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# Synergistic anti-allodynic effects of nociceptin/orphanin FQ and cannabinoid systems in neuropathic mice ☆

Ozgur Gunduz, Hakan C. Karadag, Ahmet Ulugol *

*Department of Pharmacology, Faculty of Medicine, Trakya University, 22030-Edirne, Turkey*

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ABSTRACT

Combinations of analgesics from different classes are commonly used in the management of chronic pain. The goal is to enhance pain relief together with the reduction of side effects. The present study was undertaken to examine the anti-allodynic synergy resulting from the combination of WIN 55,212-2, a cannabinoid CB1 receptor agonist, and JTC-801, a nociceptin/orphanin FQ receptor antagonist, on neuropathic pain. Mice were tested for behavioral effects before and 2–4 weeks after the surgery, in which a partial tight ligation of the sciatic nerve was made. Nerve injury-induced mechanical allodynia was assessed with Dynamic Plantar Aesthesiometer, and a hot/cold plate was used to assess cold allodynia. Both WIN 55,212-2 and JTC-801 produced dose-dependent mechanical and cold anti-allodynic effects. As shown by isobolographic analysis, WIN 55,212-2/JTC-801 combinations interacted synergistically at all three ratios studied in the mechanical allodynia assay. In conclusion, co-administration of a cannabinoid with a nociceptin/orphanin FQ receptor antagonist resulted in a synergistic interaction, which may have utility in the pharmacological treatment of neuropathic pain.



## 1. Introduction

Nerve injury that affects peripheral nerves leads to abnormal pain states referred to as neuropathic pain. This chronic pain condition is generally accepted to be relatively refractory to drug therapy, including the potent analgesics, opioids (MacFarlane et al., 1997; Benedetti et al., 1999; Watson, 2000; Przewlocki and Przewlocka, 2005). Gabapentin and tricyclic antidepressants are often prescribed, with variable responses and effectiveness (MacFarlane et al., 1997). Thus, there is apparently a need for effective drugs for relieving chronic pain complaints.

Decreased drug efficacy in the treatment of neuropathic pain states directed researchers to search for alternative strategies. A strategy is to combine low doses of analgesic drugs from different pharmacological classes (Hernandez-Delgadillo et al., 2003; Ulugol et al., 2002, 2006b; Zelcer et al., 2005; Guneli et al., 2007). This combination approach not only minimizes specific adverse effects of each of the drugs at a higher dose, but also sometimes leads to enhanced pain relief (Raffa, 2001; Pelissier et al., 2003). Therefore, new strategies based on drug combinations need to be considered.

Cannabinoids and opioids are potent analgesics that have similar pharmacological properties, such as analgesia, sedation, hypothermia and hypoactivity (Fuentes et al., 1999; Pertwee, 2001; Ulugol, 2009). Accumulating evidence suggests a role for cannabinoids in nociception, particularly in chronic pain conditions (Herzberg et al., 1997; Bridges et al., 2001; Fox et al., 2001); however, their clinical usage is limited by serious adverse effects, such as sedation, memory impairment, and psychotropic effects (Fuentes et al., 1999; Piomelli et al., 2000; Pertwee, 2001). Nociceptin/orphanin FQ (N/OFQ), on the other hand, is the endogenous ligand for N/OFQ peptide receptor (NOP), the fourth member of opioid receptor family. Results obtained from studies on its effect on nociception are contradictory, but it is generally accepted that N/OFQ exerts spinal analgesic and supraspinal hyperalgesic effects (Heinricher, 2005). Moreover, activation of the endogenous N/OFQ system after nerve injury has been suggested (Mika et al., 2004; Obara et al., 2005), and selective NOP receptor antagonists have been shown to exert anti-allodynic and anti-hyperalgesic effects in neuropathic rats (Tamai et al., 2005).

The aim of this study was to determine if the combination of WIN 55, 212-2, a cannabinoid agonist, and JTC-801, a N/OFQ antagonist, showed synergism in a mouse model of neuropathic pain. This attempt may provide clinicians the use of low dose cannabinoid–N/OFQ antagonist combination as a new treatment option in neuropathic patients.

