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Evans Blue is an inhibitor of nuclear factor-kappa B (NF-κB)-DNA binding

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Abstract—Nuclear factor-kappa B (NF-κB) is an important transcription factor, involved in many immune and inflammatory responses. It is critical in HIV gene expression as it has kappa B binding sites in the HIV-1 long-terminal repeat. Hence, targeting NF-κB to prevent its DNA binding holds a significant therapeutic potential. In this context, we report Evans Blue as a novel inhibitor of NF-κB-DNA binding. Evans Blue was found to be inhibiting DNA binding of NF-κB at a low concentration of 100 μM. Further, molecular modeling studies using docking and generation of electrostatic potential maps predicted a possible binding mode of EB to the DNA binding region of NF-κB, consistent with the experimental activity.

1. Introduction

Nuclear factor-kappa B (NF-κB), an inducible eukaryotic transcription factor of the *rel* family, normally exists in an inactive cytoplasmic complex, whose predominant form is a heterodimer composed of p50 and p65 (Rel A) subunits, bound to inhibitory proteins of the IkB family.[1–4] The IκBs bound to dimerized NF-κB factors, block their nuclear translocation, until signaling processes lead to induced IκB degradation and nuclear translocation of NF-κB.[4–7]

NF-κB is activated in response to primary (viruses, bacteria, UV), or secondary (inflammatory cytokines) pathogenic stimuli.[2,8] The targets of NF-κB, that is, the κB sites are present in the regulatory regions of the genes involved in immune (IL-1, IL-2) and inflammatory responses (IL-1, IL-6, TNF-α, TNF-β), and in genes of viruses, NF-κB/Rel members, IκB members, growth control proteins (p53, c-*myc*, Ras) and adhesion molecules.[2]

NF-κB is considered an immediate early mediator of immune and inflammatory responses and is involved in many pathological events, including progression of AIDS, by enhancing the transcription of human immunodeficiency virus type 1 (HIV-1).[9,10] Specific to HIV, a correlation has been observed between activation of NF-κB and the transcription of HIV long-terminal repeat (LTR) reporter sequence.[2,10] The strict involvement of NF-κB in LTR expression is further supported by the fact that mutations in the NF-κB binding sites result in the loss of LTR activation of several cell lines.[10,11] Moreover, a selective loss of NF-κB-DNA binding confers resistance to HIV-1 infection in a T-cell.[23]

The interesting correlation between NF-κB and HIV has made it a novel and potential target for anti-HIV chemotherapy. Its significance is magnified by the fact that other targets of HIV, especially the enzymes reverse transcriptase, and protease, present the problem of mutations and drug resistance.[12] On the other hand,

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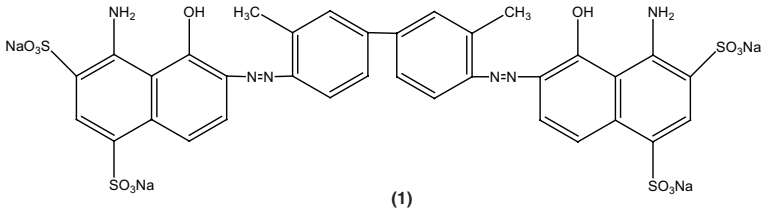
targeting NF-κB evades resistance, as it is a normal part of the T-cell and is not subject to mutations.

A limited class of drugs has been investigated against NF-κB, out of which the inhibitors of NF-κB-DNA binding seem to be very specific and potent.[13,14]

In this context, we report a potent, novel inhibitor of NF-κB-DNA binding, Evans Blue (EB) (**1**)



(1)

EB is a polysulfonated dye, having structural analogy with suramin and trypan blue.[15] EB has been reported as an inhibitor of HIV entry,[16,17] and also as an inhibitor of TNF-α binding to its receptor.[18] As a part of our ongoing search for new anti-NF-κB leads, we evaluated EB as an inhibitor of NF-κB-DNA binding. We have recently reported another polyionic compound, aurin tricarboxylic acid as an inhibitor of NF-κB-DNA binding.[13]

2. Methods and results

EB was obtained from Fluka, Switzerland. All other chemicals used were of analytical grade.

2.1. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotide containing a κB site from the mouse immunoglobulin κ light-chain enhancer was phosphorylated with polynucleotide kinase in the presence of [γ-^{32}P]ATP (Amersham >5000 Ci/mmol) and purified by a G-50 Sephadex spin column.

