Evaluation of Three COMPANY Compounds in a Rat Model of Diabetes-Induced Neuropathic Pain

This study was conducted under the terms of a Services Agreement between NeuroDetective Inc. and Company dated...
1. Introduction

This study evaluated a number of compounds belonging to a class of XXX that are used clinically for the treatment of systemic hypertension. This evaluation was conducted using a rat model of chronic pain. The model involves diabetic neuropathy induced by injection with streptozotocin (STZ). The endpoint measures are blood glucose levels, body weight, and a standard behavioral measure of pain threshold.

2. Methods and Experimental Design

a. Subjects

A total of 254 male Sprague-Dawley rats (250-300g), obtained from a commercial supplier, were initially entered into the study. Of these, 200 were excluded from data analysis for one of three reasons: (1) 52 STZ-injected animals did not develop hyperglycemia; (2) 11 animals either died or were sacrificed due to poor health conditions following STZ injection; and (3) 137 animals (of the remaining 191 hyperglycemic animals) did not develop neuropathy.

No animals died following XXX treatment. Therefore, the remaining 54 animals, weighing 298.8± 7.5 g at the time of STZ injection, were randomly assigned to one of 8 groups. The final sample sizes for each condition were: STZ/0.1 mg/kg xxx, n = 7; STZ/0.3 mg/kg yyy, n = 5; STZ/0.3 mg/kg yyy, n = 6; STZ/0.3 mg/kg zzz, n = 6; STZ/0.3 mg/kg ppp, n = 8; STZ/1.0 mg/kg jjj, n = 7; STZ/34% DMSO, n = 6; Saline/34% DMSO, n = 9. Animals were housed one per cage and maintained on a 12 h/12 h light/dark cycle with food and water available ad libitum.
b. *Induction of Diabetes*

Animals were injected with streptozotocin (60 mg/kg, i.p.) dissolved in 0.9% sodium chloride. Blood glucose measurements were assayed from samples taken from the tail vein using standard test strips and colorimeter. Only animals with blood glucose levels $> 15$ Mm were considered diabetic.

c. *Behavioral Testing*

Paw withdrawal testing was performed 4 weeks following the induction of diabetes using the well-established up/down method (Dixon, 1980). Animals were retrieved from the colony room and habituated to a Plexiglas test chamber on top of a mesh screen for 15 min. The size of the chamber allows for free movement of the animal and the mesh screen allows for application of calibrated von Frey monofilaments to the plantar surface of each hindpaw. The force applied to each paw by the monofilaments ranged from 3 mN to 202 mN. For each trial, the 50% withdrawal threshold for each hindpaw was calculated using the following formula: $[X_{th}]_\log = [vFr]_\log + ky$, where $[vFr]$ is the force of the last von Frey used, $k = 0.2268$ which is the average interval (in log units) between the von Frey monofilaments, and $y$ is a value that depends upon the pattern of withdrawal responses (Dixon, 1980). The paw withdrawal threshold for each animal was calculated as the average across the 3 trials of the test session. Values approaching zero indicate hyperalgesia.

d. *General Procedure*

On Day One of the study, following acclimation of the animals to the laboratory, baseline measurements of mechanical paw withdrawal threshold, body weight, and blood glucose level were recorded. The animals were then marked for identification under light isoflurane anesthesia. Animals were food deprived overnight. Then, on Day Two, the animals were administered STZ (60 mg/kg, i.p.) or saline. One week later (Day Nine) blood glucose level was again measured. If hyperglycemic, animals remained in the protocol. On Day 28, one day prior to the first post-STZ behavior test, blood glucose was measured a third time, to confirm hyperglycemia prior to dosing with one of the test compounds. On the morning of Day 29, mechanical paw withdrawal threshold was measured. If the animals were hypersensitive (i.e., they withdrew their paw following stimulation with a force $< 205.97$ mN, a 60% decrease in threshold compared to their mean baseline measurement), the animals were weighed and assigned to receive an i.p. injection of one of 8 coded solutions (see Subjects). Final testing
for paw withdrawal threshold was performed 30 min later, followed by the final measure of blood glucose level.

e. Drug Dosing and Preparation

Animals received a single dose of a coded drug solution four week following the induction of diabetes. All compounds were administered i.p., 30 min prior to behavioral testing. All compounds were stored and prepared according to the directions provided by COMPANY. Behavioral testing was performed “blind” and was ensured by: (1) having one person create and code the drug solutions, randomization of animals to drug treatment, and breaking of drug code; and (2) having a second person perform the behavioral testing, plus other daily project related duties (daily monitoring of animal health, etc).

