is expressed by enteric neurons^{5,6} and activation of the M1r increases enteric neuron excitability and transmitter release.⁷

Positive allosteric modulators (PAMs) facilitate the actions of endogenous receptor ligands and thus provide an enhancement of physiological control that is preferred over the generalised stimulation provided by direct agonists, that is independent of the physiological state of engagement of receptors and is not necessarily appropriate to the condition of the animal or patient. M1PAMs have been developed as compounds to enhance cognitive function, with the specific aim of treating Alzheimer's disease, and are safe for human use.^{8,9} There is also evidence that M1PAMs enhance propulsion of content and fluid secretion in the colon of humans, dogs, rats and mice, although not all M1PAMs that cause cognitive enhancement have significant effects on the colon.⁹⁻¹³ Thus, M1PAMs have the potential to facilitate physiological actions of ACh within enteric reflex pathways, for example augmenting propulsive reflexes. M1r are also prominent on the mucosal epithelium of the colon, where their activation increases

fluid secretion.¹⁴ Thus, M1PAMs could enhance colonic propulsion and fluid secretion, both actions being of potential benefit in treating constipation. However, there is scant detail of the sites and mechanisms of action of the colokinetic and prosecretory actions of M1PAMs.

An M1PAM that has been shown to increase fecal output is T662 (3-((1S,2S)-2-hydrocyclohexyl)-6-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)methyl) benzo[h]quinazolin-4(3H)-one).¹¹⁻¹³ In this study, we have used T662 and a potent novel M1PAM, T532 (1,5-anhydro-2,3-dideoxy-3-[(7-{[4-(1-methyl-1H-1,2,3-triazol-4-yl)phenyl]methyl} furo[3,2-b]pyridine-5-carbonyl)amino]-L-threo-pentitol) that we have synthesised.

2 MATERIALS AND METHODS

Experiments were conducted on mice, rats and dogs. Procedures were approved by the University of Melbourne Animal Ethics Committee (approval 1613996) and the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited (approvals AU-00020327, AU-00020311 and AU-00020478).

2.1 In vitro IP1 assay

The effectiveness of M1PAMs to potentiate ACh activation of M1r was measured using IP-One assays (CisBio, Chiba, Japan) according to the manufacturer's instructions. This system detects the accumulation of inositol monophosphate, a stable downstream metabolite of IP3 whose levels are increased by activation of a phospholipase C (PLC) cascade. CHO-K1 cells were transfected with human, mouse or canine muscarinic acetylcholine receptor M1r gene using lipofection or electroporation. Transfected cells were cultured with complete medium supplemented with 500µg/mL of G418 for longer than 14 days. Polyclonal pools of stable cells were suspended in the stimulation buffer containing 0.1% BSA. Then, cells were added (10,000 cells/well) in white 384-well plates containing test compounds and ACh (EC₂₀ ACh in presence of PAM). The plates were incubated for 60 minutes at 37°C in the presence of 5% CO₂. IP1-d2 solution and anti-IP1-cryptate Tb conjugate were added, and then incubated for 60 min at room temperature. Accumulation of IP1 was measured with Envision (PerkinElmer, Kanagawa, Japan). Data are expressed as the M1PAM EC50 values for each condition.

2.2 Recording colorectal propulsion in anesthetized rats

These experiments were conducted to determine whether M1PAMs augmented propulsive contractions and emptying of the colorectum. Experiments were conducted as previously described.¹⁵ Rats were sedated with ketamine hydrochloride (50-60 mg.kg⁻¹, i.m.), following which anesthesia was induced with α -chloralose (60 mg.kg⁻¹, i.v.). The femoral artery was then cannulated for the infusion of anesthetic and blood pressure recording, and the femoral vein was cannulated for delivery of drugs. Drugs were delivered in volumes of 0.1mL/100g. M1PAMs were dissolved in DMA/PEG (dimethylacetamide : polyethylene glycol 400 : water; 1 : 1 : 3). Each rat received a single dose for the determination of the dose-response relation. Blood pressure and heart rate were recorded with a Power Lab recording system using Chart 5 software (both from ADInstruments, Sydney, Australia). Anesthesia was maintained by intra-arterial infusion of α -chloralose (12-20 mg.kg⁻¹.h⁻¹) plus ketamine (3-5 mg.kg⁻¹.h⁻¹) in phosphate buffered saline (PBS; 0.15M NaCl containing 0.01M sodium phosphate buffer, pH 7.2). For colorectal recording, the distal colon was cannulated at the colonic flexure, and a second cannula was inserted into the anus. The colon remained *in situ*, and the muscle and skin were closed around the proximal cannula. The oral cannula was connected to a Mariotte bottle filled with warm PBS, and the distal cannula to a pressure transducer via a one-way valve. The baseline intraluminal pressure was maintained at 7-8 mmHg by adjusting the heights of the Mariotte bottle and outlet. Expelled fluid was collected in a cylinder distal to the one-way valve, and measured by weighing with a force transducer.

To determine whether the M1PAMs had a central or peripheral site of action, the spinal nerves were severed below the conus medullaris, within the spinal canal, while rats were under anaesthesia, and the M1PAM was then administered.

2.3 Recording urinary bladder emptying in anesthetized rats

Because the organisations and pharmacologies of colon and urinary bladder control are similar, and some colokinetics act on both, we investigated the effects of M1PAMs on bladder emptying by recording cystometrograms from anaesthetized rats. To record cystometrograms from the urinary bladder, a 2 cm incision was made through the midline of the lower abdomen and the bladder was exteriorised using cotton tipped applicators to minimise stress or damage.¹⁶ The bladder was instrumented for continuous filling cystometry with buffered saline at room temperature by the insertion of a catheter into the dome of the bladder. The catheter was secured

by a purse string suture using 5-0 silk thread. The bladder was returned to the abdomen, the catheter was passed through the abdominal incision and the muscle and skin were sutured around the catheter using 3-0 silk. The external end of the catheter was connected via a three way tap to an infusion pump and to a pressure transducer. Blood pressure and heart rate measurements were made continuously. At the ends of experiments, anesthetized rats were killed with a lethal dose of sodium pentobarbitone (300 mg/kg, i.v.), while still under anesthesia.

2.4 Oral administration by gavage

These experiments were designed to determine whether the M1PAMs were effective in stimulating defecation in conscious animals, and whether, at doses effective in conscious animals, any adverse effects were observed. Mice and rats were maintained in individual cages in a quiet room from the day prior to gavage. On the morning of experiments, test compounds were given by oral gavage and animals were placed in the home cage and the feces produced were collected and counted for up to 4 hr after the administration of drug. Gavage volumes were 0.5-1 mL/100g body weight. M1PAMs were dissolved in 0.5% methylcellulose for oral administration. On any one day, only a single dose was administered.

