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L-NAME, a nitric oxide synthase inhibitor, and WIN 55212-2, a cannabinoid agonist, interact to evoke synergistic hypothermia

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Synergistic interactions between NO and CB_1 receptors

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List of Abbreviations:

Nitric oxide, NO

POAH, preoptic anterior nucleus of the hypothalamus

WIN 55212-2, [(4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6H-pyrrolo[3,2,1ij]quinolin-6-one]

L-NAME, N-nitro-L-arginine-methyl ester

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Abstract

Cannabinoids evoke profound hypothermia in rats by activating central CB_1 receptors. Nitric oxide (NO), a prominent second messenger in central and peripheral neurons, also plays a crucial role in thermoregulation, with previous studies suggesting pyretic and antipyretic functions. Dense nitric oxide synthase (NOS) staining and CB_1 receptor immunoreactivity have been detected in regions of the hypothalamus that regulate body temperature, suggesting that intimate NO-cannabinoid associations may exist in the CNS. The present study investigated the effect of L-NAME, a NO synthase inhibitor, on the hypothermic response to WIN 55212-2, a selective cannabinoid agonist, in rats. WIN 55212-2 (1-5 mg/kg, i.m.) produced dose-dependent hypothermia that peaked 45-90 min post-injection. L-NAME (10-100 mg/kg, i.m.) by itself did not significantly alter body temperature. However, a non-hypothermic dose of L-NAME (50 mg/kg) potentiated the hypothermia caused by WIN 55212-2 (0.5-5 mg/kg). The augmentation was strongly synergistic, indicated by a 2.5-fold increase in the relative potency of WIN 55212-2. The inactive enantiomer of WIN 55212-2, WIN 55212-3 (5 mg/kg, i.m.), did not produce hypothermia in the absence or presence of L-NAME (50 mg/kg), confirming that cannabinoid receptors mediated the synergy. The present data are the first evidence that drug combinations of NOS blockers and cannabinoid agonists produce synergistic hypothermia. Thus, NO and cannabinoid systems may interact to induce super-additive hypothermia.

Key Words: cannabinoid, NO, WIN 55212-2, L-NAME, hypothermia, body temperature

Cannabis and its derivative compounds, collectively called cannabinoids, evoke an array of pharmacological symptoms in rodents, including hypothermia, analgesia, catalepsy, and hypolocomotion. The discovery of endogenous ligands, such as anandamide and 2-arachidonyl glycerol, which act at cannabinoid receptors, has intensified the interest in the therapeutic potential of cannabinoids (Bisogno et al., 1997). CB_1 receptors are located primarily in the central nervous system (Howlett, 1995) while CB_2 receptors are expressed by peripheral immune cells (Dragic et al., 1996).

The development of cannabinoid agonists and antagonists has facilitated the characterization of cannabinoid receptor subtypes and their pharmacological profiles. One such agonist is the aminoalkylindole, (+)-WIN 55,212-2 [(4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)-6H-pyrrolo[3,2,1ij]quinolin-6-one] (WIN 55212-2), which exhibits high selectivity for cannabinoid receptors and interacts negligibly with other neurotransmitter systems and ion channels (Martin et al., 1991; Compton et al., 1992). Several reports indicate that WIN 55212-2 elicits hypothermia in rodents via a CB_1 receptor mechanism (Compton et al., 1992; Fan et al., 1994; Fox et al., 2001). Recently, our laboratory confirmed those results by demonstrating that the systemic injection of WIN 55212-2 produces hypothermia that is dependent on CB_1, but not CB_2, receptors (Rawls et al., 2002a). Moreover, the injection of WIN 55212-2 into the preoptic anterior nucleus of the hypothalamus (POAH), which is thought to be the central site of thermoregulation, evoked CB_1-sensitive hypothermia, indicating that intra-hypothalamic CB_1 receptors play a critical role in the hypothermic response to cannabinoids (Rawls et al., 2002a). CB_1 receptor immunoreactivity, binding, and mRNA are also present in the POAH (Moldrich and Wenger, 2000; Mailleux and

Vanderhaeghen, 1992), underscoring further the involvement of the cannabinoid system in thermoregulation.

Nitric oxide (NO) has captured the attention of neuroscientists because of its role as a prominent second messenger in the central and peripheral nervous systems (Breder and Saper, 1996). The enzyme nitric oxide synthase (NOS) catalyzes the production of NO and L-citrulline from the substrate, L-arginine. Three isoforms of NO have been discovered. The neuronal and endothelial forms are constitutive, and the third form is inducible (Lowenstein et al., 1992). An accumulating body of evidence suggests that NO participates in thermoregulation. Some studies provide strong evidence that NO production is involved in the hyperthermia evoked by prostaglandin E2, lipopolysaccharide, and morphine (Amir et al., 1991; Minano et al., 1997; Benamar et al., 2001). Furthermore, the injection of N-nitro-L-arginine-methyl ester (L-NAME), a NO synthase inhibitor, abolishes the fever produced by interleukin-1-beta and lipopolysaccharide (Roth et al., 1998). Other studies, however, suggest that NO has an antipyretic function (Gourine, 1995) and participates in hypothermia (Steiner et al., 1998; Almeida and Branco, 2001; Benamar et al., 2002).

Although the hypothermic effects of cannabinoids have been investigated extensively, the contribution of other neurotransmitter systems to this hypothermia is not entirely clear. Recent evidence suggests that glutamatergic, dopaminergic, and opioidergic systems modulate cannabinoid-evoked hypothermia (Rawls et al., 2002b; Ledent et al., 1999). The aim of the present study was to ascertain the role of NO in cannabinoid-evoked hypothermia. We investigated the hypothermic effects of WIN 55212-2 by itself and in combination with L-NAME.

