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# Inhibition of Tumor Necrosis Factor-α (TNF-α)/ TNF-α Receptor Binding by Structural Analogues of Suramin

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**ABSTRACT.** Suramin, a symmetrical polysulfonated urea derivative, promotes the dissociation of trimeric human tumor necrosis factor-α (TNF-α) into biologically inactive subunits and prevents the interaction of TNF-α with its cellular receptors. The aim of this work was to identify compounds structurally related to suramin which inhibit the binding of TNF-α to its receptor. Molecular modeling studies were performed on suramin and TNF-α molecules and likely interaction sites were identified in the docked complex. On this basis, Evans blue, trypan blue, sulfonazo III, beryllon II, and 1,3,6-naphthalenetrisulfonic acid trisodium salt were identified as polysulfonated compounds endowed, to various extents, with the structural characteristics responsible for interaction with TNF-α. *N*,*N*-bis(3,5-di-*tert*-butylphenyl)-3,4,9,10-perylenedicarboximide was used as an unrelated structure. The capacity of these molecules to inhibit the binding of TNF-α with its receptor p55 was tested *in vitro* by means of a specific immunoenzymatic assay using suramin as reference compound. Evans blue and trypan blue inhibited TNF-α/p55 binding with an $IC_{50}$ of 0.75 and 1.00 mM, respectively (suramin $IC_{50}$: 0.65 mM); no effect was observed with the other molecules. Molecular modeling analyses on Evans blue and trypan blue docked into the TNF-α molecule support these experimental results by demonstrating that these compounds share with suramin a similar binding mode to TNF-α. The results of this work provide a new insight into and useful hints for the design of new chemical entities endowed with a potent and selective activity on TNF-α. BIOCHEM PHARMACOL **58**;5:851–859, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** tumor necrosis factor-α; p55 receptor; suramin; Evans blue; trypan blue; molecular modeling

TNF-α§ belongs to a family of secreted cell surface proteins that mediate immune and inflammatory responses. The primary sources of TNF-α are activated macrophages and monocytes [1], but this cytokine is also produced by a number of additional cell types, including several tumor cells [2]. Mature TNF-α molecules are released as 17 kDa monomers which associate non-covalently to form a biologically active homotrimer, as represented in Fig. 1 [3, 4]. Biological activities of TNF-α are mediated by two distinct receptors of 55–60 kDa (TNF-RI) and 70–80 kDa (TNF-RII), which are expressed independently on the membranes of most somatic cell types [5, 6]. TNF-α is a pleiotropic cytokine, with both detrimental and beneficial activities, and its overproduction has been implicated in a number of diseases, including septic shock syndrome [7], cachexia [8], AIDS [9], and in the pathogenesis of certain autoimmune diseases [10].

Suramin is a symmetrical polysulfonated urea derivative which has been widely used to treat trypanosomiasis and onchocerciasis since 1920 [11]. It has been shown to possess some antitumor activity as an inhibitor of tumor growth factors; in fact, melanoma-derived heparanase, a heparan sulfate-specific endo-β-D-glucuronidase that plays an important role in metastatic melanoma cell invasion through basement membranes, is inhibited by suramin [12]. Furthermore, suramin has recently been found to specifically promote dissociation of the biologically active trimeric form of TNF-α into inactive subunits, thus inhibiting the binding of TNF-α to its cellular receptor [13].

Several compounds structurally related to suramin were studied. In order to understand the mechanism of dissociation of TNF-α trimer by suramin, Evans blue, trypan blue, sulfonazo III, beryllon II and 1,3,6-naphthalenetrisulfonic acid trisodium salt, whose structural formulae are given in Fig. 2, were selected and the importance of sulfonic acid groups, of molecular length, and of the symmetry in the interaction between suramin and TNF-α was analyzed. To assess the capacity of these polysulfonated molecules to inhibit the binding of TNF-α to its receptor p55, a specific immunoenzymatic assay was developed and the compounds were tested using suramin as reference inhibitor.

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§ *Abbreviations:* TNF-α, tumor necrosis factor-α; and MC/EM, Monte-Carlo/energy minimization.

