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A molecular modeling study of inhibitors of nuclear factor kappa-B (p50) – DNA binding

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Summary

Nuclear Factor-kappa B (NF-κB) is an inducible transcription factor of the *Rel* family, and is sequestered in the cytoplasm by the IκB family of proteins. NF-κB can exist in several dimeric forms, but the p50/p65 heterodimer is the predominant one. Activation of NF-κB by a range of stimuli including viral products, and oxidative stress, leads to phosphorylation and proteasome dependent degradation of IκB, leading to the release of free NF-κB. This free NF-κB then binds to its target sites (κB sites in the DNA) to initiate transcription. These κB sites are also present in the Long Terminal Repeat (LTR) of HIV-1, and hence NF-κB (p50 subunit) binding to LTR–DNA is critical in viral replication. Targeting direct p50-DNA binding, in this regard, is a novel approach to design anti-HIV gene expression inhibitors, which do not have the problem of resistance unlike in other anti-HIV strategies. The present study is a part of our search for leads for the specific inhibition of p50-DNA binding. We have been experimentally studying different types of these inhibitors, and in this work, we attempted to get a common definition of their structural mechanism onto p50-DNA binding. Using three different classes of inhibitors, we modelled their association with the DNA-Binding Region (DBR) of the p50 subunit of NF-κB. Docking studies were carried out using a genetic algorithm based program (GOLD). Further, to compare electrostatic complementarity in the association of the inhibitors with the DBR, Molecular Electrostatic Potentials (MEPs) were generated for the DBR and each inhibitor. The results of docking revealed a strong network of hydrogen bonding interactions for every active inhibitor, and the contrary for the less active ones. Further, the MEPs revealed that the DBR of p50 represents a surface of electropositive potential, and the active inhibitors represent a complementary electronegative surface. With the present modelling study we conclude that the principal properties to be possessed by the new leads against p50-DNA binding should be that of having the ability to make a strong network of hydrogen bonds with the DBR of p50, and preferably, having electronegative potentials in their peripheral surface.

Introduction

Nuclear Factor-Kappa B (NF-κB) belongs to a family of transcription factors (*Rel*/NF-κB) that are sequestered in the cytoplasm by specific IκB proteins [1–6].

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NF-κB was originally characterized as a lymphoid specific protein that interacts with the immunoglobulin κ light chain gene enhancer sequences [7]. Nowadays, it has been established that NF-κB is ubiquitously expressed and has a key role in regulating the expression of many genes involved in immune, inflammatory, and developmental processes [8]. NF-κB represents a group of structurally related and evolutionary conserved proteins [1–6, 9]: so far, five

mammalian NF-κB proteins named Rel (c-Rel), Rel-A (p65), Rel-B, NF-κB1 (p50), and NF-κB2 (p52 and p100) [10–12]. With such a diverse family of proteins, NF-κB can exist in homo- or heterodimeric forms. Structurally, all NF-κB/Rel proteins share a highly conserved NH_2-terminal Rel homology domain (RHD), that is responsible for DNA binding, dimerization, and association with inhibitory proteins known as IκBs [13–15].

NF-κB can be activated by different stimuli such as microbial and viral products, proinflammatory cytokines, T- and B-cell mitogens, and physical and chemical stresses [16–20]. In turn, NF-κB regulates the inducible expression of many cytokines, chemokines, adhesion molecules, acute phase proteins, and antimicrobial peptides [21–26]. As mentioned above, before activation, NF-κB/*Rel* dimers are bound to IκBs, and retained in an inactive form in the cytoplasm [27]. Following activation, IκB is phosphorylated by IκB kinases [28, 29], polyubiquitinated by a ubiquitin ligase complex [30, 31], and further degraded by the 26S proteasome [32, 33]. Finally NF-κB is released and translocates into the nucleus, where it binds to the target DNA (κB sites), and initiates gene expression [34, 35].

By binding to the κB sites mentioned here, NF-κB mediates gene expression pathways, and is involved in a number of pathological events [36, 37], including progression of AIDS, by regulating the first step transcription and hence multiplication of human immunodeficiency virus type 1 (HIV-1) [38, 39]. As soon as the virus binds to the T-cells, the viral RNA is incorporated into the host DNA, following reverse transcription. This viral DNA (the viral genome) is expressed, eventually, giving way to multiplication of viral particles, and further, infection. It is the viral gene expression where NF-κB plays a crucial role by binding to the HIV long-terminal repeat (LTR) reporter sequence (the κB site) [5, 39]. Biochemical studies have shown a correlation between the activation of NF-κB and the transcription of HIV-LTR [5, 39, 40].

Since HIV has been characterized as a disease-causing virus, several strategies have been applied to develop drugs against it. These include mainly the inhibitors of reverse transcriptase and protease enzymes of the virus. For a comprehensive review on all inhibitors, see Reference 41. However, most of these inhibitors have shown certain problems, including that of resistance due to viral mutation [42–44].

