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Ultra-low doses of the transient Receptor Potential Vanilloid 1 Agonist, resiniferatoxin, prevents vomiting evoked by diverse emetogens in the least shrew (*Cryptotis Parva*)

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Abstract

Published studies have shown that the transient receptor potential vanilloid 1 (TRPV1) receptor agonist, resiniferatoxin (RTX), has pro- and anti-emetic effects. RTX can suppress vomiting evoked by a variety of non-selective emetogens such as copper sulfate and cisplatin in several vomit competent species. In the least shrew we have already demonstrated that combinations of ultra-low doses of RTX and low-doses of the cannabinoid CB_{1/2} receptor agonist delta-9-tetrahydrocannabinol (⁹-THC), produce additive anti-emetic effects against cisplatin-evoked vomiting. In the current study, we investigated the broad-spectrum anti-emetic potential of very low non-emetic doses of RTX against a diverse group of specific emetogens including selective and non-selective agonists of serotonergic 5-HT₃ receptor (5-HT and 2-Me-5-HT), dopaminergic D₂ receptor (apomorphine and quinpirole), cholinergic M₁ receptor (pilocarpine and McN-A-343), as well as the selective substance P neurokinin NK₁ receptor agonist GR73632, the selective L-Type calcium channel agonist FPL64176, and the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) inhibitor thapsigargin. When administered subcutaneously, ultra-low (0.01 µg/kg) to low (5.0 µg/kg) doses of RTX suppressed vomiting induced by the aforementioned emetogens in a dose-dependent fashion with ID₅₀ values ranging from 0.01 – 1.26 µg/kg. This study is the first to demonstrate that low nanomolar non-emetic doses of RTX have the capacity to completely abolish vomiting caused by diverse receptor specific emetogens in the least shrew model of emesis.

Keywords

emesis; resiniferatoxin; Least Shrew; TRPV1 Receptor; serotonin 5-HT₃ receptor; neurokinin NK₁ receptor

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Conflicts of Interest

There are no conflicts of interest.

Introduction

Numerous stimuli, including neurotransmitters such as serotonin (5-hydroxytryptamine = 5-HT), dopamine and substance P (SP), ingested chemical irritants, as well as drugs including cytotoxic chemotherapeutics (e.g. cisplatin), can evoke vomiting (Darmani, 2010; Darmani et al., 2009; Darmani and Ray, 2009). The evoked vomiting mainly occurs via direct or indirect activation of the dorsovagal complex emetic loci in the brainstem and/or the vagal afferent nerves in the gastrointestinal tract. Cisplatin is a highly effective and widely used agent for cancer treatment, but it has associated side-effects such as chemotherapy-induced nausea and vomiting (CINV) that largely depreciate patient quality of life (Dasari and Tchounwou, 2014; Hartmann and Lipp, 2003; Lipp and Hartmann, 2005). Unfortunately, current prophylactic anti-emetic regimens are still unable to completely suppress nausea and vomiting in all patients. Furthermore, their use in the clinic is accompanied by high-costs, rendering approximately 25–50% of patients unable to continue prophylactic anti-emetic treatment (Annemans et al., 2008; Bloechl-Daum et al., 2006; Broder et al., 2014; Burke et al., 2011; Du et al., 2016; Sanger and Andrews, 2006; Sommariva et al., 2016; Tamura et al., 2016).

Notwithstanding the multitude of structures involved in the emetic reflex, brainstem innervation by the vagal afferent nerves from the gastrointestinal tract is essential for the induction of vomiting caused by cisplatin-like chemotherapeutics. The majority of vagal afferent nerves are capsaicin (the hot ingredient of chili peppers)-sensitive, and it was postulated that desensitization of vagal afferents by capsaicin can alleviate emesis (Andrews et al., 2000; Darmani et al., 2014a; Rudd et al., 2015; Yamakuni et al., 2002). Indeed, published research supports the latter notion since administration of the ultra-potent analog of capsaicin, resiniferatoxin (RTX), not only has been shown to desensitize vagal afferent fibers, but can suppress CINV (Aghazadeh Tabrizi et al., 2016; Andrews et al., 2000; Darmani et al., 2014a; Iadarola and Mannes, 2011; Rudd et al., 2015; Szolcsanyi et al., 1990; Yamakuni et al., 2002). Thus, RTX may have the potential to be a broad-spectrum anti-emetic (Aghazadeh Tabrizi et al., 2016; Andrews et al., 2000; Darmani et al., 2014a; Iadarola and Mannes, 2011; Rudd et al., 2015; Szolcsanyi et al., 1990; Yamakuni et al., 2002). In fact, 100 µg/kg dose of RTX prevents both centrally (loperamide)- and peripherally (copper sulfate)-mediated vomiting in ferrets and house musk shrews (*Suncus murinus*) (Rudd et al., 2015). The most likely mechanism for the anti-emetic effects of RTX appears to be depletion of SP, possibly in the nucleus tractus solitarius in the brainstem (Rudd et al., 2015) and probably in the GIT, since the selective SP neurokinin NK1 receptor (NK₁R) agonist GR73632 can evoke vomiting via both of these emetic loci (Darmani et al., 2008; Ray et al., 2009; Rudd et al., 2015).

Relative to capsaicin, RTX is a more selective and ultra-potent agonist of the transient receptor potential vanilloid 1 receptor (TRPV1R) (Rudd et al., 2015). The TRPV1R is a non-selective cation channel and is expressed on neuronal membrane as well as in the membrane of intracellular organelles (e.g. mitochondria, endoplasmic reticulum, and more) (Fernandes et al., 2012; Gallego-Sandin et al., 2009; Hurt et al., 2016; Imler and Zinsmaier, 2014; Lang et al., 2015; Szolcsanyi et al., 1990; Zhao and Tsang, 2016). TRPV1R plays a crucial role in maintaining intracellular Ca²⁺ homeostasis (Fernandes et al., 2012; Gallego-

Sandin et al., 2009; Hurt et al., 2016; Imler and Zinsmaier, 2014; Lang et al., 2015; Szolcsanyi et al., 1990; Zhao and Tsang, 2016).

