Modifying TNFα for Therapeutic Use: A Perspective on the TNF Receptor System

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Abstract: TNFα is an inflammatory mediator that is relevant to several autoimmune diseases. Macromolecular inhibitors of TNFα have proven therapeutically useful in some preliminary studies. We have developed small molecule TNFα antagonist based on the crystal structure of TNF receptor complex. The TNFα inhibitor is specific and mediates biological function similar to the inhibitory soluble TNF receptor. This review focuses on development of small molecule anti-TNFα mimetics by us and current status of other agents.

INTRODUCTION

The main function of immune system is to eradicate foreign organisms such as viruses or bacteria. Defense against foreign organisms is mediated by innate immunity and by specific (or adaptive) immunity. The effector phases of both innate and specific immunity are large part mediated by protein hormones called cytokines. In innate immunity, the effector cytokines are mostly produced by mononuclear phagocytes and are therefore called monokines. Phagocytes accumulate at the site of infection and secrete monokines that include interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12) and tumor necrosis factor-α (TNFα). Most of these molecules are pleiotropic (i.e. affect different biological functions) and have effects on immunological processes such as inflammation and cellular responses such as apoptosis.

In the last century, Coley [1] observed beneficial inflammatory effects in the terminally ill cancer patients. Much later, in 1985, Old identified a protein in the serum of endotoxin-treated rabbits that was responsible for the hemorrhagic necrosis of tumors [2]. It was named tumor necrosis factor (TNF) for its ability to trigger necrosis and involution of transplantable tumors, and later named TNFα after the discovery of lymphotoxin or TNFβ. TNFα was highly toxic to both humans and animals [3]. In unrelated experiments, cachectin isolated from waste body fluids of animal and human with chronic disease were found to be identical to the necrosis factor. Finally the study of lipopolysaccharide (LPS) induced biological functions led to the conclusion that TNFα is mediator of the shock, disseminated coagulation, metabolic acidosis and end-organ damage brought about by LPS.

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Several biochemical and biological properties of TNFα have been elucidated since the mid-1980s when TNFα was cloned, sequenced and purified [4-7]. The major source of TNFα is the activated monocytes/macrophages. TNFα is synthesized as a 26 kDa soluble TNFα molecule and observed as homotrimer under physiological conditions [8].

Most of the cellular actions of TNFα have been attributed to the activities of two distinct receptor molecules TNF receptor I (TNFRI, p55) and TNFRII (p75) [9] which are expressed ubiquitously. TNFRI-knock out mice are resistant to endotoxin shock but succumb to infection, indicating that TNFRI plays an important role in defense against microorganisms [10]. The extracellular portions of both TNF receptors can also be shed from the cell surface through proteolytic cleavage and exist in soluble form and moreover soluble receptors retain the ability to bind TNFα and thus may act as physiological modulators of TNF activity *in vivo* [11,12].

Binding of TNFα to its membrane-bound receptors induces diverse effects in different organs and tissues. Recently several TNF receptor-associated proteins have been cloned. The cytoplasmic domains of TNF receptors do not have any intrinsic enzymatic activity, and hence they signal by inducing aggregation of intracellular adaptor molecules. The cytoplasmic domains of TNFRI bear a motif termed as 'death domain' (DD). The DD is a protein-protein interaction motif that allows two proteins with DD to bind to each other. Binding of TNF to TNFRI induces recruitment of the DD-containing protein TRADD to the DD of TNFRI [13]. Overexpression of TRADD also induces TNF-regulated responses apoptosis and activation of the transcription factors NF-κB and Jun kinase [14]. The group of TNF receptor-associated factors (TRAF) also interact with members of the TNFR family [15]. Most of TRAF proteins interact with receptor molecules either directly, or indirectly through binding to other TRAF, or through binding to TRADD. TNFRII contains cytoplasmic TRAF binding motifs and is able to bind directly to TRAF proteins. Because TRAF2 can bind to TRADD, which in turn can

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associate with TNFRI, TRAF2 can indirectly participate in signaling from this receptor as well. Studies of TRAF2 and TRAF3 knockout mice have revealed that TRAF proteins are required for activation of Jun/AP-1 signaling by TNF receptors. However, the details of signal transduction via TNF receptors are still unknown. Further studies are expected.

High concentrations of plasma TNFα have been found in a variety of infections and inflammatory disease. TNFα play a critical role in the development of autoimmune processes such as Rheumatoid arthritis (RA) and Crohn's disease [16-20] as well as other disease conditions associated with bone resorption [21-23], sepsis syndrome [24,25] and AIDS [26,27] is also exacerbated in the abnormal production of TNF.

The therapeutic application of TNFα has been investigated in several malignant diseases, but has remained limited due to severe side-effects. On the other hand, multiple roles mediated by TNFα in the development of autoimmune diseases prompted efforts to refocus on TNFα as a viable therapeutic agent for diseases [28]. Inhibition of TNFα has proven to be useful in some preliminary general studies [29,30]. Experimental studies have shown that TNFα blockade by monoclonal antibodies or by soluble TNF receptor reduced the extent and severity of arthritis both in collagen-induced arthritis in mice and transgenic mice overexpressing TNFα, which develop a rheumatoid-like destructive arthritis [31,32]. Also, anti-TNFα agents may be valuable in the treatment of bone resorption [22,33], obesity due to insulin resistance [34-36] and eye injury [37-39].