The strong dependence of the HIV gene expression on NF-κB has made it an important and potential drug target. Further, targeting NF-κB evades the problem of resistance, as it is a normal part of the T-cell and is not subject to mutations. In this context, a very limited class of drugs have been developed. We recently reviewed these drugs targeting NF-κB, specific to HIV [45]. Briefly, most of the inhibitors of NF-κB either inhibit the activation or IκB phosphorylation and degradation, thus preventing the release of free NF-κB. But approaching direct inhibition of NF-κB–DNA binding, by interfering with the DNA binding region in the NF-κB, seems to be more potent and amenable to design specific inhibitors. However, a limited class of drugs have been studied using this approach [45]. In case of HIV-1, the predominant NF-κB complex in the T-cells is the p50-p65 heterodimer [46]. Further, recent studies have shown that the p50 subunit of this complex is the one which mainly interacts with the HIV-1 LTR [47, 48]. The LTR κB site contains the sequence 5′-GGGACTCCTC-3′ [35]. The three-dimensional structure of NF-κB (p50-p65), bound to a κB site DNA is shown in Figure 1 [49]. The specific amino acids that are responsible for interaction with the DNA are residues 59–71 of the p50 subunit (59:Arg, Tyr, Val, Cys, Glu, Gly, Pro, Ser, His, Gly, Gly, Leu, Pro:71) [35, 49–51]. This particular p50 sequence is hereby referred to as the DBR (DNA Binding Region).

We have been experimentally studying different types of inhibitors which inhibit NF-κB–DNA binding [57, 61, 62]. Some of the inhibitors studied by us using Electrophoretic Mobility Shift Assay (EMSA) have given good activities. In the EMSA, a recombinant protein construct or nuclear extract from cells is incubated with the inhibitor. This mixture is further incubated with a labelled (biotinated) double stranded oligonucleotide, which in the present case contained a κB site from the mouse immunoglobulin κ light chain enhancer. Finally, using electrophoresis and chemiluminescence, the level of protein-DNA binding and hence the activity of the particular inhibitor is quantized. In order to find new anti-HIV leads which inhibit the NF-κB–DNA binding by specifically interacting with the DBR of the p50 subunit, we undertook the present computer modeling study. The purpose of this work was to get an idea of the common structural mechanism of the action of the existing group of p50-DNA binding inhibitors.

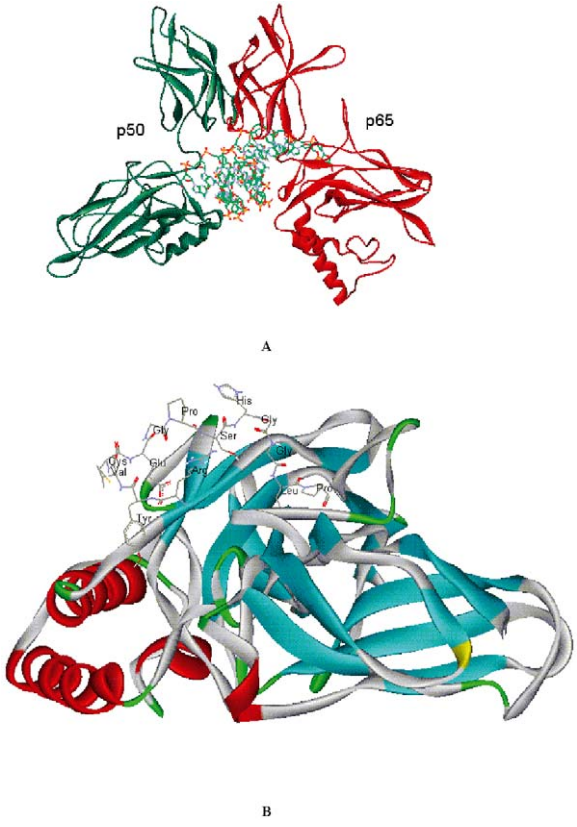


Figure 1. (A) Model of NF-κB (p50-p65) heterodimer bound to a κB site DNA. (B) A p50 molecule, displaying the specific amino acid residues (DBR) involved in DNA binding.

Inhibitors

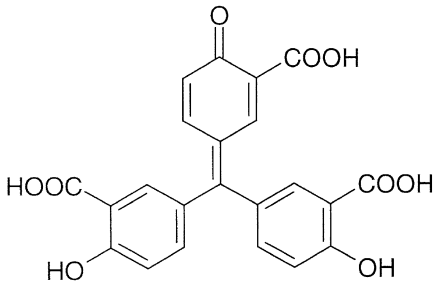
Three different classes of NF-κB–DNA binding inhibitors were used in the present study (Figure 2). Inhibitor **1** belongs to the first class, inhibitors **2**–**4** belong to the second class, and inhibitors **5**–**8** belong to the third.

