Neuropharmacology and Analgesia

Geranylgeraniol and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester isolated from Pterodon pubescens Benth.: Further investigation on the antinociceptive mechanisms of action

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The crude alcoholic extracts obtained from Pterodon pubescens Benth. seeds are widely used in Brazilian folk medicine as anti-inflammatory, analgesic, anti-rheumatic tonics and depurative preparations. We previously demonstrated the antinociceptive activity on writhing capsaicin, glutamate, and hot-plate tests of two compounds isolated from P. pubescens: geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2). This work is a continuation of the previous study investigating the possible mechanisms of action for compounds C1 and C2, and the differences between them. The present study demonstrated that when administered intraperitoneally (i.p.): i) compounds C1 and C2 produced significant anti-allodynic activity during the acute phase of the Complete Freund’s Adjuvant (CFA)-induced persistent pain model; ii) compound C1 produced significant anti-hypernociceptive activity in the carrageenan-induced pain model; iii) compound C2 presented a significant loss of activity after p-chlorophenylalanine methyl ester hydrochloride (PCPA) [5-HT synthesis inhibitor] treatment, suggesting that the mechanisms of action could be related to either the synthesis or release of serotonin; iv) compound C1 presented a significant loss of activity after ondansetron (5-HT3 receptor antagonist) treatment suggesting activity upon 5-HT3 serotonin receptors; v) compound C1 presented a significant loss of activity after efaroxan (mixed I1 imidazoline/α2-adrenoceptor antagonist) treatment suggesting the participation of this compound upon imidazoline I1 receptors; and vi) both compounds C1 and C2 did not appear to exert their activity via 5-HT1A, 5-HT2A, 5-HT2C, α1-adrenoceptor, nitric oxide, GABAA, acetylcholine muscarinic, and nicotinic receptors when evaluated in acetic acid-induced nociception.

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1. Introduction

Pterodon pubescens Benth. (Leguminosae) seeds are commercially available at the Brazilian medicinal flora market and the crude alcoholic extract of this plant is used in folk medicine in anti-inflammatory, analgesic, and anti-rheumatic preparations (Pio Correa, 1975; Lorenzi, 1998). Phytochemical studies of Pterodon genus have revealed the presence of alkaloids, isoavlones, and diterpenes. Furan diterpenes were identified and isolated from Pterodon species (Mahjan and Monteiro, 1973; Fascio et al., 1975; Campos et al., 1994; Arriaga et al., 2000; Spindola et al., 2009). Our previous studies and those by other research groups have demonstrated that furan diterpenes possessing vouacapan skeleton are involved with the anti-inflammatory, antinociceptive and antiproliferative properties of P. pubescens seed oil (Nunan et al., 1982; Carvalho et al., 1999; Silva et al., 2004; Spindola et al., 2009, 2010). Diterpenes 6α-hydroxyvouacapan-7β-17β-lactone and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester, found in P. emarginatus and P. polygalaeorus seeds, respectively, were previously reported to be associated with the anti-inflammatory activity of these species (Nunan et al., 1982). Different authors (Duarte et al., 1996; Silva et al., 2004; Coelho et al., 2005; Spindola et al., 2010) have suggested a participation of vouacapan compounds in antinociceptive and anti-inflammatory activity.

We have previously demonstrated the antinociceptive properties of compounds geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) isolated from P. pubescens Benth. when evaluated on writhing, capsaicin, glutamate and hot-plate animal experimental models (Spindola et al., 2010). In the present study, we...
examined the potential anti-allodynic, anti-hypernociceptive effects, and some of the mechanisms involved in the antinociceptive properties (writhing test) of compounds C1 and C2. Considering that different states of pain evoke different changes, models of pain in integrated systems are essential to determine the potential analgesic activity of new drugs (Suzuki and Dickenson, 2005). For this reason, different animal models were used to better understand the antinociceptive properties observed for the compounds.

2. Materials and Methods

2.1. Phytochemistry

2.1.1. Plant Material

Seeds were previously collected in São Paulo state (São Carlos city), on March 2006 and identified by Prof. Dr. Jorge Yoshio Tamashiro from IB-UNICAMP (Department of Botany) who identified the plant species. A voucher specimen was deposited at the University of Campinas (UEC) Herbarium, under number 1402 (Spindola et al., 2009, 2010).