In contrast to macromolecular TNFα inhibitors, we have recently developed a novel anti-TNF peptidomimetic [40] based on a detailed structural knowledge of the TNF receptor (55 kd) and its complex with TNFβ [41-43]. In this chapter, we have focused on current strategies used in the development of anti-TNF by our laboratory as well as others.

CURRENT STRATEGIES IN ANTI-TNFα DESIGN

Attempts to disable TNFα functions are targeted at several levels. TNFα can be inhibited at three stages: (1) synthesis, (2) processing, (3) effects on the target cell. Some approaches have shown promise for therapeutic values in certain type of disease and failed in other disease condition setup. For example, anti-TNFα antibody which has an effect against arthritic problems [44,45], but was not effective in others [28,30]. Nevertheless, the usefulness and curative potential of anti-TNFα agents may only be improved: (1) by understanding the role of TNFα in development of diseases processes and (2) balancing the TNFα concentration by precise intervention. To this purpose, it may be worthwhile to review current approaches and examine their strengths and weaknesses.

(A) Inhibition of TNFα Synthesis

Several TNFα synthesis inhibitors have been reported. This include a phosphodiesterase inhibitor [46,47], prostanoids [48,49], thalidomide [50], IL-10 [51,52] and antisense oligonucleotides [53,54]. The expression of TNFα is increased after activation of the transcription factor NF-κB. Inhibition of interaction of NF-κB with its motif has been reported with the phosphodiesterase inhibitor pentoxifylline [55]. Several animal studies have shown the efficacy of specific phosphodiesterase inhibition *in vivo*: in a rat model of experimental autoimmune encephalomyelitis (EAE). Supression of TNFα synthesis and enhanced survival have been reported after treatment with a phosphodiesterase inhibitor rolipram in a rat model of acute respiratory distress syndrome [56].

Several drugs, such as thalidomide of pentoxifylline, were evaluated in patients with active RA [57,58]. Although effective inhibition of TNFα production was demonstrated, the benefit/adverse effects ratio was poor due to a number of adverse effects, while the clinical improvement was modest [57]. These approaches are being actively pursued for improvement and it may be too early to assess their merits.

(B) Inhibition of TNFα Processing

Membrane-bound tumor necrosis factor, is proteolytically processed and released as a soluble mature form. TNFα are processed by proteases, known as matrix metalloproteinase (MMP) [59-62]. Members of a family of enzymes mainly involved in degradation of the extracellular matrix. Their biological role and relevance as therapeutic targets have been reviewed by Parsons *et al*. [63].

A relevant tumor necrosis factor-alpha converting enzyme (TACE), which is a metalloproteinase disintegrin, has been identified in mice lacking the TACE gene. These mutant mice exhibited reduced production of TNFα suggesting that TACE is critical for releasing membrane bound TNFα [64,65]. Subsequently, the gene was cloned [65]. Natural and synthetic metalloproteinase inhibitors have been identified and some are in early clinical testing [63,64].

Small molecular inhibitors such as MMP inhibitors are nonselective agents. It has been shown that certain MMP inhibitors can not only block TNFα synthesis, but also TNFR cleavage resulting in an increased expression of membrane receptors and an enhanced activation of cells [66]. MMP inhibitors' clinical usefulness are being evaluated [67]. Recently the crystal structure determination of MMP [68] may help in the design of more selective inhibitors.

(C) Inhibition of TNFα Effects

A classical approach in the development of antagonists are either ligand-mimics or substrates analogs as in the case of enzymes. Antagonists may be discovered using high-throughput screening. These approaches require little knowledge of structure and function. Monoclonal anti-human TNFα antibody and recombinant human soluble TNF receptor fusion protein have been generated as TNFα antagonists [69,70]. Some of these macromolecules have already been evaluated in the early clinical studies.

(1) Monoclonal Antibody

Monoclonal antibodies have been proven successful in the treatment of several diseases [71]. A chimeric human/mouse monoclonal anti-TNFα antibody (cA2), consisting of the constant region of human IgG_1, coupled to the Fv region of a high-affinity neutralising anti-human TNFα antibody has been developed, and tested in clinical trials [69,72]. RA patients received a single infusion of cA2 showed a significant improvement in multiple clinical assessment of disease activity [73,74]. In a trial with repeated application of the monoclonal antibody over a period of up to 95 weeks, three out of seven enrolled patients with RA developed antibodies against cA2 and a higher rate of antibody-associated side-effects was suspected [73,74]. Another type of humanized monoclonal antibody has also been engineered and tested in clinical studies [75,76]. Though the monoclonal antibody approach has been promising, it often less desired as a long term therapeutic agents due to expense of production, humanization and other related disadvantages associated with macromolecules [77].

(2) Soluble Receptor

Soluble TNF receptor species have been detected in the plasma. It is thought that a soluble receptors play a role of controlling the TNFα concentration and are necessary for normal immune regulation [78,79]. Large clinical studies on the effect of recombinant human soluble TNF receptor immunoglobulin fusion proteins (sTNFR-Fc), such as sTNFR-Fc p55 or sTNFR-Fc p75, have recently been reported [80-82]. Significant improvement has been shown in RA patients, but not in sepsis or multiple sclerosis patients. Neutralizing antibodies against sTNFR-Fc were detected in a substantial number of treated patients [80]. Fusion proteins containing the constant domain of Ig (with soluble receptor) may be particularly immunogenic, because they bind to the Fc receptors of antigen presenting cells, thereby facilitating uptake antigen presentation [83].

