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European Journal of Pharmacology xx (2007) xxx–xxx
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Inhibition of angiogenic tubule formation and induction of apoptosis in human endothelial cells by the selective cyclooxygenase-2 inhibitor 5-bromo-2-(4-fluorophenyl)-3-(methylsulfonyl) thiophene (DuP-697)

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Received 9 October 2006; received in revised form 20 June 2007; accepted 26 June 2007

Abstract

There are indications that inhibitors of the cyclooxygenase-2 (COX-2) enzyme may cause inhibition of angiogenesis, proliferation of endothelial cells and induce apoptosis in cell systems. The concentrations of inhibitors required for such effects are however much higher than those needed to inhibit COX-2, suggesting that the latter may not be involved in these actions of the drugs. We have however generated data that strongly indicates a critical role for COX-2 suppression in the inhibition of angiogenesis and induction of apoptosis in human cultured umbilical vein endothelial cells (HUVECs) by the selective cyclooxygenase-2 (COX-2) inhibitor 5-bromo-2-(4-fluorophenyl)-3-(methylsulfonyl) thiophene (DuP-697). DuP-697 concentration-dependently inhibited prostaglandin E_2 (PGE_2) production by HUVECs and at its known IC_{50} for COX-2 inhibition of 10 nM inhibited basal and vascular endothelial cell growth factor (VEGF)-induced PGE_2 production by 80% and 85% respectively. DuP-697 also induced apoptosis as shown by FACs analysis, an increase in chromatin condensation and DNA laddering in HUVECS treated with the drug. Moreover, these effects were reversed by PGE_2 and by VEGF. In parallel studies, DuP-697 induced caspases 3, 8 and 9, with the caspase-3 specific inhibitor N–Acetyl–Asp–Glu–Val–Asp–al (DEVD–CHO) blocking the induction of apoptosis. Capillary-like tubule formation by HUVECs cultured on Matrigel was inhibited by DuP-697 and this inhibition was prevented by PGE_2 but not by DEVD–CHO. These results indicate that the induction of apoptosis and inhibition of tubule formation by DuP-697 involves the inhibition of COX-2 and that whereas the induction of apoptosis is caspase-dependent, the inhibition of tubule formation occurs through a caspase-independent mechanism.

Keywords: Angiogenesis; Apoptosis; Human umbilical vein endothelial cell; DuP-697; Cyclo-oxygenase-2

1. Introduction

Cyclooxygenase (COX) enzymes convert arachidonic acid to prostaglandin H_2 (PGH_2) and exist as two distinct isoforms referred to as COX-1 and COX-2. The COX-1 enzyme is mainly constitutively expressed, but it can be induced by some growth factors such as vascular endothelial growth factor (VEGF) (Akarasereenont et al., 2002; Murphy and Fitzgerald, 2001). COX-1 is the predominant isoform in most tissues including the vascular endothelium, renal system and gastric mucosa and in platelets, where arachidonic acid is converted to thromboxane A_2 (Parente and Perretti, 2003; Vane et al., 1998). By comparison, COX-2 is only constitutively expressed in a few tissues including the rat cecum (Kargman et al., 1996), brain (Breder et al., 1995), renal system (Harris et al., 1994), but it is inducible in a wide variety of cells (Vane et al., 1998) and in the vasculature under conditions of shear stress (Inoue et al., 2002).

In contrast to the physiological role played by COX-1 in the body, expression of COX-2 is associated mainly with the induction of inflammation (Colville-Nash and Gilroy, 2000; Masferrer et al., 1995; Parente and Perretti, 2003; Seibert and Masferrer, 1994; Vane et al., 1998) or angiogenesis (Carmeliet, 2000; Masferrer et al., 2000). Prostaglandins catalysed by COX-2 also control vasodilatation and blood pressure in areas of inflammation causing an increase in swelling, an influx of

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0014-2999/$ - see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.ejphar.2007.06.057

immune cells, and an increase in pain in the area (Masferrer et al., 1995; Seibert and Masferrer, 1994). However, in the gastric mucosa, COX-2 may catalyse the formation of cytoprotective prostaglandins such as prostacyclin and prostaglandin E_2 that could maintain gastric blood flow and mucosal integrity (Takeeda et al., 2004).

The increase in swelling and vasodilatation associated with inflammation can be prevented by inhibiting the COX-2 enzyme pharmacologically. The first generation of compounds found to inhibit COX-2 were the non-steroidal anti-inflammatory drugs (NSAIDs). These compounds are, however, non-selective and effectively inhibit both COX-1 and COX-2 (Mitchell et al., 1993). As a consequence of this, chronic treatment with NSAIDs may result in severe undesirable side effects such as gastrointestinal toxicity and ulcer formation due to the inhibition of COX-1 and/or COX-2 derived cytoprotective prostaglandins (Allison et al., 1992; Mitchell et al., 1993). Indomethacin, a first generation NSAID, inhibits both COX-1 and COX-2, but it is selective for the inhibition of COX-1 at low concentrations and only inhibits COX-2 at ≥ 3 μM (Mitchell et al., 1993). Indomethacin has also been shown to induce apoptosis in *in vivo* gastric cancer models (Sawaoka et al., 1998) and *in vitro* in HT-29 cells (Hong et al., 1998).

Recently, a new generation of selective COX-2 inhibitors have been introduced and include 5-bromo-2-(4-fluorophenyl)-3-(methylsulfonyl) thiophene (DuP-697) (Gierse et al., 1995). This new class of inhibitors binds tightly to the COX-2 active site and dissociate slowly, thus having a longer lasting action. Moreover, their selectivity for COX-2 means that the activity of COX-1 remains unaffected, thereby preventing gastrointestinal injury and ulcer formation (Schmassmann et al., 1998).

Expression of COX-2 can be induced by various growth factors such as VEGF (Akarasereenont et al., 2002; Hernandez et al., 2001; Wu et al., 2006) which may act through the p38 MAP kinase and Jun kinase (JNK) signalling pathways (Wu et al., 2006) and subsequently activate transcriptional regulators on the COX-2 promoter including the nuclear factor of activated T-cells (NFAT) (Hernandez et al., 2001; Liu et al., 2003). The increase in COX-2 protein expression may enhance the production of prostaglandin E_2 (PGE_2), resulting in either an autocrine or paracrine action that enhances expression of VEGF through the early regulating kinase (ERK) 2 and/or the generation of hypoxia induced factor (HIF)-1α (Calviello et al., 2004; Huang et al., 2005).