2.5 Mouse colon organ bath assay

A length of 1-1.5 cm of distal colon was removed after the mouse was killed by cervical dislocation, the colon was rinsed with Krebs solution (mmol/L: NaCl 120.7, KCl 5.9, NaHCO₃ 15.5, NaH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 2.5, glucose 11.5). The segment of colon was suspended in oxygenated Krebs solution at 37°C and connected to an isometric force transducer (ADInstruments). A moderate tension (1-1.5 g) was applied to the colon and the colon was left to stand for 30 min. Then, KCl 30 mM was added to the Krebs solution to induce a contraction and the Krebs solution was refreshed 3 min after KCl application. This step was repeated one more time. Electrical stimulation (10 Hz for 10s, 20V) was applied in the presence or absence of drugs. The magnitude of the contraction induced by electrical stimulation was normalized to the magnitude without drug.

2.6 Loperamide-induced constipation and aging-associated constipation in mice

Two common associations with constipation in humans are the use of opiates and aging. These have been chosen as models to test the effectiveness of M1PAMs.

For the opiate-induced constipation, mice were acclimated to the experimental environment by the experimenter's handling 2-3 times in the week before the experiment. Mice were moved

to a mesh bottom cage from a regular home cage the day before. On the day of the experiment, 30 min after the treatment of loperamide (1 mg/kg, s.c.), mice were given drugs orally and a tray was placed in the bottom of the cage to collect feces. Two hours later, fecal pellets on the bottom tray were counted and collected. Wet and dry weights of the fecal pellets were measured.

In the experiment of aging constipation, 18- and 2-month-old ICR mice were used as an aging constipation model and young control, respectively. General procedure of this experiment was the same as the loperamide-induced constipation model. Two hours after oral drug administration, fecal pellets were counted and collected.

The feces were collected from mice in the 2 or 4 hours after they were placed individually in a mesh bottom cage. Fecal pellets were counted and they were inspected for scoring and weighed immediately after collection. Water content in feces was measured by drying the pellets overnight at 65°C in an oven and reweighing the tubes.

The condition of the feces was scored by an observer blinded to the treatment of the mice, using a scoring scale with well-formed externally dry pellets (as produced normally by healthy mice) = 1; well-formed but moist = 2; unformed adhering moist pellets = 3; unformed wet pellets (diarrhea-form) = 4. These correspond, approximately, to human Bristol scale 3, 4, 5 and 6, respectively.

2.7 Spinal cord injury (transection)

Spinal cord lesions disrupt control of defecation in patients. Here we investigated whether there was an effect on the responses to MIPAMs. Spinal transections were made at the T10 level using aseptic conditions under inhaled isoflurane anesthesia, using procedures previously described.¹⁷ T10 was chosen to minimise cardiovascular effects of spinal lesion. The muscles connecting to the spines of the vertebrae were detached and a laminectomy was performed to remove the dorsal aspect of the T10 vertebra. The exposed spinal cord was raised using a curved probe and the cord was transected with sharp scissors. After closure and recovery from anesthesia, the animals were examined to confirm paralysis of the hind limbs as an index of the completeness of the lesion.

2.8 Experiments on dogs

Six adult male beagle dogs (9.9-10.6 kg, Kitayama Labes, Nagano, Japan) were used for colonic motility studies. These dogs were surgically implanted with force transducers prior to the experiments.

Anesthesia was induced by butorphanol tartrate (0.1 mg/kg, i.m.) and sodium thiopental (20-25 mg/kg, i.v.), and a tracheal catheter was inserted into the respiratory tract to allow mechanical ventilation to be performed using an animal ventilator (ACOMA Medical Industry, Tokyo Japan). Anesthesia was maintained by inhalation of 0.5-3% isoflurane. Atropine sulfate (0.1 mg/kg, s.c.) was administered to relax the smooth muscle. Under these conditions, the abdomen below the xiphoid process was incised and the colon was exposed. Force transducers (F-12IS, Star Medical, Tokyo Japan) were implanted on the ileum (approximately 10 cm oral to the ileocecal region), ascending colon (approximately 3-5 cm from the ileocecal region), transverse colon (middle region of the colon) and descending colon. The electrical leads attached to each transducer were tunneled from the lateral side of the chest wall to the region between the scapulae and joined to a telemetry transceiver (GTS-850, Star Medical, Tokyo, Japan). The incision was sutured and protected by a jacket. After the surgery, antibiotic (ampicillin sodium, 100 mg/animal, i.m.) was administered for 3 days, to prevent infection.

Once the animals recovered to a stable state (wound healed and GI motility recovered by observed fecal output), colonic motility was assessed using telemetry. Dogs that showed stable contraction wave patterns (n=4) were selected for further studies.

These animals were housed in individual cages and were provided with solid food every morning. On the day of experiment, which was 10-14 days after surgery, the animals were not given food in the morning but were fed later in the day, when the study was completed. Colonic motility was measured by the telemetry system with the dogs in a freely moving state. After stabilization of the baseline, T-523 suspended in vehicle (0.5% methylcellulose), or vehicle alone, was administered by oral gavage and colonic motility was measured for 4 hr after administration. The numbers of high amplitude contractions were counted and defecation episodes were also counted.

2.9 Data and statistics

Data are expressed as mean $\pm$ SEM. Statistical comparisons are made using Student's t-test, one-way ANOVA with Tukey's multiple comparisons test or by Shirley-Williams multiple comparison test followed by one-way ANOVA, as indicated in the text. Results were considered to differ significantly when $P \leq 0.05$.

3 RESULTS

3.1 Activities of M1PAMs in transfected cells

To determine EC₅₀ for the positive allosteric actions of the compounds, CHO-KI cells transfected with the genes for M1 receptors of human, dog, rat and mouse were exposed to graded concentrations of T662 or T523 in the presence of ACh (Supplementary Table 1, Supplementary Figure 1). The agonist actions were also investigated in the absence of ACh, but because of the very low agonists potencies full concentration-effect relationships were not determined. EC₅₀ values for PAM effects were in the range of 0.7 to 1.8 nM for T662 and 8 to 10 nM for T523. The compounds had greater than 1000 fold less potency as agonists, compared with PAM effects (Supplementary Table 1, Supplementary Figure 1).

3.2 Colorectal motility in rat

Intravenous injection of a bolus of either T523 or T662 to anesthetized rats caused a prompt and sustained increase in the numbers and amplitudes of phasic colorectal contractions, accompanied by fluid propulsion through the segment of colorectum from which the pressure records were taken (Figure 1). The M1PAMs did not affect baseline tone in the muscle, although when phasic contractions occurred very close together the pressure did not completely return to baseline between contractions (Figure 1A).