(3) Disadvantages of Macromolecular Therapeutics

While the advantage of macromolecules as drugs are observed and indicated as the following: (1) highly specific, (2) often do not require extensive analysis of biodistribution and toxicity as in the case of small molecules and (3) have a long half-life. They also have some drawbacks: (1) commercial-scale production may be either difficult or costly, (2) purity may be difficult to achieve and microheterogeneity may be inevitable, (3)conformational stability may vary with environment of body fluids, (4) they may be excluded from some compartment such as blood/brain barrier, and (5) they may lead to the development of neutralizing antibodies etc. [77].

Most of the above disadvantages of macromolecule can be overcome by creating small molecular inhibitors targeted to the surface receptors/ligands. Small molecules have their own limitation including the biodistribution and half-life. Often peptides are created first to assess biological effects, while when used as a template lead to further development of viable therapeutic agents [84-86]. One potential side effect with macromolecular anti-TNFα agents treatments is that they have longer half-life, and prolonged inhibition of critical inflammatory agents such as TNFα might compromise the natural immune response. A small molecule inhibitor targeted to cell surface molecules may therefore be more suitable not only for the development of viable drugs, but also for more controlled intervention of TNFα in the plasma.

RATIONAL DESIGN OF SMALL MOLECULE ANTI-TNFα

A knowledge based approach to design of pharmaceutical drugs has been shown to be effective. The design of the HIV protease inhibitor is one illustrative example, where the three dimensional structure of the HIV protease enzyme was used to design clinically useful therapeutics. Unlike conventional approaches, rational design offers not only a viable lead at less cost, but may also provide the opportunity to understand functional mechanisms of target molecules. Our approach to design antagonists stem from a combined structural analysis of relevant molecules, antibodies, ligand and receptors. An understanding of molecular recognition features at the atomic level has allowed to design molecules that modulate the receptors' signaling function. Analysis of macromolecular structure from the design of small molecule differ from the analysis for understanding the protein-protein interactions. In the following section, these features are discussed.

Three dimensional structure of macromolecules can be viewed (for small molecule design) in general to consist of: (1) Scaffolds: secondary structures required for stability of the folding. For example, array of β-sheets in arranged in Greek key topology, Rossmann fold, and immunoglobulin fold etc., (2) recognition sites: These are mainly small regions in a molecule which are involved in inter-molecular contacts. For example, CDR loops in an antibody, β-turns, or a long flexible loops, etc (3) Active sites (surfaces/cavities): A folded protein surface may contain clefts, cavities which are required for their functions. For example, active sites of enzymes.

Protein crystallographic and NMR studies have shown that the folding topology of macromolecule is highly conserved across different species than the primary sequence. This is evident from the fact that the immunoglobulin fold is shared by many proteins of immunological interest and by large number of cell surface molecules of receptors and growth factors. Current structural knowledge on various receptors show that they often possess either immunoglobulin folds or cystine-knot repeats. Though these two structural fold seem dissimilar in overall comparison, synthesis of structural elements at the subdomain level show that they share similar secondary structural elements; these two structural domain contain 4-6 β–strands, and loops at the junctions. Both folds are stabilized by disulfide bonds. The arrangement of β–strands is however very distinct. In the immunoglobulin fold, the β–strands are sandwiched against each other. In the cystine-knot, the β–strands are arranged in a head-to-tail of elongated fashion in the receptors studied to date. Thus in macromolecules individual subdomains or group of structural elements can be

considered as frame work or scaffolds and some as functional units.

In a macromolecule, the secondary structures, α-helix, β-sheet and β-turn are well defined, classified and to some extent predictable except loops. The loop structures are highly variable and often mobile. Loop structures have been attributed to their role in molecular recognition and binding [87,88]. An attempt to classify loops in a protein is still complex and elusive, but in certain domain structures it is possible to predict the conformations due to the small length of the amino acid sequence involved [89,90]. Thus, it is reasonable to consider that loops and reverse turns in a macromolecule to be the functional units for the purpose of design of peptidomimetics.

In designing anti-receptor small molecules, several features such as structures of antibody, receptors, ligands, relevant biochemical and biological data were considered. We have designed small molecule antagonists of CD4 [91] and TNF receptors [40]. The structure of CD4 contains immunoglobulin domain similar to an antibody and TNF receptor contains "cystine-knot" repeating domains. In the following sections, a design is illustrated for the TNFα receptor. These approaches can be used for other related receptors also [85].

(A) General Strategy in the Design of Peptidomimetics

Linear peptides suffer from inherent flexibility. Studies have shown that constraining the peptides enhances their stability and in some cases affinity. Achieving stability, solubility, and bioviability are necessary properties for the therapeutic use. We have modified cyclic peptides to increase stability and bioviability by addition of aromatic residues at the terminus.

(1) Placement of Constraining Cysteine Residues

Precise placement of constraining cysteine residues in the loop or β-turn structure is critical. For this purpose, cysteine residues are placed as pairs systematically at residues (Cα atoms) separated at least by 6.2 Å away from the critical residues. The effect of disulfide closure on the loop structure and its loop size have been evaluated by a conformational search [90] followed by molecular energy minimization and dynamics (INSIGHT, Biosym Technologies, Inc).