In the first class, Aurine Tricarboxylic Acid (ATA) (**1**) has been reported in the recent past as having activity against all HIV strains [52]. Also, ATA has been a well established enzyme inhibitor [53–56]. This motivated us to experiment it as an inhibitor of NF-κB–DNA binding, and consequently we found it to be inhibiting the DNA binding of NF-κB in the nuclear extract of TNF-α stimulated Jurkat human T-cell line at a very low concentration of 30 μM [57]. The second class of inhibitors are the polyhydroxycarboxylate derivatives of phenolic compounds. These include Gallic Acid (GA) (**2**), 5,7-dihydroxy-4-methylcoumarin (5,7 DHMC), and 7,8-dihydroxy-4-methylcoumarin (7,8 DHMC) (**3**). The polyhydroxycarboxylate derivatives too, have been reported as inhibitors of the cytopathicity of HIV-1 in MT-4 cells at non-toxic concentrations [58–60]. Recently, we analyzed these drugs for their activity against NF-κB–DNA binding, using a recombinant p50 protein for EMSA [61]. We found that GA is more potent in p50-DNA binding inhibition than the coumarins (5,7 DHMC and 7,8 DHMC). The third class of inhibitors have proved to be quite interesting as metal chelators. We reported inhibitors **5**, **6**, **7**, and **8** as having dual activity in the inhibition of NF-κB, as well as another κB binding site zinc finger protein called HIV-Enhancer Binding Protein-1 (HIV-EP1) [62]. We have been modifying these chelators, but just in order to have better zinc chelating properties [63, 64]. However, inhibitors **7** and **8** should be better designed, specifically against p50-DNA binding, as they were found also to be more potent in inhibiting p50-DNA binding than **5** and **6** [62]. Specifically, the inhibition profile of these inhibitors against p50-DNA binding was in the following order: **5**<**6**<**8**<**7**, in equimolar concentrations. Further, inhibitor **5** was approximately 2%, **6** was 10%, **7** was 90%, and **8** was found to be around 75% active [62]. Inhibitors **7** and **8** are the diastereomers of each other, with **7** being (S,S), and **8** being (R,S). With this experimental basis, we investigated, theoretically, the binding of these inhibitors with the DBR of p50, in order to get a definition of the probable common structural mechanism of their activity.

Computational methods

Docking inhibitors with p50

The 3D structure of p50 was obtained from the Protein Data Bank (PDB code: 1NFK). All the water molecules were removed and just one p50 subunit was used for modelling. The modelling studies were performed using QUANTA [65], INSIGHT [66], and MOLDEN [67]. Inhibitors **1**–**8** were subjected to docking onto the p50 protein, with the amino acids 59–71 (DBR), defined as the docking site. The protonation states of all inhibitors and the p50 DBR were considered at physiological pH (EMSA experiments being done at the same pH) [57, 61, 62, 68, 69]. The docking studies were performed using GOLD 2.0 (Genetic Optimization for Ligand Docking) [70]. GOLD 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 fitness function. This



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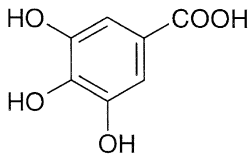

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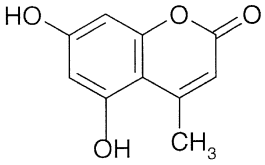

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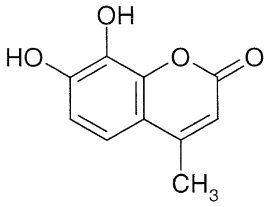

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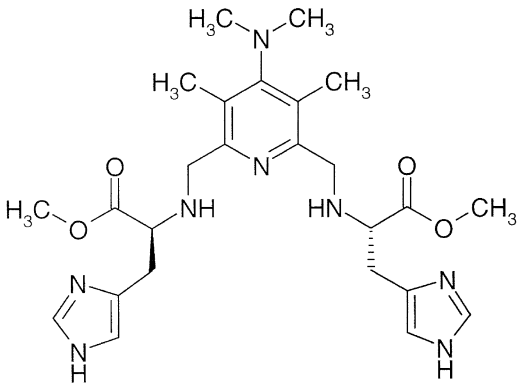

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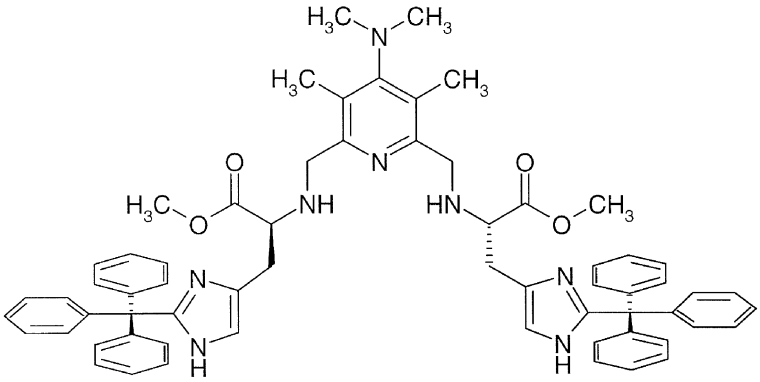
Figure 2. Structures of NF-κB-DNA binding inhibitors: (**1**) aurine tricarboxylic acid, (**2**) gallic acid, (**3**) 5,7-dihydroxy-4-methylcoumarin, (**4**) 7,8-dihydroxy-4-methylcoumarin, (**5**) N,N′-[4-(dimethylamino)-2,6-pyridinylidenedimethyl] bis (S,S) (histidine)-dimethylester, (**6**) N,N′-[4-(dimethylamino)-2,6-pyridinylidenedimethyl] bis (S,S) (2′-tritylhistidine)-dimethylester, (**7**) N,N′-[4-(dimethylamino)-2,6-pyridinylidenedimethyl] bis (S,S) (2′-tritylperoxyhistidine), and (**8**) N,N′-[4-(dimethylamino)-2,6-pyridinylidenedimethyl] bis (R,S) (2′-tritylperoxyhistidine).