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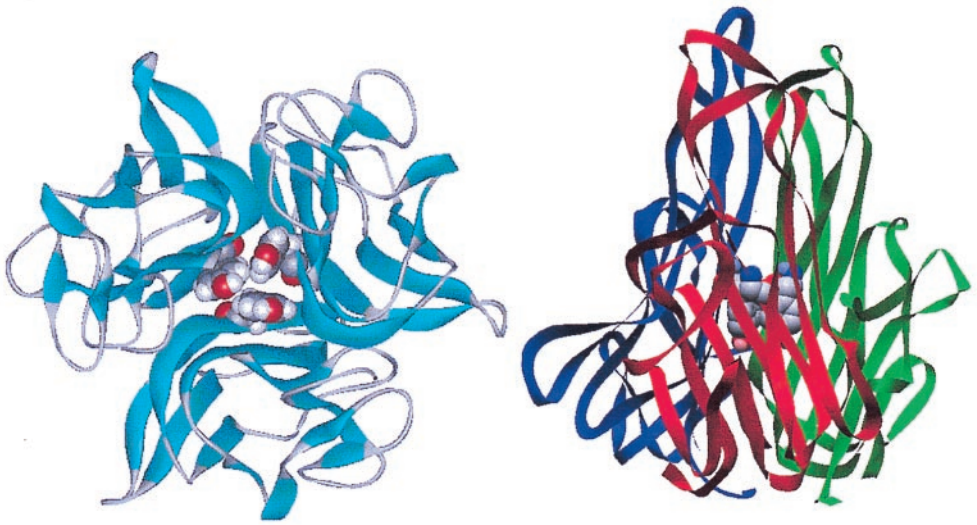


**FIG. 1. Representation of three TNF-α monomeric subunits [14] non-covalently associated to form a compact, conical trimer about 55 Å in length and 50 Å maximum breadth. The interaction between the subunits is through a simple edge-to-face packing of the β sandwiches. The hydrophobic residue cluster lies in the core of the molecule while charged residues are on the surface. At the top of the trimer, the interaction between subunits involves charged side chains (Arg 103, Glu 104 and 116, and Lys 98 and 112), whereas near the center it is mediated by polar side chains (Tyr 59, 115, 119, and 151, Gln 61 and 140, Ser 147, and Asn 34) and at the base by a patch of large apolar residues (Phe 124, Ile 155, Val 13 and 123, and Leu 36, 93, 94, 55, 57, and 157). The threefold interaction at the heart of the trimer is produced by the edge-to-face packing of the aromatic rings from a nest of tyrosine residues (59, 119, and 151) centered on Tyr 119 (space-filling representation).**

The interactions between trypan blue, Evans blue, and TNF-α were also evaluated by means of molecular modeling techniques.

## MATERIALS AND METHODS

### *Materials*

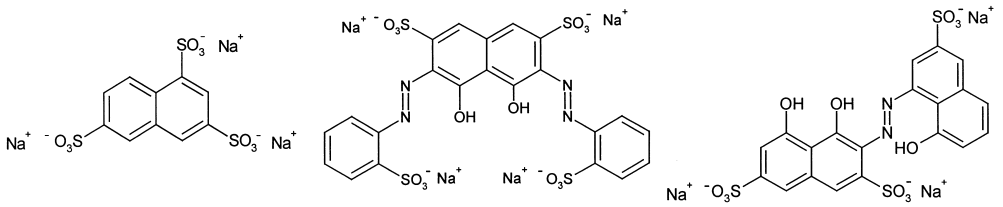
Materials were obtained from the following sources: flat-bottom 96-well plates from Falcon, Becton Dickinson; human TNF-RI receptor p55, human biotinylated TNF-α, and soybean trypsin inhibitor, biotinylated to the same degree as TNF-α, from R&D Systems; BSA and alkaline phosphatase-conjugated avidin from Sigma Chemical Co.; gelatin, Evans blue and Tween-20 from Merck; PBS from Flow Laboratories; suramin from Calbiochem-Novabiochem Corporation; trypan blue, 1,3,6-naphthalenetrisulfonic acid trisodium salt and *N,N'*-bis(3,5-di-*tert*-butylphenyl)-3,4,9,10-perylenedicarboximide from Aldrich; sulfonazo III and beryllon II from Lancaster.

### *Solid Phase TNF-α/TNF-α Receptor Binding Assay*

The assay was performed in 96-well microtiter plates introducing several modifications to the method described in [13]. Wells were coated overnight at 4° with TNF-RI p55 at the concentration of 1 μg/well in 0.1 M carbonate-bicarbonate buffer pH 9.6 (50 μL/well). After one wash with PBS–Tween buffer (PBS containing 0.1% Tween-20), the plate was incubated with 200 μL of 3% milk, 0.25% gelatine in PBS for 1 hr at 37° to saturate non-specific binding sites. After four washes with PBS–Tween buffer, 30 ng of biotinylated TNF-α (or biotinylated negative control reagent), preincubated for 15 min with different concentrations of suramin and other polysulfonated compounds, was added to the wells in PBS containing 0.1% BSA (100 μL/well) and the plate was incubated for 2 hr at room temperature. After four washes with PBS–Tween, 100 μL of alkaline phosphatase-conjugated avidin 20 ng/mL diluted in PBS containing 1% BSA was added. The plate was incubated for 1 hr at 37° and washed four times, after which "Sigma 104" alkaline phosphatase substrate in diethanolamine 1 M pH 9.8 (1 mg/mL, 100 μL/well) was added. The amount of bound TNF-α was directly proportional to optical density values measured in a Titertek Multiskan Plus apparatus (Flow) at a wavelength of 405 nm after 20 hr of incubation with the substrate at room temperature.