Materials and Methods

Animals

All animal use procedures were conducted in strict accordance with the *NIH Guide for the Care and Use of Laboratory Animals* and were approved by the Temple University Animal Care and Use Committee. Male Sprague-Dawley rats (Zivic-Miller, Pittsburgh, PA, USA) weighing 200-250 g were housed 2 per cage for a minimum of 5 days before experimental use. Rats were maintained on a 12-hr light/dark cycle and fed rat chow and water ad libitum.

Drug preparation and administration

WIN 55212-2, WIN 55212-3, and L-NAME were purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs and drug combinations were dissolved in a 10 % cremophor/saline solution and injected intramuscularly into the right thigh.

Experimental protocol

The present research project was conducted over a 3-month period. In all cases, rats were used in only one experiment and received only one injection. No more than 12 rats were tested per day. Body temperature measurements were recorded from each rat over time. On the morning of the experiment, rats were weighed and placed one per cage into an environmental room, which was maintained at a constant temperature of 21 ± 0.3 ˚C and relative humidity of 52 ± 2 %. Body temperature experiments were always started between 9 and 10 a.m. All rats were allowed to acclimate to their environment for 60 min prior to measuring the first body temperature value. Baseline temperature measurements were taken for 60 min at 30 min intervals (-60, -30, and 0 min) using a thermistor probe (YSI series 400, Yellow Springs Instrument Co., Yellow Springs, OH, USA), which was

lubricated and inserted approximately 7 cm into the colon. A digital thermometer (Model 49 TA, YSI) was used to record body temperature. Rats were unrestrained during the temperature readings, with only the tail being held gently between two fingers. To allow adaptation to the technique, the first body temperature value was discarded in each rat. Following the baseline interval, rats were administered a drug or drug combination at 0 min. Thereafter, body temperature was recorded at 15, 30, 45, 60, 90, 120, 180, 240, and 300 min post-injection.

Effect of WIN 55212-2 on body temperature

The initial set of experiments used a total of 23 rats to determine the effect of WIN 55212-2 alone on body temperature. Different rats were used for each treatment group. A maximum of 12 rats were included in a single experiment, and rats received an injection of either vehicle or one of 3 doses of WIN 55212-2 (1, 2.5, or 5 mg/kg). Following a 60-min baseline interval, we injected rats at 0 min with vehicle (n=5), 1 mg/kg WIN 55212-2 (n=6), 2.5 mg/kg WIN 55212-2 (n=6), or 5 mg/kg WIN 55212-2 (n=6). Body temperature was recorded from each rat for 300 min. These doses of WIN 55212-2 have been reported to produce hypothermia in rats (Rawls et al., 2002 a, b; Fox et al., 2001).

Effect of L-NAME on body temperature

The second set of experiments used a total of 31 rats to determine the effect of L-NAME alone on body temperature. Different rats were used for each treatment group. A maximum of 12 rats were included in a single experiment, and rats received an injection of either vehicle or one of 3 doses of L-NAME (10, 50, or 100 mg/kg). Following a 60-min baseline interval, we injected rats at 0 min with vehicle (n=8), 10 mg/kg L-NAME (n=7), 50 mg/kg L-NAME (n=9), or 100 mg/kg L-NAME (n=7). Body temperature was recorded from each rat for 300 min. We selected doses of L-NAME based on previous studies, which have mostly reported that L-NAME by itself does not produce significant hypothermia (Benamar et al., 2001, 2002; Almeida and Branco, 2001; Kamerman et al., 2002; Spina et al. 1998; Thorat and Bhargava, 1994).

Effect of WIN 55212-2 and L-NAME on body temperature

For L-NAME, none of the doses (10-100 mg/kg) used in the present study produced significant alterations in body temperature as compared to vehicle. L-NAME was considered, therefore, to be inactive across the entire dose range used here, and 50 mg/kg was chosen as an inactive and intermediate dose for combination with WIN 55212-2. Thus, in a third set of experiments, we used a total of 97 rats to determine the effect of an inactive dose of L-NAME (50 mg/kg) on the hypothermia produced by various doses of WIN 55212-2 (0.5, 1, 2, 2.5, or 5 mg/kg). Different rats were used for each treatment group. A maximum of 12 rats were included in a single experiment. Following a 60-min baseline interval, rats received an injection of L-NAME (50 mg/kg) alone, WIN 55212-2 (0.5, 1, 2, 2.5, or 5 mg/kg) alone, or a combination of L-NAME (50

mg/kg) plus a single dose of WIN 55212-2 (0.5, 1, 2, 2.5, or 5 mg/kg). Body temperature was recorded from each rat for 300 min.

Effect of WIN 55212-3 and L-NAME on body temperature

To verify that cannabinoid receptors mediated the hypothermic actions of WIN 55212-2, we investigated whether WIN 55212-3, the inactive enantiomer of WIN 55212-2, altered body temperature when administered by itself or in combination with L-NAME. A total of 24 rats were used in this final set of experiments. Different rats were used for each treatment group, and a maximum of 12 rats were included in a single experiment. Following a 60-min baseline interval, we injected rats at 0 min with vehicle (n=6), 5 mg/kg WIN 55212-3 (n=6), 50 mg/kg L-NAME (n=6), or a combination of 50 mg/kg L-NAME plus 5 mg/kg WIN 55212-3 (n=6).