T523 was about 10-fold more effective than T662, with a threshold dose of about 0.003 mg/kg and a maximum effect at about 0.03 mg/kg (Figure 1C). Responses to maximally effective doses of T662 or T523 began in the first minute after drug application, peaked at 15-20 min and declined to around baseline over about 2 hours. The phasic contractions that occurred in the first 15-20 minutes after drug application were effective in propelling fluid from the colon. Later contractions, although of similar amplitude, were sometimes ineffective in causing propulsion. A second dose administered after the response to the first had declined was effective in eliciting propulsive contractions. The M1 receptor agonist, RO168703 (0.3 to 3 mg/kg, intravenous), elicited phasic colon contractions accompanied by propulsion of contents.

3.3 Effects of compounds given orally to conscious mice

Compounds were given by oro-gastric gavage to conscious mice and feces were collected, scored for appearance (see Methods) and weighed. Significant increases in fecal pellet numbers and weight were observed for T662 and T523 at a dose of 0.3 mg/kg (Figure 2). Feces became more moist and soft with increasing doses, resulting in increases in fecal scores at 1.0 mg/kg (T662). There were no behavioural effects observed (aversion, stress, piloerection, vocalisation).

3.4 Effects of compounds given orally to conscious dogs

T523 was administered to dogs that had been instrumented to record contractile activity in the distal ileum, proximal, mid and distal colon in the morning after overnight fasting. Ten to 20 minutes after drug administration, dose-dependent co-ordinated high amplitude propagated contractions (HAPCs) were recorded in the ileum and colon (Figure 3A). These were associated with increased numbers of defecation episodes (Figure 3B). The threshold doses were 0.01 to 0.03 mg/kg (Figure 3B, C). The contractions were most prominent in the ileum, proximal and mid colon (Figure 3C). The dogs showed no observable reactions to the compound; they did not have any piloerection, vocalization or signs of stress.

3.5 Sites of action of M1PAMs

We investigated the effects of T662 on contractions of the mouse colon, *in vitro* (Figure 4A, B) and, in anaesthetised rats, we compared the effects of T662, intravenous, on colorectal motility with the connections between the lumbo-sacral spinal cord and the large intestine intact, or lesioned bilaterally, by cutting the spinal nerves within the spinal canal, below the conus medullaris (Figure 4C).

In vitro, T662 dose-dependently increased contractile responses of the mouse colon to electric field stimulation (trains of 10Hz, 20V for 10 sec, each 5 min). The threshold concentration was between 10 and 100 nM (Figure 4B). However, the M1PAM did not increase the baseline tension in the muscle.

In vivo, there were no differences in the responses with the connections of the pelvic nerve with the central nervous system severed, compared to responses with connections intact (Figure 4C). These experiments indicate that M1PAMs act peripherally and that there is no significant component attributable to a central action.

3.6 Effects on fluid secretion in the colon

We observed a stool softening effect of M1PAMs in the in vivo experiments, suggesting that M1PAMs may enhance water and electrolyte secretion. This possibility was investigated using sheets of rat colon mucosa mounted in Ussing chambers.

When added to the serosal side of the mucosa, T662 (1-100 nM) had no effect on the background electric current (short circuit current, I_{sc}). However, T662 over the same concentration range enhanced the increase in transmucosal current caused by acetylcholine (ACh; Figure 5). The effects of ACh were blocked by the M1 selective antagonist, pirenzepine, 1 μ M (Figure 5). Transmucosal resistance was not changed by T662 (Figure 5), indicating that gut mucosal leakiness was not changed.

3.7 Effects on urinary bladder

Compounds that stimulate or enhance motility of the colon commonly also affect the urinary bladder. In order to test this possibility, we have investigated effects on cystometrograms in the rat (Supplementary Figure 2). T662 at doses that had consistent strong effects on the colorectum, 0.1 to 0.3 mg/kg, by increasing colorectal contractile activity and propulsion, were not effective in the bladder.

3.8 Effects on constipation

The effects of M1PAMs in two constipation models were tested, loperamide induced constipation in mice and age-associated constipation, also in mice.

Loperamide (1 mg/kg, s.c.) was administered 30 min prior to measuring fecal output from the mice. Numbers of fecal pellets produced by loperamide pretreated mice in the two hours after drug administration were about 20% of the number produced by untreated mice. The numbers of fecal pellets were increased by oral gavage of T523 (Figure 6) and T662 (data not shown). The increase was dose-related and was associated with increases in fecal wet and dry weight and in fecal scores. Increases were observed with 0.3 mg/kg and increased with doses up to 3 or 10 mg/kg. It is notable that higher doses were required to show a clear efficacy in constipated compared to normal mice (see Figure 2).

We compared fecal output in mice aged 2 and 18 months. At 18 months, the numbers of fecal pellets produced, and their total weight, were less than half that of 2 month old mice (Figure 6). The deficiency in fecal output at 18 months was reversed by T523 (0.3 mg/kg, p.o.) and fecal output was further increased by increasing the doses of T523 (Figure 6).

3.9 Effects of T662 on fecal output after spinal cord injury

When the spinal cord is cut and animals are left to recover, triggers for defecation that act at centres in the brain (cortex and brain stem) fail to elicit defecation. In humans, this means that the bowel cannot be emptied voluntarily. Moreover, responsiveness to colokinetics can be increased after spinal cord injury, which is believed to be due to a denervation hypersensitivity.¹⁷ We have investigated the effect of T662 (1 mg/kg, oral) at 5 weeks following spinal cord transection at T10 (Figure 7). T662 was equally effective when given by gavage to spinal cord injured or sham operated rats. The conscious rats did not react adversely to the compound.

4 DISCUSSION

In this study, we investigated the colokinetic actions of two M1PAMs, T662 and T523, in rodents and dogs. These 2 compounds exhibited highly potent M1PAM activities in mouse, rat and dog, and no direct agonist activities were found for either compound in transfected cells, including cells transfected with the human M1r, or *in vivo*. Therefore, the observation we have made are deduced to be purely due to PAM activities of the compounds. Both T523 and T662 triggered defecation in dogs, mice and rats. The potencies of the compounds given orally to conscious animals were similar in the three species, with T662 being effective at about 1 mg/kg. Oral T523 was tested in dogs and mice. It was effective at about 0.3 mg/kg. In each species, there were no adverse behavioural effects exhibited by the conscious animals at doses that were effective in eliciting defecation.

