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2920 *Current Medicinal Chemistry,* **2015,** *22,* **2920-2942**

Small Molecules as Anti-TNF Drugs

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Abstract: Tumor necrosis factor (TNF, TNF-α, cachectin) is a pleiotropic, proinflammatory cytokine with multiple biological effects, many of which are not yet fully understood. Although TNF was initially described as an anti-tumor agent more than three decades ago, current knowledge places it central to immune system homeostasis. TNF plays a critical role in host defense against infection, as well as an inhibitory role in autoimmune disease. However, TNF overproduction generates deleterious effects by inducing the transcription of genes involved in acute and chronic inflammatory responses including asthma, rheumatoid arthritis, Crohn´s disease, and psoriasis. Direct inhibition of TNF by biologics, such as monoclonal antibodies and circulating TNF receptor constructs, has produced effective treatments for these disorders and validated the inhibition of this proinflammatory cytokine as an effective therapy. Unfortunately, these biological therapies suffer from several drawbacks, including high cost and the induction of autoantibody production. Thus, the development of small molecules able to modulate TNF production or signaling pathways remains a central challenge in Medicinal Chemistry. Considerable efforts have been made over the past two decades to develop such inhibitors, which could potentially be administered orally and would presumably be cheaper. This review is focused on the recent development of compounds that modulate the activity of this cytokine by acting at different levels, such as TNF expression, processing, binding to its receptors and direct inhibition. These approaches will be compared and discussed.

Keywords: Cytokines, inflammation, protein-protein interactions, TNF, TNF inhibitors, TNF-TNFR interaction.

1. INTRODUCTION

In 1891 William Coley, a surgeon from New York, injected isolates of streptococcus into a patient with inoperable cancer with the hope that the infection he produced would have the side effect of shrinking the malignant tumor [1]. His successful treatment was one of the first examples of immunotherapy. But the idea of the tumor-necrotizing activity of the injection was abandoned until 1975, when an ‘‘endotoxin-induced substance’’ was isolated from the serum of mice infected with bacillus Calmette-Guerin (BCG) and treated with endotoxin. This substance was proven to be secreted by macrophages and showed an *in vitro* toxic effect on neoplastic cell lines. Thus, it was named Tumor Necrosis Factor (TNF-α or TNF, Fig. **1**) [2]. Due to its jelly roll-like structure, which is commonly found in viral coat proteins, it is hypothesized that TNF and viruses may have originated from a common ancestor cell [3].

After TNF had been cloned and characterized in 1984 [4], its central role in immune response regulation was widely studied [5]. TNF also plays a primary role in normal physiology and pathology, operating in several networks and regulating cellular function in an autocrine and paracrine manner. Although macrophages are the main producers of TNF, it is secreted by a number of other cell types, depending on the stimulus. TNF expression is tightly controlled by positive and negative feedback loops that include TNF-induced IL-1, IFN-γ and IL-2 production, which, in turn, are regulated by the production of TNF. An archetypal negative feedback loop is the TNF-induced production

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of the anti-inflammatory cytokine IL-10, which then inhibits TNF production.

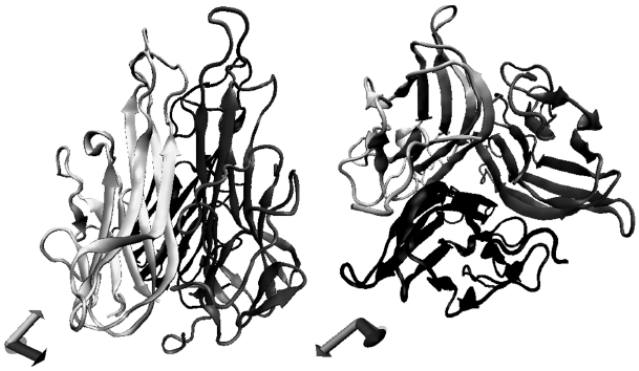


Fig. (1). The figure shows the homotrimeric arrangement that TNF forms. Left: side view. Right: Top view (Structure from Protein Data Bank (PDB) entry: 1TNF [21].

This regulation is essential because TNF is responsible for maintaining the host-defense against infections, including bacteria and viruses. However, dysregulation of TNF causes excessive inflammation and tissue injury. Systemic overproduction of TNF activates inflammatory responses to infection and injury and mediates hypotension, diffuse coagulation, and widespread tissue damage, and can even lead to septic shock. Thus, blocking TNF action has been considered an attractive therapeutic approach.

