Evidence for direct modification of NFκB by the tyrosine kinase inhibitor, herbimycin A.

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Nuclear factor kappa B (NFκB) is a transcription factor which regulates the expression of genes involved in the immune and inflammatory response [1]. In resting cells, the predominant form of NFκB comprises two polypeptides of molecular weight 50kD (p50) and 65kD (p65) complexed to an inhibitory protein IκB [2]. Upon stimulation with such agents as interleukin 1 (IL1) or phorbol myristate acetate (PMA), the IκB protein is phosphorylated and proteolytically degraded. The active NFκB complex can then translocate to the nucleus where it binds to the DNA consensus sequence in target genes and causes a change in gene expression. Protein kinases C and A and tyrosine kinases have been shown to be involved in the activation process. In this study we have investigated the role of tyrosine kinases in the activation of NFκB by IL1 and PMA in T cells. A specific tyrosine kinase inhibitor, herbimycin A, was employed. Herbimycin A reverses transformation caused by various src-related oncogenes. Evidence has indicated that its mechanism of action involves covalent modification via a benzaquinone moiety of reactive thiol group(s) of p65-src [3]. Herbimycin A has been shown to block IL1 activation of NFκB in EL4 T cells while having no effect on PMA activated NFκB in 70Z/3 cells [4]. We have repeated this observation but have also found inhibition of PMA-activated NFκB in both EL4 and Jurkat 1 cells and another T cell line, Jurkat 5. Furthermore, we have found preliminary evidence suggesting that herbimycin A may act through direct modification of a thiol on the p50 subunit of NFκB. NFκB was assessed by incubating cells (1-5x10^6/ml) with herbimycin A (0.02-2μM) for 1 hour, washing and then stimulating with 10ng/ml (10ng/ml) for 1 hour or PMA (100ng/ml) for 24 hours. Nuclear extracts were then prepared and assessed for NFκB as described [6]. Identical studies were carried out in Jurkat T lymphoma cells with PMA (100ng/ml) with a treatment time of 24 hours. Nuclear extracts from IL1- or PMA-activated cells were also treated with herbimycin A in vitro for 10 minutes at 2OC prior to assessing NFκB activity. The recombinant p50 subunit of NFκB (Promega) was similarly treated. Tyrosine phosphorylation changes in the cells were assessed by incubating cells (5x10^6/ml) in the presence or absence of 2μM herbimycin A for 1 hour followed by exposure to IL1 (10ng/ml) or PMA (100ng/ml) for 5 and 10 minutes. After washing, cell lysates were prepared as outlined and tyrosine phosphorylation monitored by carrying out anti-phosphotyrosine immunoblotting as described [7]. The blot was developed using Amersham's enhanced chemiluminescence system.

As we have reported previously, herbimycin A (0.2-2μM) inhibited the activation of NFκB by IL1 in EL4 cells and PMA in both EL4 and Jurkat (not shown) [5]. IL1 caused a weak increase in tyrosine phosphorylation in EL4 NOB-1 cells on several proteins ranging from 21kD to 66kD molecular weight. The effect was detectable at 5 minutes. Herbimycin A (2μM) inhibited this increase in phosphorylation (Figure 1).

**Figure 1. Inhibition of IL1-activated tyrosine phosphorylation by herbimycin A.**

EL4 NOB-1 cells were pretreated for 1h with 2μM herbimycin A (lanes 4-6) and then stimulated with IL1 (10ng/ml) for the indicated times (lanes 2,3,5,6) or left unstimulated (lanes 1,4). Phosphotyrosine analysis was then carried out as described [7]. Molecular weight standards are shown and arrows indicate the major changes occurring. Abbreviations: IL1, interleukin 1; PMA, phorbol myristate acetate; 2 ME, 2-mercaptoethanol; DTT, dithiothreitol.

![Image](image-url)

**Figure 2** Direct modification of NFκB by herbimycin A

4μg of nuclear extract from IL1-stimulated (lanes 1-9) EL4 A cells were incubated with 2 ME (240mM) for 10 minutes at 20°C (lanes 5-7) or left untreated (lanes 1-4). Herbimycin A (2μM) was then added at the concentrations indicated for a further 10 minutes at 20°C (lanes 2-7). NFκB was then assessed as described [7]. Lane 8 was treated with vehicle (dimethylsulphoxide) and lane 9 was treated with 2 ME (240mM) alone.

The pattern of tyrosine phosphorylation of proteins in response to PMA in EL4 NOB-1 cells differed to that obtained with IL1 (not shown). A similar pattern was seen in Jurkats although with weak changes occurring between 29kD and 45kD molecular weight. The changes in tyrosine phosphorylation were similarly inhibited by herbimycin A. Because IL1 and PMA elicited only weak effects on tyrosine phosphorylation it was possible that the inhibitory effect of herbimycin A on NFκB was not solely due to inhibition of tyrosine kinases. Given that herbimycin A has a thiol reactive benzaquinone moiety [3], we explored whether it could directly modify NFκB, as the p50 subunit has been shown to contain a thiol group on cysteine 62 which is essential for DNA binding [8]. We tested this by incubating nuclear extracts from IL1-activated cells with herbimycin A. As shown in Figure 2, treating extracts with herbimycin A interfered with the ability of NFκB to bind to its consensus sequence. Maximum inhibition of binding occurred at 250μM. This inhibition could be prevented if herbimycin A was first mixed with 240mM 2-mercaptoethanol (2-ME) (Fig. 2) or 2mM dithiothreitol (DTT) (not shown). These results indicate that the in vitro inhibitory effect of herbimycin A may involve covalent attachment of the benzaquinone moiety of herbimycin A to a thiol group on NFκB, possibly cysteine 62 on p50. A similar effect has previously been shown in vitro with p60-src and herbimycin A [5]. The presence of p50 in both IL1 and PMA-activated EL4 and PMA-activated Jurkat was demonstrated using specific antisera (not shown). We next tested for effects of herbimycin A on recombinant p50 and similarly found that it inhibited binding to DNA. This effect could similarly be prevented by first treating with 2-ME (not shown).

In conclusion, these results suggest that herbimycin A reduces IL1- and PMA-induced tyrosine kinase activity and NFκB activation in EL4 NOB-1 T cells. However, the ability of herbimycin A to directly interfere with the p50 subunit of NFκB questions the importance of tyrosine kinase activity for the activation of NFκB by IL1.

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**References**