(2) Aromatic Modification of Peptides

Cyclization of peptides confer structural and comformational rigidity which are critical for optimal interaction with macromolecules, but it does little to improve solubility. To increase stability, solubility and other properties, a variety of strategies [86] have been adopted. For example, mixed anhydride coupling has been used [92] and DeGrado and colleagues have used a semi-rigid linker m-aminomethyl benzoic acid which links the two ends of the peptide in a simple reaction [93]. These simple chemistries have allowed the development of different forms of constrained readily synthesised small molecules of known structure.

Distribution of hydrophilic and aromatic residues at the protein-protein interface, and within the antibody combining site has been observed [94-96]. A general mechanism to explain the distribution is their role stabilizing interactions due to their hydrophobicity which precludes water near the binding site. Thus the exclusion of water reduces entropy and favour strong hydrophobic interaction for binding. Based on these observations, we proposed that bulky aromatics such as phenylalanine (Phe) and tyrosine (Tyr), known to decorate the binding surface, would protrude from the antigen binding surface. Their low affinity for water molecules leads to a ordered water binding to main chain residues. These bound waters then lead to a network of water chains that become involved in bridging antigen and antibody. This concept provides a basis for more rational structural modeling and has been used to aid our creation of small forms of molecules.

Based on our studies of the dominant features of aromatics in the antigen binding surface and their ability to lead to propagated water networks that facilitate binding and increase complementarity, we have used aromatic residues in our macrocyclic constructs. In our early studies, we found that addition of aromatic residues Phe or Tyr to the termini of peptides greatly increased the efficiency of cyclization 50% to almost 100%. Furthermore, the aromatically modified cyclic-peptides behaved far more efficiently than any small compounds tested to date. The thermodynamic consequence of the bulky hydrophobic residues on ordering water molecules may be responsible for the improved relative flanked by aromatic residues at both N and C terminus but clearly other thermodynamically equivalent modifications could be employed.

(3) Molecular Modeling

There are two major approaches: (1) De novo folding design using energy minimization and molecular dynamics, and (2) Comparative modeling followed by energy minimization and molecular dynamics. These two approaches differ only in developing the trial or initial structures. The folding patterns are studied using energy minimization and molecular dynamics. Various parameters used by us during modeling of peptide mimics, are similar to described by our group [97] with minor modifications. A detailed description of various methods is beyond the scope of this chapter. A brief summary of the strategy used by us is discussed.

Initial trial structures are developed using a database consist of loops from proteins in the Brookhaven protein database [98]. Several trial structures were generated by fixing the ends of the residues where cystine well be introduced and searching the database for loop structures. Based on the sequence similarity and the loop size, trial structures were selected. Each of the structures were evaluated for the loop size, relative orientation of the side chains and solvent effects using a combination of energy minimisation and molecular dynamics. In the simulation studies, both room temperature (300 K) and high temperature (900 K) are

employed. Low energy conformers are then subjected to further minimisation and compared with the native conformation of the template. Each assigned score is based on the similarity (as measure by Cα atoms), relative disposition of critical amino acids with respect to their neighbouring residues, predicted solubility and ability to form oligomers. When required, original amino acid residues in the template are replaced in a iterative manner to conform to the above criteria.

(B) Design of TNFα Inhibitor

(1) Structure of TNF Receptor Complex

Active tumor necrosis factor is a trimeric molecule. Upon trimerization, it binds to TNF receptor which is also a trimeric molecule. The crystal structure of the TNF receptor both in complexed and uncomplexed forms provides a general understanding of mechanism by which these receptors bind to their ligands [42,43] (Fig. **1**). TNF receptors are characterized by the repeat of cysteine residues.

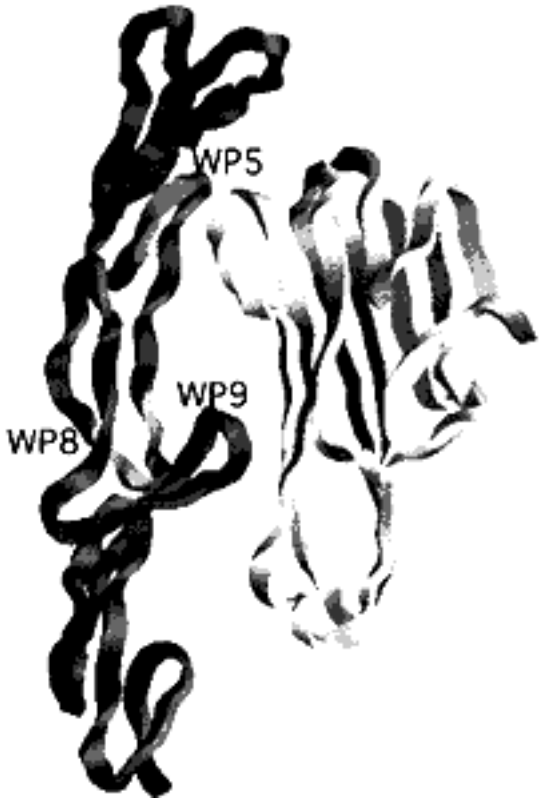

Fig. (1). Three dimensional structure of TNFRI and TNFα complex is shown. The model of TNFα was based on the crystal structure of TNFβ [43]. Crystal structure of TNFα [42] was superimposed on TNFβ to create the complex. The three major sites considered for peptidomimetic design are shown: WP5 in domain 1, WP8 in domain 2 and WP9 in domain 3.

