WE594

A Cream Formulation of TRPV1 agonist Resiniferatoxin (Resinizin™) for the treatment of painful diabetic peripheral neuropathy

Padmamalini Baskaran[1], Adithya Mohandass[1], Somaja Louis[4,5], Brenda Alexander[2], Mikhail Nemenov[3], Baskaran Thyagarajan[1] and Louis S. Premkumar[4,5]

[1]School of Pharmacy, [2]Dept. of Animal Sciences, University of Wyoming, Laramie, WY, USA, [3]Department of Anesthesia, Stanford University, Palo Alto, CA, USA, Lasmed LLC, Mountain View, CA, USA, [4]Department of Pharmacology, SIU School of Medicine, Springfield, IL, [5]Ion Channel Pharmacology LLC, Springfield, IL, USA.

Abstract

Painful Diabetic Peripheral Neuropathy (PDPN) is one of the major complications of diabetes. Currently, the centrally acting drugs such as antidepressants, anticonvulsants, opioids, and topical analgesics are used for treating PDPN. However, the use dependence and addiction potential of opioids and inefficacy of non-opioids are serious limitations. Recent research suggests that targeting Transient Receptor Potential Vanilloid 1 (TRPV1) receptor, a non-selective cation channel protein expressed in the peripheral sensory nerve terminals is an emerging option to treat pain. Blocking TRPV1 using specific antagonists induces hyperthermia in clinical trials leading to their abandonment as a therapeutic strategy. TRPV1 agonists are useful to treat pain by virtue of their ability to cause calcium influx, which subsequently leads to nerve terminal desensitization/ablation. Recently, an 8% capsaicin containing patch (Qutenza) has been approved to treat painful DPN in Europe. Since activation of TRPV1 depolarizes the nerve terminal and generates and action potential leading to pain, lidocaine, a local anesthetic is applied to numb the area before application of capsaicin patch. Here, we report the effect of an ultra-potent TRPV1 agonist, resiniferatoxin (RTX), in a topical formulation to alleviate PDPN in animal models of diabetes by inducing nerve terminal depolarization block in the short-term, which prevents pain during application and leading to nerve terminal desensitization/ablation in the long-term resulting in long lasting pain relief. RTX application suppressed the thermal hyperalgesia and mechanical allodynia in diabetic animals. Our data provide compelling evidence for developing RTX topical formulation as an effective replacement for the use of 8% capsaicin to treat painful conditions, which is both cumbersome and painful during application.

Materials and methods

Streptozotocin-induced diabetes in rats and mini-pigs

Male and female wild type (WT) Wistar rats (n = 24 per group) purchased form Jackson Lab (USA) were housed in specific pathogen-free barrier animal facility, and rodent laboratory chow and drinking water were provided *ad libitum*. The rats were starved overnight and were injected intraperitoneally with freshly prepared streptozotocin (STZ; 55 mg/kg; Cayman, USA) in saline (pH 4.5with 0.1 N citrate buffer). Control rats received saline (pH 4.5 with 0.1N citrate buffer). The food was introduced to the cages after 1-hour post injection. Blood glucose levels were measured everyday post injection and the animals that show blood glucose levels greater than 300 mg/dL were considered diabetic.

Male/female mini-pigs (20-25 kg; n = 5; Waldo Genetics, De Witt, NE, USA) were housed in large animal facility. The mini-pigs were fed *ad libitum* on a commercial finisher diet and had free access to water. STZ was freshly prepared by dissolving in saline (pH 4.5 with 0.1N citrate buffer) at a concentration of 80 mg/mL. The mini-pigs were starved overnight and then injected with freshly prepared STZ (150 mg/kg body weight) via the ear vein (~1 mL/s) within 2 min, while the animal was anesthetized. The blood glucose levels were measured by using a commercial glucometer and blood glucose levels greater than 300 mg/dL were considered diabetic.

Preparation of RTX Cream

We prepared a proprietary cream formulation of RTX that contains RTX encapsulated by an FDA approved copolymer poly-l-lactide coglycolide (PLGA) by solvent evaporation method. An accurately weighed quantity of RTX was dissolved in ethanol. This ethanolic solution was mixed with 1% PLGA (50:50) copolymer dissolved in dichloromethane. The resultant mixture is evaporated on a rotavap and dried to remove the solvents. The resultant PLGA-encapsulated RTX was mixed with an absorption base to produce RTX-cream containing various concentrations of RTX. Placebo cream was prepared by using the same method without RTX.

