Pharmacological Effects of SB 220025, a Selective Inhibitor of P38 Mitogen-Activated Protein Kinase, in Angiogenesis and Chronic Inflammatory Disease Models

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ABSTRACT

Chronic inflammatory diseases often are accompanied by intense angiogenesis, supporting the destructive proliferation of inflammatory tissues. A model of inflammatory angiogenesis is the murine air pouch granuloma, which has a hyperangiogenic component. In this model, we explored the regulation of inflammatory angiogenesis using SB 220025, a specific inhibitor of human p38 mitogen-activated protein (MAP) kinase, with an IC50 value of 60 nM and 50- to 1000-fold selectivity vs. other kinases tested. In vivo, this compound reduced the lipopolysaccharide-induced production of tumor necrosis factor at an ED50 value of 7.5 mg/kg. In the inflammatory angiogenesis model, over the course of granuloma development, we observed elevated levels of interleukin-1β and tumor necrosis factor-α during the chronic inflammatory phase when intense angiogenesis occurs. SB 220025 at 30 mg/kg b.i.d. p.o. was able to greatly reduce the expression of these cytokines and inhibit angiogenesis by ~40%. To further study the effects of p38/CSBP MAP kinase inhibition in angiogenesis-dependent chronic inflammatory disease, SB 220025 was tested in murine collagen-induced arthritis. In this model, SB 220025 was able to prevent the progression of established arthritis. Thus, this p38/CSBP MAP kinase inhibitor, which can reduce inflammatory cytokine production and inhibit angiogenesis, is an effective treatment for chronic proliferative inflammatory disease.

Proliferating tissues require angiogenesis to support their growth, and thus diseases such as cancer and chronic inflammation are thought to be angiogenesis dependent (Folkman, 1995; Jackson, 1996). In the case of chronic inflammation, angiogenesis may be required not only to support the proliferation but also to allow the massive cellular infiltration associated with the chronically inflamed state. Angiogenesis is normally under very tight control. In the normal adult, the majority of the vasculature is stable, with endothelial cell turnover on the order of thousands of days. Nevertheless, these quiescent cells can rapidly switch to an angiogenic phenotype under certain conditions, as, for example, in wound healing. Once a new capillary bed is established, however, the endothelium normally returns to its quiescent state.

Understanding the signals that regulate angiogenesis is key to controlling it under pathological conditions. Growth factors such as VEGF and FGF are clearly able to induce angiogenesis and, in the case of VEGF, appear to be regulated by physiological signals like hypoxia. Hypoxia is not always necessary, however; some inflammatory mediators can potently induce angiogenesis in vivo even in the absence of hypoxia. Both IL-1α and TNF-α can induce angiogenesis in the normally avascular cornea (BenEzra et al., 1990; Ben-Ezra and Maftzir, 1996; Fajardo et al., 1992). These two cytokines have numerous activities, including upregulation of other cytokines, such as IL-8; upregulation of adhesion molecule expression; stimulation of matrix metalloproteinase expression; and increased prostaglandin production (Dinarello, 1991). Many of these activities may contribute to the angiogenic activity of these cytokines. Thus, inhibition of the activity of IL-1β and TNF-α can have an obvious benefit in angiogenesis-dependent inflammatory diseases. One means of inhibiting IL-1β and TNF-α activity is by decreasing their production. SB 220025 is a new compound belonging to the CSAID™ class of cytokine biosynthesis inhibitors (Cuenda et al., 1995; Lee et al., 1994), which act specifically on p38/CSBP MAP kinase to block a cascade, resulting in decreased production of IL-1β and TNF-α as well as other mediators, such as IL-6 and prostaglandins (Beyaert et al., 1996; Pouliot et al., 1997).

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ABBREVIATIONS: CSBP, CSAID™ binding protein; EGFR, epidermal growth factor receptor; Erk, extracellular regulated kinase; FGF, fibroblast growth factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; MAP, mitogen-activated protein; PK, protein kinase; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
Results