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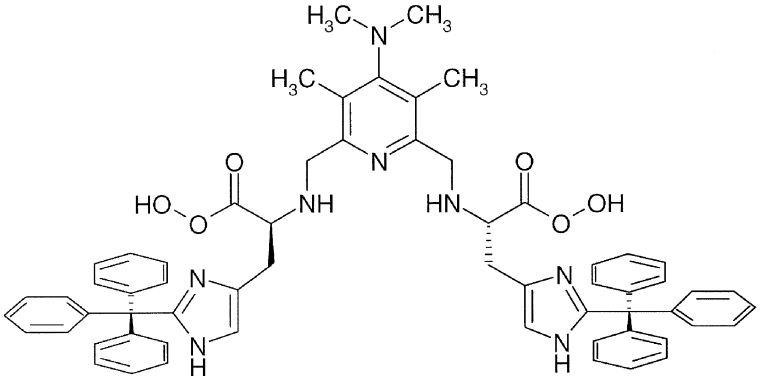

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Figure 2. (Continued).

provides docking of flexible ligands and a protein, and in this way GOLD is a useful method to fit the ligands conformationally and energetically to the binding site of the protein. The predefined function mentioned here comprises four components, viz., protein-ligand hydrogen bond energy, protein-ligand van der Waals energy, ligand intramolecular hydrogen bond energy, and ligand internal van der Waals energy [70]. The interpretation of docking results is based on this fitness function in the form of scoring (GoldScore). Docking calculations were performed in the default settings for the best possible predictive accuracy, with ten docking runs for each inhibitor. The best scored solution for each inhibitor was considered, and viewed in DS Viewer Pro 5.0 [71]. For further analyses, the geometry of each inhibitor docked with DBR, exclusively, was extracted from the final, best solutions. This complex (i.e., the inhibitor bound to the DBR) was subject to energy minimization using the semiempirical quantum mechanics method PM3MM (PM3 hamiltonian including the molecular mechanics correction for HCON linkages) for each inhibitor. These calculations were performed using the GAUSSIAN 98 program [72]. The energy minimized complex was then analyzed for non covalent interactions between each inhibitor and the DBR.

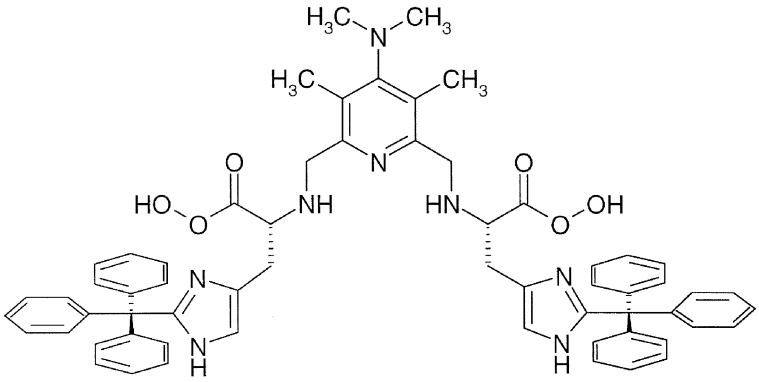

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Figure 2. (Continued).

Generating electrostatic potentials

The electrostatic potential that is created in the space around the molecule by its nuclei and electrons has been proven to be a very useful tool in explaining molecular interactive behaviour [73–75]. The theoretically calculated Molecular Electrostatic Potential (MEP), using *ab initio* quantum mechanics methods, has been described to be very predictive [76, 77]. In order to compare electrostatic complementarity in the association of the inhibitors with the DBR, we generated the MEP for each inhibitor, and the p50-DBR. The partial charges were calculated using Density Functional Theory with the Becke 3-Lee-Yang-Parr (B3LYP) functional, and the 6-31+G(d) basis set, by performing single point calculations onto the minimized geometries previously generated with PM3MM, of DBR and corresponding bound inhibitors. These calculations were also performed using GAUSSIAN 98 [72]. With the aid of MOLEKEL 4.2 [78], the electrostatic potential was mapped onto the electron density surface for the inhibitor and the DBR.

Results

Docking studies

The docking using GOLD gave interesting results. The energy minimization of the docked complexes for each inhibitor resulted in an improvement in RMSDs to the order of 10^{-8} from initial RMSDs of the order of 10^{-2}. For each inhibitor, a close view of the docking interaction, depicting the hydrogen bonds, is shown in Figure 3. Hydrogen bonds are considered up to 3.50 Å between the hydrogen bonded atoms. For inhibitors **5**–**8**, due to their size and complexity of interactions, only the amino acid residues involved in interactions docked with the respective inhibitors are depicted.