Since VEGF is critical for angiogenesis (Breier et al., 1992), its regulation by COX-2 suggests that this enzyme may act as an important mediator in this process. Indeed, selective inhibition of COX-2 activity has been shown to inhibit angiogenesis dose dependently and this was associated with a decrease in growth factor (VEGF and bFGF) expression, inhibition of proliferation of endothelial cells both *in vitro* and *in vivo* and induction of apoptosis (Hernandez et al., 2001; Leahy et al., 2002; Sawaoka et al., 1999; Yazawa et al., 2005). However the concentrations of drugs required for these effects were much higher than those required to inhibit COX-2, suggesting perhaps that the effects of the inhibitors on angiogenesis may be independent of their ability to inhibit COX-2 and that the two processes may not be linked. To address this issue, we have examined the effects of DuP-697 on capillary like tubule formation of human umbilical vein endothelial cells (HUVECs) at concentrations that selectively inhibit COX-2 and compared the effects with those of indomethacin used at concentrations that selectively inhibit COX-1. We report that DuP697 inhibits angiogenesis via specific inhibition of COX-2 and augments the induction of apoptosis at concentrations that are pharmacologically relevant.

2. Materials and methods

2.1. Materials

All chemicals and cell culture ~~medium was~~ supplied by Sigma (UK) unless stated. ELISAs for PGE_2 and 6-keto-$PGF_{2\alpha}$ were supplied by R & D systems (Europe). DuP-697 was supplied by Tocris-Cookson. Anti-COX-2 primary antibody and the anti-goat HRP conjugate antibody were supplied by Insight Biotechnology Ltd (UK). The anti-caspase 3, 8 and 9 antibodies, VEGF and PGE_2 were supplied by Merck Biosciences (UK). ~~DuP-697 was from Tocris-Cookson, UK.~~ β-actin antibody was from Merck Biosciences, UK. BCA kit was from Pierce Ltd, UK.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated according to standard procedures (Hallam et al., 1988) and cultured in gelatin-coated T25 flasks in Medium 199 supplemented with 20% heat-inactivated foetal calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and L-glutamine (2 mM). Cells were maintained at 37 °C in 5% CO_2 humidified tissue culture incubator. Cell were routinely passaged when 80 to 90% confluent and were used between passages 1 and 4.

2.3. $VEGF_{165}$ treatment of quiesced HUVECs

Confluent monolayers of HUVECs were quiesced for 16 h in serum free Medium 199 (SFM). $VEGF_{165}$ (50 ng/ml) was then added and cells were further incubated for up to 24 h.

2.4. Cell treatments

Cell monolayers (passage 1–4) were treated with DuP-697 or indomethacin for up to 24 h at the concentrations indicated. In parallel experiments, cells were incubated for 24 h with DuP-697 simultaneously with prostaglandin E_2 (PGE_2; 10 μM), $VEGF_{165}$ (50 ng/ml) or N–Acetyl–Asp–Glu–Val–Asp–al (DEVD–CHO; 10 μM).

2.5. Staining for condensed chromatin

HUVECs were plated at 3×10^5 cells/ml in gelatinised 24 well plates and cultured in 20% foetal bovine serum (FBS), 2 mM L-glutamine and 100 units/ml penicillin, 0.1 mg/ml streptomycin supplemented Medium 199 (complete medium

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199). The cells were treated with DuP-697 (10 nM) or indomethacin (3 μM) diluted in serum free medium (SFM). In corresponding experiments PGE_2 (10 μM) or $VEGF_{165}$ (50 ng/ml) was added simultaneously with DuP-697 (10 nM). After 24 h, the cells in the supernatant were counted and resuspended in sterile phosphate buffered saline (PBS) at 1×10^4 cells/ml. The cells were cytospun onto glass slides at 750 rpm for 10 min and fixed with 3.7% formaldehyde. The slides were washed, allowed to dry at room temperature before staining with acridine orange (5 μM) for 5 min. Excess stain was washed off and the slides again dried before placing a coverslip over the cells for visualisation at 405 nm under a fluorescent microscope. Cells showing condensed chromatin were counted as positive for apoptosis.

2.6. Flow cytometry analysis of apoptosis

HUVECs (3×10^5/ml) were plated in gelatinised 6 well plates and treated with DuP-697 as above. After 6 h, the cells in the supernatant were removed and stored. The adherent cells were removed from the monolayer using Accutase ® solution (Sigma) for 1 min at 37 °C. The adherent cells were pooled with the cells in the supernatant and centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in binding buffer (supplied as part of the annexin-V FITC apoptosis detection kit (Sigma)) at 1×10^6 cells/ml. To the cell suspension 5 μl of annexin-V FITC and 10 μl propidium iodide was added and incubated for 10 min at room temperature. Fluorescence of the cells was determined using the Coulter flow cytometer.

2.7. Apoptotic DNA laddering

HUVECs (1×10^5/ml) were plated in gelatinised 24 well plates and treated as above. Cells in the supernatant were centrifuged and lysed in 10 mM EDTA, 50 mM Tris-HCl, 0.5% SDS, and 0.5 mg/ml proteinase K on ice for 30 min. Cell lysate was treated with RNase A (1 μg/ml) and DNA was extracted using phenol/chloroform. DNA samples were run on 2% agarose gels at 80 V until the dye front was 3 cm from the bottom of the gel. Gels were visualised by staining in ethidium bromide (1 μg/ml) for 20 min and exposure to ultraviolet light.

2.8. Quantification of prostaglandins by ELISA

HUVECs were plated and treated as above and the supernatant removed for analysis. PGE_2 and 6-keto-$PGF_{2\alpha}$ were quantified by ELISA according to the manufacturer's instructions.

2.9. Tubule formation

Matrigel ECM (40 μl) was added to pre-cooled (−20 °C) sterile 96 well plates and allowed to set at 37 °C for 30 min. HUVECs (100 μl diluted to 2×10^5 cells/ml in supplemented Medium 199) were added to each well together with DuP-697 (10 nM) and $VEGF_{165}$ (50 ng/ml) and PGE_2 (10 μM) as required. Cells were incubated at 37 °C. Tubule formation was assessed 8 h later under light microscopy at ×400 magnification. Tubule formation was positively identified when HUVECs had migrated to make physical contact with each other to form a full tubule (adapted from (Scappaticci et al., 2001; Smith and Hoffman, 2005).