2.1.2. Compound Isolation

The compounds were isolated and identified in a previous work by our group (Spindola et al., 2009, 2010). Briefly, compounds C1 and C2 were obtained under successive column chromatography of the crude dichloromethane extract obtained from P. pubescens seeds (Fig. 1).

2.2. Pharmacology

2.2.1. Drugs

All drugs and compounds C1 and C2 were diluted with Tween® 1% (Sigma-Aldrich, U.S.A.) in 0.9% saline solution (NaCl diluted in distilled water). The following substances were used: acetic acid, Complete Freund’s Adjuvant (CFA), p-chlorophenylalanine methyl ester hydrochloride (PCPA), pindolol, ketanserin, ondansetron hydrochloride, yohimbine hydrochloride, clonidine hydrochloride. Nω-nitro-ω-arginine (L-NAME), L-arginine hydrochloride (L-ARG), efaroxan hydrochloride, idazoxan hydrochloride, bicuculline methiodide, atropine, mecamylamine, and carrageenan (Sigma-Aldrich, U.S.A.).

2.2.2. Animals

Male Swiss mice (25–35 g) and Wistar rats (150–250 g) were kept at 25 ± 2 °C exposed to 12 h light–dark cycles (the light phase starting at 7:00 am) and maintained in animal facilities (10 and 5 animals per cage, respectively) with water and food ad libitum, for at least 7 days prior to the assays. Separate groups of mice and rats were used only once for each experiment, except on the mechanical allodynia and hyperalgesia evaluation, where the same groups were used in each treatment during the whole experiment. The intraperitoneal (i.p.) route was used for all experiments, based on a previous report (Spindola et al., 2010). The studies were carried out in accordance with the current guidelines for veterinary care of laboratory animals (Voipio et al., 2008) and were performed under the consent and surveillance of Unicamp’s Institute of Biology Ethics Committee for Animal Research (1076–1).

2.2.3. Mechanical Allodynia Induced by Complete Freund’s Adjuvant (CFA)

The procedures were developed and standardized in our laboratory based on the method previously described (Villeti et al., 2003) with changes in protocol and data analysis. Different groups of rats (n = 5) were used during the whole experiment and inflammation was induced with a solution of CFA (1 mg/ml of heat killed Mycobacterium tuberculosis in 85% paraffin oil and 15% mannide monoooleate) injected (0.1 ml) into the plantar surface of the right hind paw. The left hind paw received the same volume of saline solution (NaCl 0.9% diluted in distilled water) in order to equalize the sensitivity of the animals caused by the injection. Mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer apparatus (Ugo Basile, mod 37450, Italy) which consisted of an elevated wire mesh platform to allow access to the ventral surface of the hind paws. A steel rod (diameter 0.5 mm) was pushed against the hind paw with ascending force (touch stimulator). The force ranged from 0 to 35 g over a 20-s period. When the animal withdrew the hind paw, the mechanical stimulus was automatically stopped, and the force applied by the animal to withdraw the paw was recorded to the nearest 0.1 g. An allodynia score was determined after four consecutive measurements using the touch stimulator sequentially on the left and right hind paw and calculated considering the formula below determined by the authors:

Left hind paw value / Right hind paw value = Allodynia score.

The basal score was measured before CFA injection on day 0, and the animals considered for testing were those with a mean value nearest to 1 (demonstrating no significant difference between both paw stimuli). After CFA injection, measurements were carried out considering three different phases, as follows: 4 h on day 0 (acute pain); 24 h on day 1 (sub-acute pain) and on day 14 (chronic pain). Vehicle (10 ml/kg) or compounds C1 and C2 were administered (30 mg/kg, i.p.) 30 min prior to touch stimulation, in order to evaluate the possible anti-allodynic activity observed for each phase. A positive control was not employed, as the aim of this test was to evaluate the activity of compounds using the same doses to those used in the tests described below for evaluating potential antinociceptive mechanisms.

