Biological profile and bioavailability of imidazoline compounds on morphine tolerance modulation

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ABSTRACT

Tolerance to opioid administration represents a serious medical alert in different chronic conditions. This study compares the effects of the imidazoline compounds **1**, **2**, and **3** on morphine tolerance in an animal model of inflammatory pain in the rat. **1**, **2**, and **3** have been selected in that, although bearing a common scaffold, preferentially bind to α_2-adrenoceptors, imidazoline I_2 receptors, or both systems, respectively. Such compounds have been tested *in vivo* by measuring the paw withdrawal threshold to mechanical pressure after complete Freund's adjuvant injection. To determine the ligand levels in rat plasma, an HPLC-mass spectrometry method has been developed. All the compounds significantly reduced the induction of morphine tolerance, showing different potency and duration of action. Indeed, the selective imidazoline I_2 receptor interaction (**2**) restored the analgesic response by maintaining the same time-dependent profile observed after a single morphine administration. Differently, the selective α_{2C}-adrenoceptor activation (**1**) or the combination between α_{2C}-adrenoceptor activation and imidazoline I_2 receptor engagement (**3**) promoted a change in the temporal profile of morphine analgesia by maintaining a mild but long lasting analgesic effect. Interestingly, the kinetics of compounds in rat plasma supported the pharmacodynamic data. Therefore, this study highlights that both peculiar biological profile and bioavailability of such ligands complement each other to modulate the reduction of morphine tolerance. Based on these observations, **1-3** can be considered useful leads in the design of new drugs able to turn off the undesired tolerance induced by opioids.

Keywords: α_2-adrenoceptors, imidazoline I_2 receptors, inflammatory pain, morphine tolerance, bioavailability.

Chemical compound studied in this article: 1, allyphenyline (PubChem CID 24906198); **2** (PubChem CID 3086491); **3** (PubChem CID 44269006); morphine (PubChem CID 5288826).

1. Introduction

Therapeutic use of opioids represents the standard of care in the treatment of severe chronic pain and cancer-related pain. The reduction of the analgesic effect and the need to minimize the abstinence syndrome require an increased and continued opioid dosing (Veilleux et al., 2010). Tolerance and dependence after chronic opioid exposure are the final result of a complex network of adaptation at molecular, cellular and neural level. Such adaptation concerns both opioid and non-opioid systems (Wu et al., 2008). Therefore, agents affecting indirectly the opioid network might represent useful tools in opioid management. Indeed, some of them, behaving as "biphasic opioid function modulators", enhance opioid analgesia and inhibit opioid tolerance and dependence (Su et al., 2003).

α_2-Adrenoceptors have been demonstrated to be extremely sensitive to opioid exposure (Streel et al, 2006). They have been classified into α_{2A}, α_{2B}, and α_{2C} subtypes: α_{2A} receptor mediates hypotension, sedation and analgesia, α_{2B} vasoconstriction, while α_{2C} contributes to adrenergic-opioid synergy (Tan and Limbird, 2006). Clonidine, an α_2-adrenoceptor agonist devoid of α_2 subtype selectivity, has been clinically used in pain management but, due to its α_{2A} subtype activation, is responsible for sedation and hypotension side effects. Therefore, selective α_{2C}-adrenoceptor agonists might represent alone or in combination with opioid analgesics an improvement over current therapies with clonidine-like drugs.

To overcome the side effects of opiate drugs, the synergism with compounds interacting with imidazoline I_2 receptors has been reported (Dardonville and Rozas, 2004). The imidazoline receptor family includes I_1 receptors regulating cardiovascular function, I_2 involved in central nervous system pathologies such as Parkinson's disease, depression, tolerance and addiction to opioids, and I_3 representing a potential target for the treatment of diabetes (Dardonville and Rozas, 2004; Nikolic and Agbaba, 2012; Reynolds et al., 1996; Ruiz-Durántez et al., 2003). Moreover, I_2 receptors are present in brain areas involved in perception and response

to painful stimuli (Ruggiero et al., 1998). Since it has been observed a potentiation of the analgesic effect of morphine by agmatine (Regunathan, 2006) (a possible endogenous ligand of imidazoline receptors) and a significant decrease of the imidazoline receptor density in different brain regions after chronic morphine treatment (Su et al., 2001), it is reasonable to hypothesize the involvement of I_2 receptors in the modulation of pain and in the pharmacological effects of opioids.

