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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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**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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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 $\geq 3$ 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 media were 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 Inc (UK). 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). 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 $3 \times 10^5$ 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 199). The cells were treated with DuP-697 (10 nM) or indomethacin (3 μM) diluted in serum free medium (SFM). In corresponding