2.2.4. Mechanical Hyperalgesia Induced by Carrageenan

The procedures used for this study were similar to those described previously (Randall and Selitto, 1957) with some changes in the protocol and data analysis. Different groups of rats (n = 6), used during the whole experiment, were submitted to pressure stimulus (0 to 500 g) on the right hind paw using an Analgesy-meter (Ugo Basile, mod 37215/372116, Italy) prior to carrageenan injection, in order to determine the basal value. The hypernociceptive response was considered when animals vocalized or withdrew the paw from the equipment, demonstrating pain. After this first measurement, animals received a carrageenan (0.1 ml) intraplantar (i.pl.) injection (2.5% in saline) into the right hind paw surface. After 2:30 h, they were submitted to pressure again, to evaluate whether the hypernociceptive state was reached (40% decrease). Animals were treated with vehicle (negative control), indomethacin (30 mg/kg, i.p. — positive control) and compounds C1 and C2 (30 mg/kg, i.p.), and the mechanical hyperalgesia was evaluated after 30 min, 1 h, 2 h and 3 h. Compound doses were defined according to previous experiments (Spindola et al., 2010). The value demonstrating mechanical
hyperalgesia was obtained after each measurement, and the results were shown as decreased percentage compared to the pressure tolerated in the basal (100%) value for each group.

2.2.5. Abdominal Constriction Induced by Acetic Acid (Writhing Test)
Abdominal constrictions were induced according to procedures previously described (Spindola et al., 2010), and resulted in abdominal muscle contraction and concomitant stretching of the hind limbs, in response to an i.p. injection of 0.8% acetic acid (10 ml/kg) at the time of the test. The number of abdominal constrictions during 15 min was indicative of nociception. For these evaluations, a number of male Swiss mice (6 to 8) were used.

2.2.5.1. Investigation of the Antinociceptive Mechanisms of Compounds C1 and C2. To address some of the antinociceptive mechanisms of C1 and C2, male Swiss mice were pre-treated with different receptor antagonists in the writhing test. Response thresholds were measured 30 min after the second injection. The doses of each receptor antagonist were selected based on other experiments reported in the literature (Santos et al., 2005; Dalbó et al., 2006; Yue et al., 2007) and on our preliminary experiments (Spindola et al., 2010).

2.2.5.1.1. Involvement of Serotonergic System. To explore the possible participation of the serotonergic system in the antinociceptive action of compounds C1 and C2, mice were pre-treated with either PCPA (a 5-HT synthesis inhibitor, once a day, during 4 consecutive days before testing, 100 mg/kg, i.p.), pindolol (a 5-HT1A receptor antagonist, 1 mg/kg, i.p.), ketanserin (a 5-HT2A receptor antagonist, 0.3 mg/kg, i.p.), ondansetron (a 5-HT3 receptor antagonist, 0.2 mg/kg, i.p.), or vehicle (10 ml/kg), and 20 min after they then received compounds C1 and C2 (30 mg/kg, i.p.) or vehicle, 30 min prior to acetic acid injection.

2.2.5.1.2. Involvement of Imidazoline System. Aiming to evaluate the involvement of imidazoline receptors in the antinociceptive action of compounds C1 and C2, the mice were pre-treated with efaroxan (a mixed 1imidazoline/α2-adrenoceptor antagonist, 2 mg/kg, i.p.), idazoxan (a mixed 2imidazoline/α2-adrenoceptor antagonist, 10 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.), 20 min before administration of compounds C1 and C2 (30 mg/kg), clonidine (an α2-adrenoceptor agonist, 0.1 mg/kg, i.p.), or vehicle (10 ml/kg, i.p.), and after 30 min they received the injection of acetic acid.

2.2.6. Statistical Analysis
All results were submitted to one way analysis of variance (ANOVA) with repeated measurements. \( P \leq 0.05 \) was considered the critical level for evaluating significant difference between the control and treated groups, followed by Duncan’s test, using StatSoft® software. Graphs were designed using the Origin® software.

3. Results
3.1. Mechanical Allodynia
The results presented in Fig. 2 show that compounds C1 and C2 (30 mg/kg, i.p.) produced significant anti-allodynic activity reducing the allodynic score compared to the control (vehicle) during the acute phase (4 h post CFA) of the test. The compounds exerted no activity during the sub-chronic and chronic phases.