2.10. Western blotting

Total cell protein in lysates generated from experiments was determined by the bicinchoninic acid (BCA) assay and western blot analysis performed as described previously (Smith and Hoffman, 2005). Equal concentrations of protein were loaded for each sample (20 μg for COX-2; 90 μg for caspase analysis). COX-2 was identified using a specific polyclonal goat anti-COX-2 primary antibody (0.2 μg/ml) and a horse-radish peroxidase conjugated anti-goat secondary antibody (0.08 μg/ml). Caspases were identified using mouse anti-caspase primary antibody (0.15 μg/ml) selective for either caspase 3, 8 or 9. A horse-radish peroxidase conjugated anti-goat IgG (0.08 μg/ml) was used as the secondary antibody. Levels of β-actin were analysed to confirm that equal concentrations of protein were loaded. Bands were quantified by densitometry using a Gene Genius Bioimaging system (Syngene).

2.11. Statistical analysis

Statistical significance of apoptosis, tubule formation and PGE_2 production was carried out using two-way ANOVA and confirmed with an unpaired student's *t*-test. All graphical data are the mean of at least three separate experiments with three replicates for each data point; for which the standard error was calculated.

3. Results

3.1. Expression of COX-2 in HUVECs

HUVECs grown in medium containing 20% serum expressed low levels of COX-2 protein, as determined by western blot (Fig. 1). When cells quiesced in SFM were subsequently stimulated with VEGF there was a time-dependent increase in COX-2 expression with maximal expression occurring by 8 h and COX-2 expression was maintained for 24 h after the addition of VEGF (Fig. 1).

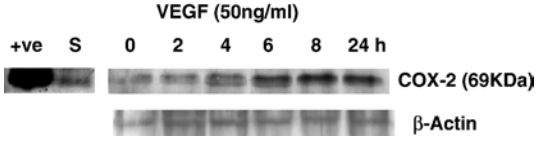

Fig. 1. Cyclooxygenase-2 expression in HUVECs. HUVECs were quiesced for 16 h in 1% FCS Medium 199 and then exposed to VEGF for 0–24 h. COX-2 expression in HUVECs cultured in Medium 199 containing 20% FCS for 24 h is also shown (S). Positive control for the antibody was assessed using J774 murine macrophages treated with lipopolysaccharide for 24 h (+ve). The image is representative of 3 separate experiments.

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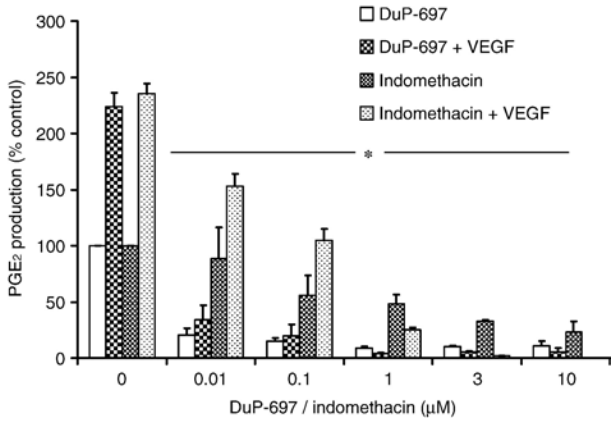

Fig. 2. Inhibition of prostaglandin E_2 production by DuP-697 or indomethacin in non-stimulated and VEGF stimulated HUVECs. Control cells were incubated with 0.01% DMSO or 0.01% methanol for 24 h. PGE_2 levels were determined by ELISA. Values are mean±s.e.m. of 3 independent experiments each with 3 replicates. Statistical significance was determined using Student's *t*-test compared to either the DMSO or methanol controls. *denotes $P<0.05$.

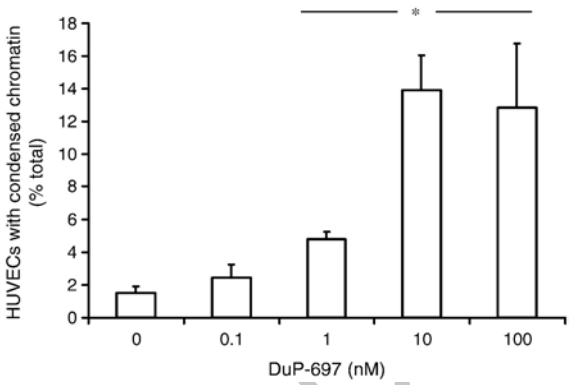

Fig. 4. Induction of chromatin condensation in HUVECs by DuP-697. HUVECs cultured in serum free media were treated with DuP-697 (0.1–100nM) for 24 h at 37 °C prior to cytospinning and staining of the DNA with acridine orange. Control cells were incubated with DMSO (0.01%). Values are means±s.e.m. of 3 independent experiments with 2 replicates in each. Statistical significance was determined using Student's *t*-test compared to the DMSO controls. *denotes $P<0.05$.

3.2. Inhibition of prostaglandin production by DuP-697and Indomethacin

Under basal control conditions, PGE_2 production by HUVECs cultured in SFM for 24 h was 124 pg/ml. Incubation with VEGF for 24 h increased PGE_2 production to 262 pg/ml. DuP-697 (0.01–10 μM) inhibited in a dose-dependent manner both basal and VEGF-stimulated PGE_2 production (Fig. 2). DuP-697 at 10 nM (the IC_{50} for COX-2 inhibition) inhibited basal and VEGF-stimulated PGE_2 production by approximately 80% and 85% respectively and concentrations of DuP-697 of 1 μM and above inhibited both basal and VEGF-stimulated PGE_2 production by>90% (Fig. 2). Indomethacin (0.01–10 μM) also inhibited basal and VEGF-stimulated PGE_2 production although higher concentrations were required for inhibition than was seen for DuP-697 (Fig. 2).

Levels of 6-keto-$PGF_{2\alpha}$ were measured as a marker of prostacyclin production. DuP-697 inhibited 6-keto-$PGF_{2\alpha}$ production by ~60% at concentrations of 0.01 μM and 0.1 μM in the non-stimulated cells. However, at the higher concentrations of DuP-697, 6-keto-$PGF_{2\alpha}$ production appeared to return to basal levels. VEGF-stimulated cells exhibited a dose dependent inhibition of 6-keto-$PGF_{2\alpha}$ with a maximal inhibition of 93% at 10 μM (Fig. 3).