This study compares the effects of three imidazoline compounds (**1**-**3**) on morphine tolerance in an animal model of inflammatory pain in the rat. These compounds were selected in that, though bearing a common pharmacophore, were able to provide preferential recognition of α_2-adrenoceptors (**1**) (Del Bello et al., 2013), I_2 receptors (**2**) (Gentili et al., 2008a) or both systems (**3**) (Del Bello et al., 2013) (Fig. 1, Table 1). To determine the ligand levels in rat plasma, an HPLC-mass spectrometry method has also been developed.

2. Materials and methods

2.1. Drugs

Compound **1** (2-(1-(2-allylphenoxy)ethyl)-4,5-dihydro-1*H*-imidazole, allyphenyline) was obtained from 2-(2-allylphenoxy)propanenitrile by treatment with sodium methoxide and ethylenediamine (Gentili et al., 2008b). Compound **2** (2-(2-(naphthalen-1-yl)ethyl)-4,5-dihydro-1*H*-imidazole) was obtained starting from methyl 3-(naphthalen-1-yl)propanoate by treatment with ethylenediamine and trimethyl aluminium (Gentili et al., 2008a). Compound **3** (2-((2-allylphenoxy)methyl)-4,5-dihydro-1*H*-imidazole) was obtained by condensation of 2-allylphenol with 2-(chloromethyl)-4,5-dihydro-1*H*-imidazole in the presence of sodium ethoxide (Brasili et al., 1995).

2.2. Animal subjects

Male Wistar rats (Harlan, S. Pietro al Natisone, UD, Italy) weighing 250-300 g were housed with ad libitum access to food and water, in a temperature-controlled room with a 12-hour light/dark cycle. All the experimental procedures described were in compliance with international laws and policies (Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

2.3. Analgesic assay

Unilateral inflammation was induced by injecting 150 µl of a 50% solution of Freund's adjuvant (CFA) (Sigma Aldrich, Milan, Italy) in physiological saline into the plantar surface of the right hind paw of the rat. CFA was injected 24 h before test drugs administration. A sham control group injected with saline was always present for comparison. Paw withdrawal threshold to mechanical pressure was measured with a Randall-Selitto analgesymeter (Ugo Basile, VA, Italy) before CFA injection (healthy animal basal threshold), 24 hours after CFA injection (inflamed paw basal threshold) and at different time after drugs administration. Morphine tolerance was induced by administering morphine 5 mg/kg subcutaneously (s.c.), twice a day, for 4 consecutive days after CFA induced inflammation in the paw. To assess the effects on morphine tolerance, **1**-**3** were administered twice a day for 4 days at a dose of 0.5 mg/kg intraperitoneally (i.p.) 15 min before morphine.

Unless otherwise noted, all experimental and control groups contained six animals per group. Antinociceptive effect was expressed as percent of the maximum possible effect (MPE) according to the following formula: %MPE = (measured threshold – mean vehicle threshold/cut off - mean vehicle threshold) x 100. All data were expressed as mean ± S.E.M.

2.4. Determination of the ligand levels in rat plasma

2.4.1. Blood sample collection

For the pharmacokinetic analysis of the compounds **1** and **2** blood samples (200-300 µl), were taken from the rat tail vein at 30, 60, 90 and 120 min after drugs injection and collected in heparinised eppendorf tubes. The samples were kept on ice and then immediately centrifuged at 4 °C for 15 min at 2000 g to allow plasma separation: then they were stored at 4 °C until analysis.