From the docking results, it was observed that ATA, despite being a very potent inhibitor of NF-κB DNA binding in human Jurkat T-cell nuclear extract, is interacting rather weakly with the DBR. It is making a weak hydrogen bond with the His67 of the DBR, and just one strong hydrogen bond with the amino terminal end of Arg56 (Figure 3A). Further, in the second class of inhibitors, in accordance with our experimental observations, GA is interacting most strongly with the DBR among the inhibitors in its class (Figure 3B). GA is able to make a network of very strong hydrogen bonds with the DBR, especially with Ser66, and various parts of the protein backbone ranging from Arg59 through Pro65. Contrastingly, the DHMCs are able to make relatively weaker hydrogen bonds with the DBR (Figures 3C, 3D). Lastly, the third class of inhibitors also show a profile correlative with the inhibition data. Inhibitor **5** is making the least number of hydrogen bonds, whereas **6** could make hydrophobic interactions (with the aid of its bulky trityl groups) with Pro65 and Pro71 of the DBR, besides making weak hydrogen bonds (Figures 3E, 3F). Further, **7** and **8**, which are diastereomers of each other, are making the maximum number of hydrogen bonding interactions with the DBR, in their class. Interestingly, **7** is making stronger hydrogen bonds than **8** (Figures 3G,

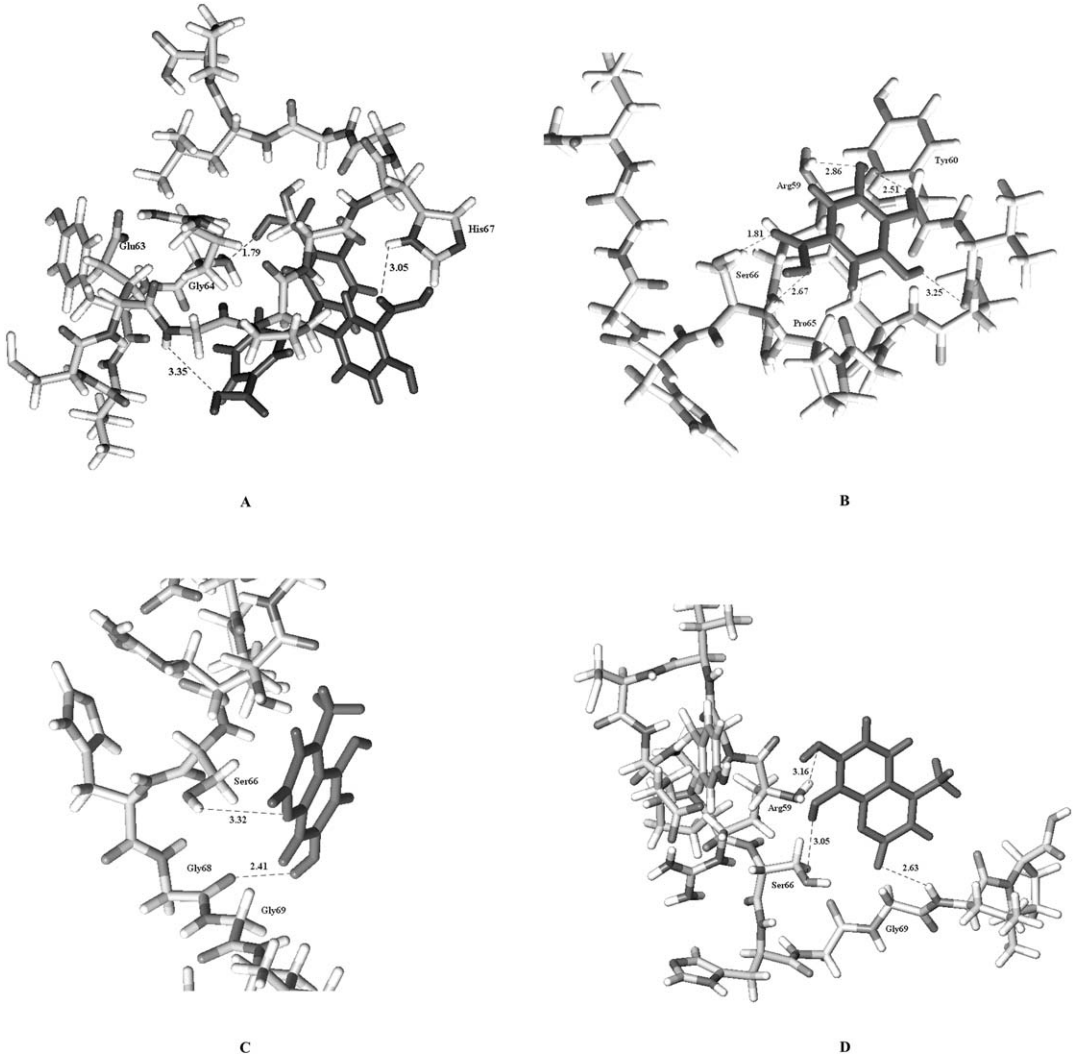

Figure 3. Inhibitors of p50-DNA binding docked with the DBR. A–H represent the docking results for inhibitors **1**–**8**, respectively. Hydrogen bonds are considered at distances less than or equal to 3.50 Å.

