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# Synthesis and antinociceptive properties of *N*-phenyl-*N*-(1-(2-(thiophen-2-yl)ethyl)azepane-4-yl) propionamide in the mouse tail-flick and hot-plate tests

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ABSTRACT

The goals of this study, were to synthesize *N*-phenyl-*N*-(1-(2-(thiophen-2-yl)ethyl)azepane-4-yl)propionamide (**1c**) and determine its antinociceptive properties. The effect of clonidine on **1c** antinociception and the involvement of opioid, $\alpha_2$-adrenergic, and $I_2$ imidazoline receptors in **1c** antinociception were studied. Also examined was the effect of an endothelin $ET_A$ receptor antagonist on **1c** antinociception. Synthesis of **1c** was accomplished in two steps using modifications of previously reported methods. Antinociceptive (tail-flick and hot-plate) latencies were measured in male Swiss Webster mice treated with **1c**; antagonists + **1c**; clonidine + **1c**; or antagonists + clonidine + **1c**. Mice were pretreated with naloxone (opioid antagonist), yohimbine ($\alpha_2$-adrenoceptor antagonist), idazoxan ($\alpha_2$-adrenoceptor/$I_2$-imidazoline antagonist), BU224 ($I_2$-imidazoline antagonist) or BQ123 (endothelin $ET_A$ receptor antagonist) to study the involvement of these receptors. Compound **1c** produced a dose-dependent increase in antinociceptive latencies; $ED_{50}$ values were 0.15 mg/kg and 0.16 mg/kg, respectively, in the tail flick and hot plate tests. Naloxone, but not yohimbine, idazoxan or BU224, blocked **1c** antinociception. Neither clonidine nor BQ123 potentiated **1c** antinociception. Results demonstrate that **1c** is 15-times more potent than morphine. The antinociceptive effect of **1c** is mediated through opioid receptors. The $\alpha_2$-adrenergic, $I_2$-imidazoline and endothelin $ET_A$ receptors are not involved in **1c** antinociception.



The synthesis and antinociceptive activity of a series of perhydroazepine (PHA) analogs (**1a**, Fig. 1) was reported previously.[1] The PHAs were designed as ring expanded analogs of the 4-anilidopiperidine analgesics, of which fentanyl (Fig. 1) is the prototype.[2] Studies indicated that the PHAs possessed significant antinociceptive properties. The most potent compound in this study, **1a**, exhibited antinociceptive potency that was comparable to that of morphine and about 100-fold lower than the potency of fentanyl (Table 1) in the mouse tail-flick test.[1] The antinociceptive actions of **1a** were blocked by the opioid antagonist naloxone, confirming its actions via the opioid receptors.[1] In a subsequent study, an improved synthetic method of the PHAs was employed to synthesize additional analogs and the synthesis and antinociceptive activity of **1b** was reported.[3] Compound **1b** was designed by introducing a hydroxyl group in the *N*-phenethyl side chain of **1a** (Fig. 1). This structural modification resulted in a 10-fold increase in the antinociceptive potency of **1b** in the mouse tail flick test compared to **1a** (Table 1).[3] In view of the possibility of further enhancing the antinociceptive potency of the PHAs by modifying the *N*-substituent, and expanding the scope of the structure–activity relationship of the PHAs, we synthesized the thiophen-2-yl analog **1c** (Fig. 1) and tested its antinociceptive activity in the mouse tail-flick and hot-plate tests. A similar modification to the structure of fentanyl resulted in the synthesis of NIH 10505 (Fig. 1) which proved to be almost as active as fentanyl in the mouse tail-flick assay (Table 1).[4] The involvement of opioid receptors in mediating **1c** antinociception was studied using opioid antagonist naloxone.