2.4.2. Materials and standards

Individual stock solutions of **1** or **2** were prepared by dissolving 5 mg of each compound in 5 ml of methanol and stored in glass-stopper bottles at 4°C. Standard working solutions, at various concentrations, were daily prepared by appropriate dilution of aliquots of the stock solutions in methanol. HPLC-grade methanol and HPLC-grade acetonitrile were supplied by Sigma-Aldrich (Milano, Italy) and HPLC-grade formic acid was supplied by Merck (Darmstadt, Germany). Deionised water (>18 MΩ cm resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA). All the solvents and solutions were filtered through a 0.45-µm PTFE filter from Supelco (Bellefonte, PA, USA) before use.

2.4.3. Extraction procedure for rat plasma samples

To 0.05 ml heparinised plasma samples 0.05 ml of acetonitrile was added, the organic phase was vortexed for 30 sec. and then centrifuged at 13000 rev/min for 20 min. The supernatant was evaporated, made back with acetonitrile and transferred to a vial with 250 µl micro-volume insert (polypropylene). Afterwards, 1 µl was filtered and then injected onto the LC-MS system.

2.4.4. LC-MS conditions

Analytical: the analysis of compounds was achieved on an analytical column Synergi Hydro-RP 80Å (250 x 4.60 mm I.D., 4 µm) from Phenomenex (Chesire, U.K.). The mobile phase for LC/ESI-MS (single quadrupole) analyses was a mixture of (A) water with 0.1% formic acid, and (B) acetonitrile with 0.1% formic acid, flowing at 0.8 ml min^{-1} in isocratic conditions:

60% A, 40% B. LC/MS studies were performed using a Hewlett Packard (Palo Alto, CA, USA) HP-1090 Series II, made of an autosampler and a binary solvent pump, with a mass spectrometer detector equipped with an ESI interface in positive ionization mode. The optimized parameters of the ESI interface were: vaporizer temperature, 325 °C; nebulizer gas (nitrogen) pressure, 50 psi; drying gas (nitrogen) flow rate, 13 ml min-1; temperature, 350°C; capillary voltage, 3500 V. Data were acquired using the selected ion monitoring (SIM) mode. The SIM ions monitored during the run were 231.1 m/z for **1** and 225.1 m/z for **2** both with Fragmentor 75 eV.

2.4.5. Method validation

The method was validated by determining linearity, recovery at three fortification levels and limits of detection (LODs) and limits of quantification (LOQs). Calibration curves of the analyzed compound were constructed injecting 1 µl of mix standard solutions at six different concentrations, i.e. 0.01, 0.05, 0.1, 0.5, 1, and 5 mg l^{-1} in LC/MS technique. Three replicates for each concentration were performed, and the relative standard deviations (RSDs) ranged from 1.1 to 2.2% for run-to-run precision, and from 3.0 to 4.7 % for day-by day precision. The calibration curves of the analyzed compounds showed a correlation coefficient equal to 1.0000 (**1**) and 0.9995 (**2**). The LOD and LOQ, defined as the peak giving a response equal to a blank signal plus three and ten times the standard deviation of the noise were calculated, respectively. The LODs and the LOQs of the studied compounds, expressed in ng ml^{-1}, were calculated injecting in LC/MS standard solutions of both analytes at various concentrations. LODs value for **1** and **2** was 1 ng ml^{-1}, while LOQs value for **1** and **2** was 3 ng ml^{-1}. The recovery percentages of **1** and **2** were investigated by spiking with the standard mixture of **1** and **2** the plasma samples before extraction, for a final concentration level of 5, 10 and 50 ng ml^{-1}. Mean recoveries of the two compounds ranged from 88 to 92% with n = 5 and RSDs <4.3% for plasma samples. Retention time stability was utilized to demonstrate the specificity

of the method. Reproducibility of the chromatographic retention time for each compound was examined five times per day over a 5-day period (n = 25). The retention times using this method were stable with a percent RSD value of ≤1.82%.