We compared the effectiveness of T662 in eliciting colorectal propulsive contractions in rats with and without intact connections from the spinal cord to the colorectum. Responses were unchanged when the colorectum was denervated. When added to preparations of mouse colon in which muscle contractions were recorded, the M1PAMs had no effect on baseline activity. However, they increased responses to cholinergic nerve activity or the application of

acetylcholine. Thus, at doses in which they were effective colokinetics, the M1PAMs did not have detectable direct agonist effects. Consistent with the site of action being in the colorectum, in both human and laboratory animals, M1r are expressed by the cell bodies and processes of enteric neurons.^{5,6} They were not found on the muscle cells. Previous studies showed that the effects of M1PAMs on defecation were not observed if M1r were knocked out.¹² We also investigated the effects of T662 in rats that had the spinal cord transected at T10, 5 weeks before. In a previous study,¹⁷ colorectal propulsive responses to a compound that acts at the spinal defecation centres, the ghrelin receptor agonist HM01, were significantly greater in rats that had spinal transection 5 weeks previous to the test. This was attributed to a denervation supersensitivity, the descending spinal pathways innervating the defecation centres having been severed. In the current study there was not a supersensitivity to T662. This supports the conclusion above that T662 acts on the enteric nervous system, not on central pathways of colorectal control. It also suggests that there are not trans-synaptic effects, through which changed responsiveness of neurons in the spinal defecation centres caused downstream effects on enteric neurons. The transection was made at T10, to avoid effects on blood pressure control that occur when sites higher in the thorax are transected. Higher level transection has more profound effects on the colon.¹⁸ Transection at T3, which causes blood pressure to drop and which removes control of the abdominal wall muscle, reduces the level of spontaneous contractile activity in the colon and causes loss of enteric neurons.¹⁸ Under these circumstances, it would be expected that the effectiveness of colokinetics could be reduced. Thus, M1PAMs might be less effective in treating colorectal dysfunction in quadriplegics, compared to paraplegics.

These studies show that two potent M1PAMs, T523 and T662, trigger defecation in mice in a dose dependent manner, suggesting that the compounds caused propulsive colonic contractions, as we confirmed directly in the rat. The potency of T523 was greater than that of T662 *in vivo*, even though *in vitro* potencies of T523 was 5 times weaker than that of T662. More importantly, although the two compounds have different chemical structures they both showed strong effects in the gastrointestinal tract. The different order of potencies of two compounds *in vitro* and *in vivo* might be explained by differences in allosteric mechanisms. Recently, Sako et al¹³ reported that TAK-071, a unique M1PAM with a low α -value, showed less GI effect, while T662 with a high α -value, exerted strong GI effects. Different PK profiles of these compounds, e.g. different C_{max} , distributions between tissue compartments and available concentrations may

also contribute. Therefore, it is considered that colokinetic action could be a general property of M1PAMs, although the effects may differ due to differences in allosteric mechanisms and receptor exposure.

Colonic high amplitude propagated contractions (HAPC) are observed in the small intestine and migrate to the proximal and distal colon. HAPC can transfer colonic contents over long distances and often precede defecation.¹⁹ These migrating colonic contraction can be monitored by intracolonic pressure measurement (manometry) and direct measurement of smooth muscle contraction of the colon using implanted force transducers.²⁰ We selected the implantation of force transducers, because colonic contractions can then be monitored by telemetry in freely moving conscious dogs. In the basal condition, fasted overnight in this study, only small contraction occurred prior to a dose of T523. When T523 was given orally, marked contractions were generated. These contractions occurred at the distal ileum and migrated from the proximal to the distal colon. Such migrating contractions were highly correlated with defecation episodes. This is consistent with the outcomes from the rat studies. Each event was not phasic, but consisted of a burst of contractions (Figure 3). In between were quiescent phases when no contractions were recorded. One possible explanation is that subthreshold events occur intermittently in the fasted condition in the dog colon. These intermittent events may not reach sufficient amplitude to cause defecation. However, such events can be amplified by M1-PAMs, so that the strong high amplitude contraction were recorded intermittently. We counted every spike-like contraction as one contraction, so that number of contractions does not correspond to the number of manometrically recorded HAPCs (Figure 3B, C). We conclude that M1-PAMs can enhance the colonic propulsive contraction in rodents and dogs by amplifying the naturally occurring propagated events. Because the M1PAMs that we have tested do not drive receptor activity in the absence of the natural ligand, ACh, they do not indiscriminately stimulate colorectal functions. Instead, they enhance physiological control that is exerted through the M1r.

Opiate-induced constipation is burdensome for patients.²¹ In experimental animals, loperamide-induced constipation is one of the major model for constipation and is utilized for assessing the anti-constipation drugs. We found that T523 was highly efficacious in reversing constipation induced by loperamide. A low dose of T523, 0.3 mg/kg, almost returned the constipation condition induced by loperamide to normal and a higher dose more than

compensated for loperamide. Therefore, a minimal effective dose of T523 to cause defecation in a healthy individual might be enough to show the efficacy in opiate-induced constipation.

Aging is also a risk factor for constipation.^{22, 23} In our facility, 18 month-old mice (C57BL/6) showed reduced fecal pellet output, compared to young mice (2 month-old). Similarly to opiate-induced constipation, T523 reversed the constipation of aged mice. In these studies in aged mice, the fecal score remained at 1, no change from control. Mouse feces are basically packed tightly and the normal feces has low water content, similar to feces of constipated mice. Furthermore, the small number of fecal pellets produced in the constipated condition would contribute minimal changes to the fecal score. Since rat feces are more moist, compared with mice, it will be worthwhile to investigate the effect in a rat constipation model.

Effects on stool water were investigated in mouse and rat. Given *in vivo*, the M1PAMs increased stool moisture, and *in vitro* experiments using the isolated mucosa from the rat colon showed that T662 enhanced electrolyte secretion elicited by acetylcholine. Thus, M1PAMs possess both colokinetic and, at higher *in vivo* doses, stool softening properties, which makes them, potentially, ideal compounds to treat constipation. It is notable that in the isolated mucosa, the M1PAM did not have an effect on basal currents, but that it increased the fluid secretion that was stimulated by acetylcholine. Moreover, transmucosal electrical resistance was not changed. Thus, the M1PAMs did not make the mucosa more leaky, and are predicted to enhance cholinergic nerve-mediated water and electrolyte secretion, that is to enhance physiological control. This is different to other stool softeners that are in clinical use. Lubiprostone is an opener of CLC-2 (chloride channel type-2) channels of enterocytes in the epithelial lining, its anti-constipatory effects result from its stimulation of fluid secretion²⁴ and linaclotide acts on guanylate cyclase receptors in the intestinal enterocytes and also increases secretion.²⁵

Conclusion: Positive allosteric modulators of muscarinic M1 receptors are of potential value as colokinetics because of their selective enhancement of physiological colorectal propulsive motor patterns.

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DISCLOSURES

Yasuo Itomi and Yasuhiro Tsukimi are employees of the Takeda Pharmaceutical Company Limited. The other authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

YT, JBF and RVP conceptualized and designed the study. RVP, YI, SD, X-YC, RMMcQ performed animal studies and analysed the data. MR conducted Ussing chamber studies and analysed the data. JBF and YT integrated data and wrote the manuscript. All authors contributed discussion and approved the manuscript.