In vitro release characteristics of RTX from cream

Either 5 mg of pure drug or 350 mg of RTX cream was placed and spread on a Strat-M Membrane (SKBM02560; EMD Millipore, USA). RTX diffusion across membranes was investigated using a Franz diffusion cell. The diffusion of RTX cream across the Strat-M membrane is predictive of diffusion in human skin. The membrane was mounted between the donor and receptor compartment and either 5 mg of pure RTX or 350 mg of RTX cream was placed on the membrane surface inside the donor compartment. The receptor compartment was filled with a buffer solution, pH 7.4 and stirred at 100 rpm. The buffer samples were collected from the receptor compartment at 0, 5, 15, 30, 60, 90 and 120, 180, 240 and 300 min. and analyzed for RTX concentration by measuring the absorbance at 210 nm in a JASCO V670 spectrophotometer. The cumulative amount of RTX diffusion over 5 hr. for each membrane was plotted against time. From the data, a calibration curve was plotted with known concentrations of RTX ranging from 30 µg/L to 1 mg/L in pH 5.5 buffer. Experiments were performed in triplicates and analyzed. The formula given below was used for calculating the cumulative percent release of RTX in phosphate buffered saline pH 5.5.

Cumulative % Release = C_1V_1 X CF / 100 X C_2

where, C_1, Concentration of drug in final solution (g/mL); C_2, Drug content (mg) used for dissolution study; CF, Correction Factor; V_1, Volume of dissolution medium (mL).

Stability testing and efficacy analysis

Accelerated stability analyses of RTX-cream was performed by storing the cream preparations in a Shell SCH10R humidity temperature-controlled stability chamber. The visual appearance and chemical stability of the product were analyzed at the end of 30, 60, ad 90 days of storage at 37 °C at a relative humidity (RH) of 60 ± 5%. At the end of the specific storage period, the efficacy of the cream was evaluated by measuring thermal sensitivities by Hargreaves method. The goal was to maintain the efficacy of RTX-cream close to 100% with an allowable deviation of 10% throughout the shelf-life period. We also determined the concentration of RTX diffused from the cream across the Strat-M using a Franz Diffusion Cell as described above.

Determination of thermal and mechanical sensitivities in control and diabetic rats

Placebo or RTX cream was applied on the plantar areas of animals (n = 8 / group) for different durations and the rats were subjected to pain testing by the Hargreaves (thermal hyperalgesia) or von Frey (mechanical allodynia) method[20,39]. The concentrations of RTX in the cream that delays paw withdrawal latency (PWL) and increases paw withdrawal threshold (PWT) were determined. Placebo or RTX-cream applied on the plantar surface was protected by Tegaderm film and licking of the paws was prevented by facial cones. All the experiments were performed in a blinded manner by randomly assigning treated and untreated groups.

Determination of thermal sensitivity in control and diabetic mini-pigs

In mini-pigs, the pain behavior was tested by drawing 5 x 5, 1 cm square grid in the upper thigh region. The diode laser fiber selective stimulation (DLss) techniques were used to test thermal pain sensitivity in animal models and humans[30-35,54]. A stepwise increase in laser current was delivered to each cell in the grid using diode laser stimulator with duration of 2s and power 1-5W using Lass 20 (LasMed, CA) similar to that has been used in diabetic patients[35] . The pain behavior indicated by a clear skin twitch, and/or an abrupt limb withdrawal and/or a characteristic tail twirl in response to laser stimulation is considered a positive painful response. The experimenter was blinded to the type of animal (control or diabetic) and the treatment (placebo or RTX-cream) while testing.

Analysis of effect of RTX-cream on body temperature

Since TRPV1 agonists cause hypothermia and antagonists cause hyperthermia[20,21], change in core body temperature was determined by measuring rectal temperature using a RET-3 rectal probe (Bioseb.com, USA). We tested the effect of RTX-cream application every week by measuring the rectal temperature within 30 minutes of application and continued every 30 min, for up to 3 hours.