Recently our group reported that combinations of ultra-low doses of RTX with low doses of the cannabinoid CB_{1/2} receptor agonist, delta-9-tetrahydrocannabinol (⁹-THC), produced additive anti-emetic effects against cisplatin induced emesis in the least shrew (*Cryptotis parva*). When 1.0 or 5.0 µg/kg doses of RTX were administered 2 h prior to concomitant exposure to varying doses of ⁹-THC, the combination caused greater suppression of mean vomit frequency and protected more shrews from vomiting evoked by cisplatin (20 mg/kg, i.p.). Until now, the anti-emetic potential of RTX by itself against diverse specific emetogens has not been investigated in a systematic manner in any species. RTX by itself causes vomiting in the least shrew at doses greater than 10 µg/kg (Darmani et al., 2014a), whereas in house musk shrews the threshold for emesis is approximately 1 µg/kg (Rudd and Naylor, 1995). On the other hand, ferrets do not vomit in response to 100 µg/kg dose of RTX (Rudd et al., 2015). Thus, in the current study, non-emetic doses of RTX (0.01 – 5.0 µg/kg, s.c.) were utilized to investigate its anti-emetic potential in least shrews.

In order to demonstrate the broad-spectrum anti-emetic potential of RTX, we utilized diverse emetogens that either selectively or non-selectively activate: i) serotonin 5-HT₃ receptors (5-HT₃R) {e.g. 2-methyl-5-HT and serotonin, respectively} (Darmani, 1998); ii) dopamine D_{2/3} receptors (D_{2/3}R) {e.g. quinpirole and apomorphine, respectively} (Darmani et al., 1999); iii) cholinergic M₁ receptors (M₁R) {e.g. McN-A-343 and pilocarpine, respectively} (Beleslin et al., 1989; Darmani et al., 2014b); iv); and SP NK₁R {e.g. GR73632} (Darmani et al., 2008; Ray et al., 2009). Since TRPV1Rs present on cellular and intracellular membranes control cytosolic calcium concentration, we also determined the anti-emetic efficacy of RTX against vomiting caused by the selective: v) L-type calcium channel agonist, FPL64176 (Zhong et al., 2018), and vi) sarcoplasmic/endoplasmic reticulum calcium ATPase inhibitor, thapsigargin (Zhong et al., 2016).

Methods

Subjects and Ethics Statement

Adult least shrews (*Cryptotis parva*) were bred in the animal facility of Western University of Health Sciences. Previous studies have demonstrated no gender differences among different emetics and therefore both male and female least shrews were used to conduct experiments. Shrews were housed in groups of 5–10 on a 12:12 light: dark cycle at room temperature 22± 1C° in a humidity controlled environment. Animals received a freely available supply of food and water as described previously (Darmani, 1998; Darmani et al., 2013, 2014a). Adult least shrews between 45–70 days old were used, weighing between 4–6 g. The protocol was approved by the Western University of Health Sciences Institutional Animal Care and Use Committee Standards (IACUC) and conducted with strict adherence to the recommendations in the guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Department of Health and Human Services Publication, revised, 2011). Pharmacological tests were limited to the fewest animals possible per dose to minimize the distress of the shrews. Animals were observed regularly for signs and symptoms of distress from the drug treatment. Any animal showing some of the following

symptoms: weight loss greater than 20% of the initial weight, abstaining from food or drink, immobility, and/or the presentation of rough or coarse fur, were euthanized via exposure to 32% isoflurane. All experiments were conducted between 09:00 and 15:00 hours.

Behavioral Emesis Studies

On the day of experiments, shrews were brought from the animal facility, separated into individual cages and allowed to adapt to the experimentation room for a minimum of two hours. Two hours before the experiments, daily food was withheld, but each shrew was given four mealworms (*Tenebrio sp.*) prior to emetogen injection to aid in identification of wet vomits, as previously described (Darmani et al., 2013).

To evaluate the extent that pretreatment with various RTX doses may diminish both the frequency of vomits and the percentage of shrews vomiting in response to a fully effective dose of each emetogen/receptor agonist, different groups of shrews were pretreated subcutaneously (s.c.) with varying doses of RTX (0.01, 0.025, 0.05, 0.25, 0.50, 1.0, 2.5, 5.0 µg/kg or the corresponding vehicle, n = 6–10 per group). Following the one-hour RTX pretreatment, and based on our previous publications, different groups of shrews received an intraperitoneal (i.p.) injection of one of the diverse emetogens at their fully effective emetic dose: 1) non-selective serotonin 5-HT₃R agonist, 5-HT (5 mg/kg, i.p.) (Darmani et al., 2014b); 2) 5-HT₃R selective agonist, 2-Me-5-HT (5 mg/kg, i.p.) (Darmani et al., 2014b); 3) non-selective D₂R agonist, apomorphine HCL (2 mg/kg, i.p.) (Darmani and Crim, 2005; Darmani et al., 1999); 4) selective D₂R agonist, quinpirole HCL (2 mg/kg, i.p.) (Darmani and Crim, 2005; Darmani et al., 1999); 5) non-selective muscarinic agonist, pilocarpine HCL (2 mg/kg, i.p.) (Zhong et al., 2014a); 6) more selective muscarinic M₁R agonist, McN-A-343 (2mg/kg, i.p.) (Darmani et al., 2014b; Zhong et al., 2014a); 7) NK₁R selective agonist, GR73632 (5 mg/kg, i.p.) (Darmani et al., 2008; Ray et al., 2009); 8) L-Type Ca²⁺ channel (LTCC) agonist, FPL 64176 (10 mg/kg, i.p.) (Zhong et al., 2014a, 2018); and 9) Sarco/endoplasmic reticulum Ca²⁺ ATPase inhibitor (SERCA), thapsigargin (0.5 mg/kg, i.p.) (Zhong et al., 2016).