3.3. Induction of apoptosis by DuP-697 and indomethacin

DuP-697 at concentrations between 0.1 nM and 100 nM caused a dose-dependent increase in chromatin condensation of non-adherent HUVECs in SFM (Fig. 4). By contrast, indomethacin only induced a statistically significant increase in chromatin condensation at 3 μM and above, concentrations that have been shown to inhibit COX-2 (Fig. 5). There was no chromatin

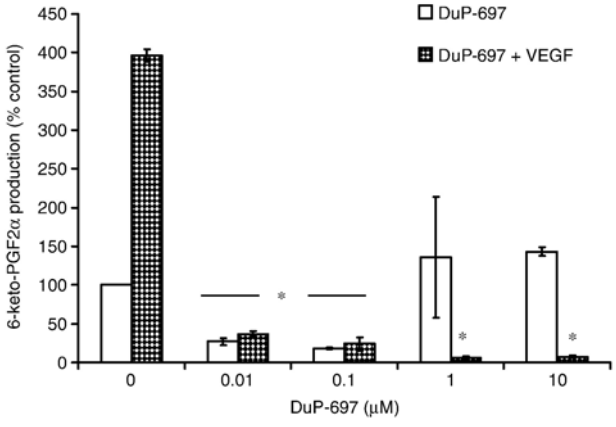

Fig. 3. Inhibition of 6-keto-prostaglandin $F_{2\alpha}$ production by DuP-697 in non-stimulated and VEGF stimulated HUVECs. Control cells were incubated with 0.01% DMSO for 24 h. $PGF_{2\alpha}$ levels were determined by ELISA. Values are mean±s.e.m. of 3 independent experiments each with 3 replicates. Statistical significance was determined using Student's *t*-test compared to the DMSO controls. *denotes $P<0.05$.

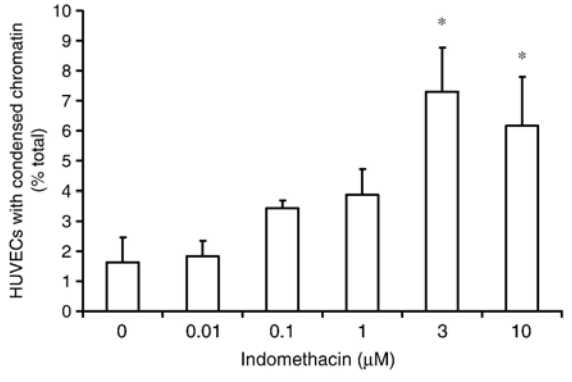

Fig. 5. Induction of chromatin condensation in HUVECs by indomethacin. HUVECs cultured in serum free media were treated with indomethacin (0.01–10μM) for 24 h at 37°C prior to cytospinning and staining of the DNA with acridine orange. Control cells were incubated with methanol (0.01%). Values are means±s.e.m. of 3 independent experiments with 2 replicates in each. Statistical significance was determined using Student's *t*-test compared to the methanol controls. *denotes $P<0.05$.

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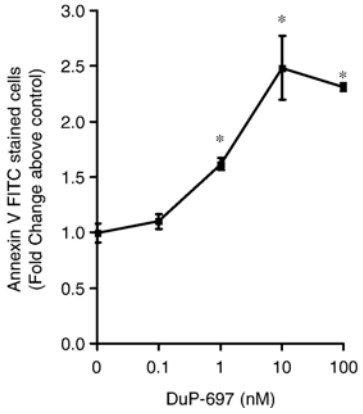

Fig. 6. Assessment of DuP-697-induced apoptosis in HUVECs by flow cytometry. HUVECs were treated with DuP-697 for 6 h at 37 °C prior to staining with Annexin-V FITC and propidium iodide and analysed by flow cytometry. Control cells were incubated with DMSO (0.01%). The data represents fold change in annexin-V FITC labelled cells and is the means±s.e.m. of 3 independent experiments with 2 replicates in each. Statistical significance was determined using Student's *t*-test compared to the DMSO controls. *denotes $P<0.05$.

condensation in adherent cells under any of these conditions (data not shown). Parallel studies conducted with flow cytometry (FACs) to confirm the pro-apoptotic actions of DuP-697 showed a concentration-dependent increase in annexin-V FITC stained cells which mirrored that in the acridine orange stained cells described above. The maximum effect, as seen with acridine orange staining, was produced by 10 nM DuP-697 which caused a 2.5-fold increase in apoptotic cells and this was not further enhanced with higher concentrations of the drug (Fig. 6). No change in staining was observed in the propidium iodide only stained cells or the cells stained by both annexin-V FITC and propidium iodide (data not shown).

The benchmark DNA laddering analysis was also carried out to evaluate apoptosis of HUVECs cultured in SFM. DuP-697

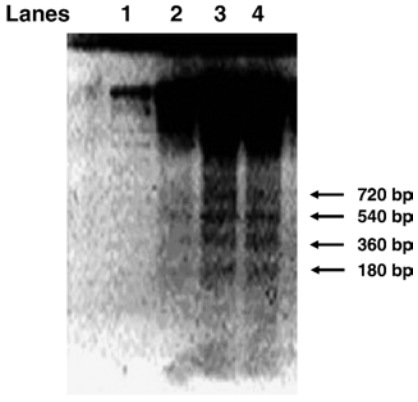

Fig. 7. Induction of apoptotic DNA fragmentation in HUVECs. HUVECs were treated with DuP-697 (10 nM) for 24 h at 37 °C prior to DNA extraction by phenol/chloroform followed by 2% agarose gel electrophoresis and staining with ethidium bromide. Control cells were incubated at 37 °C for 24 h with DMSO (0.01%). Lanes: (1) DNA ladder; (2) DMSO (0.01%) control; (3) Actinomycin-D positive control (200 nM); (4) DuP-697 (10 nM). The image is representative of 3 separate experiments.

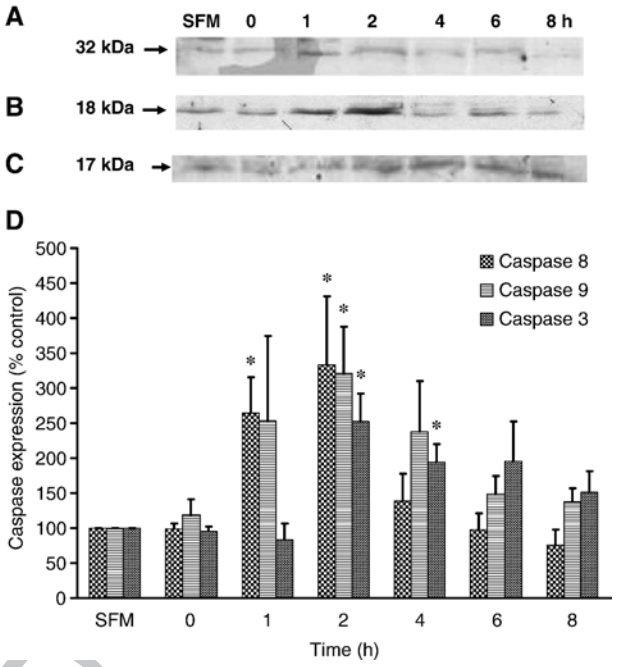

Fig. 8. Caspase expression in HUVECs. HUVECs cultured in serum free media (SFM) were treated with DuP-697 (10 nM) for 0–8 h and then assayed for (A) caspase-9, (B) caspase-8, (C) caspase-3. Control cells were incubated for 8 h with DMSO (0.01%). Caspase expression was quantified by densitometry (D). The images are representative of 3 experiments. Values represent mean±s.e.m. of 3 experiments. Statistical significance was determined using Student's *t*-test compared to the DMSO controls. *denotes $P<0.05$.