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* Corresponding author at: Trakya University, Faculty of Medicine, Department of Pharmacology, 22030-Edirne, Turkey. Tel.: +90 284 2353925; fax: +90 284 2353925.
*E-mail addresses:* aulugol@trakya.edu.tr, aulugol@yahoo.com (A. Ulugol).

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## 2. Materials and methods

### 2.1. Animals and ethics

Male Balb-c mice (Center of the Laboratory Animals, Trakya University), weighing 25–30 g at time of operation, were used. Animals were housed in groups of ten in a quiet room, and water and food were provided *ad libitum*. This study was conducted according to the guidelines of the Ethical Committee of the International Association for the Study of Pain (Zimmermann, 1983), and the experimental protocols were approved by the local "Animal Care Ethics Committee". All efforts were made to minimize animal suffering and the number of experimental animals was kept to a minimum to produce a reliable scientific data.

### 2.2. Partial sciatic nerve ligation model of neuropathic pain

#### 2.2.1. Surgical procedure

Under ketamine (100 mg/kg, i.p.)–xylazine (10 mg/kg, i.p.) anesthesia, partial sciatic nerve ligation (PSNL) was made by tightly tying 1/3 to 1/2 of the dorsal portion of the sciatic nerve, using a similar procedure to that described for rats by Seltzer et al. (1990) and for mice by Malmberg & Basbaum (1998). After confirming a complete hemostasis, the muscle and the skin were sutured and the mice were put into their cages after full recovery from anesthesia.

#### 2.2.2. Assessment of mechanical allodynia

Prior to the assessment of allodynia, animals were habituated to wire mesh bottom cages. Mechanical allodynia was assessed by using an electronic Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy). The rigid tip of the aesthesiometer, with a diameter of 0.5 mm, was applied perpendicular to the plantar surface of the hindpaw with an increasing force (0 to 5 g in 20 s) to cause brisk withdrawal, the cut-off value being 5 g. The paw withdrawal latency, defined as the time from onset of the tip to the withdrawal of the paw, was detected with the electronic aesthesiometer. Accordingly, the paw withdrawal threshold was digitally recorded in grams. Predrug latencies were assessed post-surgery. Test latencies were converted to the percentage of the maximal possible effect (%MPE) according to the following formula: $\%MPE = [(\text{postdrug latency} - \text{predrug latency}) / \text{cut-off force} - \text{predrug latency}] \times 100$.

#### 2.2.3. Assessment of cold allodynia

For assessment of cold allodynia, a cold/hot plate analgesia meter (Ugo Basile, Comerio, Italy) was used. The animals were placed on a cold plate that is maintained at a temperature of $4 \pm 0.1$ °C, and the cumulative duration of paw lifts of the injured paw were recorded for an evaluation period of 5 min (max. time). Predrug duration was assessed post-surgery. Total duration of paw lifts were converted to the percentage of the maximal possible effect (%MPE) according to the following formula: $\%MPE = [(\text{postdrug duration} - \text{predrug duration}) / \text{max. time} - \text{predrug duration}] \times 100$.

### 2.3. Drugs

WIN 55,212-2 (0.1–10 mg/kg, i.p.), and JTC-801 (0.1–10 mg/kg, i.p.) were purchased from Sigma Chemical Co. Each drug was administered 1 h before testing in a volume of 0.1 ml/10 g body weight. WIN 55,212-2 and JTC-801 were dissolved in 50% and 20% DMSO in saline, respectively. In synergy studies, drugs were co-administered in two separate injections, with an interval of 5 min. Drug doses and treatment times were chosen from previous studies (Herzberg et al., 1997; Bridges et al., 2001; Fox et al., 2001; Mabuchi et al., 2003; Suyama et al., 2003; Tamai et al., 2005; Ulugol et al., 2004, 2006b).

### 2.4. Study design and statistical analyses

In order to habituate to the environment and obtain stable responses, mice were tested for mechanical and cold sensitivity for 3 days before the testing period. Tests took place 2–4 weeks after tight ligation of the sciatic nerve, and each animal was used only once. Mechanical sensitivity was tested 5 min prior to cold sensitivity; starting with the least stressful test is known to attenuate the influence of one test on the next. Mechanical and cold allodynia were assessed immediately before, and at 1 h after i.p. injections of WIN 55,212-2 (0.1–10 mg/kg) and JTC-801 (0.1–10 mg/kg). ED50 values and drug combinations doses were going to be different in mechanical and cold allodynia tests, and using both of these tests in synergy studies was going to increase the number of the animals used. As a result, after determination of dose–response curves of the drugs, synergy studies were conducted using only the mechanical allodynia test.