Data analysis

To allow adaptation to the experimental technique, the first body temperature value for each rat was discarded. Two consecutive body temperature readings were then recorded and averaged to establish a baseline temperature prior to drug injection. Data were calculated as the mean ± S.E. of body temperature. The data were analyzed by either a 1-way repeated measures analysis of variance (ANOVA) followed by a Dunnett's *post hoc* test or a two-way (group, time) mixed-model analysis of variance (ANOVA) with repeated measures on time. Comparisons between treatment groups in the 2-way ANOVA were conducted using LS Means on the main effect of group.

The analysis of drug combinations to distinguish synergism from simple additivity followed the procedure described previously (Tallarida et al., 2001). In that procedure the graded dose-effect data of the individual drugs are first analyzed in order to

determine an effect level that is reached by both. These equi-effective doses (isoboles) are then used to determine the proportions of the combination for testing and to calculate the expected (additive) total dose of the combination needed to attain the specified effect level. This calculated quantity is then statistically compared with the total dose of the combination that produced the specified effect. If the combination total dose is less than the calculated additive total dose, the interaction is synergistic; equality means simple additivity. In cases in which one of the two drugs is inactive, its presence in a simply additive combination has no effect on the active drug's dose-effect curve. Therefore, the analysis becomes simply one in which the active drug's dose-effect curve is statistically compared before and after the addition of the inactive agent. The relative potency was determined by using the values of the individual rats in each experimental group (WIN 55212-2 or WIN 55212-2 + L-NAME) at the 60-min time point. The 60-min time point was chosen because WIN 55212-2 by itself produces maximal hypothermia 60-90 min post-injection.

Results

WIN 55212-2 produces hypothermia

Fig. 1 illustrates the effects of WIN 55212-2 (1-5 mg/kg) on body temperature. A two-way ANOVA revealed a significant effect of time [$F(3, 14)=194$, $P<0.0001$], group [$F(5, 45)=15.56$, $P<0.0001$], and group*time [$F(27, 156)=5.7$, $P<0.0001$] (Fig. 1, inset). Doses of 2.5 and 5 mg/kg WIN 55212-2 produced significant hypothermia relative to vehicle ($p<0.0001$). Conversely, a lower dose, 1 mg/kg, was ineffective. Consistent with previous studies, the onset of hypothermia was rapid, with a reduction in body temperature observed 15 min post-injection. The hypothermia peaked 45-90 min post-injection, with body temperature returning to pre-drug values thereafter. A dose of 2.5 mg/kg evoked a maximal hypothermia of $2.1 \pm 0.2\ ^{0}$C 90 min post-injection, while 5 mg/kg produced a peak drop in body temperature of $3.1 \pm 0.3\ ^{0}$C 60 min post-injection.

L-NAME does not produce significant hypothermia

Fig. 2 illustrates that L-NAME (10-100 mg/kg) does not alter body temperature significantly. A two-way ANOVA revealed that there was not an effect of group, time, or group*time on body temperature (Fig. 2, inset). The median dose of L-NAME, 50 mg/kg, was chosen for drug combination experiments with various doses of WIN 55212-2.

Drug combinations of WIN 55212-2 and L-NAME produce synergistic hypothermia

Because a dose of 50 mg/kg L-NAME by itself did not alter body temperature significantly, this dose of L-NAME was used in combination experiments with 5 doses of WIN 55212-2 (0.5, 1, 2, 2.5, or 5 mg/kg). The experimental design, in which one or both of the agents is used in a dose that is devoid of activity, allows a clear analysis of the combination. Temporal profiles of the hypothermia produced by the WIN 55212-2/L-

NAME combinations are shown in Fig. 3A, B, C, D, and E. L-NAME (50 mg/kg) significantly potentiated the actions of all doses of WIN 55212-2 except the highest dose, 5 mg/kg. Thus, we compared the dose-response effect of the active agent, WIN 55212-2, and the dose-response effect of that agent (5 doses) in combination with the inactive agent, L-NAME (Fig. 4). The depression in body temperature at 60 min was used as the effect. The two dose-response regression lines (effect on log dose) shown in Fig. 4 were generated from the temporal profiles in Fig. 1 and Fig. 3 using the body temperature drop at 60 min. A simply additive interaction would lead to the same dose-response relation, whereas a significant shift in the combination curve means that an interaction has occurred (Tallarida, 2001). It is seen that there is a pronounced leftward shift in the combination's regression line. As these lines did not differ significantly in slope ($F=7.29$, $p< 0.05$) it was possible to express this shift in terms of relative potency, R , a value computed with the assistance of Pharm Tools Pro (The McCary Group, Elkins Park, PA). R was found to have a mean of 2.51, with 95 % confidence limits (1.31-4.79). This significant leftward shift in the regression line of WIN 55212-2 means that WIN 55212-2 was 2.51-fold more potent in the presence of this non-hypothermic dose of L-NAME, a factor that quantitates the synergism. This value of R, significantly greater than unity, indicates enhanced potency and, thus, synergy for the interaction.

WIN 55212-3 does not produce significant hypothermia

WIN 55212-3 (5 mg/kg) did not cause hypothermia as compared to 10 % cremophor/saline (Fig. 5). Moreover, the combination of WIN 55212-3 (5 mg/kg) and L-NAME (50 mg/kg) was without effect on body temperature. The lack of effect of WIN

55212-3 confirms that cannabinoid receptors mediated the synergistic hypothermia caused by the combination of WIN 55212-2 and L-NAME.

