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A new pyrazolo pyrimidine derivative inhibitor of cyclooxygenase-2 with anti-angiogenic activity

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Abstract

In a previous study, we reported a new pyrazolo pyrimidine derivative, N^4-benzyl-N^6,N^6-dimethyl-1-1(*tert*-butyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine-6,4-diamine (DPP), which inhibited potently cyclooxygenase-2 activity in intact cell assays with minor activity against cyclooxygenase-1 (IC_{50} = 0.9 nM for cyclooxygenase-2 versus IC_{50} = 59.6 nM for cyclooxygenase-1). In the present work, this behaviour was confirmed in vivo by using the 24-h zymosan-injected mouse air pouch model (ID_{50} = 1.36 nmol/pouch for prostaglandin E_2 level). We also studied the possible beneficial effect of DPP in the angiogenesis-dependent murine air pouch granuloma and rat paw carrageenan-induced hyperalgesia models. DPP exerted analgesic and anti-angiogenic (52% reduction in angiogenesis at 10 mg/kg, i.p.) effects that may be associated with inhibition of cyclooxygenase-2 activity.

Keywords: Cyclooxygenase-2 inhibitor; Prostaglandin E_2; Zymosan-injected mouse air pouch; Angiogenesis; Carrageenan-induced paw oedema

1. Introduction

It has been known since the 1960s that prostaglandins play a key role in inflammation, fever and pain. These mediators are synthesized by cyclooxygenase. Cyclooxygenase-1 is a widely and constitutively expressed form, whereas the inducible enzyme, cyclooxygenase-2, is prominent at sites of inflammation (Smith and DeWitt, 1996). Cyclooxygenase-2 is also constitutively expressed in the macula densa of the kidney (Harris et al., 1994) and in the brain (Yamagata et al., 1993; Kaufmann et al., 1996). Pharmacological investigation of cyclooxygenase inhibition has shown that the classical nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit both cyclooxygenase isozymes. More recently, highly selective inhibitors of cyclooxygenase-2 have been developed that demonstrate anti-inflammatory and analgesic activities equivalent to those of dual cyclooxygenase inhibitors but with a lower gastrointestinal toxicity (Seibert et al., 1994; Chan et al., 1995).

Angiogenesis is a very complex multistep process involving a variety of biologically active substances. Of them, prostaglandins induce several growth factors, and proliferation of endothelial cells in vitro and in vivo may play a crucial role in enhancing neovascularization. Majima et al. (2000) indicated that a selective cyclooxygenase-2 inhibitor may be useful for controlling angiogenesis-related human pathological states, such as granulation tissue formation, which may induce bone absorption, causing the destruction or dysfunction of joints that is characteristic of rheumatoid arthritis.

Tissue inflammation produces spontaneous activity in otherwise silent small primary afferent axons and, consequently, evokes behavioural hyperalgesia. This peripheral hypersensitivity of the altered primary afferent can be explained in part by the local release of pro-inflammatory substances, such as bradykinin, cytokines and prostaglandins, which activate and sensitize peripheral nerve endings (Ibuki et al., 2003). This process can be inhibited by cyclooxygenase-2 inhibitors (Seibert et al., 1994; Zhang et al., 1997).

In the search for new drugs active against pro-inflammatory mediator production in different inflammatory pathologies, we have investigated the effects of a series of 6-dimethylamino 1*H*-pyrazolo[3,4-*d*]pyrimidine derivatives on murine macrophage and human leukocyte functions. In a previous work, we have shown that one of these new pyrazolo pyrimidine derivatives, N^4-benzyl-N^6,N^6-dimethyl-

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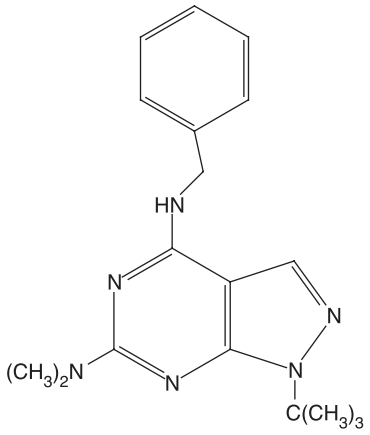

Fig. 1. Chemical structure of N^4-benzyl-N^6,N^6-dimethyl-1-1(*tert*-butyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine-6,4-diamine (DPP).