The $\alpha_2$-adrenergic and the $I_2$-imidazoline receptors influence opioid antinociception. Studies have shown a physical and functional interaction between opioid and $\alpha_2$-adrenergic receptors[5,6] and that the $\alpha_2$-adrenergic receptors play a variable role in opioid antinociception.[5,7] It was demonstrated that meperidine, remifentanil and tramadol but not sufentanil bind to $\alpha_2$-adrenergic receptors.[8] In another study, morphine, but not fentanyl, was shown to interact with $\alpha_2$-adrenergic receptors.[9] Morphine and tramadol antinociception is blocked by the $\alpha_2$-adrenergic receptor antagonist yohimbine.[10–13] Another study reported that the antinociceptive effects of tramadol or buprenorphine, but not of fentanyl or morphine, were significantly enhanced by pretreatment with yohimbine or in $\alpha_{2A}$-adrenergic receptor knockout mice.[14] Our own studies have shown that tramadol antinociception is not

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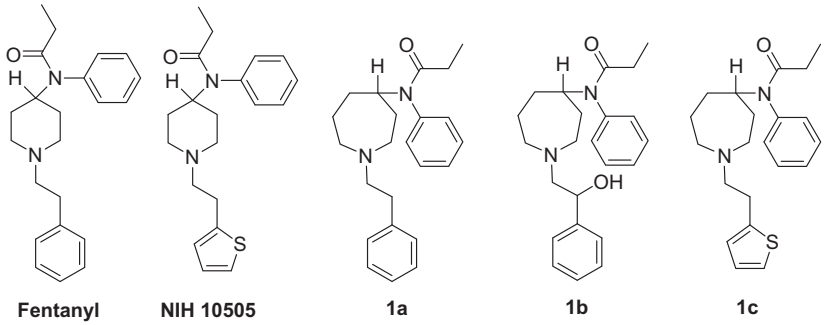



**Figure 1.** Chemical structures of fentanyl, NIH 10505, and compounds **1a**, **1b** and **1c**.

**Table 1**
Antinociceptive activities of selected compounds

| Compound | $ED_{50}$ mg/kg, sc (95% CL)[a] |
|---|---|
| Fentanyl | 0.015 (0.013–0.018)[b] |
| NIH 10505 | 0.03 (0.02–0.05)[c] |
| **1a** | 1.5 (0.9–2.6)[d] |
| **1b** | 0.13 (0.071–0.25)[b] |
| **1c** | 0.15 (0.12–0.19) |
| Morphine | 2.2 (1.5–3.2)[b] |

[a] $ED_{50}$ in mouse tail-flick assay following subcutaneous administration.
[b] Data from Ref. 3.
[c] Data from Ref. 4.
[d] Data from Ref. 1.

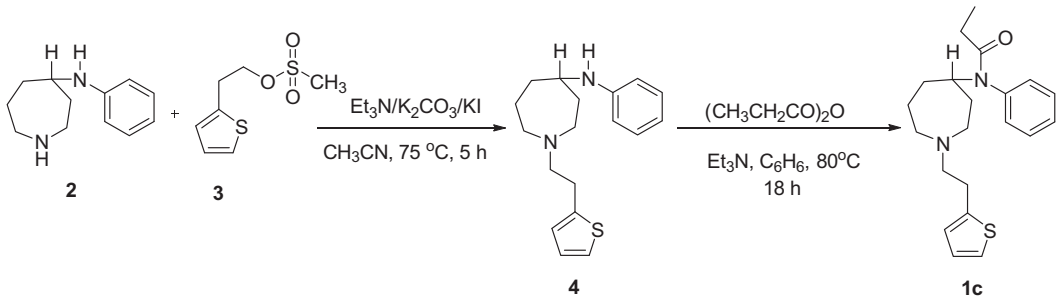
blocked by yohimbine or idazoxan ($\alpha_2$-adrenergic/$I_2$-imidazoline receptor antagonist).[15] Fentanyl antinociception was blocked by idazoxan in a model of inflammation.[16] The imidazoline $I_2$ receptor also is emerging as an important target for antinociceptives.[17] Studies have revealed a functional interaction between opioids and imidazoline $I_2$ ligands. Imidazoline $I_2$ receptor ligands enhance tramadol antinociception[18] and modulate morphine antinociception.[19] An additive antinociceptive effect was observed when morphine was co-administered with ligands selective for the imidazoline $I_2$ site.[20] Given the influence of the two receptors on opioid antinociception, we wanted to investigate the possible involvement of the $\alpha_2$-adrenergic/$I_2$-imidazoline receptors in the antinociception produced by the PHA analog **1c**. Involvement of these receptors was studied using yohimbine ($\alpha_2$-adrenergic receptor antagonist), idazoxan ($\alpha_2$-adrenergic/$I_2$-imidazoline antagonist) and BU224 ($I_2$-imidazoline receptor antagonist).[21,22]

Clonidine and other $\alpha_2$-adrenergic agonists are often used as an adjuvants to opioids.[23,24] Clonidine potentiates the antinociceptive effects of morphine,[21,25] and other opioids including meperidine, fentanyl, sufentanil and tramadol.[15,21,26–30] We have demonstrated that idazoxan or yohimbine blocks clonidine potentiation of morphine, oxycodone or tramadol antinociception implying the role of $I_2$-imidazoline/$\alpha_2$-adrenergic receptors in potentiation.[15,21] Therefore, in the present study, we were interested in examining the interaction between PHA analog **1c** and clonidine in antinociception in the absence and presence of receptor selective antagonists yohimbine, idazoxan or BU224.