Discussion

The major finding in the present study is that drug combinations of non-hypothermic doses of L-NAME and WIN 55212-2 produce synergistic hypothermia. The drug interaction was strongly synergistic, with L-NAME increasing the relative potency of WIN 55212-2 by 2.5-fold. Moreover, the inactive enantiomer of WIN 55212-2, WIN 55212-3, did not alter body temperature significantly by itself or in combination with L-NAME. The lack of effect of WIN 55212-3 confirms the synergy caused by the drug combination of WIN 55212-2 and L-NAME and strengthens our observation that inhibiting NOS activity enhances CB_1 receptor-mediated hypothermia (Iadecola et al., 1994; Traystman et al., 1995; Roth et al., 1998a).

Cannabinoid agonists produce marked hypothermia by activating CB_1 receptors (Compton et al., 1996; Costa et al., 1999; Ledent et al., 1999; Rawls et al., 2002a). The majority of previous studies have demonstrated that L-NAME does not alter body temperature in rodents (Benamar et al., 2001, 2002; Almeida and Branco, 2001; Kamerman et al., 2002; Spina et al. 1998; Thorat and Bhargava, 1994), although hypothermic responses have been reported (Scammell et al., 1996; Zarrindast et al., 2002; Kamerman et al., 2002). In our hands, 10-100 mg/kg of L-NAME by itself did not affect body temperature. NO does, however, modulate the thermoregulatory actions of other neurotransmitters. L-NAME attenuates the hypothermia produced by insulin, kappa opioids, hypoxia, and arginine vasopressin, suggesting that NO production is necessary for the development of hypothermia (Almeida and Branco, 2001; Benamar et al., 2002; Branco et al., 1997; Steiner et al., 1998). Conversely, NO generation facilitates the

hyperthermic actions of morphine, prostaglandin E2, and lipopolysaccharide (Benamar et al., 2001; Amir et al., 1991; Lin et al., 1996; Minano et al., 1997).

The effects of NO-cannabinoid interactions on body temperature have not been investigated extensively. N(G)-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NO synthase, did not affect Δ^9-THC-evoked hypothermia in mice (Thorat and Bhargava, 1994). Similarly, the acute injection of L-NAME did not alter the hypothermic, analgesic, or cataleptic response to WIN 55212-2 in mice (Spina et al., 1998). Of considerable importance, however, is that the authors reported the hypothermic effect of only one drug combination of L-NAME and WIN 55212-2. Moreover, the dose of WIN 55212, 8 mg/kg, was the highest dose of cannabinoid reported in the Spina et al. study. Although conjectural, the combination of lower, submaximal doses of WIN 55212-2 with L-NAME may have yielded greater-than-expected hypothermia in that study. It should also be noted that in mice that were tolerant to the pharmacological effects of WIN 55212-2, L-NAME injected once daily 20 min before WIN 55212-2 blocked the development of tolerance to the hypothermic and cataleptic actions but not to the analgesic effect of WIN 55212-2 (Spina et al., 1998). A more recent study demonstrated that Δ^9-THC produced antinociception, but not hypothermia or hypolocomotion, in mice lacking neuronal NO synthase (Azad et al., 2001). It is unclear why Azad et al. (2001) demonstrated that cannabinoids require NO production to produce hypothermia while we observed synergistic hypothermia following the injection of L-NAME and WIN 55212-2. One possibility is that cannabinoids exert distinct effects on different pharmacological endpoints, including hypothermia, in animals that are devoid of NOS versus animals in which NO production is blocked

acutely (Spina et al., 1998; Thorat and Bhargava, 1994). A combination of factors, such as species of animal, route of injection, type of cannabinoid agonist, method of altering NO production, or dose of cannabinoid agonist may account for the discrepancies.

Anatomical evidence supports the existence of synergistic interactions between cannabinoid systems and NO. The hypothalamus, particularly the POAH, is a central site of thermoregulation (Boulant et al., 1981). CB_1 receptor immunoreactivity is present in the lateral hypothalamic area and the POAH, indicating that intra-POAH neurons express the CB_1 receptor protein (Pettit et al.,1998; Tsou et al., 1998; Moldrich and Wenger, 2000; Mailleux and Vanderhaeghen, 1992). Because hypothalamic deafferentation did not alter cannabinoid receptor binding, it is thought that cannabinoid receptor-expressing neurons are primarily intrinsic to the hypothalamus (Romero et al., 1998). NOS-positive neurons in the ventromedial hypothalamus, another hypothalamic nucleus involved in the regulation of body temperature, also express CB_1 receptors (Bredt and Snyder, 1992; Azad et al., 2001). Those data suggest that NO and CB_1 systems are functionally linked.

The mechanism for the NO-cannabinoid synergy is unknown. Because the CB_1 receptor is located predominantly in the CNS and mediates cannabinoid-evoked hypothermia, cannabinoids appear to suppress body temperature by acting centrally (Howlett, 1995; Compton et al., 1992; Fan et al., 1994; Fitton and Pertwee, 1982; Ovadia et al., 1995; Rawls et al., 2002). The fact that NO synthase staining occurs on neurons that co-express CB_1 receptors (Bredt and Snyder, 1992; Azad et al., 2001) also suggests a central locus for the synergy between NO and cannabinoid systems. Although speculative, NO release may increase in response to cannabinoid-induced hypothermia, possibly as a compensatory reaction to the marked reduction in body temperature. In this

model, WIN 55212-2 evokes hypothermia by activating CB_1 receptors, which causes an increase in NO release in brain regions that regulate body temperature. The elevated NO levels attempt to spawn a hyperthermia that counteracts the CB_1-mediated hypothermia. Our data suggest that inhibition of NO production by L-NAME abolishes the compensatory effects of NO-evoked hyperthermia, resulting in a cumulative augmentation in the hypothermic response to cannabinoids. Indeed, endogenous cannabinoids stimulate NO release in rat kidneys, invertebrate nerve ganglia, and human immune tissue (Deutsch et al., 1997; Stefano et al., 1997, 2000). Also consistent with our hypothesis, L-NAME potentiates anandamide-induced inhibition of contractile responses in rats, prompting the authors to suggest a compensatory role for endocannabinoids in vascular function in situations where NO synthesis is chronically impaired (Mendizabal et al., 2001).