Following challenge with the emetogen, each shrew was observed for 30 minutes to record the number of animals vomiting in each group, and the frequency of vomits for each animal in a group. Each shrew was used once and euthanized via exposure to 32% isoflurane following completion of experiments.

Drugs

Apomorphine HCl, McN-A-343, quinpirole HCl, serotonin HCl and 2-methyl-serotonin maleate salt (2-Me-5-HT) were obtained from Sigma/RBI (St. Louis, MO). FPL 64176, GR 73632 and Resiniferatoxin, were purchased from Tocris (Minneapolis, MN). Resiniferatoxin was dissolved in ethanol: Tween-80: water in a 1:1:18 ratio. 5-HT, 2-Me-5-HT, GR 73632, apomorphine HCL and quinpirole HCL, pilocarpine HCL, and McN-A-343 were dissolved in water. FPL 64176 was dissolved in DMSO (Sigma) and then diluted with three volumes of distilled water to a final DMSO concentration of 25%, and thapsigargin in 10% DMSO in water. Shrews were divided into groups (n = 6–10 per group) and each shrew received a 1-h pretreatment with varying doses of RTX (µg/kg body weight, s.c.) or the corresponding

vehicle (s.c.). Following the 1-h pretreatment, each treated animal was injected with a fully effective emetic dose of either a nonselective or receptor selective emetogen.

Statistical Analysis

Mean vomit frequency ($\pm$ SEM) data was analyzed using the Kruskal-Wallis non-parametric one-way analysis of variance (ANOVA) and Dunn's multiple comparison tests. A chi squared test was used to compare the percentage of animals vomiting across groups at different doses. The 50% inhibitory dose (ID₅₀) values were calculated using non-linear regression analysis (Graph Pad PRISM version 7, San Diego, CA). We calculated the therapeutic window for RTX as the RTX dose that causes vomiting in all tested least shrews ($= 18 \mu\text{g/kg, s.c.}$) $\div$ RTX dose that completely suppressed vomiting caused by a maximally effective dose of each emetogen. In all cases, a P-value < 0.05 was necessary for statistical significance. *P < 0.05 , **P < 0.01 , ***P < 0.001 .

Results

RTX attenuates serotonergic 5-HT₃R-mediated vomiting induced by 5-HT and 2-Me-5HT

We have previously demonstrated that both the peripherally-acting (5-HT) and the centrally/peripherally-acting (2-Me-5-HT) 5-HT₃R agonists induce robust vomiting in all tested least shrews at a 5 mg/kg dose (i.p.) (Darmani, 1998; Zhong et al., 2014b). RTX pretreatment (0 – 2.5 $\mu\text{g/kg}$), significantly reduced the mean frequency of 5-HT- induced vomiting relative to the vehicle pre-treated control animals during the 30-minute observation period (KW (4, 39) = 23.48, P < 0.001) (Fig. 1a). Indeed, relative to the vehicle-treated animals, significant reductions in the frequency of 5-HT-induced vomits occurred at 0.25 $\mu\text{g/kg}$ (P = 0.036), 0.5 $\mu\text{g/kg}$ (P < 0.003) and 1.0 $\mu\text{g/kg}$ (P < 0.004), with complete blockade at 2.5 $\mu\text{g/kg}$ (P < 0.001) (Fig. 1a). Moreover RTX pretreatment significantly protected shrews from 5-HT-evoked vomiting (χ^2 4, 39) = 21.94; P < 0.001) (Fig. 1b). Significant reductions in the percentage of shrews vomiting occurred with RTX doses of 0.25 $\mu\text{g/kg}$ (56%, P = 0.006), 0.5 $\mu\text{g/kg}$ (78%, P < 0.001), and 1.0 $\mu\text{g/kg}$ (78%, P < 0.001) (Fig. 1b). Moreover complete protection from 5-HT induced vomiting was seen at the 2.5 $\mu\text{g/kg}$ RTX dose (P < 0.001) (Figs. 1a and b). RTX potently reduced both 5-HT-induced emetic parameters with respective ID₅₀ values of 0.08 (0.007 – 0.21) and 0.19 (0.078 – 0.41) ($\mu\text{g/kg}$) (Table 1).

Next, RTX was tested against the more selective 5HT₃R agonist, 2-Me-5-HT (5 mg/kg, i.p.), which generally evoked more vomits than 5-HT (see Figs. 1a and 1c) and therefore larger doses of RTX were required to cause significant reductions in both vomit frequency (KW (4, 39) = 29.63, P < 0.001) (Fig. 1c) and the percentage of shrews vomiting (χ^2 (4, 39) = 21.64; P < 0.001) (Fig. 1d). In fact, significant reductions in mean vomit frequencies were observed only in shrews pretreated with 2.5 (P < 0.001) and 5.0 $\mu\text{g/kg}$ (P < 0.001) doses of RTX (ID₅₀ = 0.23 $\mu\text{g/kg}$ (0.1 – 0.44)) (Fig. 1c). Moreover, relative to the 0 $\mu\text{g/kg}$ -treated control group, RTX caused significant decreases in the percentage of shrews vomiting at its 2.5 (67%, P < 0.0018) and 5.0 $\mu\text{g/kg}$ doses (100%, P < 0.001) (ID₅₀ = 1.26 $\mu\text{g/kg}$ (0.68 – 2.58)) (Fig. 1d, Table 1).