It has been reported that pretreatment with endothelin $ET_A$ receptor antagonists potentiated morphine and oxycodone antinociception in rats and mice[21,31] while fentanyl, tramadol and codeine antinociception was unaffected.[15,32] Potentiation of antinociception by endothelin $ET_A$ receptor antagonists was demonstrated with μ, δ and κ-selective opioid agonists.[33] Given these results with other opioids, we were interested in studying the effect of endothelin $ET_A$ receptor antagonists on **1c** antinociception. In summary, the objectives of this study were to: (1) synthesize and characterize PHA analog **1c**; (2) determine the antinociceptive properties of **1c** in the mouse tail-flick and hot-plate tests; (3) determine the involvement of opioid, $\alpha_2$-adrenergic, and $I_2$ imidazoline receptors in **1c** antinociception; (4) study the effect of clonidine on **1c** antinociception; (5) determine the involvement of opioid, $\alpha_2$-adrenergic and $I_2$-imidazoline receptors in clonidine-mediated potentiation of **1c** antinociception; and (6) determine the effect of endothelin $ET_A$ receptor antagonists on **1c** antinociception.

All synthetic and pharmacologic methods employed in this study are described in detail in the Supplementary data. The synthesis of **1c** is outlined in Scheme 1. The 4-anilino intermediate **2** was obtained from previously reported methods[3] and was allowed to react with mesylate **3**[34] using modifications as previously described,[34] to obtain the N-alkylated intermediate **4**. Treatment of **4** with propionic anhydride afforded **1c**.[3] All intermediates and final products were obtained in satisfactory yields after purification using standard methods. Intermediates and final products were characterized with IR, $^1H$, $^{13}C$ NMR, mass spectrometry and gave spectra that corroborated with the expected structures, as cited in the Supplementary data. The free base of **1c** was converted to the citrate salt and gave elemental analysis consistent with the monocitrate salt. All pharmacological studies were conducted on the citrate salt of **1c**.

We first established a dose-antinociceptive response relationship for **1c** in the mouse tail-flick and hot-plate tests. A dose-dependent increase in tail-flick and hot-plate latencies was observed (Fig. 2). Peak effect was observed 30 min after administration of **1c**; typical baseline latencies following vehicle administration were about 2 s and 11 s for the tail-flick and hot-plate tests, respectively. Significant antinociception was noted up to 4 h after administration of the highest dose of **1c** in the tail-flick test (Fig. 2). On the other hand, in the hot-plate test, significant antinociceptive effect compared to vehicle lasted only for about 90 min (Fig. 2) after administration of **1c**. Using the time of peak effect obtained from the dose–response experiments, tail-flick and hot-plate latencies were measured 30 min after administration of five different doses of **1c** and $ED_{50}$ values were calculated using the method of Litchfield and Wilcoxon.[35] The $ED_{50}$ values for **1c** were 0.15 mg/kg and 0.16 mg/kg in the tail-flick and hot-plate tests, respectively. The prototype PHA analog **1a** has an $ED_{50}$ of 1.5 mg/kg (Table 1) in the mouse tail-flick test.[1] Thus, replacement of the phenyl ring in **1a** with the thiophene ring in **1c** (Fig. 1) resulted in a significant 10-fold increase in the antinociceptive effect of **1c**. The thiophene ring is more polar and electron rich than the phenyl ring and this could contribute to altered in vivo distribution or pharmacodynamic interactions of **1c**, resulting in enhanced antinociception compared to **1a**.[36] Interestingly, the corresponding modification of fentanyl to NIH 10505 (Fig. 1, Table 1) produced a marginal decrease in antinociceptive effect. The antinociceptive effect of **1c** was blocked by naloxone (Fig. 3) in the tail-flick and the hot-plate tests, indicating that opioid receptors are involved in **1c**