Another explanation is that CB_1 receptor activation by WIN 55212-2 reduces NO release in CNS regions that regulate body temperature. This would lead to a decline in cumulative NO levels and removal of a hyperthermic tone mediated by endogenous NO. The presence of L-NAME may potentiate the hypothermic action of WIN 55212-2 by further diminishing NO production and transmission. The fact that WIN 55212-2 suppresses potassium-evoked neuronal NO synthase in cerebellar granule cells suggests that CB_1 receptor activation attenuates the activation of neuronal NO synthase and supports the hypothesis that WIN 55212-2 inhibits NO levels (Hillard et al., 1999). Yet another possibility is that L-NAME potentiated cannabinoid-evoked hypothermia by acting at sites outside of the CNS (Nagashima et al., 1994). The sites of action of L-NAME are distributed throughout the body, including brown adipose tissue, where they

are responsible for heat production, and vascular smooth muscle, where they promote heat conservation.

In conclusion, we have shown that a cannabinoid agonist and NOS inhibitor interact to evoke synergistic hypothermia. The use of body temperature, a precise metric, provides an analysis that demonstrates synergism and its statistical confirmation. These results may provide the first step in elucidating a mechanism since the same drug combination may apply to endpoints other than body temperature.

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Figure Legends

Fig. 1. The effect of WIN 55212-2 (1-5 mg/kg, i.m.) on body temperature. A single dose of WIN 55212-2 or vehicle was injected at 0 min (as indicated by arrow) and body temperature was measured for 300 min. Data are expressed as the mean ± S.E. of body temperature. n is the number of rats in each treatment group. ΔT_b is the change in body temperature from baseline (time 0). **Inset)** The ANOVA table illustrates the main effects and interaction (group, time, and group*time). A two-factor design with repeated measures on time was used. The LS Means post-hoc analysis revealed that the 2.5 and 5 mg/kg WIN 55212-2 groups differed significantly from vehicle ($p< 0.0001$).

Fig. 2. The effect of WIN 55212-2 (10-100 mg/kg, i.m.) on body temperature. A single dose of L-NAME or vehicle was injected at 0 min (as indicated by arrow) and body temperature was measured for 300 min. Data are expressed as the mean ± S.E. of body temperature. n is the number of rats in each treatment group. ΔT_b is the change in body temperature from baseline (time 0). **Inset)** The ANOVA table indicates the main effects and interaction (group, time, and group*time). The two-way ANOVA did not reveal a significant effect of group, time, or group*time on ΔT_b.

Fig. 3. A to E, The effect of a non-hypothermic dose of L-NAME (50 mg/kg, i.m.) on the hypothermia produced by WIN 55212-2 (0.5-5 mg/kg, i.m.). Rats were given a single dose of L-NAME, WIN 55212-2, or L-NAME + WIN 55212-2 at 0 min (as indicated by arrow) and body temperature was measured for at least 300 min. Data are expressed as the mean ± S.E. of body temperature. n is the number of rats in each treatment group. ΔT_b is the change in body temperature from baseline (time 0). Each data set was analyzed

by a one-way ANOVA on ΔT_b followed by a Dunnett's *post hoc* test. **(A)** [$F(2, 27)= 33.35$, $P< 0.0001$]. The 0.5 mg/kg WIN 55212-2 + L-NAME group displayed significant hypothermia relative to the 0.5 mg/kg WIN 55212-2 alone group, $p< 0.001$. **(B)** [$F(2, 27)= 32.44$, $P< 0.0001$] The 1 mg/kg WIN 55212-2 + L-NAME group displayed significant hypothermia relative to the 1 mg/kg WIN 55212-2 alone group, $p< 0.001$. **(C)** [$F(2, 27)= 40.52$, $P< 0.0001$] The 2 mg/kg WIN 55212-2 + L-NAME group displayed significant hypothermia relative to the 2 mg/kg WIN 55212-2 alone group, $p< 0.01$. **(D)** [$F(2, 27)= 20.95$, $P< 0.0001$] The 2.5 mg/kg WIN 55212-2 + L-NAME group displayed significant hypothermia relative to the 2.5 mg/kg WIN 55212-2 alone group, $p< 0.05$. **(E)** [$F(2, 30)= 62.36$, $P< 0.0001$] The 5 mg/kg WIN 55212-2 + L-NAME group did not differ significantly from the 5 mg/kg WIN 55212-2 alone group, $p> 0.05$.

Fig. 4. Regression analysis of WIN 55212-2 (0.5, 1, 2, 2.5, or 5 mg/kg, i.m.) in the absence and presence of an inactive dose of L-NAME (50 mg/kg, i.m.). The effect is the reduction in body temperature (˚C) at 60 min and is determined from the temporal profiles shown in Fig. 3A-E. ΔT_b is plotted versus the log of the dose of WIN 55212-2. The number of rats (n) for each point is shown. The log dose-response regression lines did not differ significantly from parallel ($F= 7.29$, $p < 0.05$) and revealed a shift measured as relative potency, R=2.51 (95 % confidence limits, 1.31-4.79).