Nuclear extract from Jurkat human T cell line either unstimulated or stimulated with TNF-α (10 ng/mL) for 20 min was prepared. Nuclear extract (5 ng) were used for EMSA. After incubation of each reaction mixture containing binding buffer (15 mM Tris–HCl (pH 7.5), 75 mM NaCl, 1.5 mM EDTA, 1.5 mM dithiothreitol, 7.5% glycerol, 0.3% NP-40, 1 mg/mL BSA), 0.5 μg of poly(dI–dC), nuclear extract and various concentrations of Evans Blue at room temperature for 5 min, labeled DNA probe (30,000 cpm) was added and the mixture was further incubated at room temperature for 15 min.

The sample in a volume of 20 μL was loaded onto 4% polyacrylamide gels and electrophoresed at 150 CV. Zn^{2+} (2 equiv) were introduced after the addition of 100 μM concentration of EB (Fig. 1, lane 1). Addition of Zn^{2+} before the addition of EB and addition of large excess of Zn^{2+} gave virtually the same results. Figure 1 shows the inhibitory effect of various concentrations of EB on the DNA binding of NF-κB. EB was found to inhibit the DNA binding of NF-κB at 100 μM concentration (Fig. 1, lane 2). Lane 1 shows that addition of two equivalents of Zn^{2+} inhibited the activity of EB.

2.2. Structural analysis and modeling

The first 3-D structure of NF-κB was obtained by co-crystallizing the p50 subunit with target DNA in 1995.[19,20] We obtained the 3-D structure of the NF-κB p50 homodimer bound to a κB site from the Protein Data Bank (PDB code: 1NFK) (Fig. 2) for structural analysis and modeling of the probable binding site for EB.

The κB site represents a consensus DNA sequence of 10 base pairs, 5′-GGGACTTTCC-3′, and is present in various cellular genes critical for immune or inflammatory responses, including the HIV-LTR. The structure of NF-κB directly affects its function. The specific amino acids that are classified as the binding site (hereby referred to as the DNA binding region (DBR)) for DNA are residues 59–71 of the p50 subunit of NF-κB (starting with 59: Arg, Tyr, Val, Cys, Glu, Gly, Pro, Ser, His, Gly, Gly, Leu, Pro). This portion of the p50 subunit is specific for the κB DNA sequence.[19–21]

Molecular modeling was performed using the GAUSSIAN 98[22a] (for energy calculations) and QUANTA[22b] (for visualization and analyses) packages and the genetic algorithm based automated docking program, GOLD 2.0.[22c] The three dimensional structure of p50 was obtained from the Protein Data Bank (PDB ID: 1NFK). All the water molecules were removed, hydrogens were added, and just one p50 subunit was used for modeling. EB was subjected to docking onto the p50 protein, with the amino acids 59–71 (DBR), defined as the docking site. The docking program GOLD, used in this study, uses a genetic algorithm in the evolution of a population of possible solutions via genetic operators (mutations, crossovers, and migrations) to a final population, optimizing a predefined function. Thus GOLD provides docking of a flexible ligand with the target protein site in an energetically and conformationally optimized position. The predefined function mentioned here, comprised of four components, viz., (a) protein–ligand hydrogen-bond energy, (b) protein–ligand van der Waals energy, (c) ligand intramolecular hydrogen-bond energy, and (d) ligand internal energy.[22c] The interpretation of docking results in the present study is based on this fitness function in the form of relative scoring (GoldScore). Docking runs were carried out for EB in the default settings for the best possible predictive accu-

