Exploratory analysis: Effects of RTIOXA 43 on Spontaneous physical Activity (SPA) in mice

General methods and statistics approach:

C57BL/6J mice (n=13; 7 males and 6 females, strain #:000664, JAX) were individually housed and fed chow (2018 Teklad Global 18% protein rodent diet) *ad libitum* under a 14 h light / 10 h dark schedule. After one week of acclimation to sable cages (indirect calorimetry), all mice were injected with RTIOXA 43 obtained either from MedChemExpress (Cat. No.: HY-154789) or from our collaborator, Dr. Yanan Zhang, approximately 30 minutes before the dark period began. All animals received a mock injection the day before injections began. Both compounds were evaluated for their ability to increase SPA such was reported previously for RTIOXA 47 in mice.

All mice received an intraperitoneal (IP) injection of 30 mg/kg RTIOXA 43 from MedChemExpress (RTI\_43\_M), 30 mg/kg RTIOXA 43 from Dr. Zhang (RTI\_43\_Y), and vehicle (Veh) under a repeated measures balanced Latin square design. One female mouse was excluded from the analysis due to technical issues with the Sable cage in which data was obtained.

The analysis evaluated the effects of “RTI\_43\_M”,” RTI\_43\_Y” and “veh” on SPA within 3 time points post-injection (60, 240 and 1440 minutes). Estimated marginal means (EMMs) were computed using a linear mixed-effects model, accounting for RTIOXA 43 treatment, sex, and time. Pairwise comparisons were conducted using emmeans, with the Satterthwaite approximation for degrees of freedom. All data was analyzed and plotted using R Version 2024.12.1+563.

Results:

We obtained data from sable cages of SPA over 24 hours period (figure 1)

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Figure 1. Time course of SPA in C57BL/6J mice following IP injection of RTIOXA 43 at 30 mg/kg. The graph illustrates the estimated mean of SPA in meters over time (Minutes) and the confidence interval at 95% for both young female (F) and male (M) mice. Three treatment groups are shown: RTI\_43\_M (sourced from MedChemExpress), RTI\_43\_Y (sourced from Dr. Zang), and vehicle control (98% corn oil with 2% DMSO).

We evaluated differences in SPA among treatments after 60, 240 and 1440 minutes of injection (figure 2).

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Figure 2. Effect of RTIOXA 43 on SPA at 60-, 240-, and 1440-minutes post-injection. The graph illustrates the distribution of SPA (meters) for young male (M) and female (F) mice treated with vehicle, RTI\_43\_M, or RTI\_43\_Y. Box plots represent the median (center line) and interquartile range (box edges).

After 1 hour of IP injection (60 minutes), RTI\_43\_M increased SPA in male mice by 4.8± 2.02 meters compared to the vehicle group (p = 0.04). In females, RTI\_43\_M showed a non-significant increase of 0.2 ± 2.1 meters relative to the vehicle group (p = 0.99). On contrary, RTI\_43\_Y did not significantly increase SPA in male mice (3.5 ± 2.02 meters, p = 0.18) or decreased SPA in female mice (-1.66 ± 2.10 meters, p = 0.70) compared to the vehicle group. After 4 hour of IP injection (240 minutes), RTI\_43\_M increased SPA in male mice by 21.6 ± 2.02 meters compared to the vehicle group (p < 0.0001). Also, RTI\_43\_M increased SPA in male mice 18.9±1.94 meters compared to RTI\_43\_Y (p<0.0001) which suggest different pharmacokinetics between RTIOXAs 43 in male mice. In females, neither RTI\_43\_M or RTI\_43\_Y showed significant effects compared to vehicle. These results were extended after 24 hours of RTIOXA 43 injections in which the increased SPA in male mice is not extended anymore. Curiously, only in male mice RTI\_43\_M increased SPA compared with vehicle within all time points from 1 to 23 hours after injection. In detail, after 23 hours of injection RTI\_43\_M increased SPA in 18.1 + 2.08 meters compared to the vehicle (p<.0001 (supplemental figure 1) just in male mice.

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Figure 3. Effect of RTIOXA 43 on SPA at 1380 minutes (23 hours) post-injection. The graph illustrates the distribution of SPA (meters) for male (M) and female (F) mice treated with vehicle, RTI-43-M, or RTI-43-Y. Box plots represent the median (center line) and interquartile range (box edges). RTI\_43\_M had significant effect increased SPA within all timepoint less after 1440 minutes of injection.

While Nuclear Magnetic Resonance (NMR) analysis confirmed the absence of impurities in both RTIOXAs 43 the observed differences in their effects on SPA may be attributed to variations in their physical characteristics arising from distinct purification processes. We observed a chalky texture of RTI\_43\_Y when was solubilized, likely due to slow recrystallization or rapid rotovap, suggests larger crystal formation, potentially leading to slower dissolution and absorption compared to the rapidly recrystallized RTI\_43\_M with smaller crystals. This difference in dissolution rate could explain the divergence between RTI\_43\_M and RTI\_43\_Y. The faster absorption of RTI\_43\_M might have resulted in a higher peak concentration and a more pronounced effect at 4 hours, whereas the slower absorption and potentially altered bioavailability of RTI\_43\_Y might have attenuated its effects. These effects are also sex dependent.