**Scheme 1.** Synthesis of **1c**.citrate.

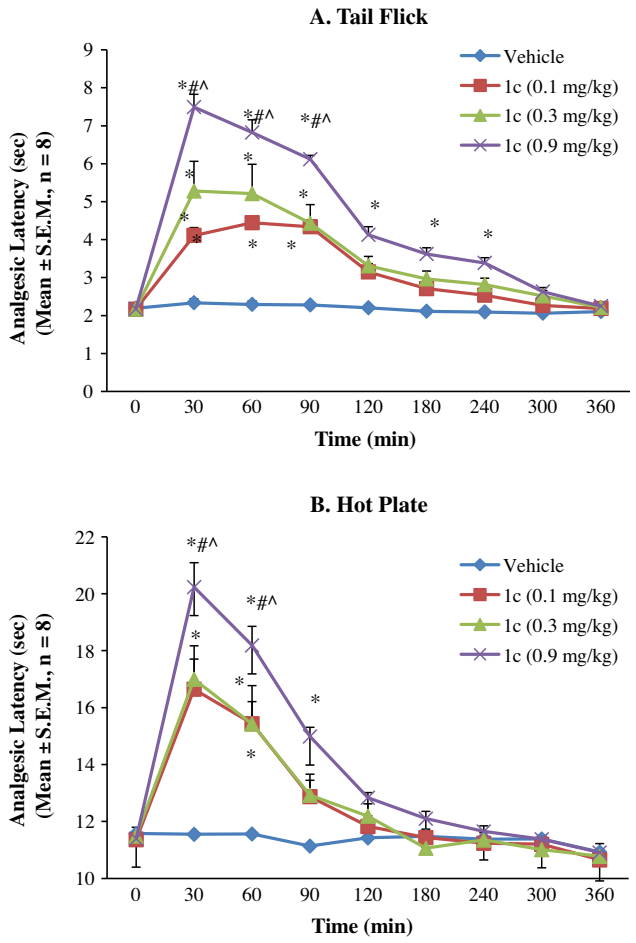



**Figure 2.** Dose–response effect of **1c**.citrate on antinociception in mice. Antinociceptive activity was determined using tail-flick (A) and hot-plate (B) tests. A dose-dependent increase in antinociception was observed. Values are means ± S.E.M.; $n = 8$ per group. $^{*}P$ <0.05 compared to control (vehicle) group, $^{\#}P$ <0.05 versus 0.1 mg/kg **1c**.citrate, $^{\wedge}P$ <0.05 versus 0.3 mg/kg **1c**.citrate.

