

# AN0128 Inhibits Pro-inflammatory Cytokine Production in a Macrophage Cell Line by Inhibiting the p38 MAP Kinase Signal Transduction Pathway

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## ABSTRACT

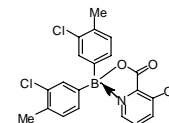
AN0128 is a novel anti-inflammatory borinic acid ester in development for several dermatological indications. AN0128 inhibits release of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 from human peripheral blood mononuclear cells and the human monocytic THP-1 line challenged with lipopolysaccharide (LPS).

The mechanism of action of AN0128 to inhibit production of TNF- $\alpha$  was investigated in THP-1 cells. RT-PCR analysis demonstrated that AN0128 did not alter transcription of the TNF- $\alpha$  gene; however AN0128 prevented synthesis of TNF- $\alpha$  protein, as demonstrated by western analysis. This inhibition was dose-dependent.

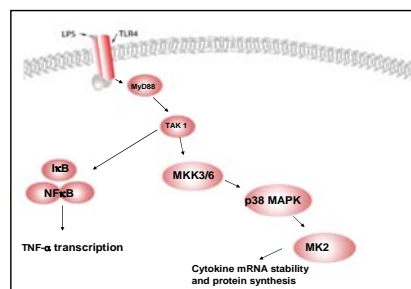
TNF- $\alpha$  mRNA stability and protein synthesis are regulated via phosphorylation through the p38 MAP kinase pathway. The mRNAs of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 are all stabilized through binding of trans-acting factors to a common AU-rich element in the 3' UTR region. Using Western blots and cytometric bead analysis with flow cytometry, we have demonstrated that AN0128 inhibits phosphorylation of p38 MAP kinase in THP-1 cells. This inhibition is likely to be an effect on the pathway upstream of p38 MAP kinase.

Competitive receptor binding assays demonstrate that 10  $\mu$ M AN0128 inhibits agonist binding to the A3 receptor between 60 and 80% with a  $K_i$  of 8.8  $\mu$ M. Binding to this receptor has been shown to inhibit p38 MAP kinase phosphorylation. Inhibition of cAMP production, an early event following A3 receptor binding, was investigated to determine whether it is affected by AN0128 binding to the G-protein-coupled A3 receptor. An AN0128-dose-related inhibition was observed.

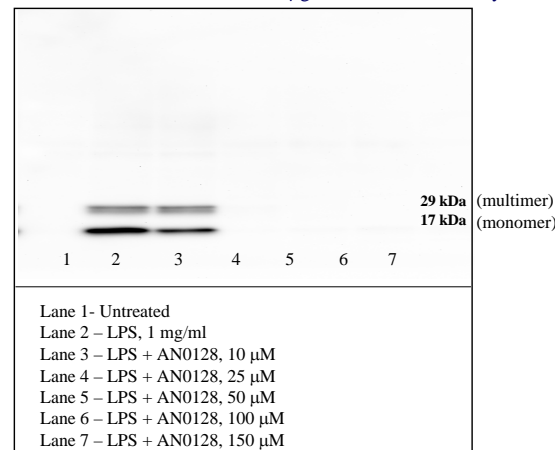
**FIGURE 1.** Structure of AN0128 (3-hydroxypyridine-2-carboxyloxy-bis (3-chloro-4-methylphenyl)-borane)



**FIGURE 2.** Signal transduction after LPS stimulation of TNF- $\alpha$  biosynthesis.



**FIGURE 4.** AN0128 dose-dependently inhibits TNF- $\alpha$  protein translation after stimulation with LPS at 1  $\mu$ g/ml. Western blot analysis



**TABLE 1.** AN0128 competes with an agonist of the A3 adenosine receptor in a competitive receptor binding assay.

Assay	Ligand	Conc.	Non-specific	Incubation	Method of detection
A3	[ <sup>125</sup> I]AB-MECA	0.15 nM	IB-MECA (1 $\mu$ M)	90 min/22°C	Scintillation counting

Receptor	Compound	IC <sub>50</sub> ( $\mu$ M)	K <sub>i</sub> ( $\mu$ M)
A3	AN0128	15	8.8
	IB-MECA (A3 agonist)	0.0011	0.00068
A2A	AN0128	75	61
	NECA (A2A agonist)	0.043	0.038

## CONCLUSIONS

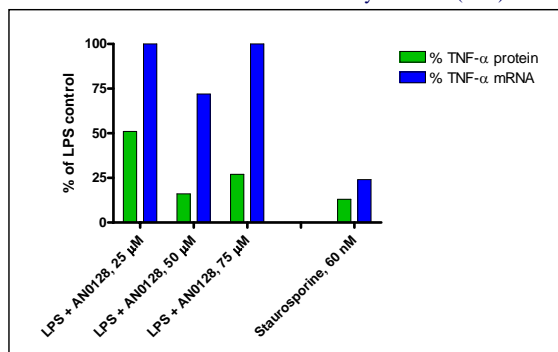
AN0128, a novel boron-containing compound, inhibits TNF- $\alpha$  production in macrophages by inhibiting phosphorylation in the p38 MAP kinase pathway, thus inhibiting TNF- $\alpha$  translation.

➤ AN0128 induced inhibition of IL-1 $\beta$ , IL-6 & IL-8 is likely also via inhibition of p38 MAP kinase phosphorylation.

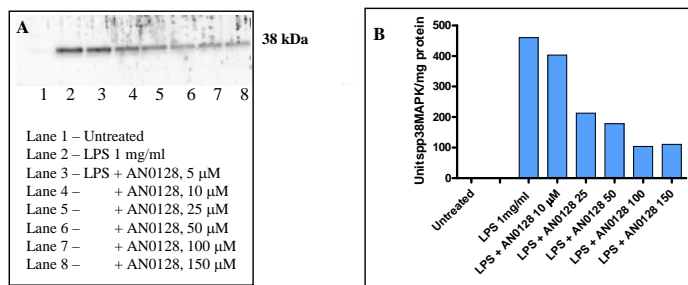
➤ AN0128 inhibits cAMP induction by forskolin, suggesting that it acts upstream of p38 MAP kinase.

## RESULTS

**FIGURE 3.** AN0128 inhibits TNF- $\alpha$  protein production as measured by ELISA (green) but does not inhibit TNF- $\alpha$  mRNA levels as measured by RT-PCR (blue).



**FIGURE 5.** AN0128 dose-dependently inhibits p38 MAP Kinase phosphorylation as measured by A) Western blotting and B) Flow cytometry with cytometry bead analysis. THP-1 cells were stimulated 1 hr with LPS. AN0128 was added 10 min prior to LPS.



**FIGURE 6.** AN0128 dose-dependently inhibits cAMP production by THP-1 cells stimulated with forskolin. Cells were treated with AN0128 in the presence of adenosine deaminase and the phosphodiesterase inhibitor Ro-20-1724 for 15 min prior to a 25-min treatment with forskolin.