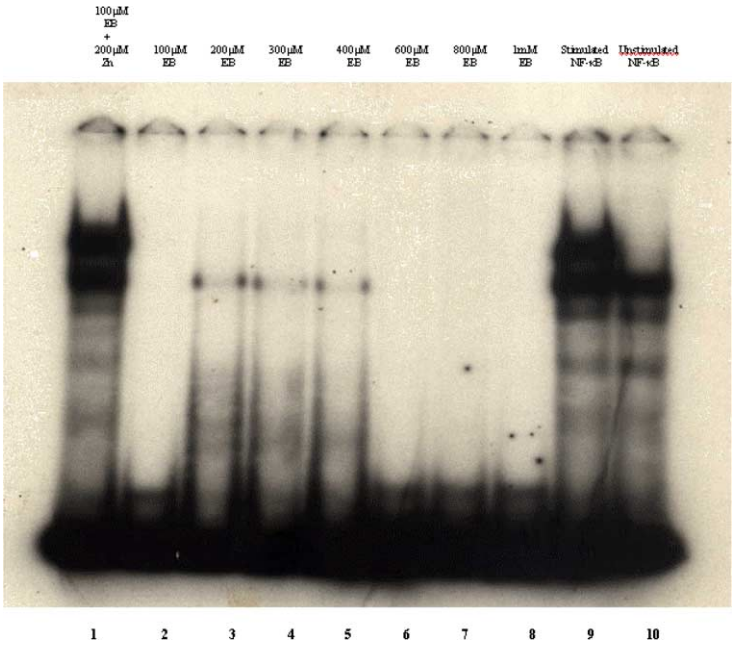


Figure 1. Effect of EB (Evans Blue) on the DNA binding of NF-κB. After stimulated NF-κB was incubated with EB in the presence of poly(dI–dC) at room temperature, a radioactive DNA probe containing a κB site from the Mouse light-chain enhancer was added. Sample was loaded onto a polyacrylamide band shift gel, and the gel electrophoresis was run.

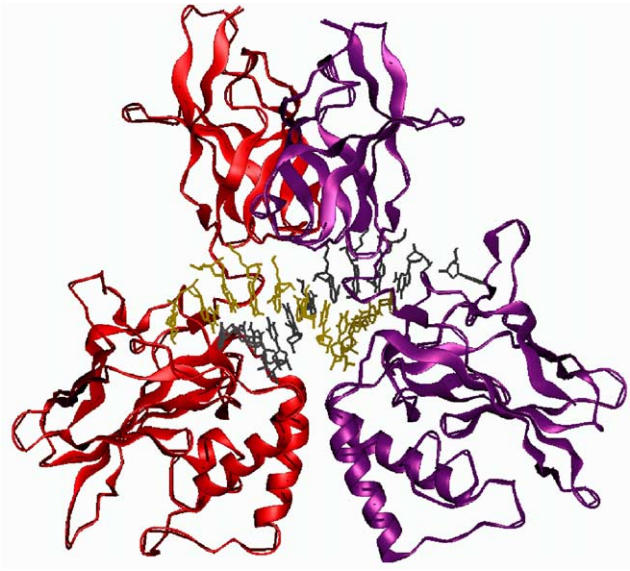


Figure 2. A p50 homodimer bound to a kappa B site DNA.

racy. The best scored solution was considered for further energy minimization with the semiempirical method PM3MM (PM3 hamiltonian including the molecular mechanics correction for HCON linkages).[22a] This modeling approach has been recently reported by us[24] with some other experimental inhibitors of NF-κB (p50)-DNA binding, interacting with the same site, and has given models in excellent agreement with the inhibition profile of the studied inhibitors. Figure 3 represents, exclusively the DBR, extracted from the p50 subunit, docked, and energy minimized with EB. EB interacts in an energetically and conformationally favorable manner with the DNA binding region, from Cys-62 through His-67.

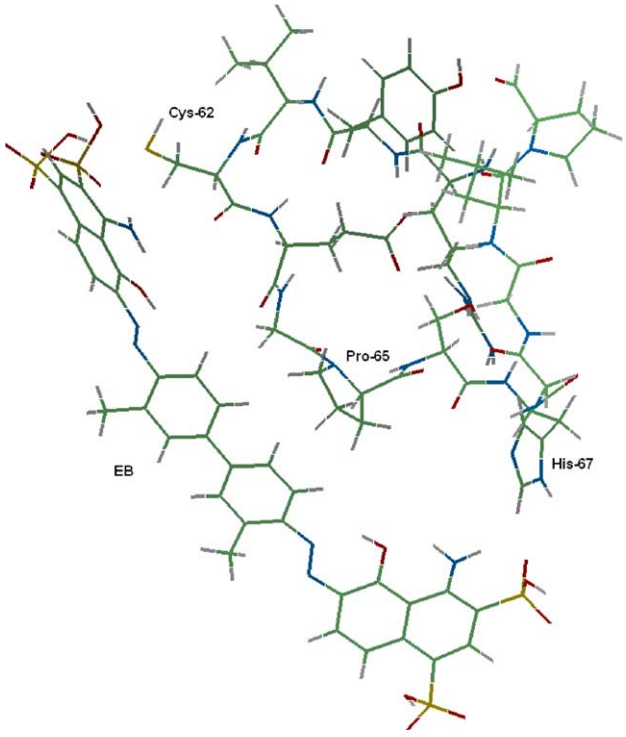


Figure 3. EB (Evans Blue) docked with the DNA binding region of p50.