Individual dose–response curves for WIN 55212-2 and JTC-801 were drawn using at least 10 animals per dose and at least 5 doses. Using mechanical allodynia test, an isobolograhic analysis was performed as described by Tallarida (2000). Briefly, $ED_{50}$ values of each agent alone were determined by linear regression analysis using the software package *Pharm Tools Pro* (The McCary Group, Emmaus, PA). Then, the 1:1, 1:3, and 3:1 fixed ratios of $ED_{50}$ values of each combination were determined. These data were evaluated as the logarithm of the total dose versus the percent maximum possible effect. The experimentally determined $ED_{50}$ value of the combination, which was expressed as the sum of the doses and denoted $Z_{mix}$, was compared to the theoretically additive $ED_{50}$ values of the combination ($Z_{add}$). The $Z_{add}$ was determined from the straight line additive isobole which connects the $ED_{50}$ values of the constituent drugs. The difference between $Z_{mix}$ and $Z_{add}$ determined whether the combination is supra-additive ($Z_{mix} < Z_{add}$), additive ($Z_{mix} = Z_{add}$), or sub-additive ($Z_{mix} > Z_{add}$) (Tallarida, 2000). Statistical analysis of combination data was described elsewhere (Codd et al., 2008; Tallarida, 2000). For the analysis, $p < 0.05$ was used as the level of significance. The calculations were made using *Pharm Tools Pro* (The McCary Group, Emmaus, PA).

## 3. Results

### 3.1. Anti-allodynic effect of WIN 55,212-2 and JTC-801

WIN 55,212-2 and JTC-801 each exhibited a dose-dependent anti-allodynic effect in both mechanical and cold allodynia tests in sciatic nerve-injured mice (Fig. 1A, B). The $ED_{50}$ values in mechanical allodynia test were determined as $0.37 \pm 0.11$ mg/kg for WIN 55,212-2 and $0.83 \pm 0.05$ mg/kg for JTC-801. In cold allodynia test, $ED_{50}$ values for WIN 55,212-2 and JTC-801 were $6.85 \pm 3.67$ mg/kg and $1.02 \pm 0.18$ mg/kg, respectively. Although we did not determine the rota rod performance or the other tetrad models, we did not observe any motor incoordination with any of the WIN 55,212-2 or JTC-801 doses tested.

### 3.2. Interactions between WIN 55,212-2 and JTC-801

Synergy studies between WIN 55,212-2 and JTC-801 were conducted in mechanical allodynia assay. As shown in Fig. 2, co-administration of WIN 55,212-2 and JTC-801 combinations produced greater anti-allodynic effects, compared with the dose–response curves of each drug alone. Accordingly, isobolographic analysis of WIN 55,212-2 and JTC-801 combination showed that the interaction of all ratios (3:1, 1:1, and 1:3) of the respective $ED_{50}$ values of the drugs were synergistic (Fig. 3). Data including specific doses, %MPE, and $ED_{50}$ values are given in Table 1.

## 4. Discussion

Although the pharmacotherapeutic treatment of neuropathic pain is constantly evolving, most of the therapeutic approaches utilized to