3H). Both **7** and **8** interact with His67, Arg56, and Ser66 with the aid of hydrogen bonds, but the trityl groups in both of them have the least possibility of any hydrophobic interactions with the DBR. Further, the His67, Arg56, and Ser66 interactions of **7** are stronger than those of **8**.

Molecular electrostatic potentials

The MEPs for each inhibitor and the DBR are depicted in Figure 4. These color coded isosurfaces represent a positive potential in blue and a negative potential in red color. The maximum potential represented is $\phi_+ = 0.5376$ au, and the minimum is $\phi_- = -0.6194$ au, over an electronic isodensity of $\rho = 0.02$ e Å^{-3}, for each surface represented. The definition of isosurfaces using other ranges of potential did not show any significant differences. DBR of the p50 subunit is found to have mainly a positive (blue) potential (Figure 4a). This potential is strongly positive on the external surface, which is the first accessible surface for any ligand or the target DNA.

Further, the major amino acids including Arg56, His67, and Ser66, which interact with the inhibitors used in this study are also having a net positive potential around them. In order to have an electrostatic complementarity with the DBR, specific inhibitors preferably should have a negative potential around them. In case of ATA it was found that this inhib-

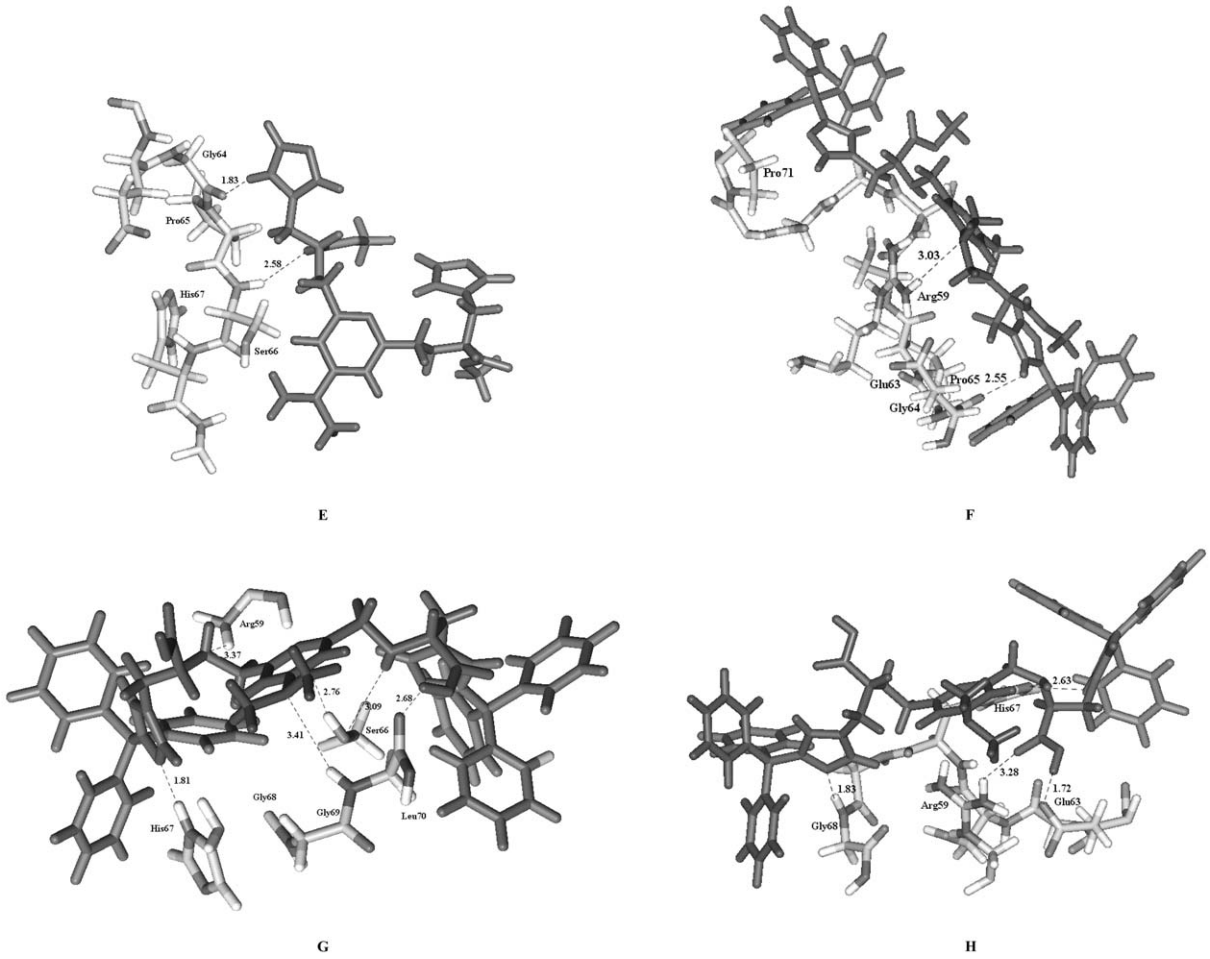

Figure 3. (Continued).