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Figure Descriptions

Figure 1 Colorectal contractile activity in the rat in response to the intravenous injection of T662 or T523. **A:** T662 (0.1 mg/kg) evoked strong phasic pressure increases that persisted for over an hour. These caused propulsion of fluid from the colorectum in the first 20 minutes, after which the efficiency of the contraction in causing propulsion waned. **B:** Response to T523 (0.03 mg/kg). This compound also caused phasic contractile activity that was associated with propulsion of fluid in the first ~15 min. **C:** Dose-response relation for T523. The numbers of phasic pressure increases > 6 mmHg in amplitude in the 30 minutes after drug application showed a dose-dependent relationship (mean $\pm$ SEM, n = 3 to 5).

Figure 2 Fecal output in mice given oral gavage with T662 or T523. Feces were collected for 4 hours after administration of drug. **A:** Fecal output was increased in a dose-dependent manner. T523 was more potent than T662. **B:** As the doses of M1PAMs were increased, there was increase in fecal weight and in fecal water content. **C:** Fecal score was also increased. This correlates with the increase in fecal water content (**B**). The data are expressed as the mean $\pm$ SEM from 9-10 mice. Statistical significance was calculated by Shirley-Williams multiple comparison test followed by one-way ANOVA. * $P < 0.05$, compared to no drug.

Figure 3 Responses in dogs to oral dosing with the M1PAM, T523. **A:** Motility responses recorded by force transducers on the serosal surfaces in response to 0.1 mg/kg T523. High amplitude propagated contractions (HAPC, example at the arrow) were evoked by T523. **B:** Numbers of defecation episodes in the 4 hr following drug gavage. Overnight fasted dogs that had no drug administered had no defecation episodes in the morning ('0'). The M1PAM evoked numerous defecations at the highest dose. **C:** Numbers of high amplitude contractions in the 4 hr following M1PAM administration. The data are expressed as the mean $\pm$ SEM from 4 dogs. * $P < 0.05$, compared with no drug.

Figure 4 Experiments to determine the sites of action of M1PAMs. **A:** Longitudinal tension recording from the mouse colon. Electrical stimulation of the strip (10 Hz for 10s at the dots), caused a brief relaxation followed by contraction. Previous studies indicate that these are nerve-mediated responses. T662 had no effect on baseline activity, but it enhanced the contraction elicited by stimulation. **B:** Quantitation of effect; contraction to stimulation with no drug = 100%. T662 between 1 nM and 10 μ M, caused a dose-related increase in the contractions. * $P < 0.05$, compared with no drug, mean $\pm$ SEM from 4 muscle strips. **C:** Contractile responses of the colorectum to T662 (0.1 mg/kg) in anaesthetised rats in which the nerves connecting the colorectum with the defecation centres were cut, or were left intact. T662 significantly increased contractile activity compared to baseline (* $P < 0.01$). Severing the nerves had no effect on responses (NS), $n = 6$ rats in each group.

Figure 5 Action of the M1PAM, T662, on fluid secretion across the mucosa of the rat colon. **A:** Record from an Ussing chamber experiment. Acetylcholine (ACh) caused an increase in short

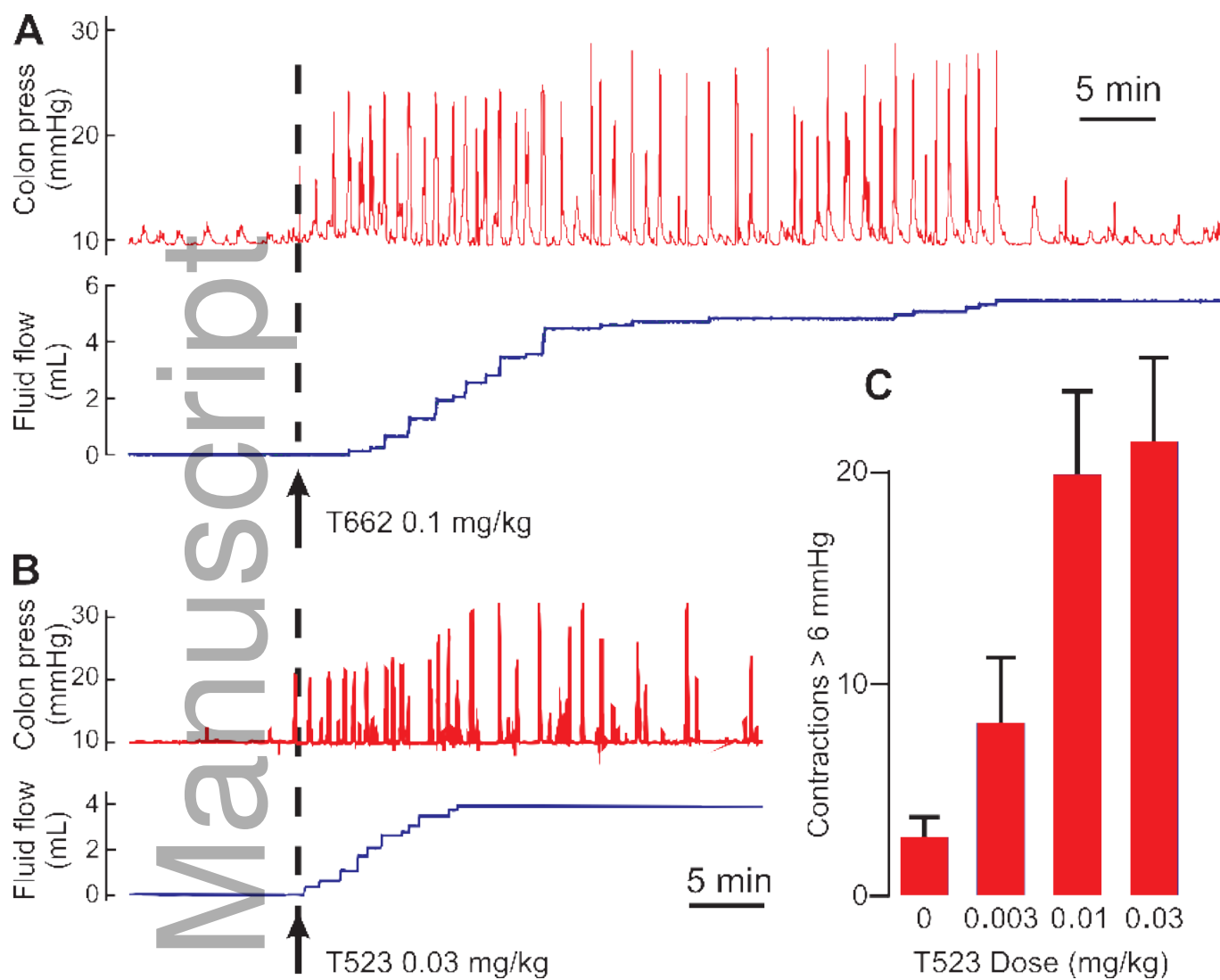
circuit current (*I*_{sc}) that faded. In the continued presence of ACh, T662 increased *I*_{sc} (although it had no effect if ACh was not present). The enhanced response was reduced by the selective M1 antagonist, pirenzepine. The upward deflections are responses to voltage pulses that were used to measure transmucosal resistance. Resistance was not changed by T662. **B:** Concentration response relation to ACh in the absence and in the presence of graded concentrations of T662. *I*_{sc} responses to ACh (100 nM) were larger when T662 was already present. Mean ± SEM, n= 6. * P<0.05, compared to no T662.