2.5. Statistical Analysis

Data analysis was performed on the crude mechanical threshold values. Data were analyzed by repeated measures (RM) two-way analysis of variance (ANOVA), with $p < 0.05$ accepted as significant. Inter-group differences were assessed by either Sidak's or Dunnett's multiple comparisons test as selected by the statistical software (GraphPad Prism version 6 for Windows, GraphPad Software, La Jolla, California, USA). Time-related profiles of treatments are presented as the mean withdrawal threshold expressed in percentage of MPE (measured threshold – mean vehicle threshold/(cut off - mean vehicle threshold) x 100) ± S.E.M. at relevant time-points.

3. Results

In acute experiments compounds **1**-**3** did not show any analgesic effect when administered i.p. at the dose of 0.5 mg/kg (data not shown). Conversely, 5 mg/kg of morphine showed a potent and significant (two-way RM ANOVA: $F(3, 30)=11.76$; $P<0.0001$) analgesic efficacy peaked 30 min ($p<0.01$ vs. vehicle; Sidak's multiple comparisons test) after subcutaneous administration (Fig. 2). However, after 4 days of twice-daily administration, rats had become completely tolerant to morphine. Thus, 5 mg/kg morphine was found to be completely inactive at day 4 (Fig. 2).

Interestingly **1**-**3**, each with a different temporal profile, significantly reduced the induction of morphine tolerance (two-way RM ANOVA: $F(3, 75)=10.28$; $P<0.0001$). In particular, a sub-chronic 4 days treatment with **2**, administered twice a day 15 min before each morphine administration significantly restored at day 4 (65-70%) the morphine analgesic response. Such

a response appeared to be maximal at t=45 min ($p<0.01$ vs. vehicle; Dunnett's multiple comparisons test) and negligible at t=90 min. The repeated treatment with **1** and **3** restored at minor extent (35-40%) the morphine response but, in this case, the maximal activity was observed at t=90 min ($p<0.05$ vs. vehicle; Dunnett's multiple comparisons test) (Fig. 3). Since the different temporal profile on the tolerance reduction displayed by **1**-**3** might be associated not only to their different target profile, but also to their bioavailability, we developed an HPLC-mass spectrometry method for the determination of the ligand levels in the rat plasma. In particular, due to the similar behaviour showed by the structural analogues **1** and **3** in the tolerance reduction assays, only **1** and **2** have been selected for pharmacokinetic studies. In rat plasma, the mean serum concentration of **1** was determined to be maximum at 60 minutes (14.71 ± 0.28 ng/ml; n=3). At 30 and 90 minutes, mean concentrations of 9.48 ± 0.04 ng/ml and 8.26 ± 0.08 ng/ml, respectively, have been found. The mean serum concentration of **2** was determined to be maximum at 30 minutes (58.00 ± 3.11 ng/ml; n=3). At 60 and 90 minutes, **2** was not found in rat plasma samples, showing a faster pharmacokinetic profile vs. **1** (Fig. 4). Plasma samples were performed in triplicate, with RSDs% lower than 1.88% and 5.36% for **1** and **2**, respectively.

4. Discussion

Our studies over the years have yielded several molecules bearing the 2-substituted imidazoline nucleus as structural motif and able to interact with the α_2-adrenoceptors and/or imidazoline receptors (Cardinaletti et al., 2009; Del Bello et al., 2013; Diamanti et al., 2012; Gentili et al., 2008a; Mammoli et al., 2012). Such molecules share the common pharmacophore reported in Fig. 1. Our structure-activity relationship studies demonstrated that the chemical nature of the bridge (X) was especially responsible for preferential or multitarget recognition (Del Bello et al., 2012, 2013), whereas that of the aromatic moiety

(Ar) appeared to modulate the functional behaviour of the ligand (Gentili et al., 2004, 2008b). In particular, the -OCH(CH_3)- bridge was suitable for ligands showing significant α_2-adrenoceptor/imidazoline I_2 receptor selectivity (e.g. **1**, allyphenyline) (Table 1). The presence of the methyl group in the bridge strongly disadvantaged the I_2 receptor interaction (Gentili et al., 2003). Conversely, the -CH_2-CH_2- bridge provided ligands endowed with high I_2 receptor affinity and high selectivity over the α_2-adrenoceptors (e.g. **2**) (Gentili et al., 2003, 2008a). On the other hand, the I_2 receptor/α_2-adrenoceptor selectivity of **2** has been also confirmed by our study performed on α_{2A}-, α_{2B}-, and α_{2C} subtypes (data unpublished). Finally, the -OCH_2- bridge appeared compatible with α_2-adrenoceptor and I_2 receptor recognition (e.g. **3**) (Gentili et al., 2003; Del Bello et al., 2013).

