Chapter 4C
(Results & Discussion)

Evaluation of novel peptide inhibitor of human TNF-α using cell culture studies
4C.1. Introduction

TNF-α plays a very crucial role in the progression of many inflammatory diseases including rheumatoid arthritis. Approaches to inhibit TNF-α induced inflammatory responses are of therapeutic values. One of the newer approaches for TNF inhibition is the use of peptide antagonists against it. Throughout the body, peptides are active regulators and information brokers with skill sets that make them interesting for drug discovery. Important virtues of peptide drugs are the chemical and biological diversity alongside high specificity, affinity, and molecular recognition. There are many advantages of peptides over the conventional drugs such as high activity, higher specificity, little unspecific binding to molecular structures other than the desired target, minimal drug-drug interaction, less accumulation in the tissues and most importantly much lower toxicity. Apart from that there are some disadvantages too like high manufacturing cost and less stability. If these can be worked upon, peptides will become the next generation drug with high potency.

Peptides inhibiting TNF-α trimerization were designed in silico using various bioinformatics tools (Agrawal, 2007). In our laboratory, purity and molecular weights of synthesized peptides were confirmed by HPLC and MALDI-TOF, respectively (data not shown). This work is focused on the designing of peptides which will inhibit the most important pro-inflammatory cytokine, TNF-α. The following sequences and molecular weights: peptide 1: YLGGVFQ (Mol. weight- 782.9 Da), peptide 2: PIYLGGVFQ (Mol. weight-993.2 Da), peptide 3: SGQVYFGIIAL (Mol. weight- 1167.4 Da) and peptide 4: ETPEGAEAK (Mol. weight- 931 Da) were considered for the study. All the four peptides were screened in vitro for their anti-TNF-α activity, using different cell lines. It was clear with the assays that the peptides are not leading to any kind of cytotoxicity. The peptide showing significant inhibition of TNF-α activity was also checked by assessing nuclear translocation of activated NF-κB. While doing the in vitro cell culture
assays, we found that peptide 2 showed significant inhibition of TNF-α consistently with all the experiments. In this report, the results of peptide 2 are presented.

4C.2. Results

4C.2.1. Peptides inhibiting TNF-α induced apoptosis in Wehi-164 cell lines

The cells after 20 hours of incubation with TNF-α in the presence of the transcription inhibitor actinomycin D (AcD) causes apoptosis of Wehi-164, a TNF-α sensitive cell line (Figure 4C.1). The peptides 1, 2, 3 and 4 were used at three different concentrations of 50, 100 and 200μM in the presence of TNF-α (2 ng/ml) and AcD (0.2%) significantly inhibited the apoptosis in a concentration dependent manner (significance *P≤0.05, **P≤0.01, ***P≤0.0001 calculated against TNFα + AcD). TNFα + AcD caused maximum apoptosis of 70% as compared to the unstimulated control cells, which were assumed to be 100% viable with no apoptosis. At 200 μM concentration the peptides 1, 2, and 3 showed maximum inhibition of apoptosis of 24.6%, 26.4% and 23.77%, respectively. Only the results of peptide 2 (Fig.5.1) is presented here for its consistent results. Suramin, a known anti-TNF compound inhibited 50.44 and 43.5% apoptosis at 200 and 100 μM concentrations. Cells incubated with all the four peptides at 200 μM concentrations showed negligible cell death, which showed the low cytotoxicity of the peptides.
Figure 4C.1. Effect of the peptide 2 and suramin on TNF-α induced apoptosis of Wehi-164 cells as evaluated by MTT assay. * p<0.05, **p≤0.01, ***p≤0.0001

4C.2.2. NF-κB activation/translocation in A549 cells using Western blotting

It is well established fact that TNF-α stimulation causes the activation of NF-κB resulting in its translocation to the nucleus. Since, p65 was the major component of NF-κB, the level of p65 subunit of NF-κB protein in the nuclear extracts of A549 cells was measured after the cells were stimulated with TNF-α, in presence and absence of peptide by Western blotting (Figure 4C.2). Beta actin, a constitutively expressed cellular protein was used as the positive control for the peptide 2 at a concentration of 25 µM, 50 µM and 100 µM significantly (45%, 46% and 55% inhibition respectively) inhibited the nuclear translocation of NF-κB. The suppression of NF-κB was found to be statistically significant (p≤0.05).
Figure 4C.2. Western blot analysis of nuclear lysate of TNF-α mediated NF-κB translocation and inhibition by peptide 2 using A549 cells at 3 different concentrations of 25, 50 and 100μM concentrations. The same concentrations of peptides when used alone did not show any cytotoxic effects or other effects as well. Beta actin was observed to be expressed equally in all the wells, as a constitutively expressed protein.

4C.2.3. Electrophoretic mobility shift assay (EMSA) confirms the inhibition of TNF-α mediated activation of NF-κB by peptide 2.

The significant suppression of NF-κB activation by the peptides was further confirmed by the electrophoretic mobility shift assay (Figure 4C.3.A). The addition of excess unlabeled consensus oligonucleotide completely prevented the band shifts demonstrating the specificity of the protein-DNA interaction.
Figure 4C.3. (A) Gel shift analysis of nuclear extracts was performed using the consensus sequence of NF-κB. Each lane contained 20 μg of nuclear extract of the unstimulated, TNF-α stimulated A549 cells in the presence and absence of peptides, for 40 minutes. (B) Densitometric analysis of the gel shift bands. (Number of observations, n=3). * p<0.05, **p<0.01

4C.2.4. Immunocytochemical analysis of the nuclear translocation of NF-κB in A549 cells

Translocation of NF-κB into the nucleus of A549 was examined using the immunocytochemical analysis of the p65 subunit (Figure 4C.4). Fluorescent green nuclei (a) were observed in TNF-α stimulated cells as opposed to the unstimulated cells. Propidium iodide (PI) was used to locate the nuclei of the cells invariably of their treatment protocols, which stained the nuclei orange (b). Figure 4C.4 (c) shows the merged figure of both the fluorescence to show the exact positions of the nuclei with respect to the whole cell. Suramin, a well established TNF inhibitor was used along
with the peptides, which showed similar inhibition of NF-κB nuclear translocation. These results were consistent with the EMSA as well as MTT assay results.