Analysis of TRPV1 expression and function

Western blotting

Skin samples and biopsies collected from control and diabetic rats and mini-pigs were lysed and centrifuged at 14000 rpm for 20 min. Lysis buffer composition (in mM) 50 Tris pH 7.5, 2 EDTA, 250 NaCl, 0.5 DL-dithiothreitol (DTT), 1 Na orthovanadate, 1 Phenylmethylsulfonyl fluoride (PMSF), 1% Nonidet P-40 (NP-40), 0.5% Sodium deoxycholate, and complete protease inhibitor cocktail and phosphatase inhibitor cocktail were added to the buffer. The supernatant was aliquoted and flash frozen in liquid nitrogen and stored at -80° C for experiments. Lysate (equivalent to 40 µg) was prepared by denaturing the protein using 6x Laemmli buffer (LB) and was resolved via SDS-polyacrylamide gel electrophoresis (SDS-PAGE). 3-color regular range protein marker was used to show molecular weights (kDa) of proteins. Proteins were resolved by running the gel at 130V in TGS (Tris/Glycine/SDS) buffer. Gels were transferred to nitrocellulose paper at constant current of 350 mA for 90 minutes using Tris/glycine buffer containing 20% methanol. Membranes were blocked in 5% non-fat dry milk powder in 1X PBS-buffered saline containing 0.1% Tween-20 (PBS-Tween) for 1 hour at room temperature (RT) and incubated with the TRPV1 primary antibody or loading control overnight at 4° C. Densitometry measurements of each protein were quantified using Image-J software (NIH)[15,59].

Immunohistochemistry

At the end of the study, the mini-pigs were euthanized by intraperitoneal injection of ketamine and xylazine (8 mg/kg and 10 mg/kg, respectively). Biopsies of upper thigh skin from the pigs were collected and processed for experiments as described[5,38]. Paraffin embedded pig skin tissues (Control or RTX treated) were cut and mounted on slides. Paraffin was removed and the tissues were rehydrated with different grades of ethyl alcohol, distilled water and PBS. Tissue sections were blocked in PBS, Triton X-100, and 5% normal goat serum. After washing with 1x PBS, the sections were exposed to rabbit polyclonal TRPV1 antibody (1:1000 dilution cat #NB-100-98897, Novus Biologicals), rabbit monoclonal PGP 9.5 antibody (1:500 dilution cat # ab-108986, Abcam, USA). Then, sections were treated with secondary antibody; goat anti rabbit IgG (1:1000; Thermofisher scientific, USA) and gently washed 3 times with PBS and cover slipped with Vectashield mounting media (Vector Laboratories). Colocalization of TRPV1 and PGP 9.5 in skin tissues was examined by confocal microscopy (Nikon, A1R Ti-E) or fluorescent microscopy (Nikon, Microphot FXA).

In vitro CGRP release measurement in isolated paw skin

To determine the function, we measured TRPV1-mediated CGRP release using commercially available ELISA kits (MyBioSource, USA and Cayman Chemicals, USA). We stimulated the isolated paw skin tissue with capsaicin (1 µM; 90 min. incubation at 37ºC) in control and diabetic animal treated with placebo or RTX cream.

Statistical analysis

Statistical analyses were performed using Origin 2020 software (Origin Labs, Northampton, MA, USA). Statistical significance was calculated using one-way ANOVA followed by Fishers Least Significant Difference (LSD) test and data were expressed as mean +/- SEM, P < .05 was considered to be significant. In all figures, statistical significance is labeled the following way: *P <0 .05, **P <0.01, and ***P < 0.001

Acknowledgment

This work was supported by funding from NIH NIDDK Phase 1 SBIR funding 1R43DK117674-01A1 to Ion Channel Pharmacology LLC.

Experimental study design

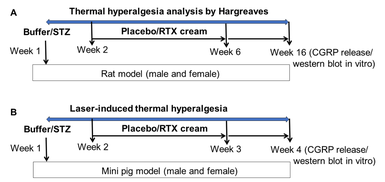

Figure 1. Experimental study design

Diffusion kinetics of RTX cream

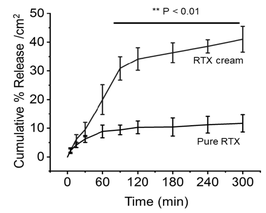

Figure 2: In vitro diffusion kinetics of RTX cream. Cumulative % release of RTX from pure RTX or cream formulation. RTX release across a Strat-M-membrane (human skin mimic) is significantly (P < 0.01) higher over several time points measured using a Frantz diffusion cell.