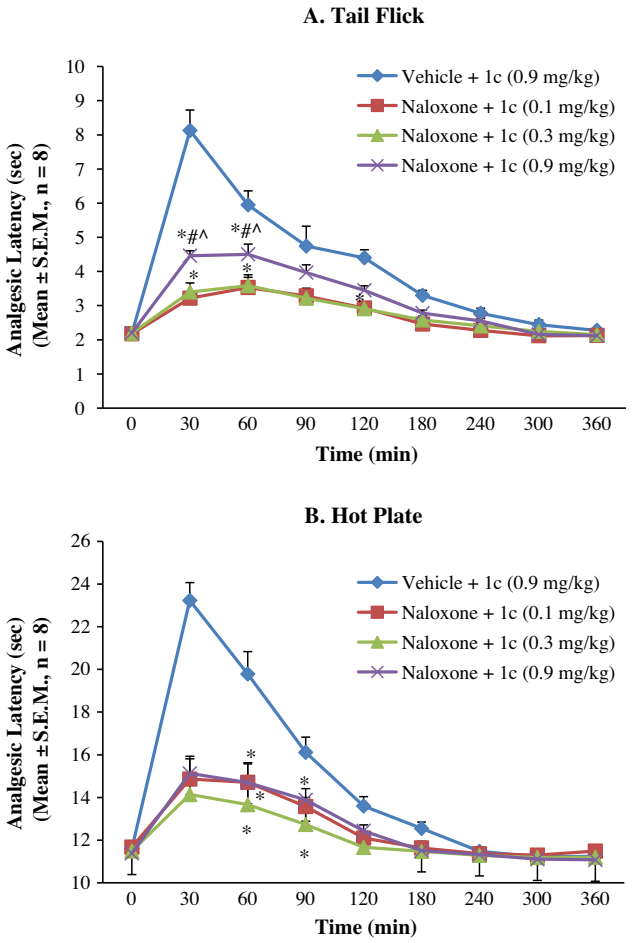



**Figure 3.** Effect of naloxone on antinociception produced by **1c**.citrate in mice. Antinociceptive activity was determined using tail-flick (A) and hot-plate (B) tests. Naloxone significantly blocked the antinociceptive effect of **1c**.citrate. Values are means ± S.E.M.; $n = 8$ per group. $^{*}P$ <0.05 versus vehicle, $^{\#}P$ <0.05 versus 0.1 mg/kg **1c**.citrate, $^{\wedge}P$ <0.05 versus 0.3 mg/kg **1c**.citrate.

antinociception. These results are consistent with previous reports on **1a** and **1b**[1,3] and suggest an overlapping mode of action for the PHAs.

To assess the involvement of $\alpha_2$-adrenergic, and $I_2$ imidazoline receptors in **1c** antinociception, mice were pretreated with either with yohimbine ($\alpha_2$-adrenergic antagonist), idazoxan ($I_2$-imidazoline/$\alpha_2$-adrenergic antagonist) or BU224 ($I_2$-imidazoline antagonist) and then given **1c**. Mice treated with vehicle produced tail-flick and hot-plate latencies of about 2 s and 11.2 s, respectively. Antinociception produced by **1c** was not blocked by any of the three antagonists (Fig. 4) indicating that $\alpha_2$-adrenergic and $I_2$ imidazoline receptors are not involved in **1c** antinociception. These results suggest that there may not be any physical or functional interaction between **1c** and the $I_2$-imidazoline/$\alpha_2$-adrenergic receptors in antinociception.

Synergistic antinociceptive interactions between clonidine and morphine have been shown to occur at the spinal level.[28,37] A decrease in antinociceptive potency of intrathecal morphine compared to wild-type mice was noted in transgenic mice lacking functional $\alpha_{2A}$ adrenergic receptors, implying a direct interaction between opioid and $\alpha_{2A}$ adrenergic receptors.[7] The morphine-clonidine synergy was found to be mediated through N-type

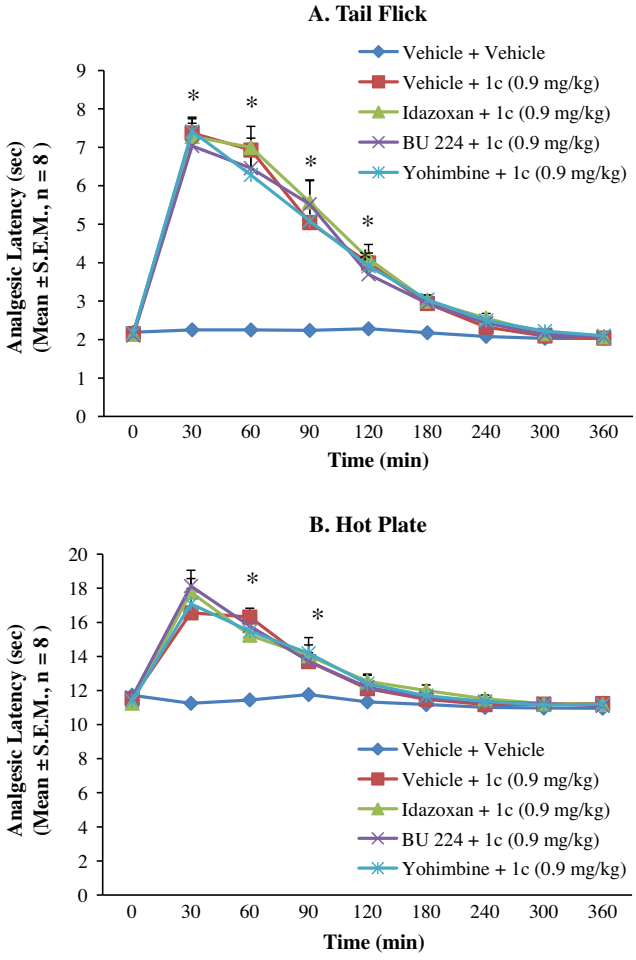



**Figure 4.** Effect of idazoxan, BU224 and yohimbine on **1c**.citrate antinociception in mice. Antinociceptive activity was determined using tail-flick (A) and hot-plate (B) tests. Idazoxan, BU224 and yohimbine did not block **1c**.citrate antinociception. Values are means ± S.E.M.; $n = 8$ per group. $^{*}P$ <0.05 compared to vehicle treated group.