3.2. Mechanical Hyperalgesia
The results presented in Fig. 3 showed that control indomethacin (30 mg/kg, i.p.) and compound C1 (30 mg/kg, i.p.) produced significant anti-hypernociceptive effect increasing the percentage of stimulus compared to the control group (vehicle), however C2 did not.
Pre-treatment with 5-HT1A receptor antagonist pindolol (1 mg/kg, i.p.), did not reverse the antinociception caused by both compounds C1 and C2 (Fig. 5);

Pre-treatment with 5-HT2A receptor antagonist ketanserin (0.3 mg/kg, i.p.), did not reverse the antinociception caused by both compounds C1 and C2 (Fig. 6);

The pre-treatment with 5-HT3 receptor antagonist ondansetron (0.2 mg/kg, i.p.) significantly reversed the antinociception caused by compound C1, however not by C2 (Fig. 7).

3.3.2. Participation of Imidazoline System

The results presented in Fig. 8 demonstrated that compounds C1, C2 (30 mg/kg, i.p.), and the α2-adrenoceptor agonist clonidine (0.1 mg/kg, i.p.) produced significant antinociception which was confirmed by the reduction of writhes in mice, compared to the control group (vehicle only). The pre-treatment with the mixed I1 imidazoline/α2-adrenoceptor antagonist efaroxan (2 mg/kg, i.p.) significantly reversed the antinociception caused by clonidine (α2-adrenoceptor agonist, 0.1 mg/kg, i.p.) and compound C1 (30 mg/kg, i.p.), however not by C2. Whereas, the pre-treatment with the mixture I2 imidazoline/α2-adrenoceptor antagonist idazoxan (10 mg/kg, i.p.), reversed only the antinociception caused by clonidine.

3.3.3. Participation of Other Systems

The results presented in Online supplement support the information that compounds C1 and C2 do not appear to exert activity via α2-adrenoceptor, nitric oxide (NO), inhibitory GABA, acetylcholine muscarinic, and nicotinic receptors when evaluated in the acetic acid-induced nociception.
against the acetic acid-induced writhing in mice. Each column represents the mean of 6 pounds geranylgeraniol (C1) and 6
4. Discussion
\[\beta\text{-dihydroxyvouacapan-17} \beta\text{-oate methyl ester (C2)} (30 mg/kg, i.p.) on the antinociceptive pro
\[\beta\text{-dihydroxyvouacapan-17} \beta\text{-oate methyl ester (C2)} (30 mg/kg, i.p.) against the acetic acid-induced writhing in mice. Each column represents the mean of 6 to 8 animals and the error bar indicates the S.E.M. \# P < 0.05, \#\# P < 0.001 compared with corresponding control values (injected with vehicle alone); * P < 0.01, **P < 0.001 comparing to the respective agonist group (reversing effect of compounds C1, C2 or clonidine 0.1 mg/kg).

4. Discussion

We recently reported a few antinociceptive properties of compounds geranylgeraniol (C1) and 6\text{o}x,7\text{β}-dihydroxyvouacapan-17\text{β}-oate methyl ester (C2) against the acetic acid-induced writhing in mice. Each column represents the mean of 6 pounds geranylgeraniol (C1) and 6
against the acetic acid-induced writhing in mice. Each column represents the mean of 6 to 8 animals and the error bar indicates the S.E.M. \# P < 0.05, \#\# P < 0.001 compared with corresponding control values (injected with vehicle alone); * P < 0.01, **P < 0.001 comparing to the respective agonist group (reversing effect of compounds C1, C2 or clonidine 0.1 mg/kg).