RTX cream suppresses thermal hyperalgesia in diabetic rats

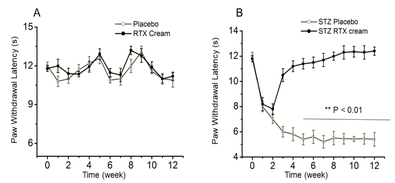

Figure 3: RTX cream suppresses thermal hyperalgesia in diabetic rats. A. There was no significant change in PWL measured following treatment of plantar surface with RTX cream in control rats over a period of time. B. STZ-induced diabetic animals exhibit hyperalgesia, indicated by a significant decrease in PWL, which was completely reversed to normal values following treatment with RTX (1 µM) cream. ** represents statistical significance P < 0.01 for n = 8-12/condition).

RTX cream reverses mechanical hypersensitivity in diabetic rats

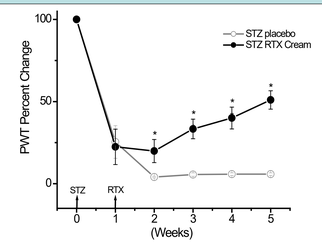

Figure 4: RTX cream reverses mechanical hypersensitivity in diabetic rats. Following STZ administration, within a week the rats became mechanically hypersensitive measured ad paw withdrawal threshold (PWT) using automated von-Frey filaments. There was significant decrease in PWT in diabetic animals. PWT measured following treatment of plantar surface with RTX cream show a reversal of mechanical hypersensitivity.

RTX cream decreases capsaicin-stimulated CGRP release and TRPV1 expression in diabetic rat paw skin

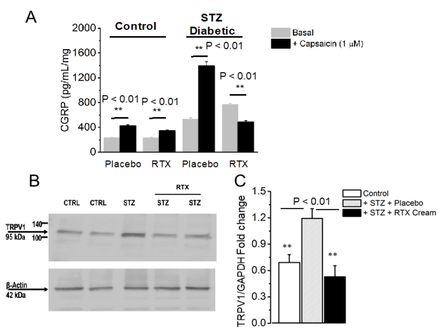

Figure 5. RTX cream decreases capsaicin-stimulated CGRP release and TRPV1 expression in diabetic rat paw skin in vitro. A. Basal and capsaicin (1 µM)-stimulated CGRP release in skin samples of control and diabetic rats showing a significant increase in CGRP release in diabetic hyperalgesic animals, which is significantly reduced following RTX cream treatment. ** represents statistical significance P < 0.01 for n = 3/condition. B. TRPV1 expression determined by immunoblotting in paw skin from control and STZ-induced diabetic animals treated with placebo or RTX cream. C. Densitometric ratio between TRPV1 expression and the respective loading control show a significant increase in diabetic hyperalgesic animals, which is significantly reduced following RTX cream application (n = 4 experiments).

RTX cream application reduces pain behavior in response to laser-induced noxious thermal stimulus in diabetic pigs

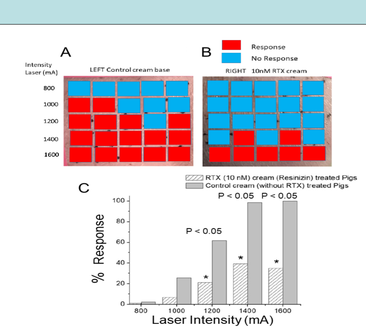

Figure 6. RTX cream application reduces pain behavior in response to laser-induced noxious thermal stimulus in diabetic pigs. A and B. The tested upper thigh region shown in the grid with red squares indicating a response and blue squares indicating lack of response to increasing laser induced noxious thermal stimulus in control and RTX cream treated regions. C. Pain behavioral measure expressed in percentage in response to a laser heat generated pain stimulus (DLss technique) following application of placebo or RTX (10nM) cream formulation to the upper thigh region of diabetic mini-pigs. * represents statistical significance (n = 4 mini-pigs).