1-1(*tert*-butyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine-6,4-diamine (DPP) (Fig. 1), inhibits potently and with a high selectivity cyclooxygenase-2 activity in murine macrophages as well as in human monocytes (IC_{50} = 0.9 nM) (Quintela et al., 2003). The present study was designed to determine the anti-inflammatory effect and the analgesic activity of DPP using the zymosan-injected mouse air pouch and the carrageenan-induced rat paw oedema models. We also studied the possible beneficial effect of DPP in the treatment of angiogenesis-dependent murine air pouch granuloma and its effect on several parameters involved in the pathogenesis of these models.

2. Materials and methods

2.1. Chemicals

N^4-benzyl-N^6,N^6-dimethyl-1-1(*tert*-butyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine-6,4-diamine (DPP) was prepared according to procedures previously described (Quintela et al., 2001). [5,6,8,11,12,14,15(n)-^{3}H]Prostaglandin E_2 and [5,6,8,9,11,12,14,15(n)-^{3}H]leukotriene B_4 were from Amersham Iberica (Madrid, Spain). Anti-mouse tumor necrosis factor-α (TNF-α) and interleukin-1β antibodies were from Immunokontact (Frankfurt, Germany). *Mycobacterium butyricum* was obtained from Difco Chem. (Michigan, USA). Gelatin was from Merck (Darmstadt, Germany). *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS398) was purchased from Cayman Chemicals (SPI-Bio, Massy, France) and other reagents were from Sigma (Missouri, USA).

2.2. Mouse air pouch

All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. The protocols were approved by the Institutional Animal Care and Use Committee. Air pouch was prepared in female Swiss mice (25–30 g) as previously described (Posadas et al., 2000). Six days after the initial air injection, 1 ml of sterile saline or 1 ml of 1% w/v zymosan in saline was injected into the air pouch. In the 4-h zymosan-injected air pouch, DPP, NS398 (0.1, 1 and 10 nmol/pouch) or saline was administered at the same time as zymosan. In the 24-h zymosan-injected air pouch, DPP, NS398 (0.1, 1 and 10 nmol/pouch) or saline was administered 2 and 10 h after the zymosan injection. After 4 and 24 h, animals were killed by cervical dislocation and the exudates in the pouch were collected. Leukocytes present in exudates were measured using a Coulter counter. After centrifugation of 4-h exudates, the supernatants were used to measure leukotriene B_4 and prostaglandin E_2 levels by radioimmunoassay (Moroney et al., 1988). In 24-h exudates, the supernatants were used to measure prostaglandin E_2 levels and nitrite by a fluorometric method (Misko et al., 1993).

2.3. Murine air pouch granuloma

Granulomatous tissue was induced in anaesthetized female Swiss mice (25–30 g) by the injection of 3 ml of air into the dorsal subcutaneous tissue on day 1, followed by

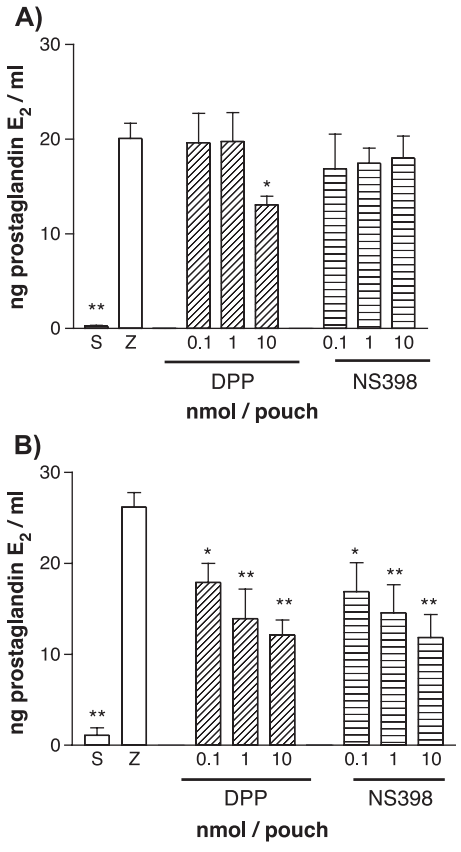

Fig. 2. Effect of DPP and NS398 (0.1–10 nmol/pouch) on prostaglandin E_2 levels in (A) 4-h zymosan-injected air pouch; drugs were administered with zymosan. (B) 24-h zymosan-injected air pouch; drugs were administered 2 and 10 h after zymosan injection. Data represent means ± S.E.M. (n = 6–12 animals); *P < 0.05, **P < 0.01. S = Saline, Z = zymosan.