### *TNF-α Modeling*

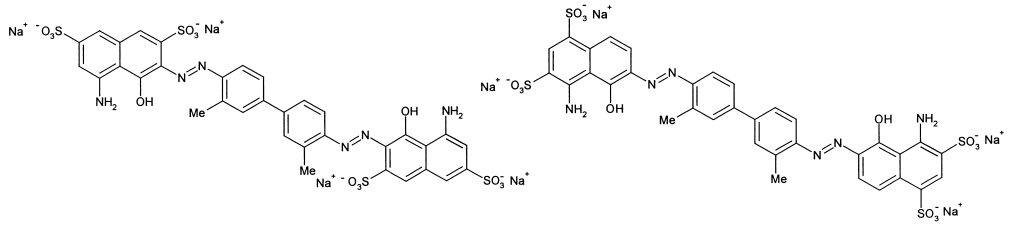
The 3D model used was derived from the coordinates of the crystal structure of TNF-α, solved at 2.6 Å resolution [14] and deposited in the Brookhaven Protein DataBank as entry 1 TNF. The PROCHECK software program [15] was then used in order to perform an in-depth analysis of the structural properties of the TNF-α model. H-bonding hydrogens were added to TNF-α, which was then minimized employing the Amber [16, 17] united atom force field implemented in MacroModel/Batchmin version 6.0. The Polak Ribiere Conjugate Gradient (PRCG) [18] energy minimization algorithm and a distance-dependent dielectric ($\epsilon = 4r$ where r is the distance in Å) Coulombic treatment of electrostatics were used. Hydroxyl and amino



1,3,6-naphthalenetrisulfonic acid trisodium salt (MW = 434.3)

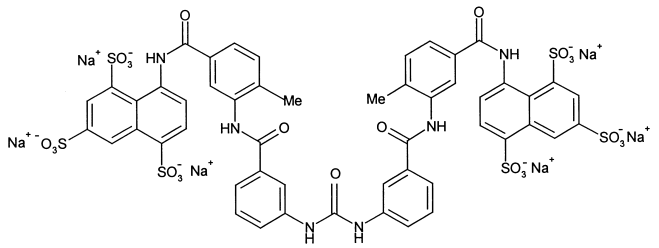
Sulfonazo III (MW = 776.6)

Beryllon II (MW = 738.5)

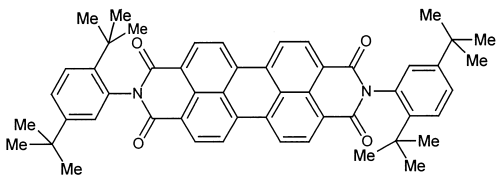


Trypan blue (MW = 960.8)

Evans blue (MW = 960.8)



Suramin (MW = 1429.2)



N,N-Bis(2,5-di-tert-butylphenyl)-3,4,9,10-perylenedicarboximide (MW = 766.9)

**FIG. 2. Structures of test compounds (MW: molecular weight).**

hydrogens were unconstrained during energy minimization to allow reorientations and thus proper hydrogen-bonding geometries. All other atoms were constrained with a force constant of 100 kJ/Å$^2$.

### *Conformational Analyses*

3D models for suramin and analogues were generated using the model building tools available within the interactive molecular modeling system MacroModel version 6.0. The geometry of each compound was minimized using the MM2 Force Field [19] implemented in the Batchmin program and the Truncated Newton Conjugate Gradient [20] algorithm with a dielectric constant of either 2r or 4r. A dielectric constant of 4r was used to calculate the electrostatic potential in all additional calculations reported herein. A Systematic Pseudo Monte Carlo Search [21] (1000 steps for variable torsion angle) using MM2 and Merck Molecular Force Field (MMFF) [22], as implemented in MacroModel, and a Systematic Search, using MMFF implemented in Spartan, were performed on the protonated and unprotonated forms of all compounds. The structures obtained were subjected to energy minimization under the same conditions described above. The minimum energy conformations for the unprotonated form were subjected to a multiminimization in water using a GB/SA Solvation Model [23].