RTX reduces apomorphine- and quinpirole-induced vomiting

Increasing doses of RTX (0.01, 0.025, 0.05, and 0.5 $\mu\text{g/kg}$) significantly attenuated both the vomit frequency (KW (4, 39) = 20.69, $P < 0.001$) and percentage of shrews vomiting (χ^2 (4, 39) = 14.92; $P = 0.0019$) in response to a 2 mg/kg (i.p.) dose of apomorphine (Figs. 2a and 2b). Significant reductions in vomit frequency were observed at doses of 0.025 ($P < 0.019$), 0.05 ($P < 0.022$) and 0.5 $\mu\text{g/kg}$ ($P < 0.001$) ($\text{ID}_{50} = 0.0095 \mu\text{g/kg}$ (0.0037 – 0.024)) (Fig. 2a). In addition, significant reductions in the percentage of shrews vomiting occurred with RTX doses of 0.01 (56%, ($P = 0.006$), 0.025 (66.67%, $P < 0.002$), 0.05 (62.5%, ($P = 0.0033$) and 0.5 $\mu\text{g/kg}$ (100%, ($P < 0.001$) ($\text{ID}_{50} = 0.19 \mu\text{g/kg}$ (0.0047 – 0.039)) (Fig. 2b, Table 1).

Next, RTX was tested against the more selective D_2R agonist, quinpirole, wherein relative to apomorphine, much higher doses of RTX were required to significantly reduce the mean vomit frequency (KW (4, 40) = 20.8, $P < 0.001$) (Fig. 2a and 2c). Indeed, statistically significant reductions in the mean frequency of vomiting occurred at 1.0 ($P < 0.024$), 2.5 ($P < 0.017$) and 5.0 $\mu\text{g/kg}$ ($P < 0.001$) with an ID_{50} of 0.48 $\mu\text{g/kg}$ (0.2 – 1.16) (Fig. 2c, Table 1). Relative to the vehicle-pretreated control group, RTX also caused significant decreases in the percentage of shrews vomiting (χ^2 (4, 40) = 20.31; $P < 0.001$) at doses of 0.5 (50%, $P < 0.001$), 1.0 (62.5%, $P < 0.003$), 2.5 (66.7%, $P < 0.002$) and 5.0 $\mu\text{g/kg}$ (100%, $P < 0.001$) ($\text{ID}_{50} = 0.61 \mu\text{g/kg}$ (0.26 – 1.6) (Fig. 2d, Table 1).

RTX reduces vomiting evoked by M_1R cholinergic agonists in a dose-dependent fashion

Varying doses of RTX were tested against the non-selective M_1R agonist pilocarpine (Fig. 3a and 3b) and the selective M_1R agonist, McN-A-343 (Figs. 3c and 3d). RTX significantly reduced both the mean frequency of pilocarpine-induced vomits (KW (4, 36) = 21.26, $P < 0.001$) (Fig. 3a) and the percentage of shrews vomiting (χ^2 (4, 36) = 19.8; $P < 0.001$) (Fig. 3b). Significant reductions in the frequency of vomiting occurred at its 0.5 ($P < 0.005$) and 1 $\mu\text{g/kg}$ ($P < 0.001$) doses ($\text{ID}_{50} = 0.15 \mu\text{g/kg}$ (0.055 – 0.44)) (Fig. 3a). Furthermore, significant reductions in the percentage of shrews vomiting (χ^2 (4, 36) = 19.8; $P = 0.001$) occurred at doses of 0.5 (75%, $P < 0.002$) and 1.0 $\mu\text{g/kg}$ (100%, ($P < 0.001$) with an ID_{50} of 0.25 $\mu\text{g/kg}$ (0.12 – 0.54) (Fig. 3b, Table 1).

RTX attenuated the mean vomit frequency of McN-A-343-induced vomiting (Fig. 3c) (KW (4, 39) = 14.83, $P < 0.005$) and provided significant and complete protection at its 1.0 $\mu\text{g/kg}$ dose ($P = 0.002$) with an ID_{50} of 0.27 $\mu\text{g/kg}$ (0.089 – 1.142) (Table 1). However, lower doses of RTX (0.1, 0.25, and 0.5 $\mu\text{g/kg}$) failed to achieve statistical significance. In addition, RTX caused significant reductions in the percentage of shrews vomiting (χ^2 (4, 39) = 21.62; $P < 0.001$) (Fig. 3d). Significant reductions in the number of shrews vomiting were seen at doses of 0.25 (50%, $P < 0.014$), 0.5 (44.4%, $P = 0.0233$) and 1.0 $\mu\text{g/kg}$ (100%, $P < 0.001$) with an ID_{50} of 0.3 $\mu\text{g/kg}$ (0.16 – 0.65) (Fig. 3d, Table 1).

RTX reduces GR 73632-induced emesis in dose-dependent manner

The SP neurokinin NK_1R selective agonist GR 73632 was previously shown to produce robust emesis in all tested least shrews at 5 mg/kg (i.p.) (Darmani et al., 2008; Ray et al., 2009). Thus, the anti-emetic potential of RTX was next tested against GR7362. Significant reductions in both the mean vomit frequency (KW (3, 37) = 13.73, $P < 0.003$) (Fig. 4a) and

percentage of shrews vomiting (χ^2 (3, 37) = 16.26; $P < 0.001$) (Fig. 4b) occurred in a dose dependent fashion. Furthermore, a significant reduction in the mean frequency of vomits was observed at the 2.5 $\mu\text{g/kg}$ dose ($P = 0.0019$) ($\text{ID}_{50} = 0.32 \mu\text{g/kg}$ (0.06 – 1.2) (Fig. 4a, Table 1); however lower doses of RTX failed to significantly attenuate vomit frequency of GR 73532-induced vomiting. Moreover, significant reductions in the percentage of shrews vomiting were seen at doses of 1.0 (62.5%, $P = 0.007$) and 2.5 $\mu\text{g/kg}$ doses (100%, $P < 0.001$) ($\text{ID}_{50} = 0.67 \mu\text{g/kg}$ (0.32 – 1.6) Table 1) (Fig. 4b).