f. Statistical Analysis

Statistical analyses of both the threshold and blood glucose level data were performed for the STZ-injected animals using repeated measures ANOVA, with time (3 time points when measures were taken) as the repeated measure and group (7 groups) as the between subjects measure, followed by post-hoc comparisons (LSD test) of group differences. Body weight data for STZ-injected animals was analyzed using a repeated measures ANOVA with time (2 time points when measures were taken) as the repeated measure and group (7 groups) as the between subjects measure, followed by post-hoc comparisons (LSD test). The data for non-STZ (saline) injected animals (the 8th group) was included to ensure internal consistency in the experimental protocol, and was analyzed separately from STZ treated animals using one-way ANOVA. Significance was set at $p \leq 0.05$. 
1. Results

a. Blood Glucose (Figure 1)

For saline treated animals (Saline/34% DMSO), blood glucose level remained normal and constant throughout the study ($F(2,16) = 1.72, p > 0.20$). The overall ANOVA for STZ treated animals showed a significant main effect for group ($F(6,38) = 2.48, p < 0.05$), a significant main effect for time ($F(2,76) = 77.39, p < 0.001$), and a significant group X time interaction ($F(12,76) = 1.94, p < 0.05$). Of primary importance for the purpose of this study are the results of the post-hoc comparisons among the STZ-treated groups; all STZ-treated animals had significantly elevated blood glucose levels prior to injection of the test compounds (Wk4 Pre-Inj vs. Baseline), and this elevated blood glucose level was not modulated by the test compounds (Wk4 Post-Inj vs. Wk4 Pre-Inj). These results demonstrate that, using the parameters of this study, STZ induced diabetes, as measured by an increase in blood glucose level, and drug treatment did not alter this.

b. Body Weight (Figure 2)

For saline treated animals (Saline/34% DMSO), body weight significantly increased during the study ($F(1,8) = 215.89, p < 0.001$). Analysis of body weight for STZ-treated animals showed a significant main effect of time ($F(1,38) = 15.94, p < 0.001$), but no significant main effect of group ($F(6,38) = 1.95, p > 0.09$) nor a significant group X time interaction ($F(6,38) = 0.84, p > 0.50$). The main effect of time for the STZ-treated groups indicates that STZ animals lost body weight following the injection, but the lack of a significant interaction with group indicates that, at the time of drug treatment, this weight loss did not differentially affect any of the groups. The lack of a significant main effect of group in the weight measure also indicates that the general health of STZ treated animals was similar at the time of drug administration.
c. **Mechanical Paw Withdrawal Threshold** (Figure 3)

The mean paw withdrawal thresholds were calculated for each animal and then averaged across subjects within each group. A lower number indicates enhanced responding to mechanical stimulation (i.e. hyperalgesia).

For saline treated animals (Saline/34% DMSO), paw withdrawal threshold remained constant during the study ($F(2,16) = 3.55, p > 0.05$). Overall ANOVA for STZ-treated animals showed a significant main effect of time ($F(2,76) = 165.04, p < 0.001$) and a significant group X time interaction ($F(12,76) = 2.39, p < 0.05$). Post-hoc comparisons showed significant attenuation of mechanical hyperalgesia following treatment with all $\alpha_2$-adrenergic agonist compounds. STZ-treated animals that received vehicle injection (STZ/34% DMSO) remained hyperalgesic. These results indicate that treatment with these $\alpha_2$-adrenergic agonists can attenuate neuropathic pain associated with an animal model of diabetes.

2. **Summary**

1. As indicated in Figure 1, STZ treatment significantly increased blood glucose level. This increase was not altered by treatment with xxx. The data from saline treated animals indicate that there was internal consistency in the blood glucose measurement in this protocol, so the elevated blood glucose in STZ-injected animals was in fact due to STZ.

2. As indicated data in Figure 2, STZ treatment significantly attenuated body weight gain. This is expected as a direct consequence of the diabetic disease state. Of importance is the lack of difference in body weight among the STZ-treated animals, suggesting that animal health was similar among groups. The data from saline treated animals indicates that normal animals gain weight during a 4-week period and further supports the idea that the lack of body weight increase in STZ animals is due to the diabetic state.