Further, to understand the role of electrostatics in the event of molecular recognition between EB and the

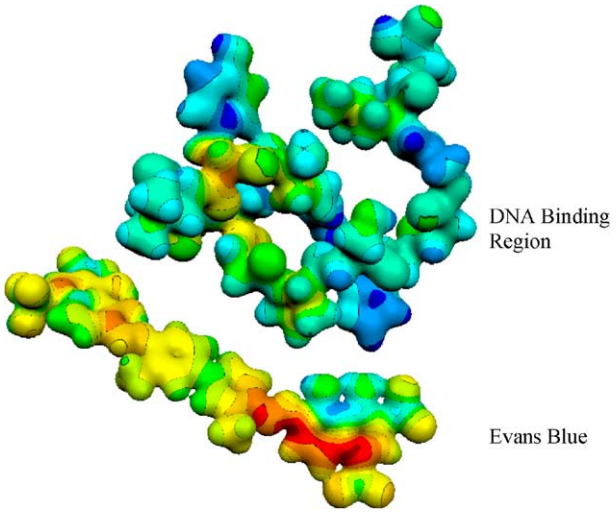


Figure 4. Molecular electrostatic potentials (MEPs) representing a maximum potential, $\phi_+ = 0.54$ au, and a minimum potential, $\phi_- = -0.62$ au are mapped onto electron density isosurfaces of $\rho = 0.02\,e\,Å^{-3}$. Negative potentials are depicted in red color and positive in blue.

p50 DBR, we calculated molecular electrostatic potential (MEP) maps using quantum chemical calculations. Density functional theory, with the B3LYP functional was used employing the 6-31+G(d) basis set, to obtain the electron density around EB and p50 DBR. These calculations were also performed using GAUSSIAN 98 program.[22a] The electrostatic potential was mapped onto the calculated electron density surface, with the aid of MOLEKEL 4.2 package.[25] The MEPs for EB and the DBR are shown in Figure 4, positioned in the docked conformation. MEPs generated in this way have been proved to be quite insightful in understanding molecular recognition immediately, prior to tight binding with several experimental inhibitors.[24,26] In the present case, the MEPs representing a maximum potential of 0.54 au, and a minimum of −0.62 au, mapped onto electron density isosurfaces of $0.02\,e\,Å^{-3}$, for both EB and the DBR clearly show that EB is quite electronegative (red regions) in its potential surface, as contrast to the DBR, which clearly is having a relatively more electropositive potential (blue regions).

Electronegative EB should achieve its initial molecular recognition with electropositive DBR, much in the same way as other experimental inhibitors[24] of the same type do, followed by several modes of possible non-covalent interactions, stabilizing the two. Specifically, there is a possibility of (1) disulfide linkage between Cys-62 and $–HSO_3$ group on one end of the EB, (2) hydrophobic interaction between Pro-64 and the central non-polar region of EB, (3) hydrogen bond between His-67 and –NH2 group of the drug. There is also a strong possibility of a network of hydrogen bonds between EB and the backbone of the DNA binding region. It is highly probable that EB could be interacting with major amino acids responsible for DNA interaction, and nevertheless, it may be affecting the conformation of p50, besides general steric hindrance, and hence, affecting its ability to bind with DNA.

Further, it is observed that addition of zinc with EB, retains the NF-κB-DNA binding. This could be possible due to the fact that zinc may be forming a complex with EB, occupying its critical sites responsible for interaction with the DNA binding region. However further studies are required to understand the zinc related mechanism of EB.

On the basis of these initial EMSA and modeling studies, Evans Blue seems to be a potent inhibitor of NF-κB-DNA binding at a low concentration. However, it is not known that EB can enter the cell to exhibit its activity against cellular factors, biochemical studies are required to support its function. Also the challenge of having p50 specific inhibition by EB remains to be addressed. These initial studies represent EB as a promising lead against NF-κB-DNA binding.

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