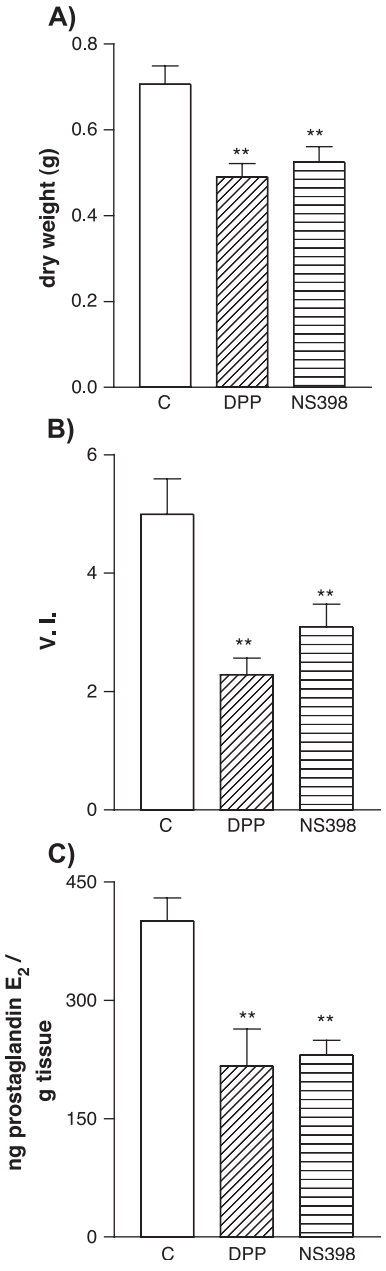

Fig. 3. Effect of DPP (10 mg/kg; i.p.) and NS398 (10 mg/kg; i.p.) on (A) tissue dry weight, (B) vascular index (V.I.) (mg of carmine dye/g of dry tissue) and (C) prostaglandin E_2 levels in homogenized granuloma. Results are expressed as means ± S.E.M. ($n = 8–12$ animals per group). $^{**}P < 0.01$ with respect to the vehicle-treated animals (C).

intrapouch injection of 0.5 ml of 0.1% v/v croton oil in Freund's complete adjuvant on day 0 (Colville-Nash et al., 1995; Jackson et al., 1998). Then 0.5 ml of DPP, NS398 (10 mg/kg) or saline was intraperitoneally administered once daily on days 0–5. The degree of granulomatous inflammation and vascular density was assessed after 6 days.

2.4. Assessment of vascularity

On day 6, a vascular cast was made by intravenous injection of 1 ml of a solution of 10% carmine red in 5% gelatin. The granulomatous air pouch linings were dissected and treated as described by Farndale et al. (1986). The dye content of samples was assayed spectrophotometrically at 490 nm against a carmine red standard curve. The results are expressed as a vascular index (V.I. = mg dye/g dry tissue).

2.5. Measurement of prostaglandin E_2 and cytokine levels in granulomatous tissues

Tissues extracts were obtained by homogenization of granulomas in 2.5 ml of 5 mM KH_2PO_4. After centrifugation at $2000 \times g$ for 10 min at 4 °C, aliquots of the supernatants were used to measure prostaglandin E_2 levels by radioimmunoassay (Moroney et al., 1988), TNF-α and interleukin-1β levels were measured by time-resolved fluoroimmunoassay (Pennanen et al., 1995).