Pain is normally a transitory unpleasant sensation subsequent to a noxious or potentially injurious stimulus generated in somatic and visceral tissues. Unlike acute pain, inflammatory and neuropathic pains are often persistent, chronic states. Patients suffering from chronic pain often experience hypersensitivity to mechanical, thermal and chemical stimulation in the form of hyperalgesia (aggravated pain response to normally painful stimuli) and/or allodynia (pain response to innocuous stimuli) (Levine and Alessandri-Haber, 2007). Our previous work demonstrated the effectiveness of compounds C1 and C2 on the writhing, hot plate, capsaicin and glutamate tests, suggesting their activities related to vanilloid and/or glutamatergic receptors (Spindola et al., 2010). A growing amount of experimental data indicates that these receptors are involved in the mechanism of pain and inflammation, and their antagonists might exhibit clinically potential relevance in the management of pathological pain states, such as chronic pain (Woolf and Thompson, 1991). Considering the diverse etiologies and the variety of molecular mechanisms underlying pain hypersensitivity, the approach of targeting ion channels in primary afferent nociceptive neurons that can contribute to the detection of physical stimuli, may be an effective approach for developing more successful therapies for clinical pain syndromes (Levine and Alessandri-Haber, 2007). To assess the effects of a 30 mg/kg (defined by previous data based on compounds ED\text{50}) doses of compounds C1 and C2 given intraperitoneally (i.p.) in persistent models of pain, we analyzed the mechanical anti-allodynic and anti-hypernociceptive actions induced by intraplantar injections of Complete Freund’s Adjuvant (CFA) and carrageenan, respectively. Both models of persistent pain used in the present study produce central sensitization in response to the release of several pro-inflammatory mediators, which increase the sensitivity of peripheral and central sensory pathways (Basbaum, 1999; Minami et al., 2006).

Persistent pain caused by CFA intraplantar injection involves central sensitization due to the release of multiple inflammatory and pain mediators that account for sensitivity increase of both peripheral sensory afferents at the injury site, and in the central nervous system (Samad et al., 2001). The arrival of sensory information from nociceptors into the dorsal horn considerably alters the level of activity within the cord as both excitatory and inhibitory systems can impinge upon spinal neuronal activity. This forms the basis of central hypersensitivity which results in increased responsiveness of dorsal horn neurons, often observed in persistent inflammatory and neuropathic states of pain (Suzuki and Dickenson, 2005). Our results showed that the mechanical anti-allodynic effects of compounds C1 and C2 (30 mg/kg, i.p.) were observed on the acute phase of the test (4 h post CFA). These effects were not observed on the sub-acute and chronic phases (Fig. 2).

Consequently, the anti-hypernociceptive effects of both C1 and C2 were evaluated in the carrageenan-induced hyperalgesia, consisting in a neutrophil-mediated acute inflammatory response that produces hind paw swelling, erythematic and localized hyperthermia induced by an injection of carrageenan into the rats paw (Cunha et al., 2005). This inflammatory method has become a widely used model for studying acute inflammation (Winter et al., 1962). In this model, carrageenan evokes a very inflammatory and nociceptive response characteristic, which is mediated by different groups of endogenous substances that stimulate chemosensitive nociceptors, thus playing a major role in the development of inflammatory pain (Posadas et al., 2004). Our results also showed that compound C1 was effective in this assay (Fig. 3) reducing mechanical hyperalgesia of rats, presenting an important difference compared to C2 which did not produce the same effect.

Moreover, in the present study we attempted to characterize further some of the mechanisms by which compounds C1 and C2 may exert their antinociceptive action in a chemical model of nociception in mice (writhing test), evaluating a few specific receptors in different pain systems. We assessed the following pathways to evaluate some receptors involved in the antinociceptive mechanisms: serotonin, α\text{2}-adrenergic, imidazoline, nitric oxide, inhibitory GABAA, neuronal nicotinic acetylcholine, and muscarinic acetylcholine systems. However, only the serotoninergic and imidazoline systems seemed to be involved, as described below.

In CNS, serotonin (5-HT) neurons are involved in nociceptive transmission as well as in pain inhibition induced by opioid agonists. Serotonin has also been observed to produce an inhibitory effect on pain transmission in the spinal cord and brain (Yaksh and Wilson, 1079). This inhibitory effect could be mediated by 5-HT receptors influencing the descending inhibitory system (Sawynok and Reid, 1996). Our findings demonstrated that compounds C1 and C2 may exert their antinociceptive properties, at least in part, via serotoninergic system. This assertion is supported by the demonstration that depletion of endogenous serotonin with the tryptophan hydroxylase inhibitor p-chlorophenylalanine methyl ester hydrochloride (PCPA) at a dose known to decrease the cortical content of serotonin and to significantly reverse the morphine antinociception, largely antagonized the antinociception of C2 (though not C1), suggesting that the action of the compound could be due to 5-HT synthesis or release influencing (Fig. 4). In addition, specific 5-HT receptors 5-HT\text{1c}, 5-HT\text{2A}-,