Figure 6 Effect of the M1PAM, T523, on fecal output in loperamide treated and aged mice. **A:** Loperamide, 1 mg/kg s.c., 30 min before the test, reduced fecal output in the first two hours after dosing, by over 80% compared to untreated mice (Cont). T523, 0.3 mg/kg, reversed the effect and greater fecal output was caused by 1-10 mg/kg T523 (* P<0.05, compared to control). The reduction in fecal weight caused by loperamide was reversed by T523. With doses of T523, 1-10 mg/kg, the feces had a high water content (relative heights of blue and red columns). The fecal score for loperamide treated mice was increased by T523, 1-10 mg/kg T523, consistent with the greater water content (* P<0.05, compared to control). **B:** Fecal output in the first two hours after transfer of 18 month old mice to a clean cage was about 25% of output from 2 month old mice. T523, 0.3 mg/kg, increased the fecal output of 18 month mice to the level of 2 month mice. At 1-10 mg/kg T523, greater fecal production occurred (* P<0.05, compared to 18 month mice with no drug). The reduced fecal weight of older mice was reversed by T523 (* P<0.05, compared to no T523). The feces also had higher water content. The fecal score was increased by T523 (* P<0.05, compared to 2 month or 18 month no T523). All data is for feces after 2 hr in the clean cage. Data is mean ± SEM from 9-10 animals per group.

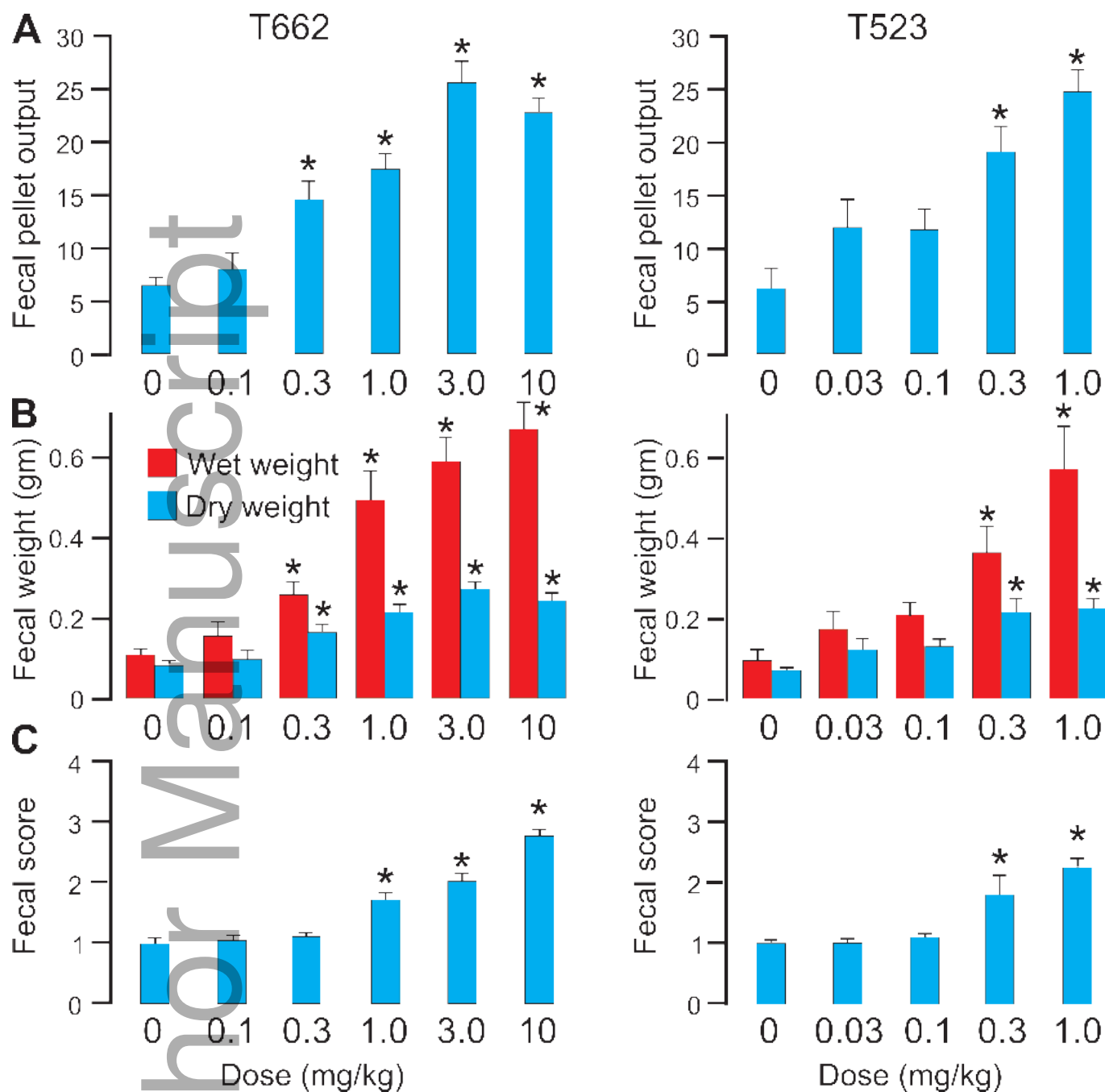
Figure 7 Effects of T662 on propulsive contractions, fecal output and fecal water content were equivalent in rats with chronic spinal transection (SCI) compared to sham operated rats. The spinal cord was transected at T10, or rats were subjected to sham spinal surgery. Colorectal function was evaluated 5 weeks after surgery. **A:** Colorectal propulsion in anesthetized rats in response to T662 (0.1 mg/kg, i.v.). There was no significant difference (NS) when responses in SCI and sham rats were compared. **B:** Responses to T662 (1 mg/kg) given by oral gavage to conscious rats. Responses in SCI and sham operated rats were not different. **C:** Fecal water

contents prior to T662 (1 mg/kg, oral) and after T662 were not different between SCI and sham rats. T662 increased water content in both groups ($P < 0.01$; $n = 8$). Data mean $\pm$ SEM.