itor does not have much complementarity with the DBR (Figure 4b). Though it has some electronegative potential, but there is a high electropositive character in the majority of its surface. In the second class of inhibitors, GA has a major distribution of negative potential around its surface, though the DHMCs too have mostly strong negative potentials (Figures 4c–4e). Specifically, the surface in GA, which is not exactly in an electronegative potential, is lying in an average, but not electropositive potential. However, in case of DHMCs, there are regions in the surface which are associated with a more electropositive character. In case of the third class of inhibitors, the MEPs are also in agreement with the experimental results. Inhibitor **5** has mostly electropositive potential in its peripheral surface, which is not complementary with the potential of the DBR surface (Figure 7f). Inhibitor **6**, with the addition of benzene ring containing trityl groups, has the same profile as that of **5**, except having an average potential in the surface covering the trityl groups, but biased toward electronegative character (Figure 4g). On the other hand, **7** and **8** are having a better and stronger distribution of electronegative potential around their surfaces. This is more prominent in the regions of their carboxylic groups, and histidines as well, which are the regions also making hydrogen bonding interactions with the DBR (Figures 4h, 4i).

Discussion

The docking study and the comparative electrostatic potentials in the present work have given an interesting idea about the profile of the DNA binding region of the p50 subunit of NF-κB and the possible profile of specific inhibitors. These two parameters used in

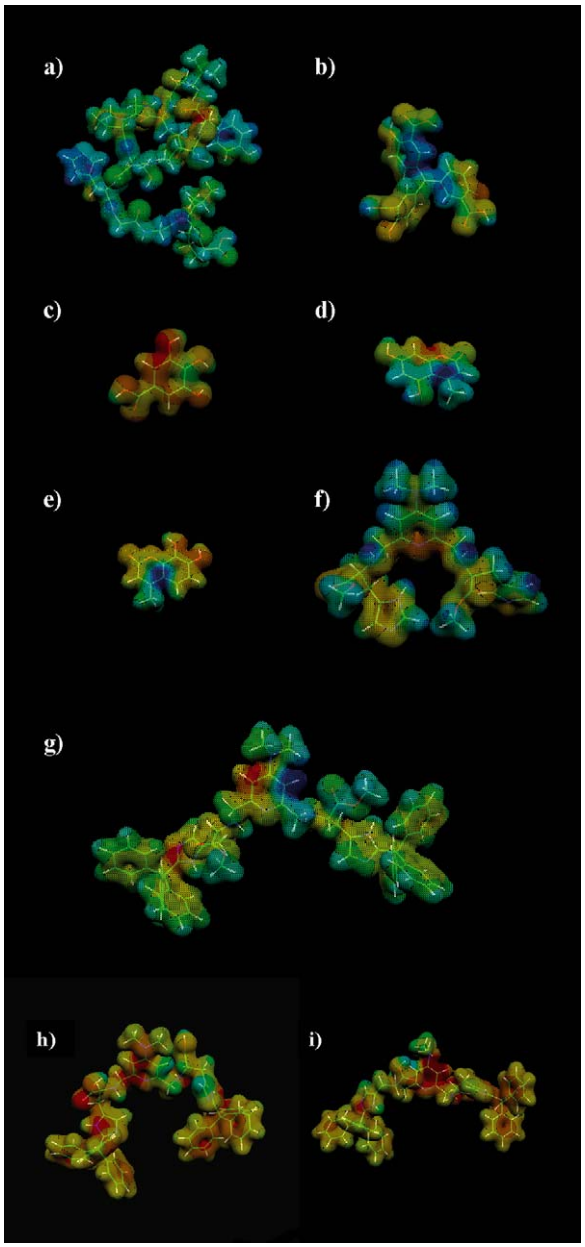

Figure 4. Molecular Electrostatic Potentials (MEPs) representing a maximum potential, $\phi_+ = 0.5376$ au, and a minimum potential, $\phi_- = -0.6194$ au are mapped onto electron density isosurfaces of $\rho = 0.02$ eÅ^{-3}. Negative potentials are depicted in red color and positive in blue. The DNA binding region of p50 is depicted in (a), and (b)–(i) represent MEPs for inhibitors **1**–**8**, respectively.

this study have been previously used in rational drug design as tools for lead optimization, and pharmacophore searches [79–84]. Further, GOLD has been successfully used previously, to predict protein recognition and binding [70, 80, 90]. However, it has certain limitations, including the one that it does not allow for protein flexibililty, and its scoring function gives priority to hydrogen bonding and van der Waals interactions but electrostatic interactions are not considered. Due to these strengths and limitations of GOLD, in the present study semiempirical energy minimizations were performed on the docked systems and electrostatic potential maps were investigated too. Energy minimization, post-docking (using GOLD) has been recently reported on several inhibitors [79].

Transcription factors like NF-κB-p50 are important in structural regulation of transcription. They can help the target DNA to bend in order to facilitate binding of the RNA Polymerase II, to initiate transcription [85, 86]. For this and other functions, a transcription factor has specific conformation(s), and interactions with the DNA [85–87]. So, the drugs which are aimed at directly interfering with the DNA binding ability of the protein should block its DNA binding sites, by interfering with the side chains, or by affecting its conformation, besides providing steric hindrances.