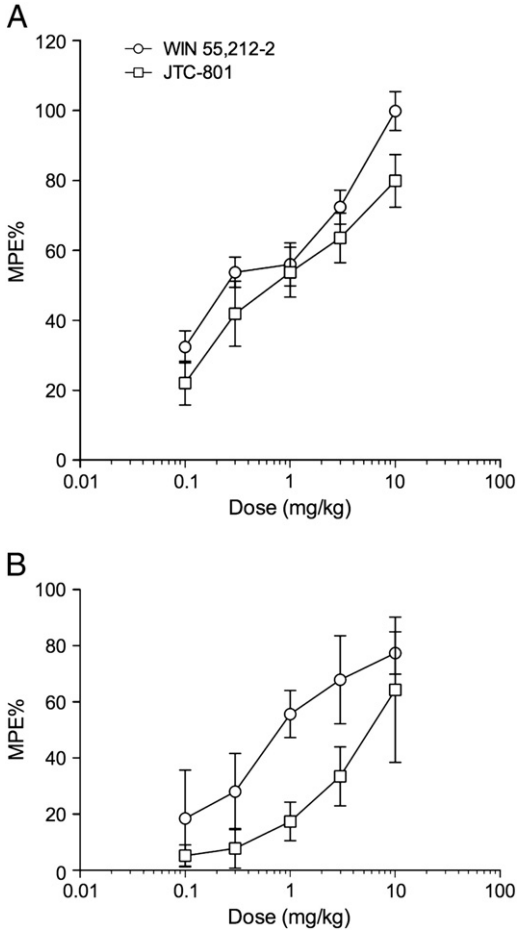


**Fig. 1.** Dose–response curves for the anti-allodynic effects of WIN 55,212-2 (0.1, 0.3, 1, 3, 10 mg/kg, i.p.) and JTC-801 (0.1, 0.3, 1, 3, 10 mg/kg, i.p.) in mechanical allodynia (A) and cold allodynia (B) tests in neuropathic mice. Each point is the mean ± S.E.M. of 10 animals.

treat neuropathic pain conditions have met with limited success, in part because of its multiple etiologies. Clinicians recommend the combination therapy for this chronic pain state; it is suggested to be

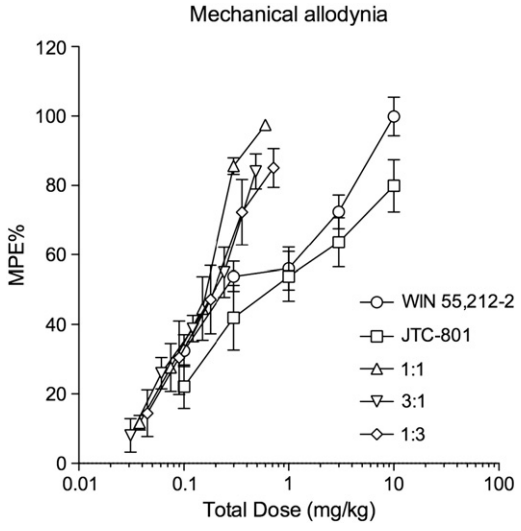


**Fig. 2.** Dose–response curves for the anti-allodynic effects of WIN 55,212-2/JTC-801 combination at 3:1, 1:1, and 1:3 ratios in mechanical allodynia test in neuropathic mice. Each point is the mean ± S.E.M. of 10 animals.

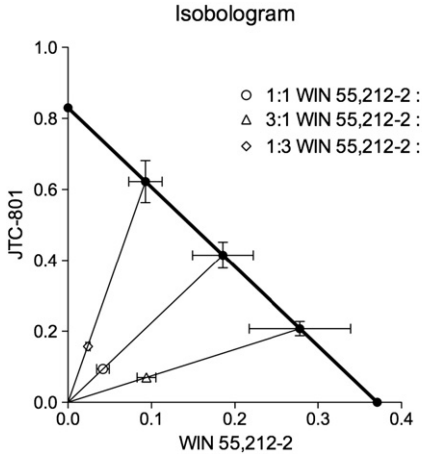


**Fig. 3.** Isobologram for the co-administration of WIN 55,212-2 and JTC-801 at 3:1, 1:1, and 1:3 ratio combinations. Filled circles above the straight line represents the theoretical $ED_{50}$ with 95% confidence limits, while open circles under the straight line represents the experimental $ED_{50}$ with 95% confidence limits.

especially useful when the selected drugs have different mechanism of action. This approach seems to be valuable not only when the drugs act synergistically, but also when the interaction is additive, since it may minimize specific adverse effects associated with the use of low doses of individual drugs. The results of the present study indicate synergy between a cannabinoid and a NOP receptor antagonist in mediating nociception in an animal model of neuropathic pain.