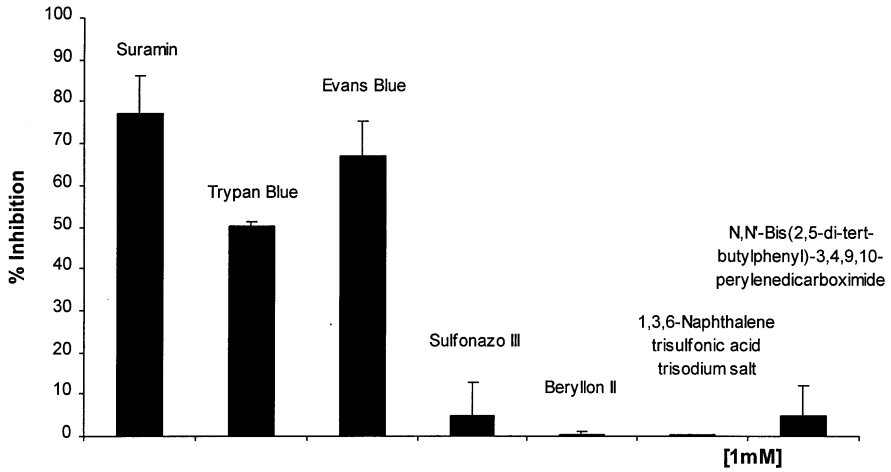



**FIG. 3. Effects of polysulfonated compounds on TNF-α/TNF-α receptor binding. Microtiter plate wells were coated with the p55 subunit of the TNF-α receptor. After saturation, biotinylated TNF-α, preincubated with suramin and other polysulfonated compounds at the concentration of 1 mM, was added. Binding reaction was revealed by incubation with alkaline phosphatase-conjugated avidin and "Sigma 104" substrate. Percent inhibition was determined by measuring the decrease in bound TNF-α. The values reported are the averages ± standard deviations of three different determinations.**

### *Docking*

Manual docking was performed for suramin and related compounds. All the complexes were energy-minimized (Polak Ribiere Conjugate Gradient) with a 4r dielectric constant. Ligand and a shell of residues surrounding the possible binding sites of the complex, located within a range of 5 Å from the ligand, were unconstrained during minimization, while all other residues were constrained with a force constant of 100 kJ/Å. The minimum energy conformation was then used as a starting point for an MC/EM. Finally, only the ligand and the hydroxyl and amino hydrogens, within a shell of 10 Å from the ligand, were unconstrained for the MC/EM calculations. All other atoms within the shell were constrained with a force constant of 100 kJ/Å$^2$. All calculations were performed on a Silicon Graphics O2 R10000 workstation. Typically, 1000 to 2000 MC/EM steps were used.

## RESULTS

### *Experimental Results*

Initial experiments performed on Evans blue, trypan blue, sulfonazo III, beryllon II, 1,3,6-naphthalenetrisulfonic acid trisodium salt, and *N*,*N*-bis(3,5-di-*tert*-butylphenyl)-3,4,9,10-perylenedicarboximide at the concentration of 1 mM indicated that Evans blue and trypan blue were able to inhibit the binding of TNF-α to its specific receptor p55; on the contrary, sulfonazo III, beryllon II, 1,3,6-naphthalenetrisulfonic acid trisodium salt, and *N*,*N*-bis(3,5-di-*tert*-butylphenyl)-3,4,9,10-perylenedicarboximide did not modify the capacity of biotinylated TNF-α to bind to its receptor p55 (Fig. 3). Further experiments performed on Evans blue and trypan blue at the concentrations of 0.1–4 mM showed that they inhibited TNF-α-p55 binding in a dose-dependent manner with an $IC_{50}$ of 0.75 and 1 mM, respectively (suramin $IC_{50}$ = 0.65 mM) (Fig. 4).