(10 nM) induced high molecular weight DNA fragmentation and the classical lower molecular weight (720 bp down to 180 bp) DNA laddering after 24 h, which is indicative of apoptosis (Fig. 7).

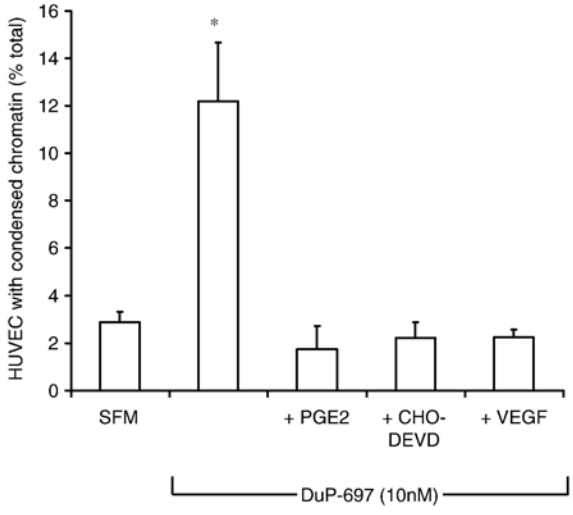

Fig. 9. Reversal of DuP-697 induced chromatin condensation by PGE_2, CHO-DEVD or $VEGF_{165}$ in HUVECs. HUVECs cultured in serum free media (SFM) were treated with DuP-697 (10nM) alone or in the presence of PGE_2 (10 μM), caspase 3 inhibitor (CHO-DEVD) (12.5 μM) or VEGF (50 ng/ml) and incubated at 37 °C for 24 h. Cells were cytospun and stained with acridine orange. Control cells were incubated for 24 h in serum free medium containing DMSO (0.01%). Values represent means±s.e.m. of 3 experiments with 2 replicates in each. Statistical significance was determined using Student's *t*-test compared to the DMSO controls. *denotes $P<0.05$.

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To further confirm the induction of apoptosis with DuP-697, caspase activation was examined using antibodies specific to the active caspases. There was induction of caspases-8 (Fig. 8A, D) and -9 (Fig. 8B, D) within 1 h of DuP-697 treatment and this induction peaked at 2 h, declining thereafter. By comparison, caspase-3 was maximally induced by 2 h with levels slowly declined thereafter (Fig. 8C, D).

Incubations of cells with PGE_2 (10 μM), the specific caspase-3 inhibitor DEVD–CHO (12.5 μM) or VEGF (50 ng/ml) completely reversed apoptosis induced with DuP-697 (10 nM) (Fig. 9). These compounds also inhibited DuP-697-induced DNA laddering (data not shown).

3.4. Effects of DuP-697 and indomethacin on in vitro angiogenesis

In vitro angiogenesis was assessed by quantifying capillary-like tubule formation of unstimulated and VEGF stimulated

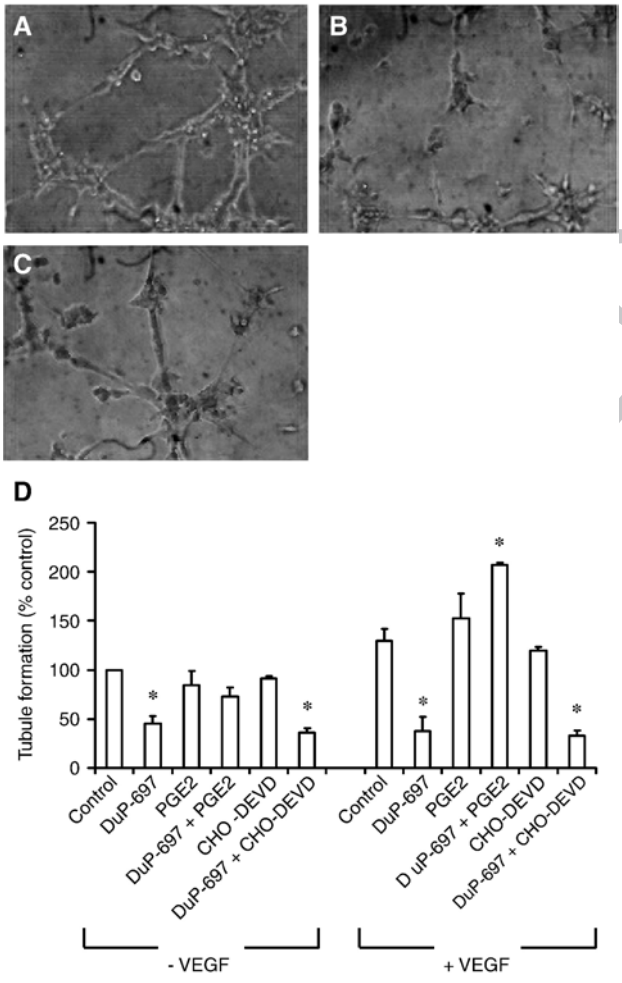

Fig. 10. Capillary-like tubule formation of HUVECs. HUVECs were incubated on Matrigel and tubules were photographed after 8 h (A–C). (A) complete media+DMSO (0.01%), (B) DuP-697 (10nM), (C) DuP-697+PGE_2 (10μM). Tubules were quantified and expressed as a % of tubule formation in control non-stimulated cells (D). CHO–DEVD was used at a concentration of 12.5 μM. Values are means±s.e.m. of 3 experiments with 2 replicates in each. Statistical significance was determined using Student's *t*-test compared to the DMSO controls. *denotes $P<0.05$.