Several attempts have been made to develop therapeutic agents able to modulate the production of this cytokine at different levels, such as TNF expression, processing, binding to its receptors or direct inhibition. Direct inhibition of TNF by biologics, such as monoclonal antibodies and circulating TNF receptor constructs, has produced effective treatments for these disorders and validated the inhibition of this proinflammatory cytokine as an effective therapy. Unfortunately, these biological therapies are accompanied by several drawbacks, including high cost and induction of autoantibody production. This review focuses on the search for drug-like, small molecule TNF inhibitors over the last decade, emphasizing direct inhibitors that could replace the current therapies based on biologics.

2. BIOLOGY OF TUMOR NECROSIS FACTOR

2.1. TNF and Its Receptors

Tumor necrosis factor alpha is mainly produced by activated macrophages and natural killer (NK) cells. Low TNF expression is also observed in a variety of other cells, including fibroblasts, smooth muscle cells and tumor cells [6].

The immature protein (transmembrane pro-TNF or tTNF) has a molecular mass of 26 kDa and is proteolytically cleaved, mainly by the metalloprotease TNF converting enzyme (TACE or ADAM17), to a 17-kDa active unit [7]. Other proteases, such as ADAM10 [8], MMP7 [9] and MMP13 [10], have been shown to cut pro-TNF and generate soluble TNF (sTNF). sTNF is a homotrimer with a molecular mass of 52 kDa [11] (Fig. **1**). The trimeric structure of TNF is essential for its biologic activity, because mutations that destabilize the trimer or attenuate monomer association result in the loss of TNF biologic activity [5].

Although they originate from the same molecule, sTNF and tTNF seem to exert different biologic actions. Both are biologically active, but have distinctive roles and act through two different receptors. The membrane-bound form of TNF mainly binds to TNFR2, while the released, soluble form binds to TNFR1. Although sTNF affinity for TNFR2 is five times higher than for TNFR1 [12], the later initiates the majority of TNF's biologic activities. TNFR1 (p55/p60, CD120a) is expressed in virtually all cell types (except for erythrocytes), while TNFR2 (p75/p80 or CD120b) expression is principally restricted to immune cells [3, 13]. The main difference between these receptors is the death domain (DD), which is present in TNFR1 and absent in TNFR2. Because of the presence of DD, TNFR1 is capable of inducing apoptosis [11].

2.2. The Role of TNF in Physiology and Pathogenia

TNF plays an important role in inflammatory disorders of both inflammatory and non-inflammatory origin [13]. This acute phase protein initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophages and neutrophils to the site of infection. TNF binding to TNFR1 leads to the activation of transcription factors involved in cell survival and inflammatory responses [3, 14, 15]. This interaction also initiates caspases activation *via* the intermediate membrane proteins TRADD (TNF receptor-associated death domain) and the Fas-associated death domain protein (FADD), which eventually leads to apoptosis (Fig. **2**) [16].

TNF homotrimer is able to bind to TNF receptors. This binding causes conformational changes in the receptor leading to the dissociation of an inhibitory, silencer of death domain (SODD) protein from the intracellular death domain. This dissociation enables the TRADD protein to bind with the DD. TNF receptor-associated death domain, a 34-kDa protein, binds to the DD of TNFR1 and recruits adaptor proteins like RIP