2.6. Carrageenan-induced paw oedema

Wistar rats (150–200 g) were used to assess the anti-inflammatory and analgesic activity of DPP and NS398 in the carrageenan-induced paw oedema test (Seibert et al., 1994; Otterness and Moore, 1988). DPP, NS398 (1 μmol/paw) or vehicle was administered (intrapaw) 15 min before carrageenan injection (0.1 ml; 1% w/v in saline) into the subplantar area of the right hind paw. The volumes of injected and contralateral paws were measured 1, 2 and 3 h after induction of oedema by using a plethysmometer (Ugo-Basile, Comerio, Italy). Carrageenan-induced hyperalgesia was measured by the response of the rat to increased pressure applied to the carrageenan-injected paw, using an analgesimeter (Ugo-Basile), at 1 and 3 h. Measurements were made by an investigator who was unaware of the

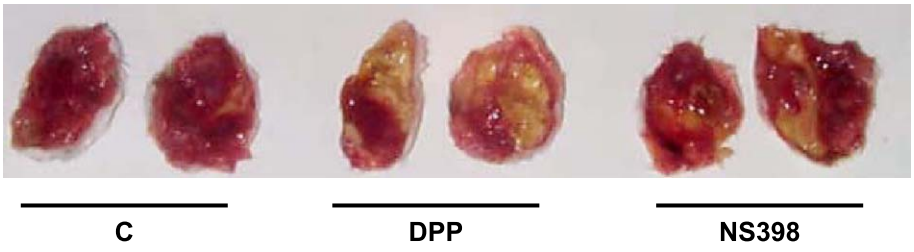

Fig. 4. Effects of once-daily intraperitoneal injection of vehicle (C), DPP (10 mg/kg) or NS398 (10 mg/kg) on angiogenesis and development of chronic granulomatous inflammation. Shown are the carmine casts of representative mice from each group, photographed on day 6.

allocation of treatments. After the last determination of paw oedema (3 h), the animals were killed by cervical dislocation and the right hind paws were cut off and centrifuged at $2000 \times g$ for 15 min in order to recover a sample of oedematous fluid to determine prostaglandin E_2 levels.

2.7. Statistical analysis

Statistical evaluation included one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. $P < 0.05$ (*) and $P < 0.01$ (**) were taken as significant. Results are shown as means ± S.E.M; *n* represents the number of experiments or animals.

3. Results

3.1. Effect of DPP on the mouse air pouch

We used the 4-h zymosan-stimulated mouse air pouch to assess the in vivo effect of DPP on leukocyte migration, leukotriene B_4 generation and prostaglandin E_2 levels derived from cyclooxygenase-1 activity (Posadas et al., 2000). As shown in Fig. 2A, prostaglandin E_2 levels were reduced by intrapouch administration of DPP at the highest dose assayed, whereas leukocytes in pouch exudates collected 4 h after zymosan challenge and leukotriene B_4 levels were unaffected (data not shown). The reference inhibitor NS398 (selective cyclooxygenase-2 inhibitor) reduced leukocyte infiltration by 36% at 10 nmol/pouch without affecting the other parameters.

In the 24-h zymosan-stimulated mouse air pouch, the expression of cyclooxygenase-2 is maximal and prostaglandin E_2 levels are increased in exudates (Posadas et al., 2000). We used this time point to assess the effect of DPP on cyclooxygenase-2 activity in vivo. Prostaglandin E_2 levels were significantly inhibited after intrapouch administration of both compounds with an ID_{50} of 1.36 (0.16–3.16) nmol/pouch for DPP and 1.15 (0.67–3.16) nmol/pouch for NS398 (Fig. 2B). Cell influx and nitrite levels were reduced by NS398 at the highest dose (57%), whereas DPP did not significantly affect these parameters (data not shown).

3.2. Effect of DPP on angiogenesis in the murine air pouch granuloma

The murine air pouch granuloma provides a model in which modulation of angiogenesis in an inflammatory bed can be quantified. Anti-inflammatory effects were indicated by a reduction in granuloma size (dry weight) (Fig. 3A). Daily intraperitoneal administration of DPP (10 mg/kg) or NS398 (10 mg/kg) caused a marked reduction in angiogenesis (52% and 38%, respectively), as measured by the vascular index of the granuloma (Fig. 3B). These results are illustrated in Fig. 4, showing the carmine casts of two mice from each group. The lack of capillaries and the inhibition of granuloma development can be seen in these representative samples of DPP and NS398-treated groups with respect to the control.