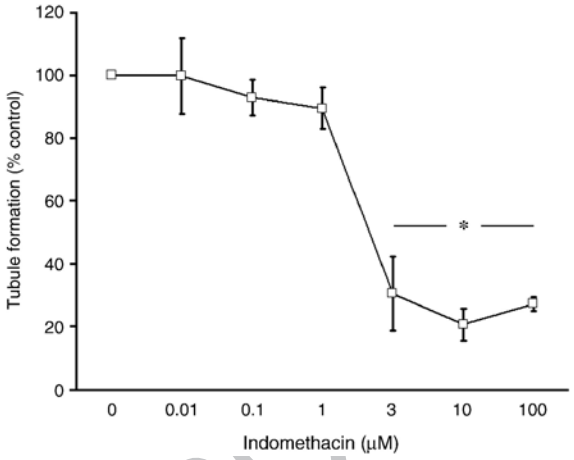

Fig. 11. Effect of indomethacin on HUVEC tubule formation. HUVECs in complete media were treated with indomethacin (0.01–100 μM) for 8 h. Control cells were incubated for 8 h in complete media with DMSO (0.01%). Values represent means±s.e.m. of 3 experiments with 2 replicates in each. Statistical significance was determined using Student's *t*-test compared to the DMSO controls. *denotes $P<0.05$.

HUVECs cultured on Matrigel. Control HUVECs formed tubules on Matrigel after an 8 h incubation at 37 °C (Fig. 10A, D). DuP-697 (10 nM) significantly inhibited tubule formation of unstimulated HUVECs (Fig. 10B, D). PGE_2 (10 μM) reversed the inhibition of tubule formation caused by DuP-697 (Fig. 10C, D). Incubation with the casapse-3 inhibitor DEVD–CHO did not prevent the DuP-697-induced inhibition of tubule formation (Fig. 10D).

Similar results were obtained when capillary-like tubule formation was assessed in VEGF-stimulated HUVECs. VEGF treatment caused a small but statistically significant increase of tubule formation relative to control levels (Fig. 10D). VEGF induced tubule formation was significantly reduced by DuP-697 (10nM) and this inhibition was reversed with PGE_2 (Fig. 10D). Indomethacin only inhibited tubule formation at concentrations of 3 μM and above (Fig. 11).

4. Discussion

The present work shows unequivocally that DuP-697 induces apoptosis and inhibits capillary-like tubule formation in HUVECs. This was confirmed using several approaches including analysis of chromatin condensation, FACs analysis, the distinctive DNA laddering and changes in caspase activation. In all these studies, the peak effects were observed at a concentration of 10 nM DuP-697, which is the IC_{50} value for inhibition of COX-2 activity *in vitro* (Gierse et al., 1995).

Results from various cell types indicate that inhibition of COX-2 is associated with the induction of apoptosis whereas the inhibition of COX-1 may not be involved. COX-2 over-expression in endothelial cells has been shown to promote cell survival (Leahy et al., 2002). In U397 cells, inhibition of COX-1 did not induce apoptosis whereas inhibition of COX-2 was required to induce apoptosis *in vitro* (Johnson et al., 2001; Riendeau et al., 1997). In our studies we have found that whereas DuP-697 induced apoptosis at concentrations specific for the

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inhibition of COX-2, the non-selective COX inhibitor indomethacin induced apoptosis only when used at concentrations known to inhibit COX-2 (≥3 μM; (Mitchell et al., 1993)) and it had no effect when used at lower concentrations that specifically inhibit COX-1. This supports the notion that COX-2 rather than COX-1 is associated with cell survival and protection against apoptosis in HUVECs.

Our studies also revealed that PGE_2 or VEGF prevented DNA laddering and chromatin condensation induced in HUVECs by 10 nM DuP-697. These findings indicate that both PGE_2 and VEGF may protect against DuP-697 induced apoptosis in these cells. Similarly, exogenous PGE_2 has also been shown to prevent apoptosis in HCA-7 human colon carcinoma cells induced by selective COX-2 inhibition (Sheng et al., 1998).

The concentration of DuP-697 that induced chromatin condensation (10 nM) was the concentration that also inhibits both PGE_2 and 6-keto-$PGF_{2\alpha}$ production. This suggests that inhibition of COX-2 is very important for the induction of apoptosis. Further work is required in order to identify the specific prostanoid(s) that when inhibited triggers apoptosis. In addition, several isoforms of prostaglandin E synthase (PGE) have been identified, including the cytosolic PGEs (Tanioka et al., 2000), microsomal PGEs-1 (Jakobsson et al., 1999) and mPGEs-2 (Tanikawa et al., 2002). Thus it will be of interest to evaluate which isoform(s) is responsible for PGE_2 production in HUVECs.

Several studies have implicated caspases as mediators of apoptosis induced by COX-2 inhibitors. For instance, Basu et al. (2005) have reported that 48 h treatment of MDA-MB-231 and MDA-MB-468 breast cancer cells with celecoxib resulted in caspase 3 and 7 dependent apoptosis. In our studies, caspases 3, 8 and 9 were induced by DuP-697. Since caspase cleavage does not always reflect activation we conducted additional studies aimed at inhibiting the activity of caspase 3 which is the effector caspase in apoptosis. These studies were carried out using the selective caspase-3 inhibitor DEVD–CHO which inhibited chromatin condensation and prevented DNA laddering, confirming that DuP-697-induced apoptosis in HUVECs is caspase-3 dependent.

Treatment of HUVECs with DuP-697 (10 nM) prevented capillary-like tubule formation *in vitro* whereas the non-specific COX inhibitor indomethacin only inhibited angiogenesis at concentrations known to inhibit COX-2 (≥3 μM). These data suggest that COX-2 is essential for tubule formation and that this process may require PGE_2 production since inhibition of tubule formation by DuP-697 was reversed by exogenous PGE_2 in our studies. This notion is consistent with a report by Leahy et al. demonstrating that PGE_2 prevented the inhibition of *in vivo* rat cornea angiogenesis induced by celecoxib (Leahy et al., 2002).