FIG. 5. The effect of WIN 55212-3 (5 mg/kg, i.m.) alone or in combination with L-NAME (50 mg/kg, i.m.). Rats were given vehicle, L-NAME (50 mg/kg), WIN 55212-3 (5 mg/kg), or L-NAME (50 mg/kg) + WIN 55212-3 (5 mg/kg) at 0 min (as indicated by arrow) and body temperature was recorded for 300 min. Data are expressed as the mean ± S.E. of body temperature. n is the number of rats in each treatment group. ΔT_b is the

change in body temperature from baseline (time 0). A one-way ANOVA revealed that there were no significant differences between groups [$F(3, 36)= 0.5745$, $P= 0.6367$].

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Footnotes

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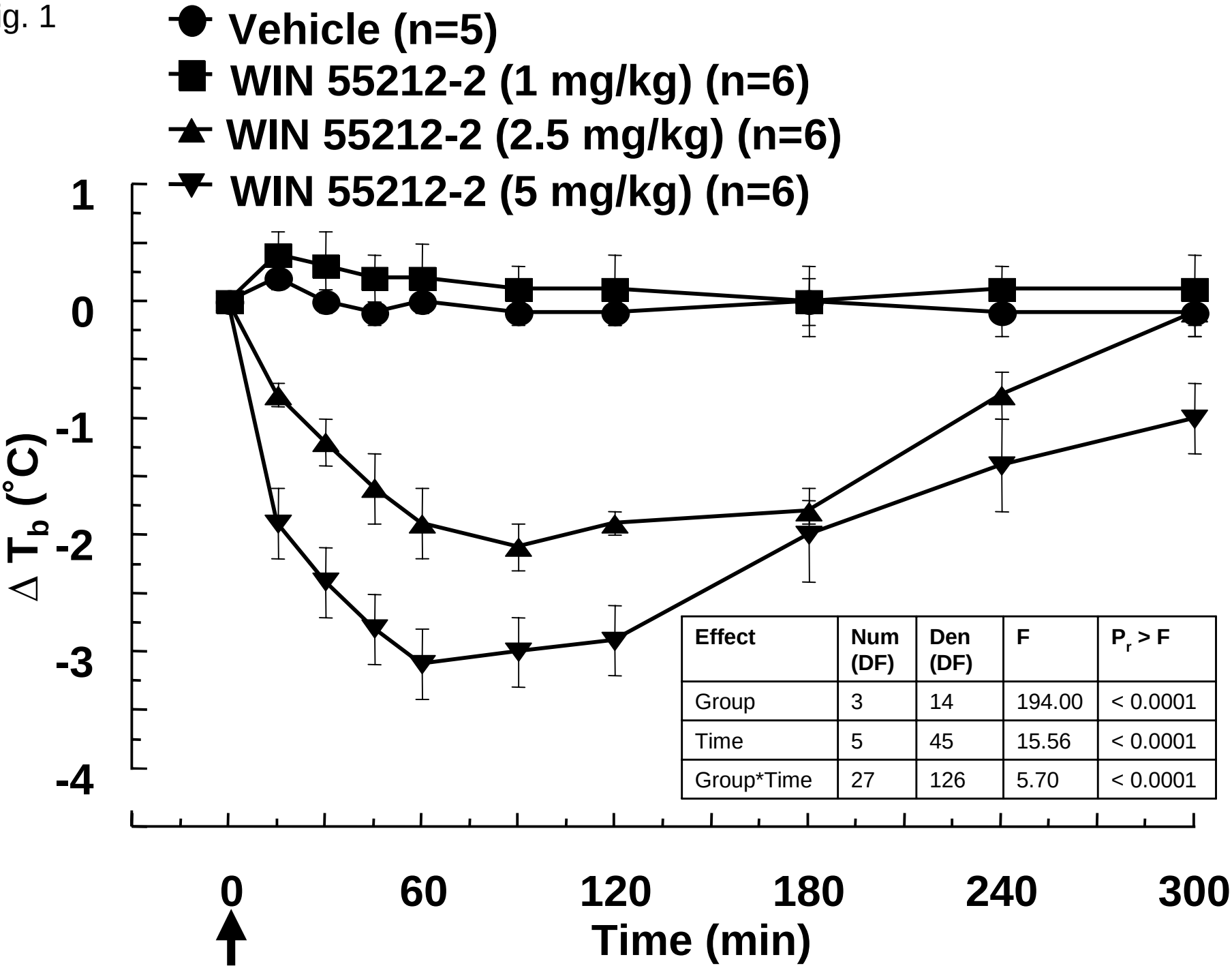
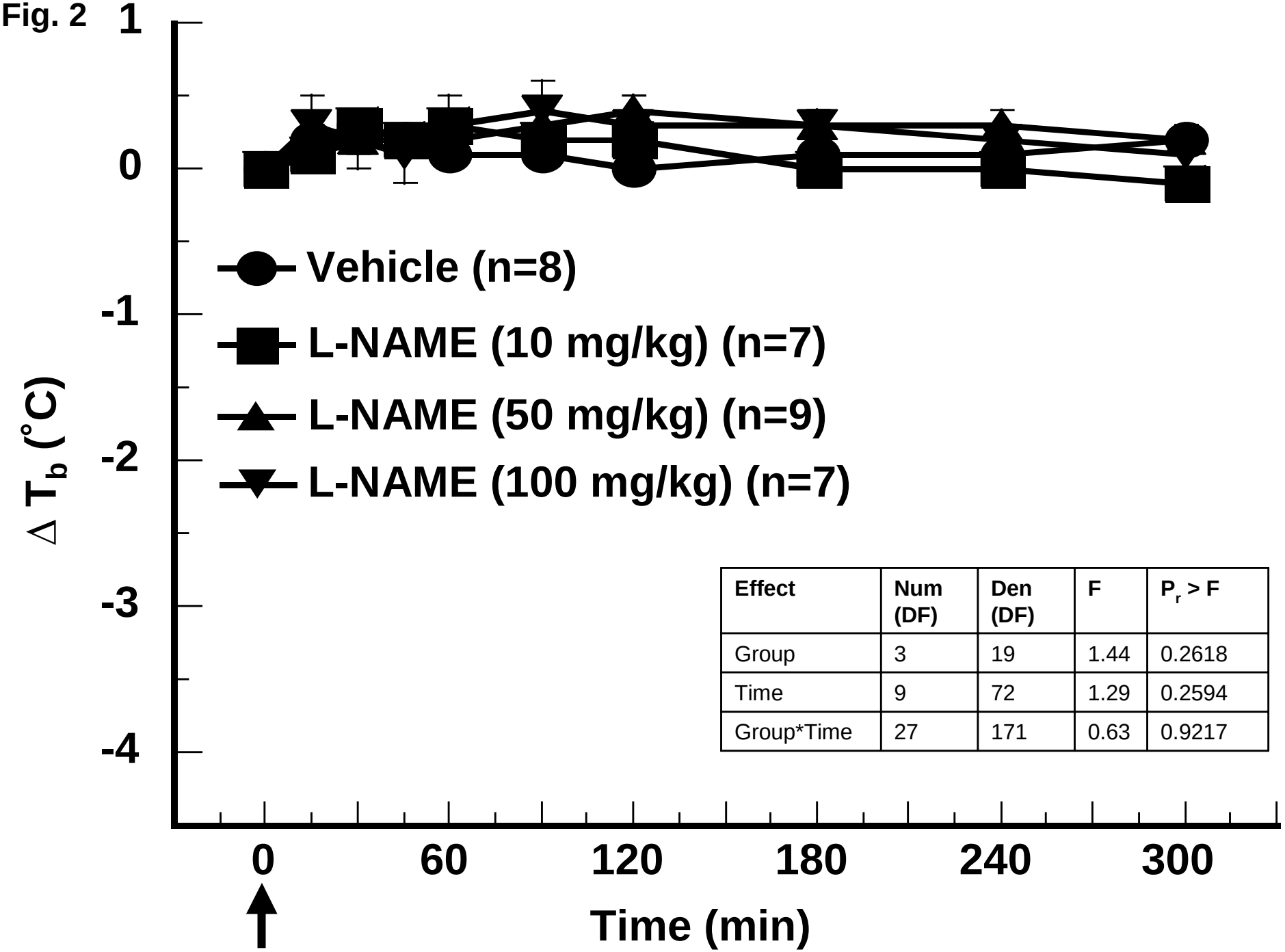
Fig. 1