It is generally accepted that the analgesic activity of cannabinoids is mediated through activation of $CB_1$ receptors, which are expressed in areas involved in nociception (Fuentes et al., 1999; Piomelli et al., 2000; Fox et al., 2001; Pertwee, 2001). Moreover, cannabinoid agonists have been shown to attenuate allodynia and hyperalgesia in animal models of neuropathic pain (Herzberg et al., 1997; Bridges et al., 2001; Fox et al., 2001; Ulugol et al., 2004). Cannabinoid induced antinociception appears to occur mainly at spinal and supraspinal sites, but a peripheral action has also been suggested (Fox et al., 2001; Dogrul et al., 2003). Our findings, indicating that the cannabinoid agonist, WIN 55,212-2, dose-dependently attenuate allodynia in neuropathic mice when administered intraperitoneally, are consistent with these reports.

Administration of N/OFQ is reported to have no effect, to produce analgesia, hyperalgesia, or anti-hyperalgesia (Heinricher, 2005). However, it is broadly accepted that i.c.v. N/OFQ produce hyperalgesia, but i.t. N/OFQ produce an analgesic effect. The effect of peripheral N/OFQ on nociception is also controversial; intradermal nociceptin has been shown to evoke excitatory responses from the dorsal horn neurons, on the other hand, it has been suggested to possess a local peripheral antinociceptive action (Kolesnikov and Pasternak, 1999; Carpenter et al., 2000; Sakurada et al., 2005). N/OFQ has been proven to produce these effects by acting on NOP receptors. We showed that JTC-801, a nonpeptidergic NOP receptor antagonist, dose-dependently exerted an anti-allodynic effect in neuropathic animals. Our results are in agreement with results of Mika et al. (2004) and Obara et al. (2005), who suggested that increased activity of N/OFQ system could be the reason for lower responsiveness of opioids in neuropathic pain. Our findings are also in line with similar studies, showing that systemic JTC-801 attenuated allodynia and hyperalgesia in neuropathic animals, and NOP receptor antagonists potentiate morphine efficacy in neuropathic pain (Mabuchi et al., 2003; Suyama et al., 2003; Tamai et al., 2005; Khroyan et al., 2009).

Several drug combinations that exert additive or synergistic antinociceptive interaction have been tested. Among these, the effect of low dose cannabinoid and opioid combination on nociception is widely studied (Reche et al., 1996; Smith et al., 1998; Smith et al., 2007). Low

**Table 1**
Anti-allodynic effects of WIN 55,212-2, JTC-801, and WIN 55,212-2/JTC-801 combination in neuropathic mice.

| $ED_{50}$ value ratios (JTC 801:WIN55,212-2) | JTC 801:WIN55,212-2 drug combinations dose (mg/kg i.p.) | | | % Maximal possible effect | ED50 (SEM) or Z value (SEM) at 1 h | |
|---|---|---|---|---|---|---|
| | JTC 801 | WIN55,212-2 | Total Dose | | $Z_{mix}$ (SEM) | $Z_{add}$ (SEM) |
| JTC 801 only | 0.100 | | | 22.11 | 0.830 (0.053) | |
| | 0.300 | | | 41.96 | | |
| | 1.000 | | | 52.79 | | |
| | 3.000 | | | 62.81 | | |
| | 10.000 | | | 80.11 | | |
| 3:1 | 0.039 | 0.006 | 0.045 | 14.45 | 0.181 (0.010)* | 0.715 (0.079) |
| | 0.078 | 0.012 | 0.090 | 30.44 | | |
| | 0.156 | 0.024 | 0.180 | 47.12 | | |
| | 0.312 | 0.048 | 0.360 | 72.33 | | |
| | 0.623 | 0.095 | 0.718 | 85.08 | | |
| 1:1 | 0.026 | 0.012 | 0.038 | 11.71 | 0.136 (0.015)* | 0.601 (0.073) |
| | 0.052 | 0.024 | 0.075 | 27.61 | | |
| | 0.103 | 0.047 | 0.150 | 44.56 | | |
| | 0.207 | 0.093 | 0.300 | 88.60 | | |
| | 0.415 | 0.185 | 0.600 | 97.50 | | |
| 1:3 | 0.013 | 0.018 | 0.031 | 8.09 | 0.164 (0.015)* | 0.486 (0.083) |
| | 0.026 | 0.035 | 0.061 | 25.98 | | |
| | 0.052 | 0.070 | 0.122 | 38.80 | | |
| | 0.104 | 0.140 | 0.244 | 54.95 | | |
| | 0.208 | 0.280 | 0.488 | 84.06 | | |
| WIN55,212-2 only | | 0.100 | | 32.44 | 0.371 (0.105) | |
| | | 0.300 | | 53.80 | | |
| | | 1.000 | | 56.05 | | |
| | | 3.000 | | 72.66 | | |
| | | 10.000 | | 100.00 | | |