This type of repeat has been found in other protein species and have come to be known as "cystine-knot" [99,100]. The cystine knot of the TNF receptor family consists of 42 amino acid residues with 6 cystine residues forming three inter chain disulfide bond to create the structural motif. Three dimensional structure of TNF receptor reveal the cystine-knots repeats to be about 30 Å in length and are arranged in a head-to-tail fashion exposing the loops on one side of the receptor. These loops appear to be involved in either oligomerization or ligand binding [42,43]. Three dimensional structure of uncomplexed TNF receptors unexpectedly shows receptors associated as dimers. In the dimeric form, the first and last cystine domains involved dimeric contacts [101]. In both crystal structures, the membrane proximal domain is disordered perhaps due to the lack of the transmembrane that normally holds this domain in a stable form.

(2) Receptor as Template

Analysis of crystal structures of TNFα, TNFβ and TNFRI-TNFβ complexes reveal a trimeric association. TNF receptors in the absence of TNFα/TNFβ are observed as dimer. However in a trimeric association, the ligands and the receptors have multiple contact sites. Crystallization artefacts may create dimeric complexes in the crystal lattice and it may be difficult to correlate the nature of the three dimensional structure of the complex to the biological form.

The trimeric complex of TNFRI receptor and TNFβ has been determined at atomic resolution [41]. TNFα and TNFβ molecules share similar 3D-structure, and 36% sequence homology. A trimeric complexes of TNFRI and TNFα were built by superimposing TNFα over TNFβ three dimensional structure. A careful analysis of both TNFR-TNFβ interaction and TNFR-TNFα interaction, the contact

Table 1. Sequences of First Generation of Anti-TNF Peptidomimetics

Template	Mimic	Sequence		
Loop (60-67) in domain 1	**WP5**	YC	**FTASENH**	CY
	WP5N	YC	**FTNSENH**	CY
	WP5R	YC	**FTRSENH**	CY
	WP5J	FC	**ASENH**	CY
	WP5JN	FC	**NSENH**	CY
	WP5JY	YC	**ASENH**	CY
Loop (76-83) in domain 2	**WP8L**	YC	**RKELGQV**	CY
	WP8J	YC	**RKEMG**	CY
	WP8JF	FC	**RKEMG**	CY
	WP98JP	YC	**KEPGQ**	CY
Loop (107-111) in domain 3	**WP9Q**	YC	**WSQNL**	CY

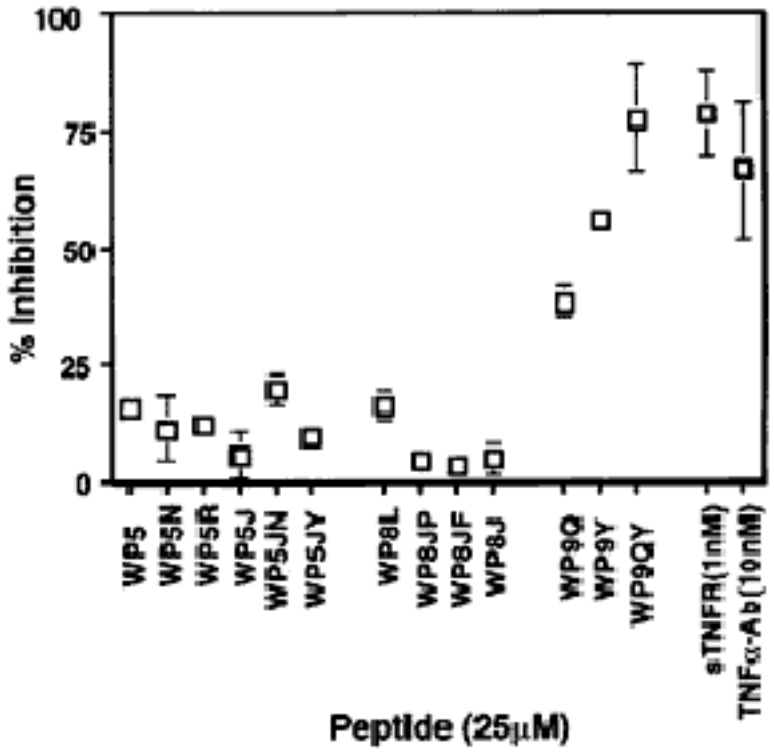

Fig. (2). Peptides designed from receptor (WP5, WP8, WP9) have been evaluated for their ability to compete with TNFα for binding to the receptor. Inhibition of ^{125}I-TNFα binding to TNFRI by exocyclic peptidomimetics in competitive radioreceptor assay. Inhibitory activities were compared at 25 μM of each peptide, 1 nM of soluble TNF-receptor and 10 nM of anti-TNFα antibody.

sites are mapped. Based on the analysis, we selected three sites, WP-5, WP-8 and WP-9, for the design of peptidomimetics (Fig. **1**). Each of these contact sites is located at different part of the receptor: WP-5 is located in the first domain of the receptor, WP-8 is located in the second domain, and WP-9 is located in the third domain. At the three interaction sites, specific loops were identified: residues 60-67 (WP-5), 76-83 (WP-8) and 108-111 (WP-9) as a template. Based on the technique described earlier, peptidomimetics were designed (Table **1**).