Not only are the VEGF and PGE_2 signalling pathways interrelated, but, in addition, down-stream effectors of these pathways regulate both apoptosis and angiogenesis. VEGF may enhance COX-2 expression forming a positive feedback loop that regulates both VEGF production and COX-2 induction (Caughey et al., 2001). VEGF binding and the production of PGE_2 have been shown to be important in αVβ3 integrin binding and cell survival (Dormond et al., 2002; Leahy et al., 2002; Yazawa et al., 2005). Inhibition of PGE_2 decreased αVβ3 integrin expression and activated apoptosis through the inhibition of Bcl-2 expression and subsequent caspase 9 activation or Fas receptor trimerisation and activation of caspase 8 (Aoudjit and Vuori, 2001; Dormond et al., 2002; Pollman et al., 1999). In relation to angiogenesis, the products of COX-2, including PGE_2 and TXA_2, play an important role in cellular migration and tubule formation with specific inhibition of PGE_2 and TXA_2 preventing proliferation and angiogenesis (Jantke et al., 2004; Wu et al., 2003). PGE_2 may induce VEGF expression through binding to the EP4 receptor and activating the JNK and HIF-1α pathways (Ghosh et al., 2000; Huang et al., 2005; Kuwano et al., 2004). PGE_2 has also been shown to increase binding of endothelial cells to the extracellular matrix (ECM) through αVβ3 dependent mechanisms (Leahy et al., 2002; Yazawa et al., 2005).

In summary, the selective COX-2 inhibitor DuP-697 has been found to induce apoptosis and prevent capillary-like tubule formation *in vitro* at pharmacologically relevant concentrations. The effects observed may possibly be due to the specific inhibition of COX-2 by DuP-697 with a subsequent decrease in PGE_2 production. Moreover, our data has demonstrated that DuP-697 induced apoptosis in HUVECs may be caspase-dependent while the inhibition of tubule formation may occur through a caspase-independent mechanism.

References

Akarasereenont, P.C., Techatraisak, K., Thaworn, A., Chotewuttakorn, S., 2002. The expression of COX-2 in VEGF-treated endothelial cells is mediated through protein tyrosine kinase. Mediat. Inflamm. 11, 17–22.

Allison, M.C., Howatson, A.G., Torrance, C.J., Lee, F.D., Russell, R.I., 1992. Gastrointestinal damage associated with the use of nonsteroidal antiinflammatory drugs. N. Engl. J. Med. 327, 749–754.

Aoudjit, F., Vuori, K., 2001. Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-flip and implications for anoikis. J. Cell Biol. 152, 633–643.

Basu, G.D., Pathangey, L.B., Tinder, T.L., Gendler, S.J., Mukherjee, P., 2005. Mechanisms underlying the growth inhibitory effects of the cyclo-oxygenase-2 inhibitor celecoxib in human breast cancer cells. Breast Cancer Res. 7, R422–R435.

Breder, C.D., Dewitt, D., Kraig, R.P., 1995. Characterization of inducible cyclooxygenase in rat brain. J. Comp. Neurol. 355, 296–315.

Breier, G., Albrecht, U., Sterrer, S., Risau, W., 1992. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development 114, 521–532.

Calviello, G., Di Nicuolo, F., Gragnoli, S., Piccioni, E., Serini, S., Maggiano, N., Tringali, G., Navarra, P., Ranelletti, F.O., Palozza, P., 2004. n-3 PUFAs reduce VEGF expression in human colon cancer cells modulating the COX-2/PGE2 induced ERK-1 and-2 and HIF-1alpha induction pathway. Carcinogenesis 25, 2303–2310.

Carmeliet, P., 2000. Mechanisms of angiogenesis and arteriogenesis. Nat. Med. 6, 389–395.

Caughey, G.E., Cleland, L.G., Penglis, P.S., Gamble, J.R., James, M.J., 2001. Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: selective up-regulation of prostacyclin synthesis by COX-2. J. Immunol. 167, 2831–2838.

Colville-Nash, P.R., Gilroy, D.W., 2000. Cyclooxygenase enzymes as targets for therapeutic intervention in inflammation. Drug News Perspect. 13, 587–597.

Dormond, O., Bezzi, M., Mariotti, A., Ruegg, C., 2002. Prostaglandin E2 promotes integrin alpha Vbeta 3-dependent endothelial cell adhesion, rac-