Figure 4C.4. Effect of the anti-TNF peptides on the TNF-mediated nuclear translocation of NF-κB in the A549 cell line challenged with TNF-α (100 ng/ml) for 40 minutes using immunocytochemistry technique.

4C.3. Discussion

Rheumatoid arthritis is a T-cell-mediated disease. However, independent studies by Duff (Di Giovine et al., 1988), Feldmann (Buchan et al., 1988) and Saxne (Saxne et al., 1988) demonstrated that pro-inflammatory cytokines were present in the synovium and plasma of patients with RA. Since these key findings were published, there have been multiple attempts to develop inhibitors of TNF-α activity and three protein based drugs have made it to the market: etanercept (Enbrel; Amgen/Wyeth), infliximab (Remicade; Centocor/Schering-Plough/Tanabe Sieyaku) and adalimumab (Humira; Abbott) have been approved by the US FDA (Taylor P C, 2003).

An FDA advisory panel recently discussed issues concerning the use of injectable protein-based TNF-α inhibitors that must also be addressed with the second-generation,
small-molecule oral inhibitors. The most prevalent concern has been an increased incidence of re-exacerbation of latent tuberculosis. Tuberculosis testing has been recommended for patients commencing anti-TNF-α therapies. Studies with infliximab and etanercept have also shown a potential risk for worsening congestive heart failure and specific warnings are now placed on the FDA labels for these products. In addition, a potentially increased incidence of lymphoma has been observed in patients treated with the injectable TNF-α blocking agent, including the recently approved fully human antibody adalimumab. Recent data indicate that RA is associated with increased risk for lymphoma and this risk is thought to increase with disease severity (Baecklund et al., 1998; Baecklund et al., 2003; Ekstrom et al., 2003). However, the contribution of TNF-α inhibitors to lymphoma risk remains to be determined.

One of the approaches of TNF-α inhibition is the use of peptide antagonists against it. Throughout the body, peptides are active regulators and information brokers with skill sets that make them interesting for drug discovery. Important virtues of peptide drugs are the chemical and biological diversity alongside high specificity, affinity, and molecular recognition. There are many advantages of peptides over the conventional drugs such as high activity, higher specificity, little unspecific binding to molecular structures other than the desired target, minimal drug-drug interaction, less accumulation in the tissues and most importantly much lower toxicity.

There are a large number of small-molecule agents that are in various stages of preclinical and clinical development that inhibit the synthesis of TNF-α. Some of the initial small molecule inhibitors that have been developed are, Thalidomide (Thaledomide, Calgene), p38 Map kinase inhibitors and TACE inhibitors. Although these inhibitors have proven to be quite successful and promising as yet, because of their property of simultaneously inhibiting IFNγ, IL-12, etc., but this property may be dangerous also, as this may immunocompromise the individual (Palladino et al., 2003).
Different peptide inhibitors are also reported against various proteins, which have an importance in the progression of many diseases. Like RDP1258 and its D-isomer, Allotrap 1258, showed enhanced efficacy in the inhibition of cytotoxicity in human and mouse T lymphocytes (Iyer et al., 2000); functional peptides that inhibit IkB degradation leading to transcriptional inactivation of NF-κB (Swaroop et al., 2001) and WP9QY a peptide that mimics a TNFR ligand contact site blocks bone resorption by interfering with recruitment and activation of osteoclasts by RANKL (Aoki et al., 2006). But all these peptides show a very high and sometimes non-specific inhibition. This is the main reason for the side effects of many known inhibitors.

In the present study, the objective was also to search for some small peptide inhibitors against TNF-α. It is well known that stimulated macrophage produce membrane bound 27kd TNF-α, first trimerizes and then can either bind directly to TNFR-55 and TNFR-75 receptors through cell-to-cell contact or undergo cleavage by TNF-α converting enzyme (TACE) and bind in its soluble form. Thus trimerization is the most important step for action of TNF-α and was targeted for designing of peptide inhibitor. The purified TNF-α was tested for its activity on Wehi-164 cells. With the increasing concentration of the peptide, there was a decrease in the TNF-α activity, with maximum effect at 200 μM peptide concentration ($p \leq 0.05$). No cytotoxicity was observed in the cells incubated with the peptides only. There was an increase in cell viability, on administration of TNF-α only i.e, in absence of actinomycin D. It happens so because; TNF-α also induces activation of NF-κB, which is found to inhibit apoptosis (Beg and Baltimore, 1996; Wang et al., 1996). It was reported by Antwerp et al., that on the loss of NF-κB inducibility, there is a pronounced decrease in cell viability. TNF-α mediated nuclear translocation of p65 subunit of NF-κB protein was also checked by immunoblotting of the nuclear extracts of A549 cells, incubated with TNF-α, with or without peptides. The present study demonstrated that the peptide prevented activation of p65/NF-κB by TNF-α and effectively inhibited
nuclear translocation of p65. Hence in the present study, the peptides show significant inhibition of the activity of TNF-α.