Our recent studies by the radiant heat tail-flick test showed that compound **1** (allyphenyline), an α_{2C}-adrenoceptor agonist/α_{2A}-adrenoceptor antagonist, administered i.p. at low dose (0.05 mg/Kg) 15 min before morphine administration, enhanced morphine analgesia (due to its α_{2C}-adrenoceptor agonism), without sedative side effects (due to its α_{2A}-adrenoceptor antagonism) (Cardinaletti et al., 2009). We also demonstrated that allyphenyline significantly reduced morphine tolerance and dependence (Del Bello et al., 2010). Interestingly, such beneficial effects were associated to a significant antidepressant action (Del Bello et al., 2012). In addition, allyphenyline at the same dose reduced the anxiety-like behaviour after alcohol intoxication (Ubaldi et al., 2015).

Even if at higher dose (10 mg/Kg), the selective I_2 receptor compound **2**, injected s.c. and evaluated by radiant heat tail-flick test, significantly enhanced morphine-induced analgesia (Gentili et al., 2008a).

Finally **3**, a multitarget compound characterized by α_{2C}-adrenoceptor agonism/α_{2A}-adrenoceptor antagonism and nanomolar affinity for I_2 receptors, similarly to allyphenyline reduced morphine-induced withdrawal syndrome and depression-like behaviour. This effect

253 was completely blocked by idazoxan, a mixed α_2-adrenoceptor/I_2 receptor antagonist (Del
254 Bello et al., 2013).
255 The present study, showing the ability of **1**-**3** to significantly reduce the induction of
256 morphine tolerance, confirms the favourable involvement of α_{2C}-adrenoceptor agonism and
257 imidazoline I_2 receptor interaction in such an effect. Interestingly, the sub-chronic treatment
258 with **2** significantly restored the lost morphine analgesic efficacy (65-70%) by maintaining the
259 same time-dependent profile displayed after a single morphine administration on day 1.
260 Indeed, the analgesic response was maximal at t=45 min and negligible at t=90 min. (Fig. 3).
261 Conversely, in the case of **1** and **3** the morphine analgesic response was restored at minor
262 extent (35-40%) but it proved to be significantly prolonged, the maximal activity being
263 observed at t=90 min (Fig. 3).
264 The modulation of morphine tolerance resulted to be not related to the morphine analgesia
265 enhancement. Indeed, though on a classical acute paradigm of pain on healthy animals
266 allyphenyline (**1**) significantly enhanced morphine analgesia (Cardinaletti et al., 2009), in the
267 present experimental protocol in animals made inflamed by a previous treatment with CFA
268 (sub-chronic pain model) **1**-**3** did not affect the analgesic effect of morphine (data not
269 shown). The discrepancies found between different experimental models are not surprising
270 and may be attributed to both the difference of species (mice vs. rats), stimulus (heat vs.
271 pressure), and condition (healthy vs. inflamed).
272 Interestingly, the pharmacodynamic behaviours of the studied compounds evidenced an
273 activity pattern that was in keeping with their pharmacokinetic profile. In fact, according to
274 the biological results, in rat plasma the mean serum concentration was maximum at 30
275 minutes (58.00 ± 3.11 ng/ml) for **2** and at 60 minutes (14.71 ± 0.28 ng/ml) for **1** (Fig. 4). This
276 observation suggested that the different temporal profile displayed on the tolerance reduction
277 could be affected by their different bioavailability. However, the role played by the peculiar *in*

vitro biological profile of the ligand in its pharmacological effect should be also considered. Indeed, whereas the selective engagement of the imidazoline I_2 receptors produced by **2** might contribute to induce an almost full restoring of morphine activity, the selective α_{2C}-adrenoreceptor activation induced by **1** or the combination between α_{2C}-adrenoreceptor activation and imidazoline I_2 receptors engagement (**3**) might promote a change in the temporal profile of morphine analgesia by maintaining a mild but long lasting analgesic effect. However, for compound **3** a slight tendency to provide a more prolonged effect can also be observed (Fig. 3).