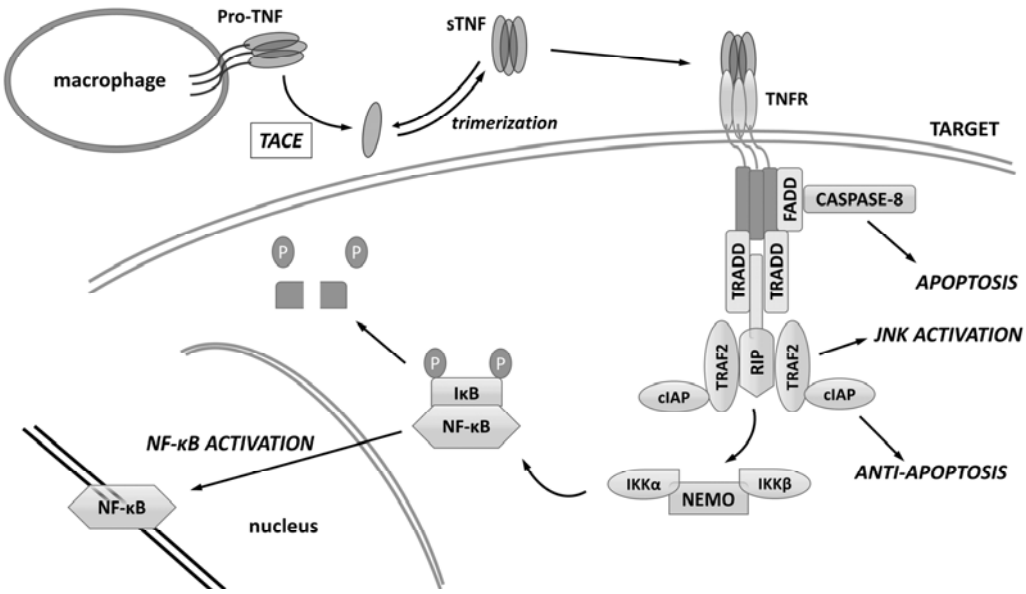


Fig. (2). Receptor binding and actions of tumor necrosis factor (TNF). Binding of TNF to TNFR1 triggers recruitment of many adaptor proteins, such as TRADD, TRAF2, cIAP and RIP. These adaptor proteins in turn recruit additional key pathway enzymes, for example caspase-8, and IKKβ, which become activated and finally could lead to apoptosis, NF-κB activation and JNK activation. Adapted from Ref [22].

(receptor interacting protein), TRAF-2 (TNFR associated factor 2) and FADD (another TRADD family member). These adaptor proteins recruit key molecules that are responsible for the activation of the following three intracellular signaling pathways: NF-κB, MAPK pathways, and induction of death signaling [3, 13, 16, 17].

NF-κB is a major survival factor that prevents TNF-induced apoptosis. The inhibitory protein IκBα, which normally binds to NF-κB and inhibits its translocation, is phosphorylated by IKK (IκB kinase) and subsequently degraded, releasing NF-κB. NF-κB is a heterodimeric (p50-p65/RelA) transcription factor that translocates into the nucleus and mediates the transcription of a vast array of proteins involved in cell survival, proliferation, inflammatory response and anti-apoptotic factors.

Among the three major mitogen-activated protein kinase (MAPK) cascades, TNF induces activation of the stress-related JNK (c-Jun N-terminal kinase) group and p38 MAPK. JNK minimally activates classical ERKs (extracellular signal regulated kinases), and then translocates into the nucleus, where it primarily targets c-Jun and c-Fos to activate the transcription factor AP-1. Finally, p38 translocates into the nucleus and activates ATF2. In molecular biology ATF-2 is a class of AP-1 transcription factor dimer. These MAPK pathways are involved in cytokine production, cell differentiation and proliferation, and are generally proapoptotic.

Like all death domain-containing members of TNFR super family, TNFR1 is involved in death signaling. TNF-induced cell death plays an important role in inflammatory process. TRADD binds FADD and recruits the cysteine protease caspase-8. At high concentrations caspase-8 induces autoproteolysis and the activation of other caspases like caspase 3, 6 and 7. All of these caspases lead to cell apoptosis.

2.3. Current TNF Inhibitors

Owing to the relation between TNF and inflammation or tissue destruction, the abnormal production of TNF plays a fundamental role in the pathophysiology of several human diseases, particularly autoimmune diseases. Therefore, research has been focused on inhibiting the effects of TNF in diseases like rheumatoid arthritis (RA), Crohn's disease and bacterial septic shock (caused by certain gram negative bacteria), as well as the prevention of alloreactivity and graft rejection. Furthermore, low levels of this cytokine may aid in homeostatic maintenance by regulating body's circadian rhythm [18], and may also promote remodeling or replacement of injured and senescent tissue by stimulating fibroblast growth [19].

Hence, development of TNF inhibitors (TNFi) and their introduction to the clinic was a milestone in the treatment of certain autoimmune diseases. TNFi was the first class of biological agents approved for the treatment of RA [20].

The currently available TNF blockers, the four anti-TNF specific monoclonal antibodies (infliximab, adalimumab, golimumab, certolizumab) and soluble TNFR2 (etanercept), work by neutralizing the activity of soluble TNF and preventing it from binding to TNFR1/TNFR2. Monoclonal antibodies, but perhaps not etanercept, also work by inducing complement-dependent cytotoxicity and antibody-dependent cytotoxicity by directly binding to transmembrane TNF. All of these drugs are given subcutaneously every 1 to 4 weeks, with the exception of the intravenous infliximab.

Anti-TNF monoclonal antibodies and etanercept are equally effective treatments for rheumatoid arthritis, juvenile chronic arthritis, ankylosing spondylitis, psoriatic arthritis and psoriasis. But only the monoclonal antibodies are efficacious in inflammatory bowel disease, and perhaps in uveitis [23].

The five approved TNFi reached annual sales of over 16 billion US dollars in 2008. The three TNFi, etanercept, infliximab and adalimumab, were the top selling drugs of any class in 2012. In the majority of cases, indications for adult patients include failure to respond to classic anti-inflammatory drugs, predominantly methotrexate (MTX). The efficacy of TNFi is less pronounced in treatment-naive patients, a percentage of which respond adequately to standard, nonbiologic therapy. For treatment-naive patients, TNFi might be an option when standard, nonbiologic treatment is not effective, or contraindicated for any reason [20].