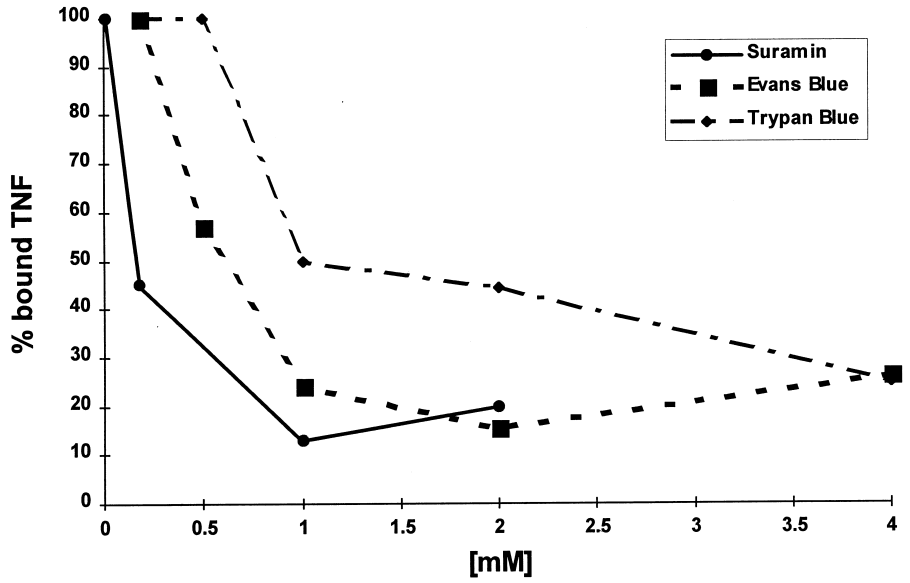
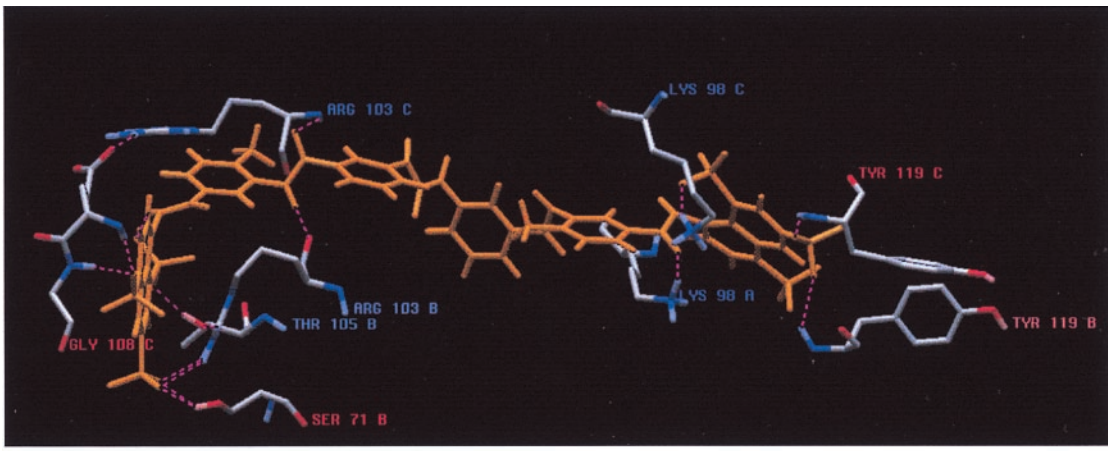
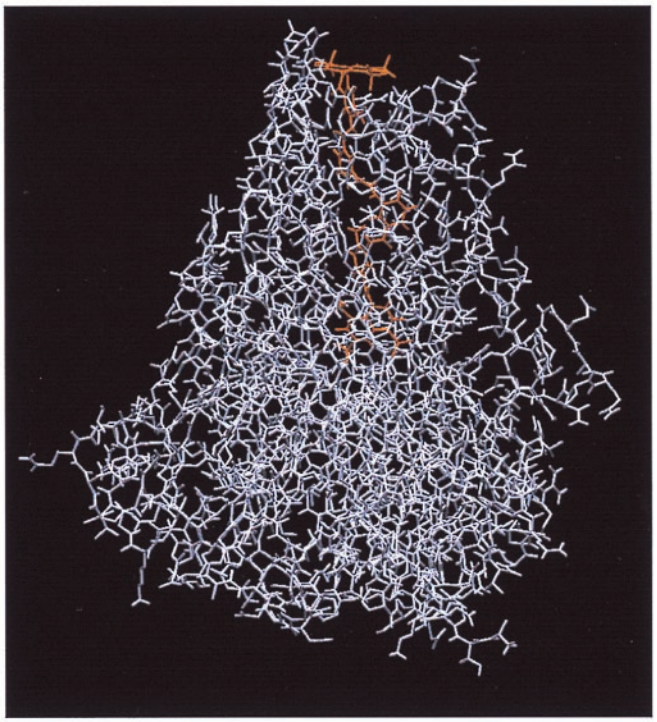



**FIG. 4. Dose–response curves of 3 polysulfonated molecules on TNF-α/TNF-α receptor binding. The curves were generated by plotting the percentage of bound TNF-α as a function of the various concentrations of suramin, Evans blue, and trypan blue. The amount of bound TNF-α is directly proportional to optical density values measured at a wavelength of 405 nm. Results are representative of one of three separate experiments performed.**