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Fig. 8. Graph demonstrating effect of pre-treatment of animals with efaroxan (2 mg/kg, i.p.) and idazoxan (10 mg/kg, i.p.) on the antinociceptive profiles of geranylgeraniol (C1) and 6\text{o}x,7\text{β}-dihydroxyvouacapan-17\text{β}-oate methyl ester (C2) (30 mg/kg, i.p.) on the antinociceptive pro
and 5-HT3 were investigated in the same visceral model, throughout the pretreatment with the respective antagonists: pindolol, ketanserin, and ondansetron.

The selective antagonist of 5-HT3 receptors ondansetron, consistently reversed the antinociception provoked by C1 administration when using the writhing test (Fig. 7). Conversely, neither compound seemed to exert antinociceptive activity upon 5-HT1A or 5-HT2A, because the pretreatment with the specific antagonists, pindolol and ketanserin (respectively), did not block the antinociceptive action of both compounds on the same model (Figs. 5 and 6). Considering that supraspinal serotonergic inputs maintain the facilitatory influences of the spinal cord following injury (Wang et al., 2002), we speculated that C1 could act directly upon the 5-HT3 receptor, and that C2 could act by increasing or decreasing the serotonin amount, influencing the descending inhibitory pathway.

The imidazoline I1-receptor has the best understood physiologic actions among the reported imidazoline receptors, and has been implicated in hypotension produced by clonidine and other imidazolines when injected into the rostral ventrolateral medulla (RVLM) or administered peripherally (Ernsberger et al., 1997; Head et al., 1997). The I1-receptor has been further implicated in the modulation of gastric acid secretion, analgesia and modulation of electrolyte kidney secretion (Smyth et al., 1995). Another interesting finding of the present work is that the results herein presented provided consistent evidence supporting imidazoline I1 receptor involvement in the antinociception caused by C1, evident due to the fact that efaroxan, at a similar dose to that known to prevent antinociception induced by the imidazoline I1 receptor agonist moxonidine, consistently attenuated both clonidine and C1 induced antinociception during the writhing test (Fig. 8) (Shannon and Lutz, 2000). Contrarily, imidazoline I2 receptors do not appear to be involved in the antinociception of C1 and C2, as the pretreatment using the preferential I2 imidazoline receptor antagonist idazoxan, largely failed to prevent the antinociception of both compounds. The present data allowed us to verify that the antinociception caused by C1 is probably, at least in part, linked to an interaction with imidazoline I1 receptors.

In summary, the data of this paper demonstrated important differences in the antinociceptive effects observed for compounds geranylgeraniol (C1) and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester (C2) obtained from P. pubescens Benth., being active against several pain states and extending our previous results which indicated synergistic activity among these compounds and corroborating the facilitatory influences of the spinal cord following injury (Wang et al., 2002), we speculated that C1 could act directly upon the 5-HT3 receptor, and that C2 could act by increasing or decreasing the serotonin amount, influencing the descending inhibitory pathway.

The present study demonstrated that: 1) compounds C1 and C2 produced significant anti-allodynic activity during the acute phase of the CFA-induced persistent pain model; 2) compound C1 produced significant anti-hypernociceptive activity upon the carrageenan-induced pain model; 3) compound C2 significantly lost activity after PCPA treatment suggesting participation of geranylgeraniol upon 5-HT3, serotonin receptors; 5) compound C1 significantly lost activity after efaroxan treatment suggesting participation of this compound upon imidazoline I1 receptors; and 6) both compounds C1 and C2 do not appear to exert activity via 5-HT1A, 5-HT2A, α2-adenoreceptor, imidazoline I2, NO, GABAβ, acetylsalicylic muscarinic, and nicotinic receptors when evaluated in the acetid acid-induced nociception (Supplementary information).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ejphar.2011.01.025.

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