SB 220025 selectively inhibits p38 MAP kinase. The pyrimidyl imidazole compounds as exemplified by SB 203580 have been previously demonstrated to specifically inhibit p38 (Cuedna et al., 1995; Lee et al., 1994; Young et al., 1997). SB 220025, a novel member of this structural class (fig. 1), was tested in a number of kinase assays to assess its use as a p38 inhibitor (table 1). This compound inhibited p38 phosphorylation of an EGFR peptide substrate with an IC_{50} value of 60 nM, which is 10-fold more potent than SB 203580 (IC_{50} = 0.6 μM; Cuedna et al., 1995). In selectivity assays, p38 inhibition by SB 220025 was >1000-fold selective over Erk (p42/p44 MAP kinase), 500-fold selective over PKA, >1000-fold selective over EGFR and 50-fold selective over PKC.
SB 220025 inhibits inflammatory cytokine production in vivo. To examine the in vivo efficacy of SB 220025, an acute model of LPS-induced TNF-α expression was used. SB 220025 at a range of doses from 3 to 50 mg/kg was given to mice orally 30 min before challenge with LPS. Serum TNF-α was measured by ELISA after 2 hr. This compound dose-dependently inhibited TNF-α production with an ED50 value of 7.5 mg/kg (fig. 2). Greater than 80% inhibition was obtained at 50 mg/kg. Thus, SB 220025 is an orally available, potent inhibitor of TNF-α synthesis.

SB 220025 inhibits angiogenesis in the murine air pouch granuloma model. The inflammatory cytokines TNF-α and IL-1β are potent inducers of angiogenesis. To test whether they are involved in the promotion of angiogenesis in chronic inflammation, we used the cytokine-suppressive p38/CSBP MAP kinase inhibitor SB 220025 in a model of inflammatory angiogenesis. The murine air pouch granuloma has been characterized as a chronic inflammatory progression with a profound angiogenic component (Colville-Nash et al., 1995). It provides a model in which modulation of angiogenesis in an inflammatory bed can be quantified. Granulomas were formed in a 3-ml dorsal subcutaneous air pouch by injection of 0.5 ml of Freund’s complete adjuvant and croton oil. Within 3 days, a cohesive granulomatous tissue encased the adjuvant mixture. The granulomas were evaluated by weight, histology and vascular index (mg of carmine dye/g of dry tissue), which was used to assess the extent of angiogenesis.

Using a range of doses, we analyzed the effect of SB 220025 on granuloma size and vascular index on day 6. This time point was chosen because it allows sufficient time for angiogenesis and the development of chronic inflammatory character but occurs before the onset of fibrotic features (Colville-Nash et al., 1995; Jackson et al., 1997). The compound caused a dose-dependent reduction in angiogenesis as measured by the vascular index of the granuloma (fig. 3). The maximum effect was a 44% reduction at 50 mg/kg. This is similar to the maximum effect we obtained with a positive control, the angiostatic steroid medroxyprogesterone (fig. 3), which was chosen for its well-documented antiangiogenic activity (Gross et al., 1981), lack of anti-inflammatory activity and consistent pharmacology in our experience with this model. Neither SB 220025 nor the angiostatic steroid had an effect on granuloma size (dry weight).

Effect of SB 220025 on the time course of angiogenesis. We evaluated the effect of the p38/CSBP inhibitor at several time points to determine whether its effects would be different at the various stages of inflammatory and angiogenic progression.
genic progression. SB 220025 was given orally, at an inter-
mEDIATE dose of 30 mg/kg twice a day starting on day 0, and
granulomas were evaluated on days 3, 5, 7 and 14. Granu-
loma size remained fairly constant and was unaffected by the
SB 220025 (fig. 4). The vascular index of the control group
rose gradually from day 3 to 14, whereas the vascular index
of the treated group remained constant. At day 3, the com-
pound did not cause a significant reduction in vascular index
compared with control; however, at days 5, 7 and 14, the
vascular index was lowered significantly by SB 220025.
Thus, the p38/CSBP MAP kinase inhibitor did not affect
the initial burst of angiogenesis but did prevent the increase in
angiogenesis that occurs after day 3.

Inflammatory cytokines such as IL-1β and TNF-α have
been implicated in the pathogenesis of angiogenesis in
chronic inflammation, and p38 inhibitors, such as SB
220025, have been demonstrated to inhibit the synthesis of
these cytokines. We measured the levels of these cytokines
over the course to granuloma development to determine
whether the modulation of their expression by SB 220025
correlated with inhibition of angiogenesis. Cytokine levels
were measured by ELISA using homogenates of granuloma
tissue. TNF-α levels rose sharply, peaking at day 7 and
dropping back down to moderate levels by day 14 (fig. 4). SB
220025 greatly reduced TNF-α levels at day 7. IL-β levels
were also high in control granulomas, peaking at day 7, and
as with TNF-α, the p38/CSBP MAP kinase inhibitor effec-
tively blocked the increased IL-1β expression. Thus, the
ability of SB 220025 to block the sharp rise in TNF-α and IL-1β
between days 5 and 7 correlated well with the ability of the
compound to prevent the increase in vascular index that
occurs over the same time points.