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activation, and spreading through cAMP/PKA-dependent signaling. J. Biol. Chem. 277, 45838–45846.
Ghosh, A.K., Hirasawa, N., Niki, H., Ohuchi, K., 2000. Cyclooxygenase-2-mediated angiogenesis in carrageenin-induced granulation tissue in rats. J. Pharmacol. Exp. Ther. 295, 802–809.
Gierse, J.K., Hauser, S.D., Creely, D.P., Koboldt, C., Rangwala, S.H., Isakson, P.C., Seibert, K., 1995. Expression and selective inhibition of the constitutive and inducible forms of human cyclo-oxygenase. Biochem. J. 305, 479–484.
Hallam, T.J., Pearson, J.D., Needham, L.A., 1988. Thrombin-stimulated elevation of human endothelial-cell cytoplasmic free calcium concentration causes prostacyclin production. Biochem. J. 251, 243–249.
Harris, R.C., McKanna, J.A., Akai, Y., Jacobson, H.R., Dubois, R.N., Breyer, M.D., 1994. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. J. Clin. Invest. 94, 2504–2510.
Hernandez, G.L., Volpert, O.V., Iniguez, M.A., Lorenzo, E., Martinez-Martinez, S., Grau, R., Fresno, M., Redondo, J.M., 2001. Selective inhibition of vascular endothelial growth factor-mediated angiogenesis by cyclosporin A: roles of the nuclear factor of activated T cells and cyclooxygenase 2. J. Exp. Med. 193, 607–620.
Hong, S.P., Ha, S.H., Park, I.S., Kim, W.H., 1998. Induction of apoptosis in colon cancer cells by nonsteroidal anti-inflammatory drugs. Yonsei Med. J. 39, 287–295.
Huang, S.P., Wu, M.S., Shun, C.T., Wang, H.P., Hsieh, C.Y., Kuo, M.L., Lin, J.T., 2005. Cyclooxygenase-2 increases hypoxia-inducible factor-1 and vascular endothelial growth factor to promote angiogenesis in gastric carcinoma. J. Biomed. Sci. 12, 229–241.
Inoue, H., Taba, Y., Miwa, Y., Yokota, C., Miyagi, M., Sasaguri, T., 2002. Transcriptional and posttranscriptional regulation of cyclooxygenase-2 expression by fluid shear stress in vascular endothelial cells. Arterioscler. Thromb. Vasc. Biol. 22, 1415–1420.
Jakobsson, P.J., Thoren, S., Morgenstern, R., Samuelsson, B., 1999. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. Proc. Natl. Acad. Sci. U. S. A. 96, 7220–7225.
Jantke, J., Ladehoff, M., Kurzel, F., Zapf, S., Kim, E., Giese, A., 2004. Inhibition of the arachidonic acid metabolism blocks endothelial cell migration and induces apoptosis. Acta Neurochir. 146, 483–494.
Johnson, A.J., song, X., Hsu, A., Chen, C., 2001. Apoptosis signaling pathways mediated by cyclooxygenase-2 inhibitors in prostate cancer cells. Adv. Enzyme Regul. 41, 221–235.
Kargman, S., Charleson, S., Cartwright, M., Frank, J., Riendeau, D., Mancini, J., Evans, J., O'Neill, G., 1996. Characterization of Prostaglandin G/H Synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts. Gastroenterology 111, 445–454.
Kuwano, T., Nakao, S., Yamamoto, H., Tsuneyoshi, M., Yamamoto, T., Kuwano, M., Ono, M., 2004. Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. FASEB J. 18, 300–310.
Leahy, K.M., Ornberg, R.L., Wang, Y., Zweifel, B.S., Koki, A.T., Masferrer, J.L., 2002. Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. Cancer Res. 62, 625–631.
Liu, D., Jia, H., Holmes, D.I., Stannard, A., Zachary, I., 2003. Vascular endothelial growth factor-regulated gene expression in endothelial cells: KDR-mediated induction of Egr3 and the related nuclear receptors Nur77, Nurr1, and Nor1. Arterioscler. Thromb. Vasc. Biol. 23, 2002–2007.
Masferrer, J.L., Leahy, K.M., Koki, A.T., Zweifel, B.S., Settle, S.L., Woerner, B.M., Edwards, D.A., Flickinger, A.G., Moore, R.J., Seibert, K., 2000. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res. 60, 1306–1311.
Masferrer, J.L., Zweifel, B.S., Colburn, S.M., Ornberg, R.L., Salvemini, D., Isakson, P., Seibert, K., 1995. The role of cyclooxygenase-2 in inflammation. Am. J. Ther. 2, 607–610.
Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1993. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc. Natl. Acad. Sci. U. S. A. 90, 11693–11697.
Murphy, J.F., Fitzgerald, D.J., 2001. Vascular endothelial growth factor induces cyclooxygenase-dependent proliferation of endothelial cells via the VEGF-2 receptor. FASEB J. 15, 1667–1669.
Parente, L., Perretti, M., 2003. Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. Biochem. Pharmacol. 65, 153–159.
Pollman, M.J., Naumovski, L., Gibbons, G.H., 1999. Endothelial cell apoptosis in capillary network remodeling. J. Cell. Physiol. 178, 359–370.
Riendeau, D., Charleson, S., Cromlish, W., Mancini, J.A., Wong, E., Guay, J., 1997. Comparison of the cyclooxygenase-1 inhibitory properties of nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors, using sensitive microsomal and platelet assays. Can. J. Physiol. Pharmacol. 75, 1088–1095.
Sawaoka, H., Kawano, S., Tsuji, S., Tsujii, M., Gunawan, E.S., Takei, Y., Nagano, K., Hori, M., 1998. Cyclooxygenase-2 inhibitors suppress the growth of gastric cancer xenografts via induction of apoptosis in nude mice. Am. J. Physiol. 274, G1061–G1067.
Sawaoka, H., Tsuji, S., Tsujii, M., Gunawan, E.S., Sasaki, Y., Kawano, S., Hori, M., 1999. Cyclooxygenase inhibitors suppress angiogenesis and reduce tumor growth in vivo. Lab. Invest. 79, 1469–1477.
Scappaticci, F.A., Smith, R., Pathak, A., Schloss, D., Lum, B., Cao, Y., Johnson, F., Engleman, E.G., Nolan, G.P., 2001. Combination angiostatin and endostatin gene transfer induces synergistic antiangiogenic activity in vitro and antitumor efficacy in leukemia and solid tumors in mice. Molec. Ther. 3, 186–196.
Schmassmann, A., Peskar, B.M., Stettler, C., Netzer, P., Stroff, T., Flogerzi, B., Halter, F., 1998. Effects of inhibition of prostaglandin endoperoxide synthase-2 in chronic gastro-intestinal ulcer models in rats. Br. J. Pharmacol. 123, 795–804.
Seibert, K., Masferrer, J.L., 1994. Role of inducible cyclooxygenase (COX-2) in inflammation. Receptor. 4, 17–23.
Sheng, H., Shao, J., Morrow, J.D., Beauchamp, R.D., DuBois, R.N., 1998. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. Cancer Res. 58, 362–366.
Smith, E., Hoffman, R., 2005. Multiple fragments related to angiostatin and endostatin in fluid from venous leg ulcers. Wound Repair Regen. 13, 148–157.
Takeeda, M., Hayashi, Y., Yamato, M., Murakami, M., Takeuchi, K., 2004. Roles of endogenous prostaglandins and cyclooxygenase izoenzymes in mucosal defense of inflamed rat stomach. J. Physiol. Pharmacol. 55, 193–205.
Tanikawa, N., Ohmiya, Y., Ohkubo, H., Hashimoto, K., Kangawa, K., Kojima, M., Ito, S., Watanabe, K., 2002. Identification and characterization of a novel type of membrane-associated prostaglandin E synthase. Biochem. Biophys. Res. Commun. 291, 884–889.
Tanioka, T., Nakatani, Y., Semmyo, N., Murakami, M., Kudo, I., 2000. Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis. J. Biol. Chem. 275, 32775–32782.
Vane, J.R., Bakhle, Y.S., Botting, R.M., 1998. Cyclooxygenases 1 and 2. Annu. Rev. Pharmacol. Toxicol. 38, 97–120.
Wu, G., Luo, J., Rana, J.S., Laham, R., Sellke, F.W., Li, J., 2006. Involvement of COX-2 in VEGF-induced angiogenesis via P38 and JNK pathways in vascular endothelial cells. Cardiovasc. Res. 69, 512–519.
Wu, G.S., Zou, S.Q., Liu, Z.R., Tang, Z.H., Wang, J.H., 2003. Celecoxib inhibits proliferation and induces apoptosis via prostaglandin E2 pathway in human cholangiocarcinoma cell lines. World J. Gastroenterol. 9, 1302–1306.
Yazawa, K., Tsuno, N.H., Kitayama, J., Kawai, K., Okaji, Y., Asakage, M., Sunami, E., Kaisaki, S., Hori, N., Watanabe, T., Takahashi, K., Nagawa, H., 2005. Selective inhibition of cyclooxygenase (COX)-2 inhibits endothelial cell proliferation by induction of cell cycle arrest. Int. J. Cancer 113, 541–548.

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