*$p<0.01$ versus corresponding $Z_{add}$ value, Student's t distribution (Tallarida, 2000).

dose cannabinoid enhanced the antinociceptive effect of morphine; a similar synergy was also shown when low dose opioid was used (Reche et al., 1996; Smith et al., 1998). On the contrary, the same synergy was not observed in neuropathic rats, indicating the existence of two distinctive antinociceptive systems in pathological pain (Mao et al., 2000). However, a synergistic effect with the combination of drugs affecting cannabinoid and N/OFQ systems might also be expected, since increased N/OFQ levels and upregulation in the NOP receptor have been shown (Briscini et al., 2002; Mika et al., 2004), and NOP receptor antagonists have been effective in neuropathic pain states (Mabuchi et al., 2003; Suyama et al., 2003; Tamai et al., 2005; Khroyan et al., 2009). Here, JTC-801 may diminish the activation of N/OFQ system, and potentiate the effect of the cannabinoid.

Both cannabinoid and opioid receptors are G-protein coupled, and these receptors are co-distributed in areas involved in nociception (Cichewicz, 2004). Similarly, the major intracellular effects of cannabinoid $CB_1$ and NOP receptor activation are inhibition of adenylate cyclase, hyperpolarization, and inhibition of voltage-dependent calcium channels (Rawls et al., 2007). However, these intracellular effects are unlikely to be responsible for the synergism between WIN 55,212-2 and N/OFQ, since one of them is an agonist and the other is an antagonist, and exert opposite effects. On the other hand, the analgesic effect of JTC-801 on neuropathic pain has been suggested to be mediated by inhibition of nitric oxide production (Mabuchi et al., 2003). Similarly, possible involvement of L-arginine/nitric oxide pathway has been implicated in the induction of tolerance to the analgesic effect of WIN 55,212-2 (Banafshe et al., 2005). Inhibition of L-arginine–nitric oxide pathway may be one of the mechanisms playing role in the synergistic antinociceptive interaction between cannabinois and N/OFQ systems. Another possibility that cannot be disregarded is a pharmacokinetic interaction between JTC-801 and WIN 55,212-2 (Raffa, 2001; Cichewicz, 2004). After i.p. administration, drugs are subjected to the first-pass by the liver and interactions at the level of CYPs are possible, which in consequence may influence the concentration of the drugs. Apart from this, there are other possible points of interactions, like transport, passing through the barriers, etc.

Mechanical and cold allodynia are suggested to be mediated through separate mechanisms and pathways. Mechanical allodynia is known to be mediated through large diameter, Aβ-afferent fibers, whereas small diameter, unmyelinated high threshold C-fibers is seemed to mediate cold allodynia (Kauppila, 2000; Ulugol et al., 2006a). Morphine would have been more effective against cold allodynia, since it is known to block small but not large diameter fiber evoked responses (Dickenson and Sullivan, 1986). Our test drugs, WIN 55,212-2 and JTC-801, each exhibited a dose-dependent anti-allodynic effect in both mechanical and cold allodynia tests. Their ED50 values were higher in cold allodynia test, suggesting that mechanical allodynia is more sensitive to administration of both WIN 55,212-2 and JTC-801.

Recently, cannabinoid and N/OFQ interactions have been investigated in studies suggesting that N/OFQ-induced feeding is blocked by cannabinoid antagonists (Pietras and Rowland, 2002), and cannabinoid-evoked hypothermia is attenuated by a NOP receptor antagonist (Rawls et al., 2007). Moreover, NOP receptor antagonists have been shown to potentiate morphine anti-allodynic activity in neuropathic rats (Khroyan et al., 2009). Regardless of the mechanism, to our knowledge this is the first demonstration indicating that cannabinoid–NOP antagonist combination reduces neuropathic pain behaviors in a synergistic manner. Further experiments are required to determine the side effect interaction of this combination and whether a similar synergy will be observed clinically.

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