**Microscopic analysis angiogenesis in the granuloma.**

Angiogenesis in the granuloma was microscopically evalu-
ated using cedarwood oil clearing. Figure 5 shows the vascu-
lature of day 6 granulomas from both untreated and SB
220025-treated mice. The profound angiogenesis in the gran-
uloma is demonstrated by the extensive vascular network in
the control tissue. There was a striking reduction in the
vasculature of the treated tissue. The fine capillaries seen in
the control tissue were completely absent in the treated tis-
sue, and only a few larger vessels remained visible.

**Effect of SB 220025 on chronic inflammatory disease.**
The anti-inflammatory and antiangiogenic activities of SB
220025 suggest that it would provide an effective treatment
in chronic inflammatory diseases such as rheumatoid arthritis,
which has both inflammatory cytokine and angiogenic
components. Thus, we tested SB 220025 in a chronic inflam-
matory disease model, murine collagen-induced arthritis.
Mice were primed with bovine collagen, and 3 weeks later,
the animals were given intraperitoneal injections of soluble
collagen and monitored for the appearance of arthritis. Dos-
ing began after arthritis was evident, usually between days 7
and 14 after collagen boost. The first day of dosing was
designated day 0. Animals treated with SB 220025 (50 mg/kg
p.o. b.i.d.) had no increase in severity of arthritis over 10
days, whereas the severity of arthritis in the control mice was
increased at days 7 and 10 (fig. 6). Thus, the p38/CSBP MAP
kinase inhibitor effectively blocked the progression of arthri-
tis.

**Discussion**

Proinflammatory cytokines such as IL-1β and TNF-α have
been shown to play a central role in many inflammatory
processes (Dinarello, 1991). This study demonstrates the im-
portance of IL-1β and TNF-α in chronic inflammatory angi-
genesis and arthritis. Angiogenesis is a normal physiological
response in wound healing, but in diseases such as rheuma-
toid arthritis and psoriasis, it can take on a pathological role.
The association between angiogenesis and chronic inflamma-
tion has led to the hypothesis that angiogenesis is induced by
inflammatory events. Indeed, it has been shown that IL-1β or
TNF-α can induce angiogenesis in the normally avascular
cornea (BenEzra et al., 1990; BenEzra and Maftzir, 1996;
Fajardo et al., 1992). We evaluated the role of these cytokines
in inflammatory angiogenesis in vivo by using a murine air
pouch granuloma model. Both IL-1β and TNF-α levels in the
tissue increased sharply over the first 7 days of
granuloma formation, the same time period in which angiogen-
sis was very active.

We modulated the activity of IL-1β and TNF-α using the
p38/CSBP inhibitor SB 220025, which inhibits their synthe-
sis. This compound is more potent than the previously re-
ported p38 inhibitor SB 203580. We observed an ED₅₀ value
of 7.5 mg/kg for LPS-induced serum TNF-α production,
which is twice as potent a value as that reported for SB
203580 (Badger et al., 1996). SB 220025 caused a significant
dose-dependent decrease in the vascular density of the gran-
uloma, and this correlated with decreases in IL-1β and
TNF-α levels. The hypothesis is that decreasing IL1 and
TNF-α levels resulted in inhibition of angiogenesis in an
inflammatory tissue bed.

When we analyzed a time course of granuloma develop-
ment, we observed that the control group granuloma size, as
measured by dry weight, increased dramatically from day 0
to 3 and then was steady from day 3 to 14. In contrast, the
control group vascular index increased steadily from day 3 to

**Fig. 4.** Time course of granuloma development in murine air pouch
angiogenesis model. Animals were orally dosed twice daily with either
vehicle or 30 mg/kg SB 220025 from day 0 until removal of granuloma
tissue at days 3, 5, 7 or 14. A, Granuloma size was determined by dry
weight to control for possible differences in edema. B, Vascular index (mg
of carmine dye/g of dry tissue) was determined as described in the text. C,
TNF-α levels within granuloma were determined by ELISA on tissue
homogenates. D, IL-1β levels were determined by ELISA in the same
tissue homogenates as C. Data are shown as mean ± S.D. (n = 5).
*Significant from control at P < .05. **Significant from control at P < .01,
calculated by Duncan's multiple-range test.
control group, angiogenesis was still increasing in this study even though TNF-α and IL-1β levels had dropped substantially. This time point represents another phenotypic change in the granuloma, when the chronic inflammatory phenotype gives way to a fibrotic phenotype (Jackson et al., 1997). Thus angiogenesis may be driven by another factor or factors, such as FGF, at this stage.