(3) Evaluation of peptidomimetics

Cyclic peptides derived from all the three surface loops of the TNF receptor, namely, loop (56-73) in domain 1, loop (76-83) in domain 2 and loop (107-114) in domain 3 on the TNF-receptor [40], have been tested for biological and biochemical functions.

The activity of the peptides were evaluated for: (1) Inhibition of TNFα binding to its receptor and (2) Measuring its ability to inhibit apoptosis. The first generation peptides were screened using binding assay for inhibition of TNFα binding to its receptor. The results for the first generation of peptides are shown in (Fig. **2**). Some of the peptides showed significantly higher activity, consistently.

The cyclic peptide, WP9Q, was the most promising as deduced from the initial screening. We have reengineered the peptide by careful analysis of the interaction site. First we observed that the interaction site (WP9) is accessible to solvents (Fig. **3**) and presence of water molecules. Also that one of the charged residue 109E in the loop (WP9) is disordered. The missing side chain of 109E is within the contact distance of 150H, 152D, 100E and 102Q. Thus, at the interface is negatively charged at WP9 site due to the clustering of acidic residues. Since the loop is exposed to solvents, water molecule may involve in the interaction between TNFβ and TNF receptor through aminoacid glutamic acid in the loop (109E). Further, charged residues have been implicated in recruiting solvents molecules [102]. One of the consequences in the presence of water molecules at the site of interaction is to provide stability [103-105]. Macromolecules have a large surface area and one or two water molecules can provide stability. However, small molecules, can not mimic the large surface area of a protein. In such cases, water molecules tend to weaken the interactions due to unstable secondary structure formation [106,107]. So, we modified the peptide with bulky aromatic residues and replaced a charged residue by polar residue. Based on our modeling, second generation peptides were designed (Table **2**).

Three dimensional structure of WP9QY by molecular modeling was verified with the solution structure of WP9QY (Fig. **4a**). The overall folding was correctly modeled (Fig. **4b**) and the orientation of Trp was found to be different. The WP9QY peptidomimetic engineered from the third domain

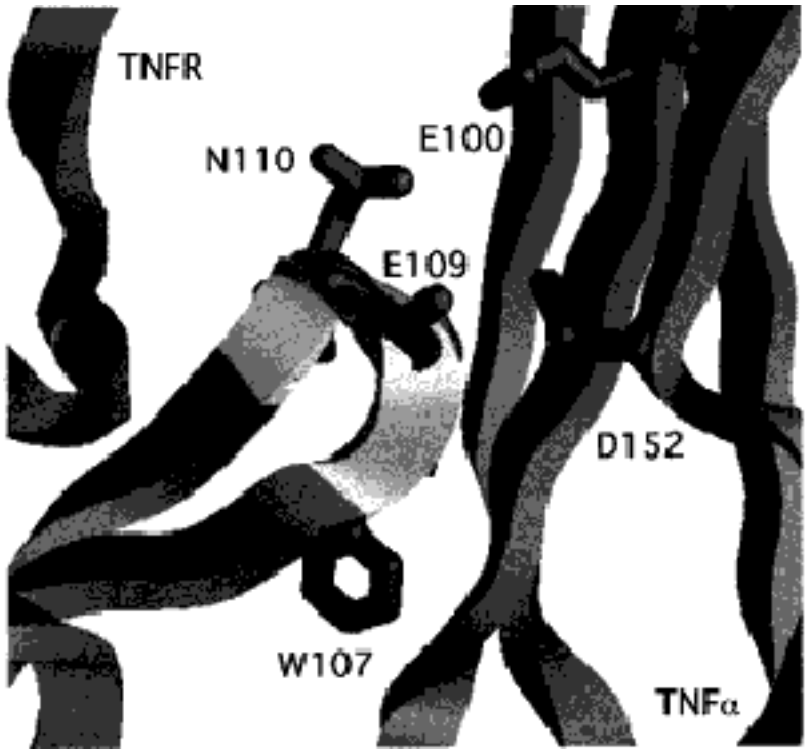

Fig. (3). Analysis of WP9 site: The interaction of TNFR and TNFα is shown. This interaction site, WP9 is solvent accessible. At this site, E109 in the loop is disordered and the side chain atoms are not visible in the electron density and built as alanine in the crystal structure [41]. The interface at this site is negatively charged due to the clustering of acidic residues from TNFα.

(WP9) inhibited TNFα binding (IC_{50}=7.5 μM) to its receptor (Fig. **5**). Also, the peptidomimetic protected cells against TNFα induced cell death suggesting that the peptide specifically bind to TNFα and inhibit TNFα activity (Fig. **6**). The peptidomimetic showed therapeutic values. These peptides are soluble and can be used *in vivo*. In a preliminary studies, WP9QY reduced the severity of EAE conditions in mice.