A

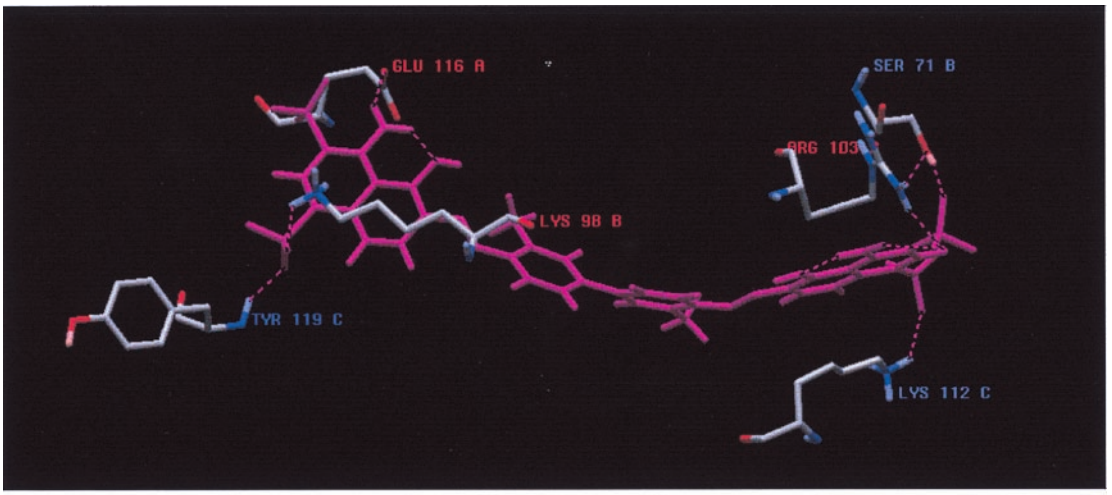


B

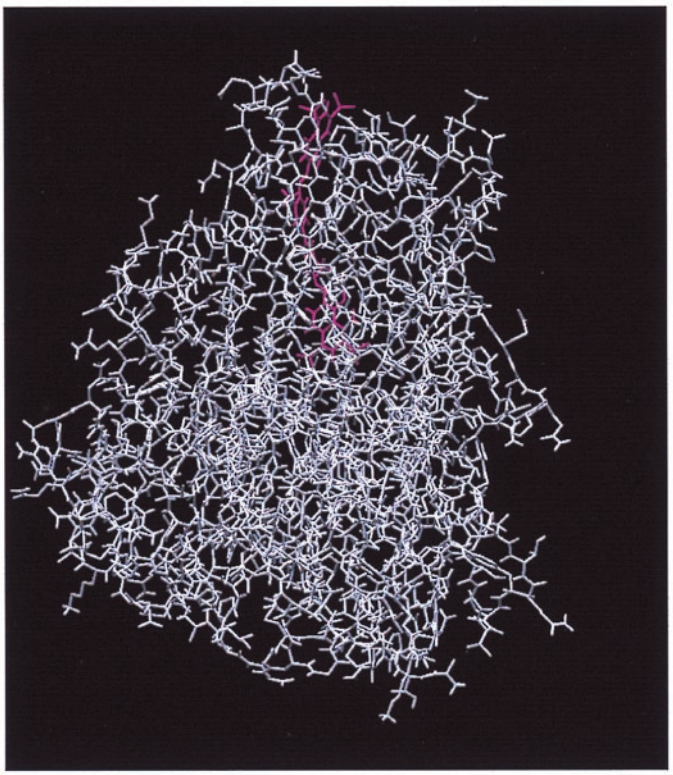
**FIG. 5. (A) Stereoview of the binding site(s) of suramin on TNF-α, emphasizing the electrostatic interactions. (B) suramin (orange) docked into TNF-α.**

### *Computational Results*

Several different docking modes were explored, since no experimental information is available on binding interactions of suramin, Evans blue, and trypan blue to TNF-α. The docked complex along the threefold axis, with one of the naphthalene rings reaching the core region (Tyr 59, 119, and 151), was identified as a likely intermediate complex geometry. A total of 68 minimum energy geometries for the TNF–α–suramin complex were formed by MC/EM after 1000 steps. Figure 5 represents the minimum energy conformation interactions with the following residues: Gly 108 C, Arg 103 B and C, Thr 105 B, Ser 71 B, Lys 98 A and C, Tyr 119 C and B. Only 5 minimum energy structures were obtained from Evans MC/EM. The global minimum (Fig. 6) presents the following interactions: the sulfonic groups (on the surface of TNF-α) interact with the side chains of Lys 112 C, Arg 103 B, and Ser 71 B; the amino group interacts with the side chain of Glu 116 A; the sulfonic groups (inside TNF-α) interact with the side chains of Lys 98 B and Tyr 119 C. MC/EM calculation on trypan yielded 11 mimima (Fig. 7) characterized by sulfonic groups (on the surface of the TNF-α) interacting with the side chains of Arg 103 B and Thr 72 B. The sulfonic group in the core of the trimer forms a hydrogen bond with Lys 98 B and the amino group interacts with the side chain of Glu 116 A.




A



B

**FIG. 6. (A) Stereoview of the binding site(s) of Evans blue on TNF-α emphasizing the electrostatic interactions. (B) Evans blue (purple) docked into TNF-α.**

## DISCUSSION

The involvement of TNF-α in the pathogenesis of several different inflammatory diseases, such as septic shock syndrome [24], multiple sclerosis [25], inflammatory bowel disease [26], and rheumatoid arthritis [27], makes this cytokine an obvious and attractive therapeutical target. Several approaches have been undertaken so far in order to inhibit TNF-α production. Lee *et al.* suggested the use of p38 mitogen-activated protein kinase inhibitors [28], thalidomide was shown to enhance TNF-α mRNA degradation [29], and recently a number of matrix metalloproteinase inhibitors have been reported to inhibit the TNF-α converting enzyme [30]. Moreover, several groups have attempted to limit TNF-α's deleterious biological effects by administering anti-TNF-α monoclonal antibodies [31] or TNF-α recombinant receptors [32].