RTX cream application reduces capsaicin-stimulated CGRP release and TRPV1 protein expression in diabetic mini- pig skin

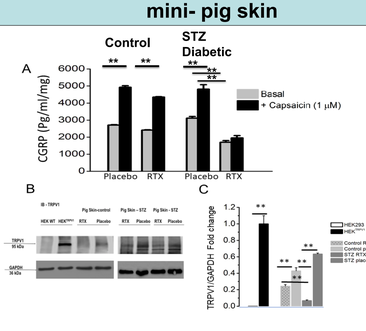

Figure 7. RTX cream application reduces capsaicin-stimulated CGRP release and TRPV1 protein expression in diabetic mini- pig skin in vitro. A. Capsaicin-stimulated CGRP release from skin samples of control and diabetic mini-pigs treated with placebo or RTX-cream. ** represents statistical significance P < 0.01. B. TRPV1 expression in skin samples from control and STZ-induced diabetic mice treated with placebo or RTX cream. C. Densitometric ratio between TRPV1 expression and the respective loading control, GAPDH (n = 4 experiments). HEK293 cells and TRPV1 stably expressing HEK293 cells (HEK^{TRPV1}) were used as negative and positive controls for TRPV1 expression. ** represents statistical significance P < 0.01 for n = 3 /condition).

Immunohistochemical staining for TRPV1 in control and diabetic mini-pig skin biopsies

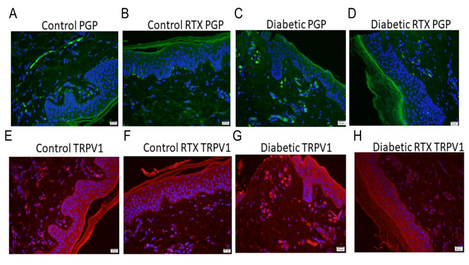

Figure 8: Immunohistochemical staining for TRPV1 in control and diabetic mini-pig skin biopsies. Immunohistochemical staining of PGP 9.5 (yellow) and TRPV1 (red) in skin biopsy samples collected from control (A, B, E, F) and diabetic (C, D, G, H) mini-pigs. In control animals, RTX cream decreased PGP 9.5 and TRPV1 expression (B, F). In diabetic animals, there is increased expression of PGP 9.5 and TRPV1 (C, G). Treatment with RTX cream decreased the expression of PGP 9.5 and TRPV1 (D, H). Scale bar 50 mm.

STZ injection induces hyperglycemia in rats and mini pigs

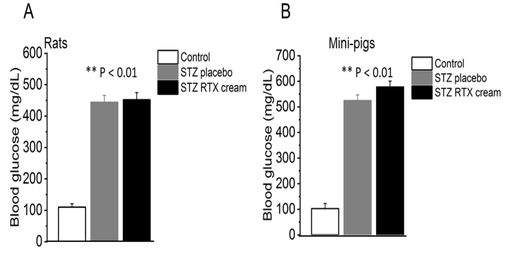

Figure 9. STZ injection induces hyperglycemia in rats and mini pigs. The blood glucose levels were significantly higher in diabetic hyperalgesic rats (A) and mini pigs (B). ** represents statistical significance P < 0.01 for n = 10/condition for rats and n = 2 to 4 mini-pigs).

Conclusions

- RTX is an ultrapotent TRPV1 agonist
- Very low concentrations of RTX induces depolarization block and prevents transmission of painful signals from peripheral nerve terminals
- Nanoparticle RTX cream formulation (Resinizin™) penetrates well into skin layers
- RTX cream reduces thermal and mechanical hypersensitivity in diabetic rats
- RTX cream reduces thermal hypersensitivity in mini-pig model of diabetes
- Our preclinical data confirm that RTX topical formulations (cream/patch) are effective treatment option for painful diabetic peripheral neuropathy.

References

Baskaran P, Mohandass A, Gustafson N, Bennis J, Louis S, Alexander B, Nemenov MI, Thyagarajan B, Premkumar LS. Evaluation of a polymer-coated nanoparticle cream formulation of resiniferatoxin for the treatment of painful diabetic peripheral neuropathy. Pain. 2023 Apr 1; 164 (4):782-790.