Ultra- Low doses of RTX attenuates FPL 64176-induced vomiting

The emetic efficacy of the L-type Ca^{2+} channel agonist FPL 64176 has been previously demonstrated in the least shrew (Darmani et al., 2014b; Hutchinson et al., 2015; Zhong et al., 2014a). We therefore tested the anti-emetic potential of RTX (0 – 0.25 $\mu\text{g/kg}$) against FPL 64176 (10 mg/kg, i.p.)-evoked vomiting. RTX reduced the mean vomit frequency in a dose dependent manner (KW (4, 39) = 19.84, $P < 0.001$) with an ID_{50} of 0.013 $\mu\text{g/kg}$ (0.0052 – 0.0036) (Fig. 5a, Table 1). Likewise, RTX caused significant reductions in the percentage of shrews vomiting (χ^2 (4, 39) = 17.36; $P < 0.002$) with an ID_{50} of 0.19 $\mu\text{g/kg}$ (0.01 – 0.07) (Fig. 5b, Table 1). In fact, significant reductions in the percentage of shrews vomiting occurred at doses of 0.01 (37.5%, $P = 0.0339$), 0.025 (55.6%, $P = 0.006$), 0.05 (45.5%, $P = 0.0146$) and 0.25 $\mu\text{g/kg}$ (100%, $P < 0.001$) (Fig. 5b).

Low doses of RTX reduces thapsigargin-induced vomiting in a dose-dependent fashion

The selective SERCA inhibitor thapsigargin evokes vomiting in all tested shrews at 0.5 mg/kg (i.p.) (Zhong et al., 2016). Thus, we tested the anti-emetic potential of RTX (0.025, 0.05, 0.1, 0.25 $\mu\text{g/kg}$, s.c.) against thapsigargin (0.5 mg/kg, i.p.)-evoked vomiting. Low doses of RTX reduced both the mean vomit frequency (KW (4, 37) = 15.41; $P < 0.004$) (Fig. 6A) and the percentage of shrews vomiting (χ^2 (4, 37) = 21.26, $P < 0.001$) (Fig. 6b). Among the tested doses, only the 0.25 $\mu\text{g/kg}$ RTX ($P < 0.001$) provided significant and complete protection from thapsigargin-induced vomiting ($\text{ID}_{50} = 0.06 \mu\text{g/kg}$ (0.016 – 0.35) (Fig. 6a, Table 1). Relative to the 0 $\mu\text{g/kg}$ control group, RTX pre-treatment significantly decreased the percentage of shrews vomiting at doses of 0.025 (55.56%, $P = 0.006$), 0.1 (50%, $P < 0.011$), and 0.25 $\mu\text{g/kg}$ doses (100%, $P < 0.001$) with an ID_{50} of 0.074 $\mu\text{g/kg}$ (0.033 – 0.2) (Fig. 6b, Table 1).

Discussion

Published literature has fully confirmed the anti-emetic potential of 100 $\mu\text{g/kg}$ s.c. dose of RTX against diverse non-specific emetogens in both ferrets and house musk shrews (Rudd et al., 2015). However, at such or even lower doses, RTX can produce unwanted side-effects such as vomiting in house musk shrews, as well as a reduction in core body temperature, stimulation of respiration, hyperthermia, and ano-genital grooming in rodents and/or house musk shrews (Rudd et al., 2015). The primary finding of the present study is that 20 – 400 times lower non-emetic doses of RTX can potently suppress vomiting in least shrews evoked by diverse emetogens that either: i) stimulate specific emetic receptors/ion channels present on the cell membrane (serotonin, 2-methy-5-HT, apomorphine, quinpirole, pilocarpine, McN-A-343, GR73632 and FPL 64176); or ii) target intracellular structures such as the ER

membrane which subsequently mobilizes intracellular calcium (thapsigargin) in the cytosol. Indeed, ultra-low and low nanomolar doses of RTX completely and dose-dependently reduced both the mean vomit frequency and the percentage of least shrews vomiting (ID_{50} values ranging from 0.01 – 1.26 $\mu\text{g/kg}$; Table 1) evoked by the following emetogens:

- i. the peripherally acting non-selective 5-HT₃R agonist 5-HT (5 mg/kg, i.p.) with complete emesis protection at 2.5 $\mu\text{g/kg}$ RTX.
- ii. The centrally/peripherally-acting and more selective 5-HT₃R agonist 2-Me-5-HT (5 mg/kg, i.p.) with total vomit protection at 5.0 $\mu\text{g/kg}$ RTX.
- iii. the non-selective dopamine D₂R agonist apomorphine (2 mg/kg, i.p.) with 100% emesis protection at 0.5 $\mu\text{g/kg}$ dose of RTX.
- iv. the more selective dopamine D₂R agonist quinpirole (5 mg/kg, i.p.) with 100% emesis protection at 5.0 $\mu\text{g/kg}$ dose of RTX.
- v. the non-selective muscarinic M₁R agonist pilocarpine (2 mg/kg, i.p.) with complete vomit reduction at 1.0 $\mu\text{g/kg}$ RTX.
- vi. the more selective M₁R agonist McN-A-343 (2mg/kg, i.p.) with complete emesis protection at 1.0 $\mu\text{g/kg}$ RTX.
- vii. the selective NK₁R agonist GR 73632 (5 mg/kg, i.p.) such that a 2.5 $\mu\text{g/kg}$ dose of RTX provided complete emesis protection.
- viii. the selective LTCC agonist FPL 64176 (10mg/kg, i.p.) where a 0.25 $\mu\text{g/kg}$ dose of RTX completely prevented the evoked vomiting.
- ix. the selective SERCA inhibitor thapsigargin (0.5 mg/kg, i.p.), wherein a 0.25 $\mu\text{g/kg}$ RTX dose completely blocked the induced vomiting.