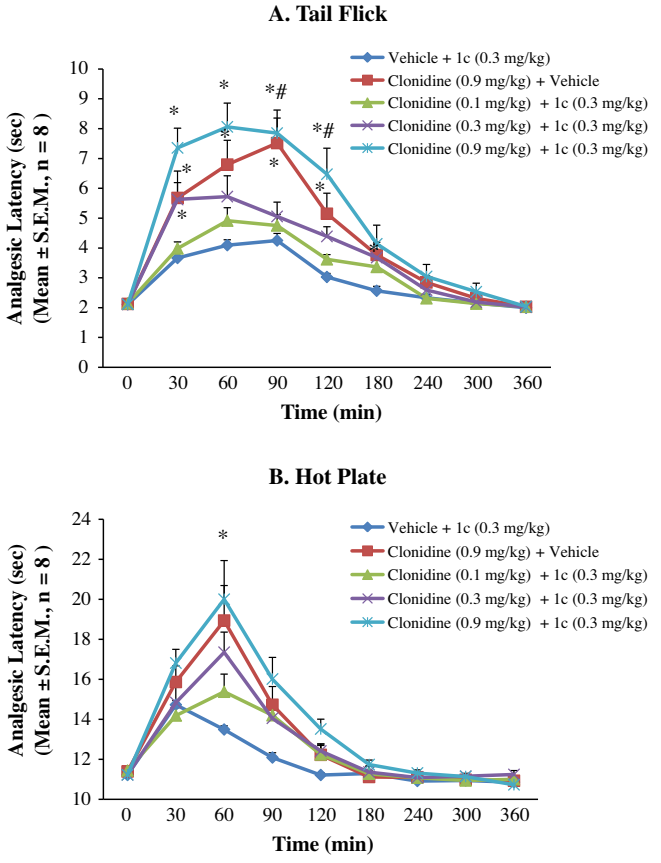



**Figure 5.** Effect of clonidine on **1c**.citrate-induced antinociception in mice. Antinociceptive activity was determined using tail-flick (A) and hot-plate (B) tests. An additive effect was observed with clonidine plus **1c**.citrate. Values are means ± S.E.M.; $n = 8$ per group. $^{*}P$ <0.05 compared to vehicle + **1c**.citrate group and $^{\#}P$ <0.05 compared to a lower dose of clonidine.

calcium channels and picrotoxin sensitive G-proteins[38] along with p38 mitogen activated protein kinase (MAP kinase) and β-arrestin 2.[39] A subsequent report showed that μ-opioid and the $\alpha_{2A}$ adrenergic receptors co-localize in hippocampal neurons where these receptors form complexes that can be activated either by a μ-agonist or an $\alpha_{2A}$ agonist, resulting in G-protein activation and an increase in MAP kinase signaling.[5] It was further understood that the μ-opioid and the $\alpha_{2A}$ adrenergic receptors form a heterodimer in brain neurons and communicate with each other via a conformational change that enables manipulation of one receptor by the other.[6] Thus, binding of morphine to the μ-opioid receptor causes a conformational change in the norepinephrine-bound $\alpha_{2A}$ adrenergic receptor that translates into inhibition of $G_i$ G-protein activation and decreased MAP kinase signaling.[6] The μ-opioid-$\alpha_{2A}$ adrenergic receptor antinociceptive synergy has been shown to occur between numerous opioids and $\alpha_2$-adrenergic agonists.[29,40] Therefore, we were interested in assessing the effect of clonidine on **1c** antinociception in mice. Administration of the combination of **1c** (0.3 mg/kg, sc) plus clonidine (0.9 mg/kg, ip) resulted in a slight increase in tail-flick and hot-plate latencies compared to the administration of **1c** (0.3 mg/kg, sc) or clonidine (0.9 mg/kg, ip) alone (Fig. 5). Therefore, this could be characterized as a mild additive effect as opposed to potentiation. Each drug probably produces its antinociceptive effect independent of the other and results in an overall increase in antinociceptive latencies. This mild additive effect was not blocked by yohimbine, idazoxan or BU 224 (data not shown). Therefore, it can be concluded that no significant interaction occurs between clonidine and **1c** through the $I_2$-imidazoline/$\alpha_2$-adrenergic receptors in antinociception. These results are in contrast to previous studies on other opioids. Clonidine has been shown to potentiate morphine,[21,25] oxycodone,[21] tramadol,[15] fentanyl,[26] sufentanil,[27] meperidine,[40] and other opioids. It is possible that **1c** may behave like fentanyl in many respects due to structural similarities. There are conflicting reports with regards to fentanyl-$\alpha_2$-adrenergic receptor interactions in antinociception. One study reports the potentiation of fentanyl antinociception by clonidine.[26] On the other hand, other studies have demonstrated that fentanyl did not exhibit affinity for the $\alpha_2$-adrenergic receptor[9] and fentanyl antinociception was unaffected in $\alpha_{2A}$ adrenergic receptor knockout mice or in wild-type mice pretreated with $\alpha_2$-adrenergic antagonists yohimbine and atipamezole.[14] Further, the $\alpha_2$-adrenoceptor has been shown to sense the conformational changes induced by various ligands differently.[41] It is possible that the changes induced by **1c** may not be sensed by the $\alpha_{2A}$-adrenergic receptors, translating to a lack of potentiation.