Effect	Num (DF)	Den (DF)	F	$P_r > F$
Group	3	14	194.00	< 0.0001
Time	5	45	15.56	< 0.0001
Group*Time	27	126	5.70	< 0.0001

Fig. 2



Effect	Num (DF)	Den (DF)	F	$P_r > F$
Group	3	19	1.44	0.2618
Time	9	72	1.29	0.2594
Group*Time	27	171	0.63	0.9217

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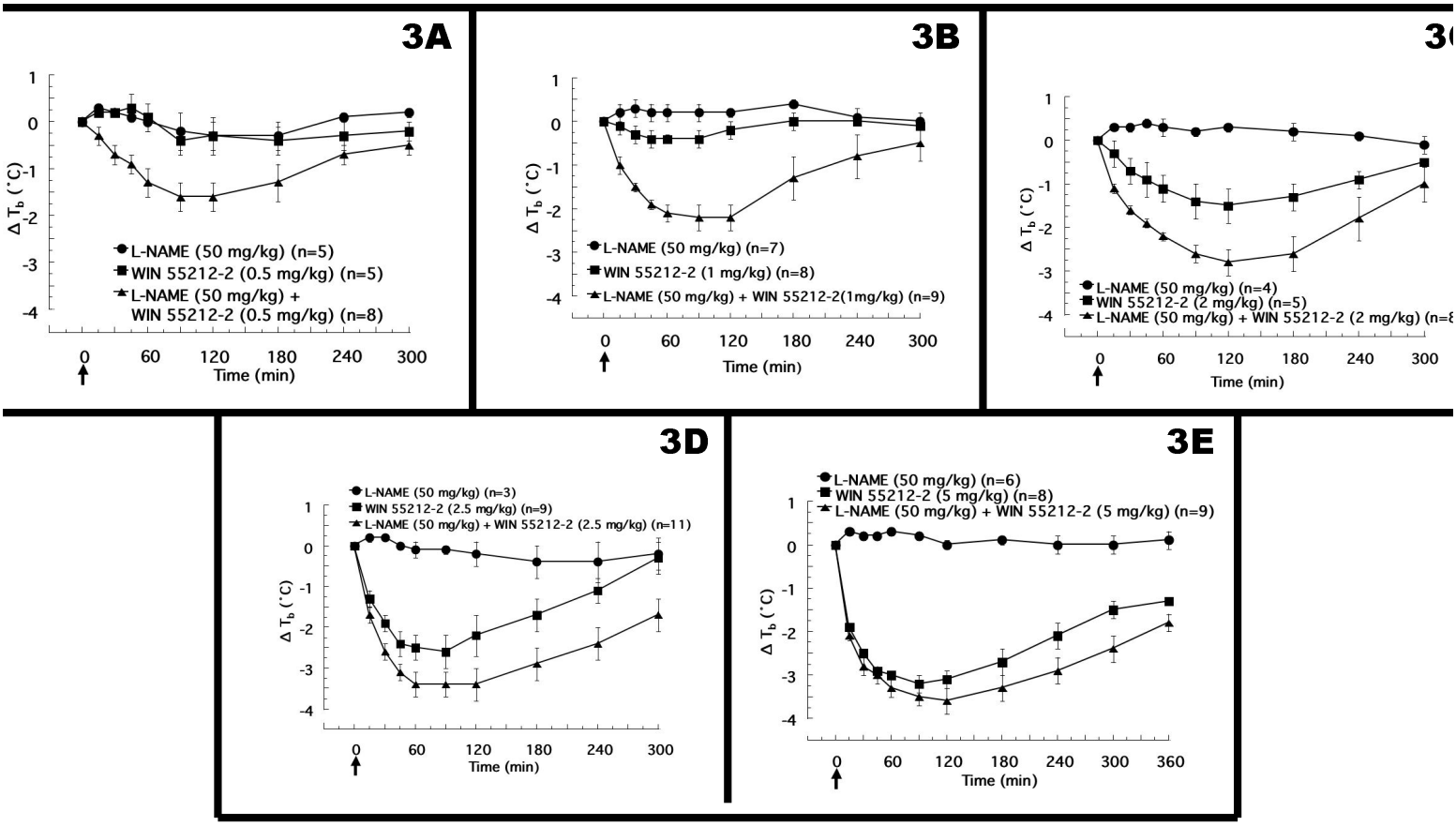