Thus far, we and others have found no emetic agent (i.e. a negative control) that would resist the antiemetic efficacy of RTX. Indeed, in the current manuscript we have tested the antiemetic potential of ultra-low doses of RTX against the above discussed receptor selective/nonselective emetogens, and have found that no agent is able to override the antiemetic efficacy of RTX. This is not surprising since we have previously shown that RTX can suppress vomiting caused by a massive dose of cisplatin (20 mg/kg, i.p.) (Darmani et al. 2014a). Furthermore, other investigators have demonstrated the antiemetic potential of a larger dose of RTX (0.1 mg/kg, s.c.) in several vomit competent species against vomiting caused by radiation, copper sulfate, loperamide (Andrews and Bhandari, 1993); nicotine, motion (Andrews et al., 2000) and cisplatin (Andrews et al., 2000; Yamakuni et al., 2002).

In order to investigate the dose-response anti-emetic potential of non-emetic doses of RTX against the above discussed emetogens, we chose variable, but a maximally effective emetic dose of each tested emetogen, to induce vomiting in all tested shrews. As can be seen from the above summary and Table 1, among the above discussed emetogens, RTX was most potent, and an equally-efficacious anti-emetic, against vomiting caused by the Ca^{2+} mobilizers FPL 64176 and thapsigargin. Notwithstanding the fact that the fully emetic tested-dose of FPL 64176 is 20 times greater than thapsigargin. Furthermore, apomorphine, pilocarpine, McN-A-343 and serotonin were the next most-sensitive emetogens for the

antiemetic effects of RTX, whereas GR 73632 and 2-Me-5-HT generally required larger doses of RTX for complete emesis protection. In the clinic varying intrathecally-administered doses of RTX (3 – 26 µg) is being tested for the treatment of chronic pain (Brown, 2016) with no major post-treatment side-effect (Brown, 2016). RTX (10 – 50 nM; intravesical administration) has also been investigated for the suppression of lower urinary tract symptoms in patients with overactive bladder (Apostolidis et al., 2006; Guo et al., 2013). Based upon these clinical findings and our calculation of the therapeutic window of RTX in the least shrew (Table 1), one could predict that RTX may be an effective antiemetic in patients, since it can completely prevent the vomiting caused by the discussed diverse emetogens at a dose range of 3.6 (against 2-Me-5-HT and quinpirole) to 72 times (against FPL 64176 and Thapsigargin) lower than its emetic efficacy (18 µg/kg) that evokes vomiting in all tested least shrews.

We have recently deciphered the intracellular mechanisms of emetic actions of the discussed calcium mobilizers in the least shrew (Zhong et al., 2016; Zhong et al., 2018). Indeed, FPL 64176 is a selective LTCC agonist and causes vomiting via the calcium-induced calcium release (CICR) process by an initial influx of extracellular Ca^{2+} through LTCCs followed by release of intracellular stored Ca^{2+} from the ER via ryanodine receptors (RyRs) (Zhong et al., 2018). The evoked intracellular Ca^{2+} mobilization was then followed by activation of diverse intracellular emetic signaling proteins (e.g. ERK1/2, PKC α /βII and Akt). These events were also accompanied by: i) c-Fos immunoreactivity in the least shrew brainstem vomit-associated nuclei including the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMNX); and ii) release of serotonin but not SP in the dorsomedial subdivision of the NTS.

On the other hand, thapsigargin is a specific and potent inhibitor of the SERCA pump which transports free cytosolic Ca^{2+} into calcium-stores in the lumen of the ER to counter-balance the cytosolic intracellular Ca^{2+} release from the ER into the cytoplasm via the inositol trisphosphate receptors (IP₃Rs) and ryanodine receptors (RyRs) present on the ER membrane (Garaschuk et al., 1997; Gomez-Viquez et al., 2003, 2010). Thapsigargin may also evoke release of stored Ca^{2+} from the ER into the cytosol. These events tend to deplete Ca^{2+} stores in the ER and subsequently evoke extracellular Ca^{2+} influx through store-operated Ca^{2+} entry (SOCE) or via LTCCs (Cheng et al., 2013; Feske, 2007; Parekh and Putney, 2005; Zhong et al., 2016). In regard to emesis, thapsigargin appears to evoke vomiting (Zhong et al., 2016) by: i) inducing an initial increase in cytosolic Ca^{2+} concentration by inhibiting SERCA as well as releasing Ca^{2+} from the ER into the cytoplasm via both RyRs and inositol triphosphate receptors (IP₃Rs); followed by ii) extracellular Ca^{2+} influx through LTCCs prior to activation intracellular emetic signals (CaMKII and ERK1/2) in the least shrew brainstem tissue, iii) increased c-Fos immunoreactivity in the brainstem emetic nuclei (area postrema (AP), NTS and DMNX), and iv) SP release in the DMNX. The selective NK₁R antagonist netupitant prevented thapsigargin-evoked vomiting, whereas the 5-HT₃R antagonist palonosetron failed to do so. On the other hand, both palonosetron and netupitant were effective against FPL 64176-induced emesis. Thus, there appear to be differences in the efficacy of netupitant and palonosetron in blocking vomiting evoked by the two calcium mobilizers.