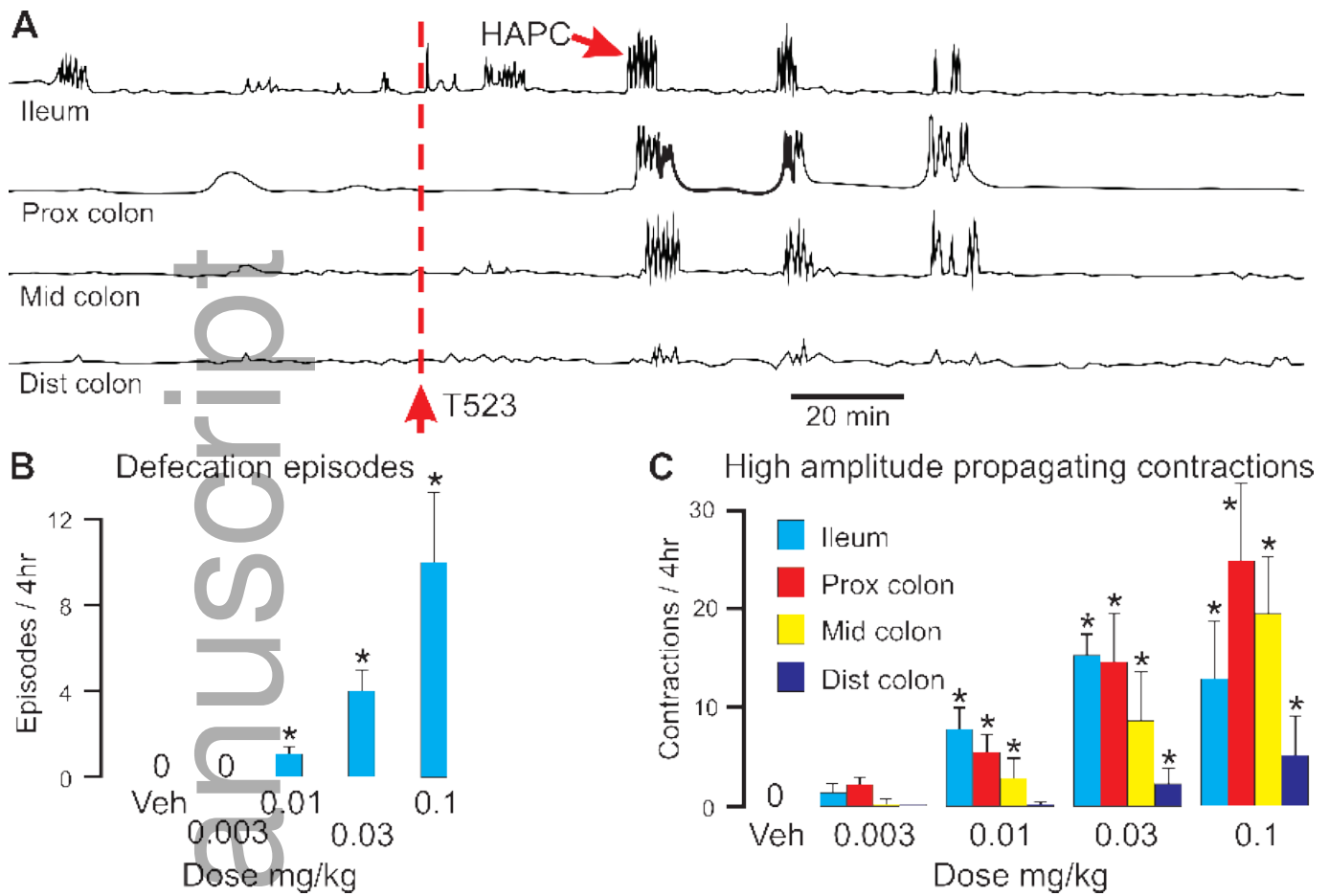
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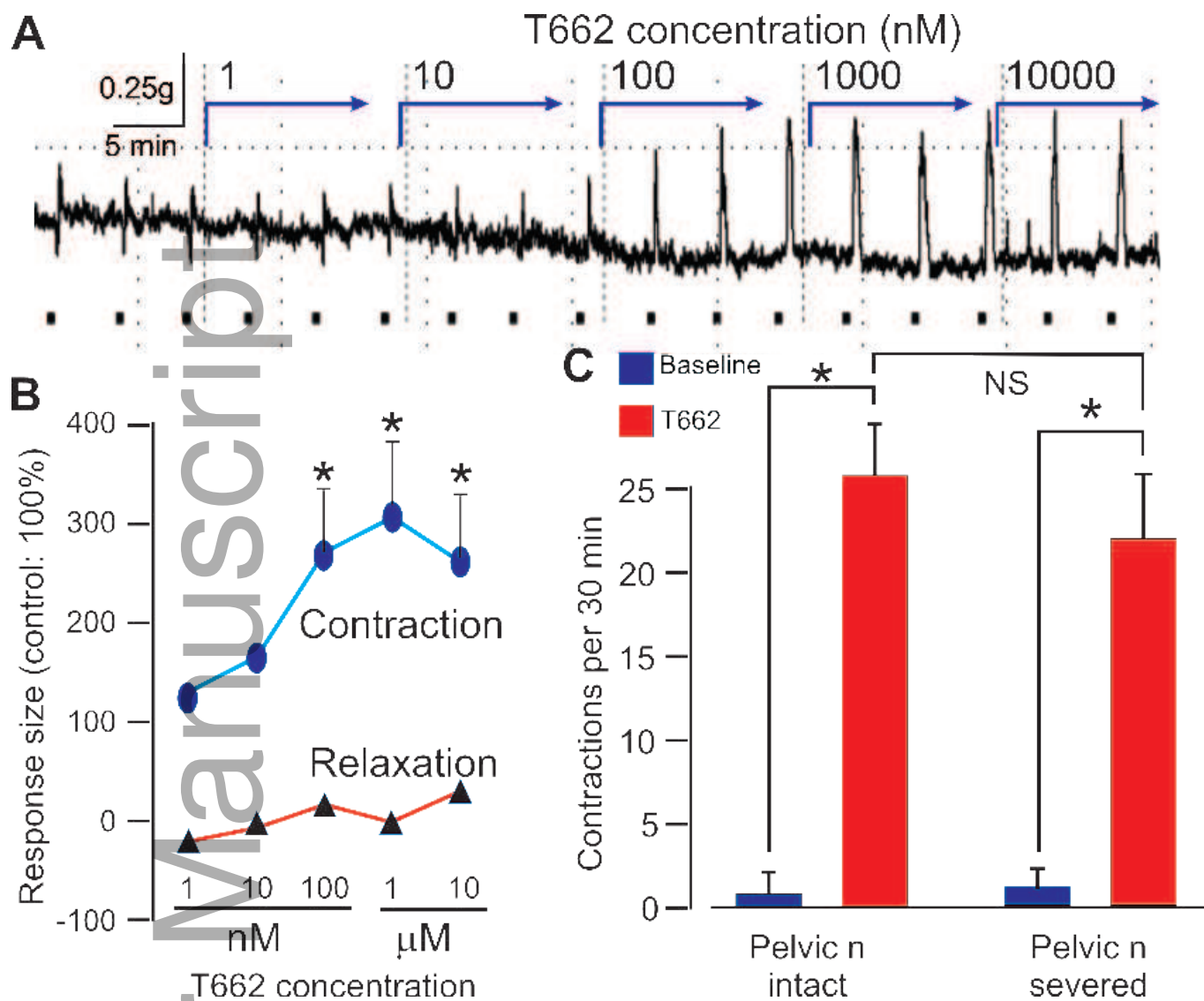
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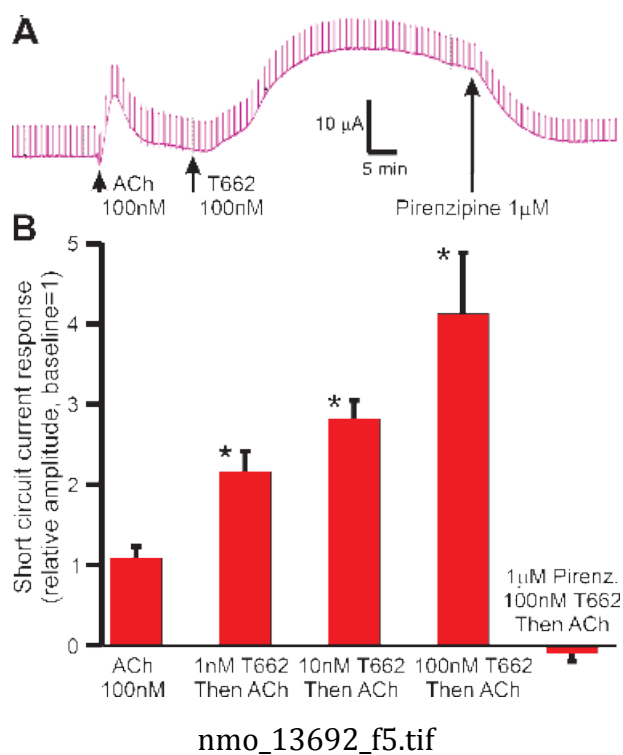
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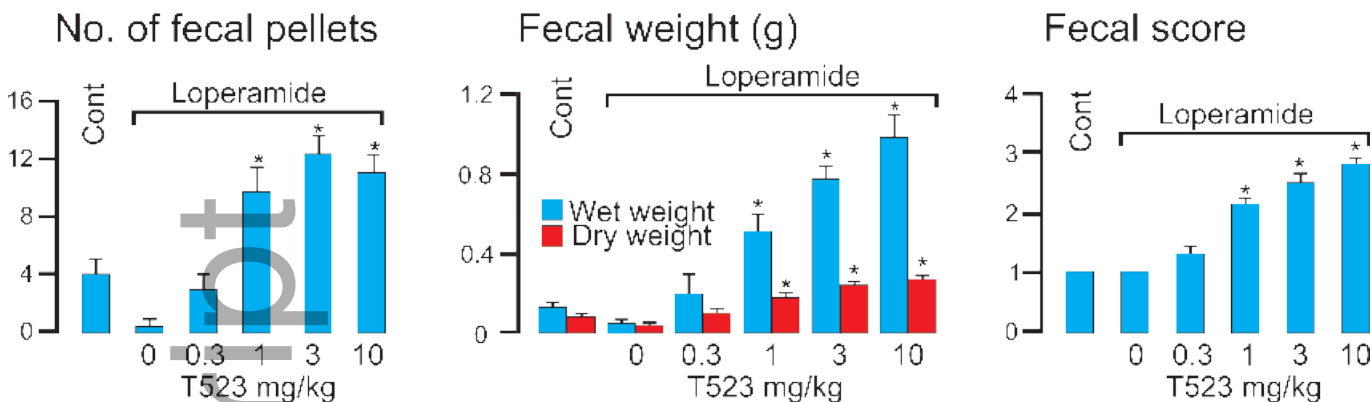