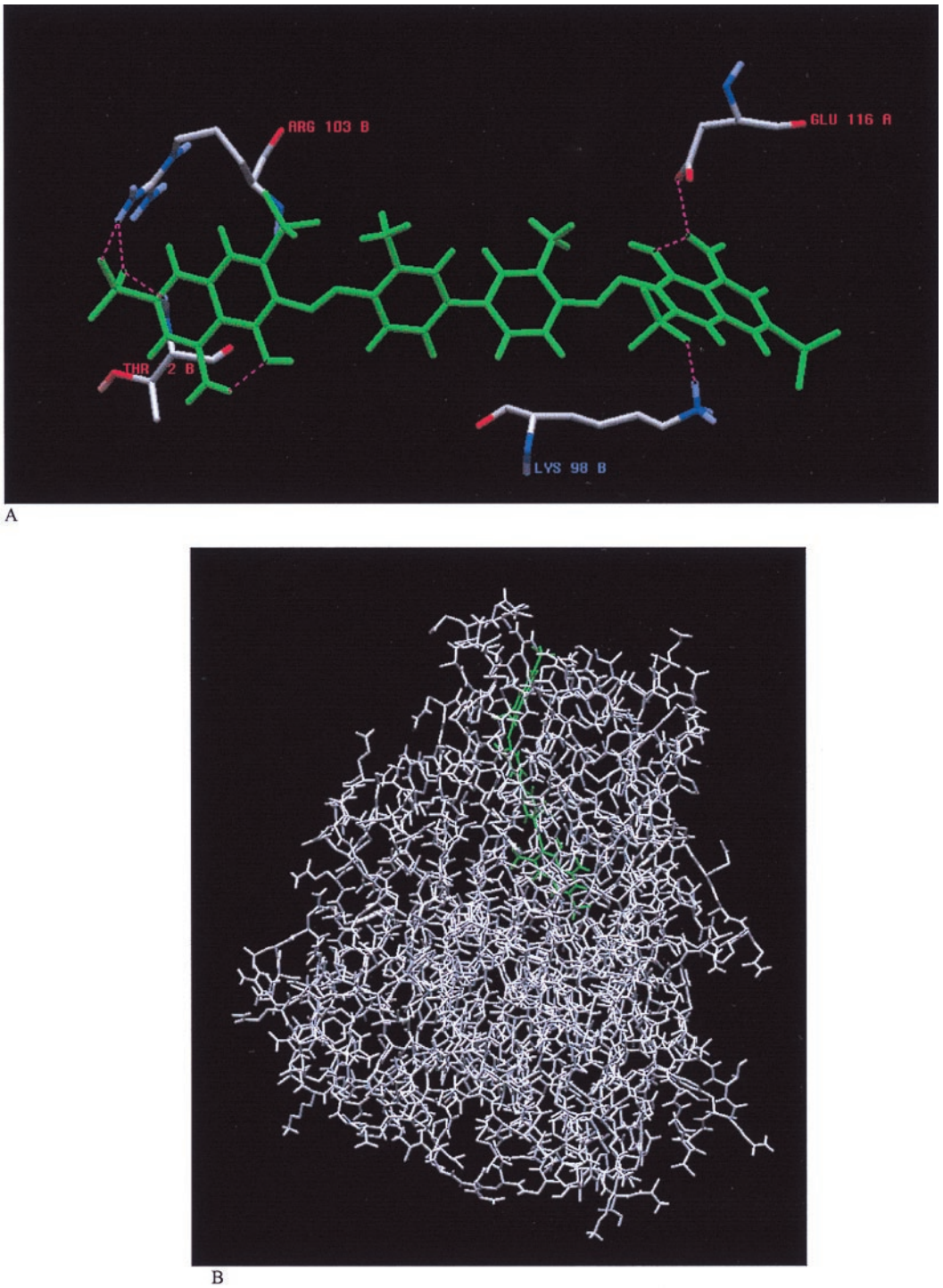



**FIG. 7. (A) Stereoview of the binding site(s) of trypan blue on TNF-α, emphasizing the electrostatic interactions. (B) trypan blue (green) docked into TNF-α.**

Suramin has been shown to inhibit the interaction of many polypeptides, including TNF-α, with their receptors, leading to a decrease in their biological activity; additional evidence has indicated that this occurs through a direct action on the ligands rather than on the receptors [33]. Working on the TNF-α molecule, Alzani *et al.* [13] have demonstrated that suramin inhibits TNF-α activity by inducing deoligomerization of its quaternary structure. However, the clinical use of suramin is made difficult by its low selectivity for TNF-α and its capacity to aspecifically interfere with the activities of several different macromolecules.