3.3. Effect of DPP on prostaglandin E_2, TNF-α and interleukin-1β levels in the murine air pouch granuloma

The effect of DPP and NS398 (10 mg/kg; i.p.) was determined in supernatants of 6-day granuloma homoge-

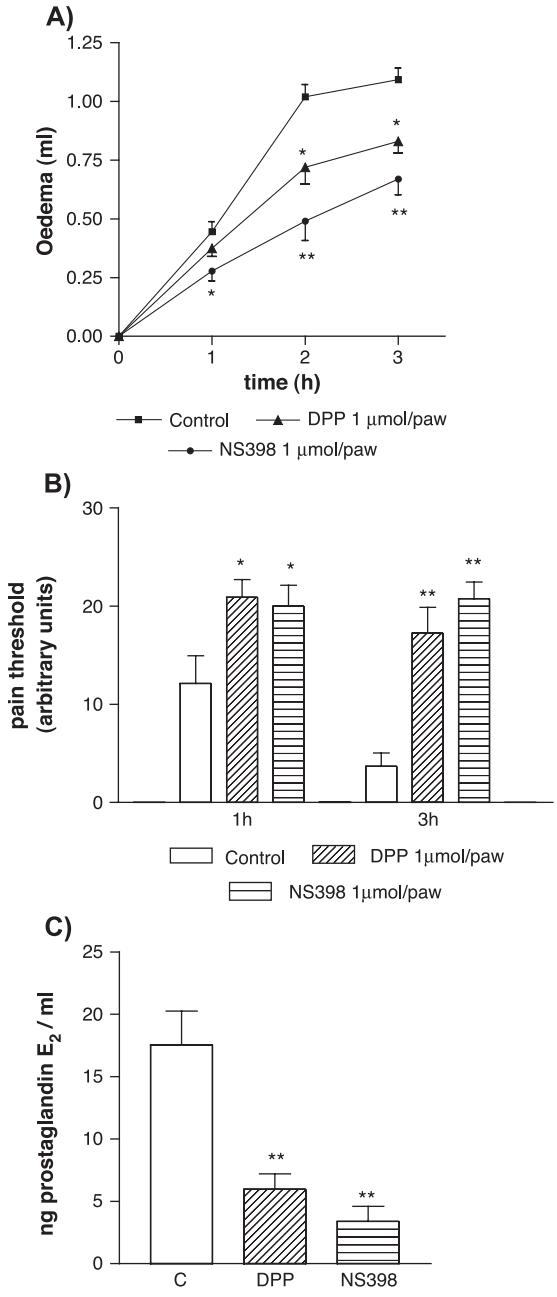

Fig. 5. Effect of DPP (1 µmol/paw) and NS398 (1 µmol/paw) on (A) carrageenan-induced rat paw oedema, 1, 2 and 3 h after the induction of inflammation, (B) pain threshold 1 and 3 h after the induction and (C) prostaglandin E_2 levels in oedematous fluid. Data represent means ± S.E.M. ($n = 6–12$). *$P < 0.05$, **$P < 0.01$.

nates. DPP and NS398 reduced prostaglandin E_2 levels by 46% and 42%, respectively (Fig. 3C), whereas TNF-α and interleukin-1β levels were not affected by either of these compounds (data not shown).

3.4. Effect of DPP on carrageenan-induced paw oedema

We first evaluated the anti-inflammatory activity of DPP on rat paw oedema (Fig. 5A). When administered 45 min before carrageenan, DPP and NS398 at 1 μmol/paw significantly inhibited swelling at 2 and 3 h after carrageenan injection.

In carrageenan-induced hyperalgesia, the pain threshold of animals treated with DPP or NS398 (1 μmol/paw) was significantly higher than that of vehicle-treated animals (control group) (Fig. 5B) at both time points (1 and 3 h).

DPP as well as NS398 at 1 μmol/paw reduced significantly the content of prostaglandin E_2 in paw oedematous fluid (Fig. 5C).