Table 2. Sequences of Second Generation of Anti-TNF Peptidomimetics

Template	Mimic	Sequence		
Loop (107-111) in domain 3	WP9Q	YC	**WSQNL**	CY
	WP9QY	YC	**WSQYL**	CY
	WP9Y	YC	**WSQNY**	CY

CONCLUSION AND PERSPECTIVE

Peptidomimetic (WP9QY) is one of the first peptides to demonstrate anti-TNFα activity *in vivo* (data not shown) and can be further improved as a substitute for anti-TNFα antibody or soluble receptor. Three dimensional structures of TNFβ [43] and TNF receptor [101] and its complex with TNFβ enabled us to design first anti-TNF small molecules that are specific and selective. Further, the structural study of TNF receptor not only enhanced our understanding of their function, but also led to realization that the TNF receptor's topology is not unique, but being shared by many other receptors [108]. TNF receptor and its ligand complex has become the template for understanding other receptors such as Fas, CD40, RANK etc. and their functions. Crystal structure of TNF receptor complex led to the molecular modeling of other receptors [109,110] allowing the development of therapeutic agonists and antagonists for other receptors [85].

After a decade, drugs discovered based on three dimensional structures are proving to be clinically useful and cost effective. Enzyme inhibitors have been successfully developed into useful drugs; HIV protease inhibitors [111,112] and anti-coagulants are some illustrative examples. Still the challenge to translate other bioactive peptides into clinically useful drugs is largely remain unsolved. But studies reported recently suggest that it may be possible to use peptides as drugs [113].

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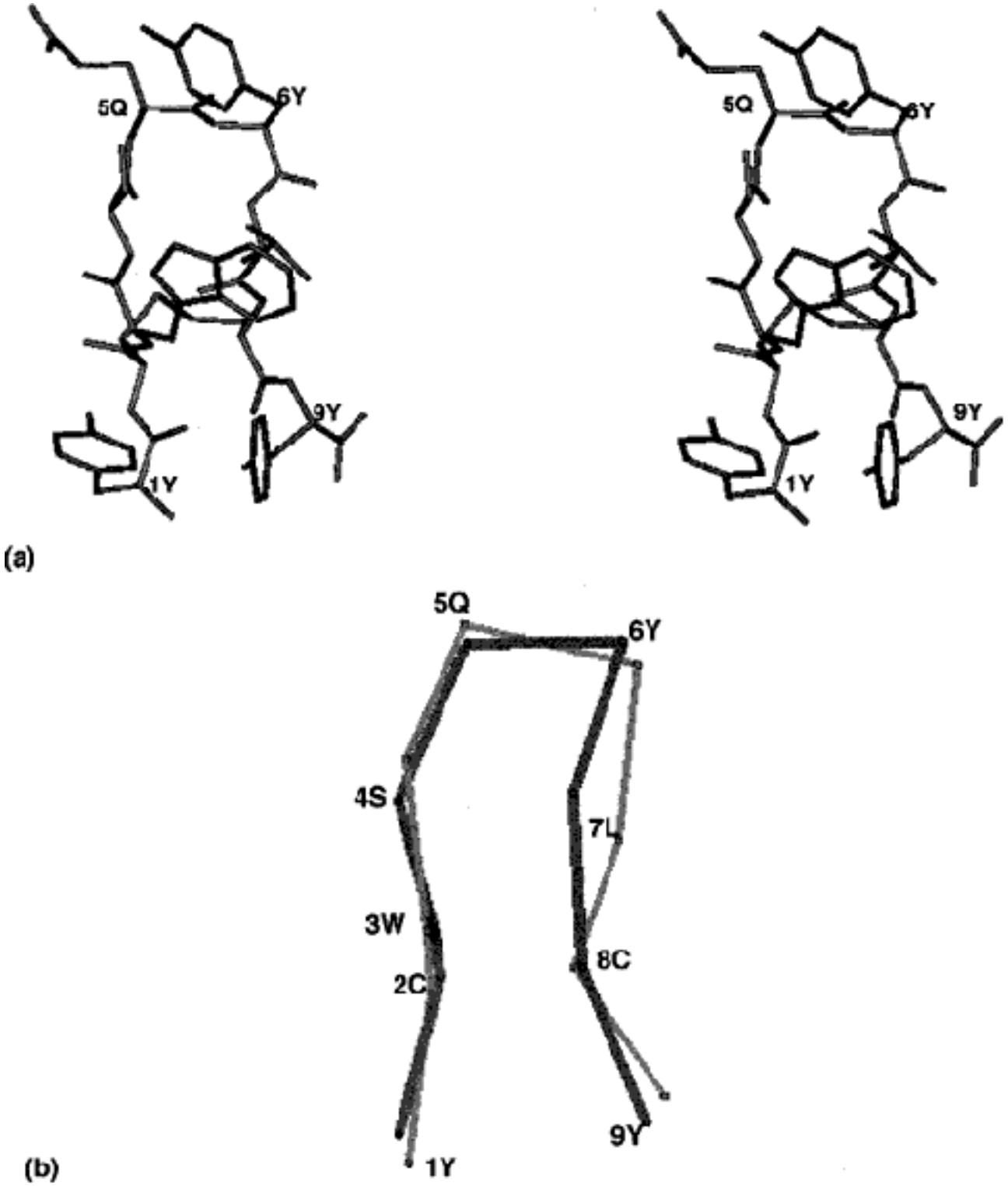

Fig. (4). Solution structure of peptidomimetic WP9QY. (a) stereoview of the solution structure of WP9QY. (b) The folding of molecular model and solution structure is similar. Superimposition of WP9QY (dark line) and the original surface loop of the TNF receptor (light line) is shown.