The purpose of this study was to examine the interactions between the TNF-α molecule and a series of compounds endowed with structural characteristics analogous to those of suramin in order to define the chemico-physical requirements of a hypothetical TNF-α inhibitor. Suramin

was chosen as reference compound because of its capacity to disaggregate the TNF-α trimeric molecule [13] and, therefore, to inhibit the biological activity of TNF-α [34]. The computer-aided docking procedure performed on suramin and TNF-α allowed us to define the chemico-physical basis of the nature of the interactions between these two molecules; the results of these studies seem to indicate that the six sulfonic acid ($SO_3^-$) groups of the suramin molecule, its molecular length (29.4 Å), and possibly its symmetrical structure are the main characteristics responsible for the capacity of the drug to interact with the TNF-α trimer. The TNF-α–suramin complex may well represent an intermediate step in the overall mechanism of suramin action leading to the dissociation of the TNF-α trimer. On the basis of this conclusion, a series of other molecules were selected and analyzed for their capacity to inhibit the binding of TNF-α to its receptor in an *in vitro* assay. Evans blue and trypan blue were considered, as they have a symmetry axis, four sulfonic groups, and a length comparable to suramin (Evans blue: 25 Å; trypan blue: 26 Å); sulfonazo III is a small (10 Å) symmetrical molecule with four sulfonic acid groups; beryllon and 1,3,6-naphthalenetrisulfonic acid trisodium salt are small (10 Å) asymmetrical compounds with three sulfonic groups; finally, *N*,*N*-bis(3,5-di-*tert*-butylphenyl)-3,4,9,10-perylenedicarboximide is a 20 Å asymmetrical molecule devoid of sulfonic groups.

The experimental results of this study show that Evans blue and trypan blue, whose chemico-physical characteristics more closely resemble those of suramin, can inhibit TNF-α/TNF-α receptor binding in a dose-dependent manner, whereas sulfonazo III, beryllon II, 1,3,6-naphthalenetrisulfonic acid trisodium salt, and *N*,*N*-bis(3,5-di-*tert*-butylphenyl)-3,4,9,10-perylenedicarboximide, which share with suramin only some of the characteristics we considered crucial for its dissociative effects on TNF-α, were devoid of activity in the in vitro assay.

These results prompted us to study the likely binding site(s) location of Evans blue and trypan blue on the TNF-α molecule in order to verify whether these two compounds can exert a mechanism of action similar to that of suramin. In fact, our docking studies on the suramin–TNF-α complex suggest that a minimal length and a particular orientation of the sulfonic groups are required for the ligand to penetrate into the channel along the trimer symmetry axis: the length of Evans blue and trypan blue in an extended conformation is similar to that of suramin and at the same time compatible with the distance between the three Tyr core residues and the entrance of the channel formed by the three TNF-α monomers assembled into the trimeric form; in fact, the distance between the $C_\alpha$ of Tyr 119A and Arg103A is about 26 Å. Furthermore, trypan blue and Evans blue share a TNF-α-binding pattern similar to suramin; indeed, according to our calculations, they interact with at least three residues (Arg 103A, Lys 98 B, and Glu 116 A) that suramin binds to.

The fact that beryllon II, sulfonazo III, and 1,3,6-naphthalenetrisulfonic acid trisodium salt do not affect TNF-α binding to its receptor seems to indicate that electrostatic interactions between sulfonic groups and TNF-α residues are not sufficient in themselves to induce the dissociation of the trimer, thus suggesting that other types of interactions (e.g. dispersion forces) and molecular size itself may play an important role in TNF-α deoligomerization. On the other hand, the lack of binding inhibition observed with *N*,*N*-bis(3,5-di-*tert*-butylphenyl)-3,4,9,10-perylenedicarboximide indicates that the effects of suramin on TNF-α are not exclusively related to its molecular size.

In conclusion, the aim of the present work was to elucidate the chemico-physical basis of the interaction between the molecules of TNF-α and suramin. Molecular modeling studies suggest that the sulfonic groups and molecular length are the main features responsible for the capacity of suramin to interact with and successively dissociate the TNF-α trimer. These hypotheses were experimentally tested and confirmed by analyzing, in an *in vitro* assay, a series of compounds selected on the basis of their increasing analogies with suramin's relevant characteristics. In summary, similar molecular sizes (as shown by Evans blue, trypan blue, and suramin) as well as similar ligand-TNF-α interactions determined by computer-assisted techniques correspond to similar TNF-α inhibition levels. Therefore, our results provide a series of new elements which should be taken into consideration in the design of new suramin-derived chemical entities in order to obtain molecules endowed with a more potent and selective activity on TNF-α.

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