4. Discussion

We recently described the time course of inflammatory mediator production in the zymosan-injected mouse air pouch as well as the participation of cyclooxygenase-2 metabolites in the later phase of this model (Posadas et al., 2000). In the present work, we used two different sets of experiments, the 4-h and the 24-h zymosan-stimulated mouse air pouch, to assess the effects of DPP on cyclooxygenase-1 and cyclooxygenase-2 activities in vivo. This compound exhibited an inhibitory behaviour that correlated well with its in vitro effects on peritoneal mouse macrophages and human monocytes (Quintela et al., 2003). DPP reduced potently and dose-dependently the prostaglandin E_2 levels measured in exudates from 24-h zymosan-stimulated mouse air pouch, indicating that it inhibits cyclooxygenase-2 in vivo with potency similar to that of NS398. However, DPP shows minor cyclooxygenase-2 selectivity because, at the highest dose assayed, it was able to reduce prostaglandin E_2 levels in exudates from 4-h zymosan-stimulated mouse air pouch which depend mainly on cyclooxygenase-1 activity (Posadas et al., 2000).

It is known that angiogenesis contributes to the pathology of chronic inflammatory diseases, such as rheumatoid arthritis (Leahy et al., 2000). As a consequence, inhibitors of angiogenesis may be beneficial in inflammatory disorders (Walsh and Pearson, 2001). The murine air pouch granuloma is a model of inflammation characterized by intense angiogenesis during the chronic inflammatory phase, with elevated levels of cytokines (interleukin-1β and TNF-α), induction of cyclooxygenase-2 and high prostaglandin E_2 production (Jackson et al., 1998; Leahy et al., 2000). Under our experimental conditions, we observed a clear reduction in granulomatous tissue and vascular index by NS398 and DPP. Our results indicate that inhibition of the endogenous production of prostaglandin E_2 participates in the modulation of angiogenesis by both compounds.

The carrageenan paw inflammation model has long been used to evaluate the anti-inflammatory activity of NSAIDs (Otterness and Moore, 1988) and hyperalgesia (Francischi et al., 2002). Paw hyperalgesia reaches a maximum between 2 and 3 h and clearly fades 4 h after carrageenan injection, whereas oedema reaches a maximum value at 3 h and resolves by 24 h (Francischi et al., 2002). We showed that DPP and the reference cyclooxygenase-2 inhibitor, NS398, significantly inhibited carrageenan-induced hyperalgesia and swelling and reduced the prostaglandin E_2 content of paw oedematous fluids.

There is compelling evidence that cyclooxygenase-2-derived prostaglandins play a major role in inflammatory reactions. It has also been shown that the selective inhibition of the inducible enzyme leads to anti-inflammatory effects in a number of inflammatory models in the rat, such as those for adjuvant arthritis (Anderson et al., 1996), carrageenan-induced air pouch (Masferrer et al., 1994) and footpad oedema (Smith et al., 1998). In this last model, selective cyclooxygenase-2 inhibitors were much more effective as anti-inflammatory agents and analgesics than selective cyclooxygenase-1 inhibitors. However, the full inflammatory response is probably sustained by prostanoids generated by both constitutive and inducible cyclooxygenases (Parente and Perretti, 2003).

Although cyclooxygenase-2 is expressed constitutively in the brain (Yamagata et al., 1993), the presence of this isozyme in the spinal cord and the increased levels of peripheral prostaglandins seem to be important for nociception and the analgesic actions of selective cyclooxygenase-2 inhibitors (Svensson and Yaksh, 2002; Zhang et al., 1997). Ibuki et al. (2003) suggested that centrally induced cyclooxygenase-2 is involved in hyperalgesia in later time periods, whereas peripherally induced cyclooxygenase-2 is essential for the initial development of hyperalgesia. Our results with local intraplantar injection of the cyclooxygenase-2 inhibitor DPP would suggest a major peripheral site of action of cyclooxygenase-2 inhibitors in mediating the observed analgesia. However, paw oedema is clearly less dependent on prostaglandin modulation as it decreased only slightly over the time period studied. Other mediators that increase vascular permeability, such as nitric oxide (Salvemini et al., 1996) and serotonin, may persist at the site (De Resende et al., 2001).

In summary, the present study has demonstrated that DPP exerts acute anti-inflammatory, analgesic and anti-angiogenic effects that may be associated with the inhibition of cyclooxygenase-2 activity and the resulting reduction of prostaglandin E_2 production.

Acknowledgements

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