It is worth mentioning that modulation of many of the above discussed intracellular signals can affect TRPV1R function (Rosenbaum and Simon, 2007). It is well established that TRPV1R is a non-selective Ca^{2+} ion-channel present on both the cell membrane and membranes of intracellular organs such as the ER, and since RTX increases cytosolic Ca^{2+} levels in neurons via both extracellular influx and intracellular release from the ER (Karai et al., 2004). Our current findings suggest that the immediate pro-emetic action of RTX could be due to increased levels of Ca^{2+} in the cytosol, whereas TRPV1R desensitization and/or internalization (Caudle et al., 2003; Moran and Szallasi, 2018) may account for the delayed anti-emetic effects of RTX. Indeed, presynaptic extracellular Ca^{2+} influx is an important factor for release of neurotransmitters (He et al., 2018) and stimulation of TRPV1Rs can evoke a rapid SP release in both mouse intestinal sensory neurons (Engel et al., 2012) and house musk shrew brainstem slices (Rudd et al., 2015). Furthermore, desensitization of TRPV1R on vagal afferents and subsequent depletion of SP in the NTS were the early proposed suggestions for the anti-emetic actions of RTX (Aghazadeh Tabrizi et al., 2016; Andrews et al., 2000; Darmani et al., 2014a; Iadarola and Mannes, 2011; Rudd et al., 2015; Szolcsanyi et al., 1990; Yamakuni et al., 2002).

Since SP and GR73632 evoke vomiting via the activation of neurokinin NK_1Rs in least shrews (Darmani et al., 2008; Ray et al., 2009), the ability of RTX pretreatment to prevent GR73632-induced vomiting in the present study suggests that desensitization of TRPV1Rs probably affects intracellular calcium dynamics (i.e. extracellular influx and intracellular release) (Karai et al., 2004) and consequently prevents emesis by blocking one or more emetic mechanisms described above for FPL 64176 and/or thapsigargin. Capsaicin and its analog nonivamide are also agonists of TRPV1Rs, and likewise can induce release of other emetic neurotransmitters such as dopamine and serotonin in a calcium-dependent manner in neural SH-SY5Y cells (Rohm et al., 2013) and in the rat nucleus accumbens (Marinelli et al., 2005). Thus, as per above discussion, TRPV1R desensitization could also account for the ability of RTX to suppress vomiting evoked by selective/non-selective agonists of serotonin $5\text{-HT}_3\text{R}$ (2-Me-5-HT and serotonin, respectively) and dopamine D_2R (quinpirole and apomorphine, respectively). Although the intracellular emetic mechanisms for D_2R remain to be established, it is well-accepted that stimulation of brainstem and intestinal $5\text{-HT}_3\text{Rs}$ in the least shrew causes increased extracellular Ca^{2+} influx through $5\text{-HT}_3\text{Rs}$ and LTCCs in both the brainstem and enterochromaffin cells in the GIT, leading to CICR from intracellular ER Ca^{2+} stores via RyRs (Zhong et al., 2014b). Furthermore, it has been shown that prior exposure to capsaicin causes vascular responses in arteries through inhibition of LTCCs (Hopps et al., 2012).

Non-selective (pilocarpine) and cholinergic M_1R preferring agonists (McN-A-343) also evoke vomiting in all tested shrews via Ca^{2+} mobilization in least shrews with a maximal efficacy at 2 mg/kg (i.p.) (Zhong et al., 2014a, 2017). In the current study, RTX pretreatment completely and potently abolished vomiting evoked by both of these cholinergic agonists. Mechanistically it has previously been shown that stimulation of TRPV1R induces an initial release of basal acetylcholine in the myenteric plexus-longitudinal muscle preparation (Geber et al., 2006), whereas TRPV1R-desensitization inhibits its release (Geber et al., 2006; Thyagarajan et al., 2014). Thus, these TRPV1R stimulation/desensitization events that differentially affect the processes of acetylcholine release in the GIT and probably in the

brainstem emetic loci, may also account for the initial pro-emetic and the delayed anti-emetic actions of RTX.

Cisplatin-evoked vomiting is the most difficult event that a drug or combination of drugs can prevent. Although it is clearly shown that RTX can suppress the acute-phase of cisplatin-induced vomiting (Darmani et al., 2014a), the antiemetic potential of RTX has not yet been demonstrated against the delayed phase of cisplatin-evoked emesis. In future studies we plan to investigate the latter issue, along with RTX effects on intracellular emetic signals, which were beyond the scope of the current study. Further, we need to find out the molecular mechanisms underlying the antiemetic role of less than 5 µg/kg RTX against its pro-emetic role at 10 µg/kg or greater doses of RTX.

Conclusion

Based upon published findings regarding the anti-emetic potential of RTX against non-specific emetogens in larger animal models of vomiting, here we confirm the broad-spectrum anti-emetic potential of low nanomolar doses of RTX (ID₅₀ range 0.01 – 1.26 µg/kg) in the least shrew against an array of specific receptor selective and non-selective emetogens, as well as cytosolic calcium modulators which induced vomiting in all tested shrews at a dosage range of 0.5 – 10 mg/kg. Our results suggest an initial enhancement of cytosolic calcium mobilization through TRPV1Rs for the early pro-emetic effects of RTX, while TRPV1R desensitization probably accounts for its delayed broad-spectrum anti-emetic properties.

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Fig. 1.