These results deserve to be replicated in follow-up studies by using animal models of chronic pain resembling closer those human conditions that need morphine or other opioid drugs as the only available drugs able to alleviate pain.

5. Conclusion

This study (i) ascertains the positive effects of **1**-**3** on the morphine tolerance induction; (ii) highlights that the biological profile and bioavailability of such ligands complement each other to govern the potency and the duration of the displayed effect and (iii) provide useful suggestions for the design of novel tools potentially suitable in the morphine tolerance management.

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Figure Legends

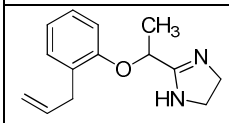
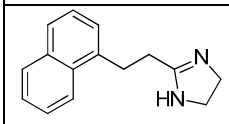
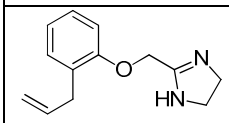
Fig. 1. Molecular structures of the imidazoline compounds **1**, **2**, and **3** sharing a common pharmacophore characterized by an aromatic moiety (Ar) linked to the position 2 of the imidazoline nucleus by a bridge (X).

Fig. 2. Effect of acute (day 1) or sub-chronic (day 4) 5 mg/kg morphine administration in a rat model of inflammatory pain (i.e. CFA- induced mechanical hyperalgesia). Day 1 label indicates rats administered acutely with morphine or saline, respectively. Day 4 label indicates rats administered twice a day, for 4 consecutive days with morphine or saline, respectively. **p<0.01 morphine-day 1 vs. vehicle–day 1. Data are expressed as mean (% MPE) ± S.E.M.

Fig. 3. Effect of sub-chronic (day 4) 5 mg/kg morphine administration in a rat model of inflammatory pain (i.e. CFA- induced mechanical hyperalgesia). Morphine was administered twice a day, for 4 consecutive days in the absence or presence of 0.5 mg/kg of **1**, **2**, and **3**, respectively. **p<0.01 morphine + compound **2** vs. morphine; *p<0.05 morphine + compound **1** vs. morphine; *p<0.05 morphine + compound **3** vs. morphine . Data are expressed as mean (% MPE) ± S.E.M.

Fig. 4. Pharmacokinetic analysis showing the concentrations of **1** and **2** (ng/ml) in rat plasma collected at 30, 60, 90 and 120 min after drugs injection. Compounds were administered i.p. at a dose of 0.5 mg/kg. Data are expressed as mean (ng/ml) ± S.E.M. of each treatment groups.

Table 1. Affinity (pK_i), Antagonist Potency (pK_b), Agonist Potency (pEC_{50}), and Intrinsic Activity (i.a.) on Human α_2-Adrenoceptor Subtypes; Affinity (pK_i) on imidazoline I_2 receptors on Rat Brain Membranes.

compound	α_{2A}		α_{2B}		α_{2C}		I_2
	pK_i	pK_b	pK_i	pEC_{50} (i.a.)	pK_i	pEC_{50} (i.a.)	pK_i
 **1**	7.24	7.40	6.47	NA[a]	7.07	7.30 (0.90)	5.82
 **2**	< 5	-	< 5	-	< 5	-	8.94
 **3**	6.90	6.50	6.15	6.01 (0.60)	7.15	7.21 (0.73)	8.88

Data were expressed as means ± S.E.M. of 3−6 separate experiments. [a]Compounds exhibiting i.a. of <0.3 were considered not active (NA).