Fig. 4

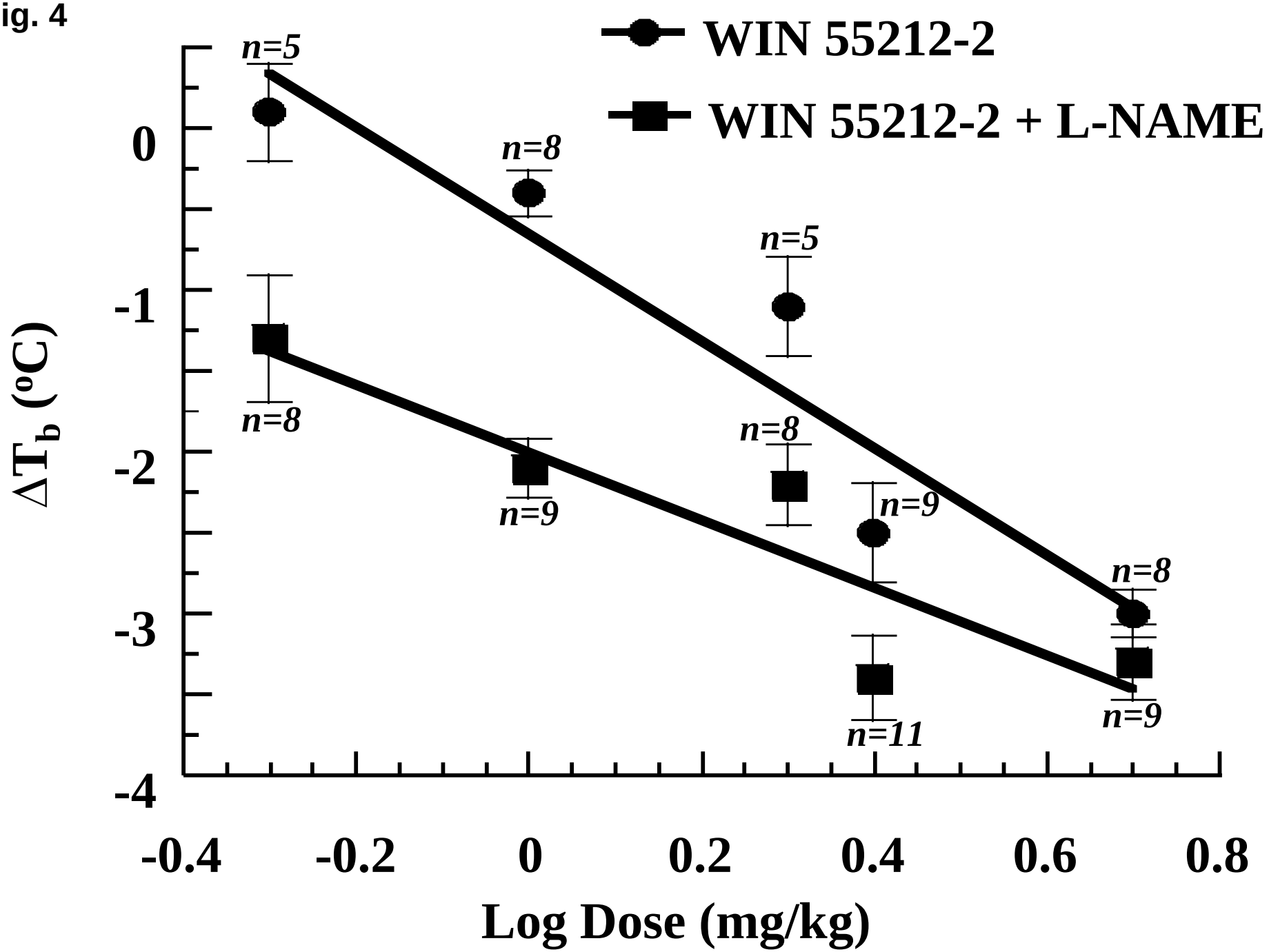

Fig. 5