Finally, the effect of endothelin-$ET_A$ receptor antagonist BQ123 on **1c** antinociception was assessed. Pretreatment with endothelin $ET_A$ receptor antagonists has been shown to potentiate morphine,[31] oxycodone[21] antinociception and also antinociception mediated by selective μ-, δ- and κ-opioid agonists.[33] It has been shown that $ET_A$ receptor antagonists cause a reversal of morphine tolerance by enhancing G-protein coupling.[32,42,43] It is possible that the same mechanisms may be involved in potentiation of opioid antinociception by endothelin $ET_A$ receptor antagonists. We were

interested in determining the effects of endothelin $ET_A$ receptor antagonist BQ123 pretreatment on **1c** antinociception. Our results indicate (data not shown) that BQ123 pretreatment did not in any way influence **1c** antinociception in the mouse tail-flick and hot-plate tests. Therefore we conclude that a functional link between **1c** and endothelin $ET_A$ receptors does not exist in antinociception. These results are consistent with those obtained previously with fentanyl. Fentanyl antinociception was unaffected by pretreatment with an endothelin $ET_A$ receptor antagonist.[32] Although opioid analgesics produce their antinociceptive effects by interacting with opioid receptors, it is known that different opioid agonists can produce distinctly unique downstream events.[44] This bias amongst opioid agonists has been observed in terms of G protein coupling, receptor phosphorylation, interactions with arrestins, receptor desensitization, internalization and signaling. It is possible that the unique interactions between **1c** and opioid receptors may have activated downstream events that do not functionally overlap with the endothelin $ET_A$ receptor signaling pathways in antinociception. This might explain the lack of potentiation of **1c** antinociception by endothelin $ET_A$ receptor antagonist BQ123.

In conclusion, we report the synthesis, characterization and antinociceptive properties of thiophene PHA analog **1c**. Replacement of the phenyl ring of **1a** with a thiophene ring in **1c** resulted in a 10-fold increase in antinociceptive potency. The PHA analog **1c** is a potent antinociceptive with an $ED_{50}$ of 0.15 mg/kg and 0.16 mg/kg in the mouse tail-flick and hot-plate tests, respectively; exhibiting potency that is approximately 15-fold greater than that of morphine and about 10-fold lower than that of fentanyl. Opioid receptors are involved, and $I_2$-imidazoline and $\alpha_2$-adrenergic receptors are not involved in **1c** antinociception. Clonidine did not potentiate **1c** antinociception; no functional interaction exists between clonidine and **1c** in antinociception. Endothelin $ET_A$ antagonist BQ123 did not potentiate **1c** antinociception indicating the absence of a functional link between **1c** and endothelin $ET_A$ receptors in antinociception. Opioid receptors exclusively mediate the antinociceptive effects of **1c** in the mouse tail-flick and hot-plate tests. This is the first report demonstrating the lack of involvement of $I_2$-imidazoline, $\alpha_2$-adrenergic receptors in PHA antinociception as well as the lack of potentiation of PHA antinociception by clonidine and endothelin $ET_A$ receptor antagonists.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.11.069.

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