The inhibitors studied in this work have all been experimented for their ability to inhibit NF-κB–DNA binding. The results of the present modelling have shown a good and interesting correlation with the inhibition profile. Beginning with ATA, in the experiment it was found to be very active at extremely low concentration [57]. However, the docking study suggests that it forms rather weak interactions with the DBR of p50. Also the MEP surface of ATA is not very complementary with the surface of the DBR. With these results, and with experimentally agreeable results of other classes of inhibitors used in this study, it seems as if ATA does not interact directly with the DBR of p50. This is quite possible because for all the inhibitors used in this study, except ATA, EMSA studies were performed using pure recombinant p50 protein, besides using whole nuclear extracts. But for ATA, results from only jurkat T-cell nuclear extract, showing NF-κB inhibition are available. It is possible that ATA is not interacting with the p50 but with the p65 subunit, or with another nuclear factor, which is affecting the DNA binding property of the NF-κB complex. In a similar study, using Jurkat T-cell nuclear extract, Hiramoto et al. [88] described a quinone derivative, suppressing NF-κB–DNA binding, and it was

experimentally described that the inhibitor interacted with an unknown nuclear factor, but not the NF-kB protein. P50 specific studies are required to further analyse the activity of ATA against p50-DNA binding, and to compare our modelling results.

Next, in case of the second class of inhibitors, they have shown a perfect agreement with the experimental results which were obtained using a pure p50 assay, unlike ATA. The EMSA studies with pure p50 suggest that the activity of inhibitors is due to complex formation with the protein [61]. Thus GA, being the most potent in this group, also makes strongest interactions in the form of a network of hydrogen bonds with the DBR, in comparison to the DHMCs, which make rather weaker hydrogen bonds. The MEPs for this group are also in accordance with the experimental results. In case of the third class of inhibitors also, pure p50 assays have been made, which suggest complex formation between them and the protein; the results of the modelling are in agreement with the inhibition profile. Structurally, inhibitors **5** and **6** are different only in that **6** has the bulky trityl groups substituted in the two symmetrical histidines. This has shown the activity of inhibitor **6** to be around 10%, whereas the activity of **5** is almost 2% [62]. With the present modelling, we attribute this increase to possible hydrophobic interactions of these trityl groups with the two prolines present in the DBR. Also, the electrostatic potential with these two trityl substitutions has been found to be biased towards negative, and hence somewhat complementary to the DBR. A significant increase in the activity was found in inhibitor **7**, which is exactly similar to inhibitor **6**, but with the ester group of **6** being substituted with a carboxylic group. With this **7** is making more hydrogen bonding interactions with the DBR, and is fitted closely with the DBR. Also the MEP of inhibitor **7**, especially near the carboxylic groups, is electronegative, and thus **7** could be approaching the DBR in an electrostatically better way. It is probable that the carboxylic groups in **7** (and also inhibitor **8**) could be affecting the overall spatial and electrostatic potential arrangements of the inhibitor. Further, inhibitor **8**, which is a (R,S) diastereomer of **7**, has a lower activity than **8**, but still better than inhibitors **5** and **6**. This further indicates that the internal and spatial arrangement of the inhibitors affect their activity. Coming back to ATA, although it has possibilities of making several hydrogen bonds with the DBR, the docking study reveals very few. Most of the ATA structure is not interacting with the putative hydrogen bonding acceptor or donor groups of the DBR. This is another case of spatial arrangement and internal flexibility of the inhibitor, affecting its binding with the DBR.

It is pertinent to state here that the docking studies suggest that trityl groups in inhibitors **7** and **8** are not of much significance in interacting with the DBR. Also, the log P values of these inhibitors have been reported to be 5.72, which is very poor and is attributed to the bulky trityl groups [89]. Hence, pharmacologically, such bulky substituents are a liability, and as suggested by the inhibitor **6**, they do not contribute significantly to the activity.

Hence, the present modelling study on different classes of p50-DNA binding inhibitors gives a good prediction about their possible structural activity. By making strong hydrogen bonds, active inhibitors may be masking important amino acids like arginine, histidine, and serine, which are important in DNA binding [35]. Further, they may be affecting the specific conformation of p50 DBR [46] required for DNA binding, besides making steric hindrances to the DBR. Also the MEPs give an idea of the importance of the electrostatic complementary behaviour of the association of the inhibitors with the DBR. It is through this potential that a molecule is first 'seen' or 'felt' by another approaching chemical species [73–75]. In the present case, it is favourable for p50-DNA binding inhibitors to have a net electronegative potential in order to better approach and fit complementarily with the DNA binding region of the p50 subunit.

The present study is a step towards lead finding by designing specific inhibitors of p50-DNA binding, and further, inhibitors of HIV-1 gene expression. The definition for the design of the next set of p50-DNA binding inhibitors should thus encompass their abilities to make a maximum number of hydrogen bonds, but with a suitable spatial arrangement and flexibility for the interaction with the DBR. Also, an electrostatic potential more electronegative on the peripheral surface of the molecule should be favourable towards better binding.

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