3. As indicated in Figure 3, there is a significant anti-hyperalgesic effect of these xxx agonists in this animal model of diabetic neuropathy. The diabetic neuropathy remained stable and was not
influenced by vehicle injection. The data from saline treated animals indicates that there was internal consistency in the mechanical paw withdrawal measurement throughout the experimental protocol and that hyperalgesia in STZ-injected animals is due to the STZ.

Additional Information and Interpretation of Side-Effects

All STZ-treated animals that were included in the final analysis appeared to be in relatively good health throughout the study, and only expressed symptoms that are expected in diabetic animals (i.e. increased urine excretion, soft feces, lack of weight gain, etc.). No negative reactions were observed to any of the α2-adrenergic agonist compounds.

An extensive number of animals were employed in the present study. In fact, only 54 of 254 (21%) of STZ-injected animals were included in the final analysis. As anticipated for this model, 20% of STZ-injected animals did not become hyperglycemic (most likely due to missed i.p. injection). Also as anticipated, 4% of the animals were lost due to health related issues following STZ injection. This loss is directly related to the dose of STZ, which must be titrated to ensure hyperglycemia without a significant incidence of death.

The remaining animals that were excluded (137) did not develop mechanical hyperalgesia, and thus could not be tested. This low incidence of neuropathy in hyperglycemic animals (28%) was not anticipated. One possible reason is the a priori decision by the COMPANY to set the criterion for hyperalgesia at a very stringent level ($< 205.97$ mN, 60% decrease in threshold). The main rationale for setting such a stringent level was to ensure detection of maximal effects of the test compounds. The downside of this decision was that the final sample size per group was lower than originally anticipated. Nonetheless, statistical significance was achieved for all drug-treated groups with the resulting sample sizes.
Figures:

Figure 1

- STZ/0.1 mg/kg (n=7)
- STZ/0.3 mg/kg (n=5)
- STZ/0.3 mg/kg (n=6)
- STZ/3 mg/kg (n=6)
- STZ/0.3 mg/kg (n=8)
- STZ/1.0 mg/kg (n=7)
- STZ/25% DMSO (n=6)
- Saline/5% DMSO (n=9)

Test Time:
- Baseline
- Wk. 4 Pre-Inj
- Wk. 4 Post-Inj

Mean Blood Glucose Level (mmol/L)
Figure 2

- STZ/0.1 mg/kg (n=7)
- STZ/0.3 mg/kg (n=5)
- STZ/0.3 mg/kg (n=6)
- STZ/0.3 mg/kg (n=6)
- STZ/1.0 mg/kg (n=7)
- STZ/34% DMSO (n=6)
- Saline/34% DMSO (n=9)

Mean Body Weight (g)

Test Time

Baseline

Wk. 4
Figure 3

Mean Mechanical Paw Withdrawal Threshold (mN)

- STZ/0.1 mg/kg (n=7)
- STZ/0.3 mg/kg (n=6)
- STZ/0.5 mg/kg (n=6)
- STZ/1.0 mg/kg (n=7)
- STZ/34% DMSO (n=6)
- Saline/34% DMSO (n=9)

Test Time:
- Baseline
- Wk. 4 Pre-Inj
- Wk. 4 Post-Inj

*** *** **
Figure Captions

Figure 1: Mean (± SEM) blood glucose levels prior to STZ injection (baseline), one day prior to week 4 testing (Wk. 4 Pre-Inj), and immediately following injection of a test compound or vehicle and behavioral testing (Wk. 4 Post-Inj). There were no statistically significant differences among groups at baseline; all animals that received STZ had elevated blood glucose levels. There were no statistically significant differences between pre- and post-injection measures for any group receiving test compounds.

Figure 2: Mean (±SEM) body weight prior to STZ injection (baseline) and immediately prior to Wk. 4 drug treatment. At baseline there were no significant differences among the groups. In control animals (Saline/34% DMSO) body weight significantly increased during the 4-week study. All animals that received STZ had reduced body weight increase *** = p < 0.001 vs. baseline.

Figure 3: Mean (± SEM) mechanical paw withdrawal threshold prior to STZ injection (baseline), immediately prior to drug injection (Wk. 4 Pre-Inj), and 30 min following the test compound injection (Wk. 4 Post-Inj). At baseline there were no significant differences among the groups. In control animals (Saline/34% DMSO) threshold remained constant. All animals that received STZ had significantly reduced thresholds prior to treatment with test compounds. All groups receiving test compounds had significantly reduced hyperalgesia (** = p < 0.05, *** = p < 0.001 vs. Wk. 4 Pre-Inj).