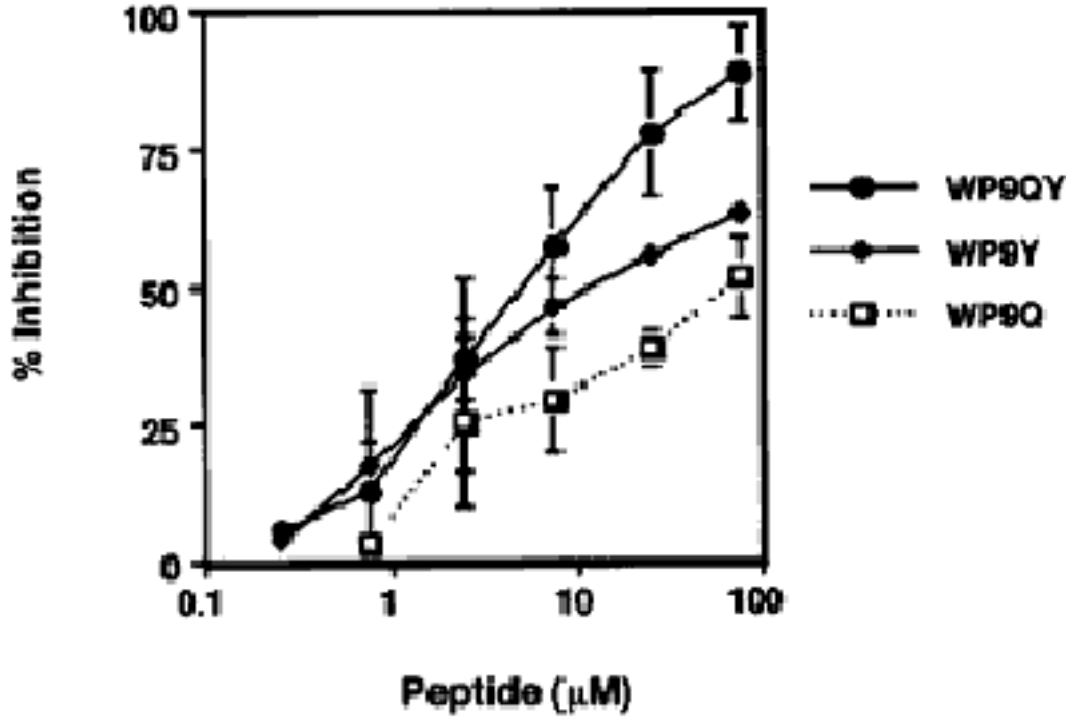

Fig. (5). Evaluation of second generation peptide mimic from WP9 site. Inhibition (%) of TNFα binding to TNFRI by several doses of peptides were calculated and plotted. The experimental conditions are same as explained in figure 2. The results indicate the means and standard deviations derived from three independent experiments.

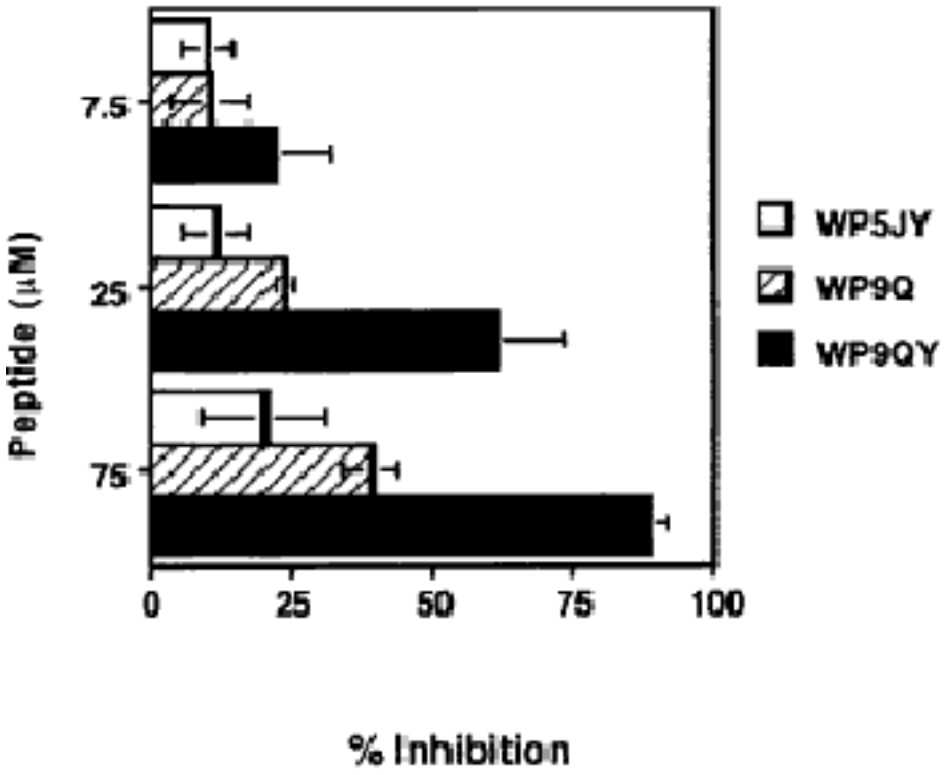

Fig. (6). Inhibition of TNFα-induced cytotoxicity on L929 cells by the antagonistic peptides. Absorbance obtained with 1 μg/ml of ACT-D alone and with ACT-D and 50 pg/ml of TNFα were referred as 100% survival and 100% cytotoxicity, respectively. The results indicate the means and standard deviations derived from three independent experiments.

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