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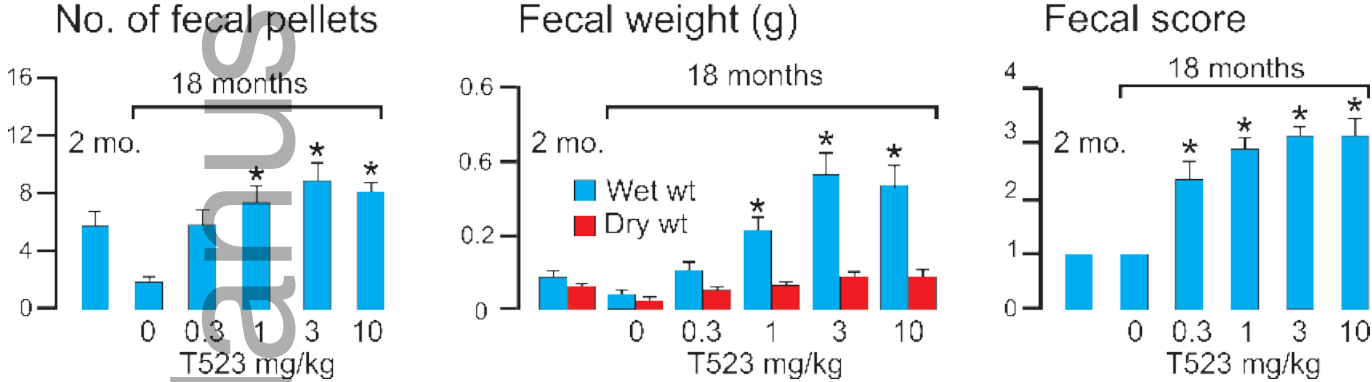
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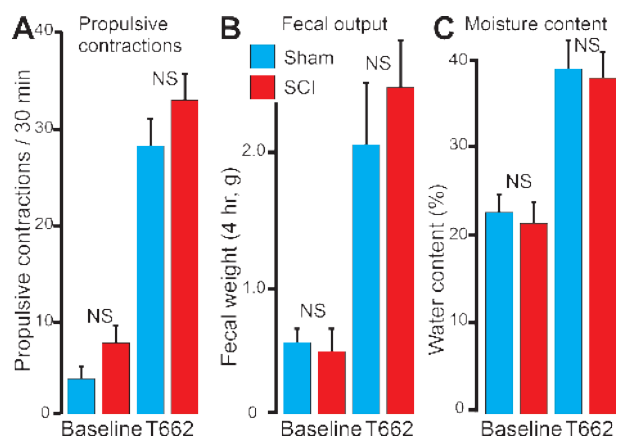
A: Effect of T523 on opiate-induced constipation



B: Effect of T523 on constipation of aging



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