Exploratory analysis: Effects of RTIOXA 43 on SPA in mice

General methods:

C57BL/6J mice (n=13, 7 males and 6 females) were individually housed and fed with chow (2018 Teklad Global 18% Protein Rodent Diets) ad libitum under 12 h/10 h light off/light on schedule. After 1 week of acclimation to sable cages the animals were injected with 30 mg/kg of RTIOXA-43 obtained from MedChemExpress (Cat. No.: HY-154789, medchem) or from our collaborator Dr Yanan Zhang ~30 minutes before the light off period began. All animals received one mock injection the day before injections began. Both compounds were evaluated in their capacity to increase Spontaneous physical activity (SPA) in mice.

All mice received and Intraperitoneal (IP) injection of 30 mg/kg of RTIOXA-43 from MedChemExpress (RTI\_43\_M), 30 mg/kg of 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 technical issues with the sable cage.

Analysis:  
1.

We compare changes in SPA (β estimate = the slope of the regression line representing the change in SPA over time) among treatments (Vehicle, RTI\_43\_M and RTI\_43\_Y) using a lineal mixed model.



Figure 1. Time course of SPA after IP injection of RTIOXA-43 30 mg/kg in young female (F) and male (M) C57BL/6J mice. RTI\_43\_M: RTIOXA-43 30 mg/kg obtained from medchemexpress, RTI\_43\_Y: RTIOXA-43 30 mg/kg obtained from Dr Zang, veh: vehicle: 98% corn oil with 2%DMSO

RTI\_43\_M increased SPA in male mice relative to vehicle (slope is bigger?).

2.

We also compared mean SPA at specific times (60, 240, 1440 min after injection) among treatments. We choose to evaluate the effect to 240 min to reproduce the effects of RTIOXA-47 on SPA in mice.



Figure 1. Estimated average in SPA after 60, 240 and 1440 min of RTI\_43 injections.

At 1 hour post-injection, male mice treated with RTI\_43\_M exhibited a higher estimated mean SPA (18.46, 95% CI: 4.38 to 32.54) compared to the vehicle group (13.70, 95% CI: -0.42 to 27.82, p < 0.0001). Male mice treated with RTI\_43\_Y also showed a significantly higher estimated mean SPA (17.20, 95% CI: 3.18 to 31.22) compared to the vehicle group (p < 0.0001). Importantly there was no statistically significant difference between the estimated mean SPA of RTI\_43\_M and RTI\_43\_Y (p = 0.05475806). Therefore, the significant increase in SPA observed in male mice at 1 hour post-injection for RTI\_43\_M and RTI\_43\_Y compared to the vehicle group is NOT observed in female mice.

At 240 minutes post-injection, male mice treated with RTI\_43\_M exhibited a significantly higher estimated mean SPA (61.52, 95% CI: 47.44 to 75.59) compared to the vehicle group (39.88, 95% CI: 25.75 to 53.99, p < 0.05). While RTI\_43\_Y also showed a higher estimated mean SPA (42.73, 95% CI: 28.65 to 56.81) than the vehicle group, this difference was not statistically significant (p > 0.05). Furthermore, the estimated mean SPA for RTI\_43\_M was significantly higher than that of RTI\_43\_Y (p < 0.05). At 240 minutes, there are no statistically significant differences in SPA among the RTI\_43\_M, RTI\_43\_Y, and vehicle groups in female mice.

At 24 hours post-injection, male mice treated with RTI\_43\_M exhibited an estimated mean SPA of 148.63 (95% CI: 133.38 to 163.89), while male mice treated with RTI\_43\_Y showed an estimated mean SPA of 148.95 (95% CI: 130.31 to 167.59). The vehicle group had an estimated mean SPA of 145.13 (95% CI: 129.09 to 160.41). However, there were no statistically significant differences between any of the treatment groups (RTI\_43\_M vs. Vehicle, p = 0.411489; RTI\_43\_Y vs. Vehicle, p = 0.380424; RTI\_43\_M vs. RTI\_43\_Y, p = 0.965749). For females, neither RTI\_43\_M (estimated mean SPA: 147.23, 95% CI: 129.58 to 164.88, p = 0.8872518) nor RTI\_43\_Y (estimated mean SPA: 144.98, 95% CI: 127.33 to 162.63, p = 0.9859563) caused a statistically increased in SPA in female mice compared to the vehicle control group (estimated mean SPA: 144.38, 95% CI: 126.73 to 162.03)."

Overall, these results indicate that both RTI\_43\_M and RTI\_43\_Y has a sex specific effect and significantly increased SPA in male mice at 1-hour post-injection, with no significant difference between the two RTIOXA-43. However, at 4 hours, only RTI\_43\_M showed a significant increase in SPA compared to the control, and at 24 hours, neither RTI-43 had a significant effect compared to control. This suggests a potential divergence in RTI-43 effects over time in a sex specific manner.

While Nuclear Magnetic Resonance (NMR) analysis confirmed the absence of impurities in both RTIOXA-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 similar significant increase in SPA seen at 1 hour for both drugs, but also the divergence at later time points. 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. Further studies, including dissolution and pharmacokinetic analyses, are warranted to confirm these hypotheses and elucidate the precise mechanisms underlying the observed differences.