The dose-response anti-emetic effects of RTX against vomiting evoked by the non-selective (5-HT) and selective (2-Me-5HT) serotonin 5-HT₃R agonists in the least shrew. Varying doses of RTX (s.c.) were administered to different groups (n = 9–10 per group) of least shrews 1 h prior to either 5-HT or 2-Me-5HT injection (5 mg/kg, i.p.). For 5-HT injection (5 mg/kg, i.p.), the mean frequency of vomits ($\pm$ SEM) is shown in graph a and percentage of shrews vomiting in graph b. For 2-Me-5HT (5 mg/kg, i.p.), the mean frequency of vomits ($\pm$ SEM) is shown in graph c and percentage of shrews vomiting in graph d. Significant differences relative to the corresponding control groups (RTX 0 µg/kg) are indicated as * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

Fig. 2.

The dose-response anti-emetic effects of RTX against vomiting evoked by the dopamine D₂ receptor non-selective (apomorphine) and selective (quinpirole) agonists in the least shrew. Varying doses of RTX (s.c.) were administered to different groups (n = 9–10 per group) of least shrews 1 h prior to either apomorphine (2 mg/kg, i.p.) or quinpirole injection (2 mg/kg, i.p.). With regards to apomorphine, graphs a and b respectively represent the mean frequency of vomits (± SEM) and the percentage of shrews vomiting. For quinpirole graphs c and d respectively represent the mean frequency of vomits (± SEM) and the percentage of shrews vomiting. Significant differences relative to the corresponding control groups (RTX 0 μg/kg) are indicated as * P < 0.05; ** P < 0.01; ***P < 0.001; **** P < 0.0001.

Fig. 3.

The dose-response anti-emetic effects of RTX against vomiting evoked by a non-selective (pilocarpine) and a selective cholinergic M_1 R agonist McN-A-343 in the least shrew.

Varying doses of RTX (s.c.) were administered to different groups ($n = 9-10$ per group) of least shrews 1 h prior to either pilocarpine (2 mg/kg, i.p.) or McN-A-343 (2 mg/kg, i.p.) injection. Graphs a and b represent mean frequency of vomits ($\pm$ SEM) and the percentage of shrews vomiting for pilocarpine, whereas c and d respectively indicate data for McN-A-343. Significant differences relative to the corresponding control groups (RTX 0 µg/kg) are indicated as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$

Fig. 4.

The dose-response anti-emetic effects of RTX against vomiting caused by the selective neurokinin NK₁R agonist GR73632 in the least shrew. Varying doses of RTX (s.c.) were administered to different groups (n = 9–10 per group) of least shrews 1 h prior to GR 73632 injection (5 mg/kg, i.p). Graphs a and b respectively represent the mean frequency of vomits (± SEM) and the percentage of shrews vomiting. Significant differences relative to the corresponding control groups (RTX 0 µg/kg) are indicated as * P < 0.05; ** P < 0.01; ***P < 0.001.

Fig. 5.

The dose-response anti-emetic effects of RTX against vomiting caused by the selective LTCC agonist FPL 64176 in the least shrew. Varying doses of RTX (s.c.) were administered to different groups ($n = 6-10$ per group) of least shrews 1 h prior to FPL 64176 injection (10 mg/kg, i.p.). Graphs a and b respectively represent the mean frequency of vomits ($\pm$ SEM) and the percentage of shrews vomiting. Significant differences relative to the corresponding control groups (RTX 0 $\mu\text{g/kg}$) are indicated as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Fig. 6.

The dose-response anti-emetic effects of RTX against vomiting caused by the selective SERCA inhibitor thapsigargin (0.5 mg/kg, i.p.) in the least shrew. Varying doses of RTX (s.c.) were administered to different groups ($n = 7-10$ per group) of least shrews 1 h prior to thapsigargin (0.5 mg/kg, i.p.). Graphs a and b respectively represent the mean frequency of vomits ($\pm$ SEM) and the percentage of shrews vomiting. Significant differences relative to the corresponding control groups (RTX 0 $\mu\text{g/kg}$) are indicated as ** $P < 0.01$; **** $P < 0.0001$.

Table 1

Anti-emetic ID₅₀ values for Resiniferatoxin against vomiting caused by diverse emetogens.

Emetogens	Resiniferatoxin ID ₅₀ (µg/kg)				Therapeutic window
	Frequency		Percent Inhibition		
5-HT	0.08	(0.007–0.21)	0.19	(0.078–0.41)	$18 \div 2.5 = 7.2$
2-Me-5HT	0.23	(0.1–0.44)	1.26	(0.68–2.58)	$18 \div 5.0 = 3.6$
Apomorphine	0.0095	(0.0037–0.024)	0.19	(0.0047–0.039)	$18 \div 0.5 = 36$
Quinpirole	0.48	(0.2–1.16)	0.61	(0.26–1.6)	$18 \div 5.0 = 3.6$
Pilocarpine	0.15	(0.055–0.44)	0.25	(0.12–0.54)	$18 \div 1.0 = 18$
McN-A-343	0.27	(0.089–1.142)	0.3	(0.16–0.65)	$18 \div 1.0 = 18$
GR 73632	0.32	(0.06–1.2)	0.67	(0.32–1.6)	$18 \div 2.5 = 7.2$
FPL 64176	0.013	(0.0052–0.0036)	0.19	(0.01–0.07)	$18 \div 0.25 = 72$
Thapsigargin	0.06	(0.016–0.35)	0.074	(0.033–0.2)	$18 \div 0.25 = 72$

Therapeutic window for RTX is defined as (RTX dose that causes vomiting in all least shrew (= 18 µg/kg, s.c.)(Darmaniet al., 2014a) $\div$ RTX dose that can completely suppress vomiting induced by different emetic agents in this study.