Granuloma size was not decreased by inhibition of angiogenesis with SB 220025. This was not surprising because granuloma growth is not angiogenesis dependent in this air pouch model (Colville-Nash et al., 1995; Jackson et al., 1997). The model provides an in vivo system for the study of hypervascularization in an inflammatory tissue but is not a model of inflammatory disease. To test the effect of SB 220025 in a model of rheumatoid arthritis, an angiogenesis-dependent chronic inflammatory disease, we used murine collagen-induced arthritis. Using a therapeutic dosing regimen, in which dosing did not begin until there was evidence of arthritic joint disease, SB 220025 was able to prevent further increases in the severity of arthritis. Thus, an inhibitor of IL-1β/TNF-α synthesis and angiogenesis was a very effective treatment for arthritis. This agrees with other studies that demonstrated that TNF-α antibodies (Piguet et al., 1992) were an effective treatment for collagen arthritis and that the angiogenesis inhibitor AGM-1470 was able to reduce the severity of collagen-induced arthritis in rats (Peacock et al., 1992). Interestingly, in a study of other anti-inflammatory drugs (Griswold et al., 1988), the nonsteroidal anti-inflammatory drug ibuprofen was not particularly effective in this model, further suggesting that the anticytokine and antiangiogenic properties of SB 220025 are key to its antiarthritic activity.

Although inhibition of IL-1β and TNF-α synthesis is strongly implicated to be responsible for the antiangiogenic and antiarthritic activities of SB 220025, it is possible that inhibition of the synthesis of other cytokines also may be involved. Other factors, such as the inducible cyclooxygenase, IL-6, IL-8 and GM-CSF, also are regulated by p38/CSBP MAP kinase (Beyaert et al., 1996; Lee et al., 1988, 1989, 1993; Pouliot et al., 1997) and thus may be affected by SB 220025. However, IL-1β and TNF-α are reported to have more potent

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**Fig. 5.** Cedarwood oil histology of air pouch granuloma. Carmine dye vascular casts were made in day-6 granulomas from mice treated with SB 220025 (30 mg/kg b.i.d. p.o.) (A) or vehicle only (B). Granulomas were fixed in ethanol, cleared in cedarwood oil as described in the text, and photographed with transmitted light under 12× magnification.

**Fig. 6.** Collagen-induced arthritis and measurement of severity are described in the text. Dosing with SB 220025 (50 mg/kg b.i.d. p.o.) or vehicle began only when the severity score reached ~4, and this was designated day 0. Data are shown as mean ± S.D. (n = 7). *Significant from control at P < .05. **Significant from control at P < .01 calculated by Student’s t test.
angiogenic activities than eicosanoids and these other cytokines, and the most effective antiangiogenic activity of SB 220025 on days 3 and 5 of granuloma development correlated well with inhibition of IL-1β and TNF-α synthesis. It is important to note that p38 inhibitors such as SB 203580 and SB 220025 also affect the signaling pathways of these cytokines and thus may work via inhibition of both cytokine synthesis and action (Badger et al., 1996; Cuenda et al., 1995). In addition, although it is a very selective inhibitor of p38 MAP kinase and we are unaware of any other activities that could account for its pharmacology, it is possible that SB 220025 may also inhibit an as-yet-unidentified kinase. Therefore, in vivo data should be interpreted with normal caution.

The association between inflammation and angiogenesis has long been observed, but until recently there has been little evidence to clearly demonstrate the link. This study shows that angiogenesis is dependent on inflammatory cytokines in a chronic inflammatory model. It is not clear whether inflammatory cytokines are involved in other angiogenesis dependent processes, such as tumor growth, and this remains to be tested. From our studies and others (Badger et al., 1996), it is apparent that p38/CSBP MAP kinase inhibition should provide an effective treatment for chronic proliferative inflammatory diseases.

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References
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