Although already approved for various indications, clinical trials of new TNFi applications are ongoing. Moreover, many indications that are currently off-label, have good prospects for approval due to increasing data adverting to potential TNFi efficacy [20].

However, marketed TNF blockers have several limitations. While about one third of patients refractory to nonbiologic treatments do not respond to current TNF blockers, less than half of the responders achieve complete disease remission [24]. In addition, safety considerations include autoimmune diseases, such as diabetes type 1, systemic lupus erythematosus, psoriasis and vasculitis; several demyelinating diseases and neurologic events, including exacerbations of preexisting multiple sclerosis, congestive heart failure and malignancies; although there is thus far no firm evidence that these drugs are associated with an increased incidence of solid tumors. Current data suggest a higher rate of lymphomas in patients receiving TNF blockers relative to the general population [23, 24]. Also, several infections may arise in some patients: tuberculosis has been the most common serious infection observed when using biologic TNF antagonists [23, 25]. Overall, the relative risk of serious pulmonary and skin infection is more than double in TNFi-treated patients compared to controls [26]. Chronic HBV infection may be reactivated, and caution is needed in patients with HCV and HIV infection. Moreover, the development of antinuclear antibodies is common, even against double-stranded DNA or cardiolipin [23]. Finally, the yearly cost of marketed anti-TNF drugs ranges between USD 13,000 and 20,000 per patient, limiting many patients' access to anti-TNF therapy [23].

Thus, the development of small molecules able to modulate TNF levels *in vivo* remains a central challenge in Medicinal Chemistry due to the lack of oral bioavailability, the severe immunological responses and opportunistic infections during treatment with biologics -as it is mentioned above- in addition to the challenge in the manufacturing and purification process of the biologics that increase their cost. Even minor changes during their synthesis can cause significant changes in efficacy or immunogenicity compared to the relatively easy classical synthesis of a small molecule drug. For this reason, considerable efforts have been made over the past two decades to develop such inhibitors which could potentially be administered orally, produce fewer undesirable effects compared to biologic therapy and would presumably be easier to obtain and then, cheaper [27, 28]. Roughly, these efforts may be grouped into four different approaches:

- Inhibition of the proteases that cleave immature TNF (tTNF).
- Inhibition of TNF production through action on intracellular signaling pathways.
- Direct inhibition of TNF.
- Inhibition of the TNF - TNFR interaction.

The most relevant results for each strategy that have been published in recent years are outlined below.

3. INHIBITION OF THE PROTEASES THAT CLEAVE IMMATURE TNF

As stated before, at the cellular level TNF is synthesized as a membrane-anchored precursor protein, which is not active as such. Two-thirds of this precursor protein is released by limited proteolysis into extracellular space as an active, soluble protein comprising the C-terminal. This proteolysis is supervised mainly by the metalloprotease TNF-converting enzyme

(TACE or ADAM17). This discovery marked TACE as a target protein that could indirectly modulate TNF production. Much effort has been devoted to the development of such small-molecule TACE inhibitors, which has recently been reviewed in detail [3, 29].

4. INHIBITION OF TNF PRODUCTION THROUGH ACTION ON INTRACELLULAR SIGNALING PATHWAYS

The primary and more traditional approach for discovering new, small and bioactive molecules is the screening of both natural and synthetic compounds searching for, in this case, potentially anti-inflammatory activity. Thus, testing inhibition of cytokine release, including TNF release, has become a major aim of current drug development and an important method for evaluating the bioactivity of new drugs proposed as potential novel therapies to treat inflammation-derived diseases [30-32].

These screenings usually consist in the evaluation of proinflammatory cytokine production after the stimulation of human or murine inflammatory cells (mostly macrophages) with bacterial lipopolysacharyde (LPS) in the presence of the compound or drug of interest [22-34]. This kind of assay is not TNF specific, and the IC_{50} values measured are highly dependent on the experimental details (cell lines, period of incubation, *etc.*). For these reasons, the values reported by different groups should be taken only as indicators, and consequently, no attempts will be made to establish a comparison between them in this review.

These assays can also be extended to other proinflammatory cytokines, such as IL-1β and IL-6, and other inflammation mediators [33, 34, 36, 37, 39-42, 45, 46]. Furthermore, many of the compounds that exhibit inhibitory activity against TNF also inhibit the production of other cytokines.

Natural products represent a promising source of new entities with high chemical and structural diversity and wide array of biological activities. Several natural compounds with anti-TNF activity have been found through the aforementioned screening strategy, namely lanostane triterpenes (*e.g.* compound **1**, Fig. **3**) [37], flavonoids (*e.g.* compounds **2**-**4**, Fig. **3**) [35, 47], drimane-type sesquiterpenoids such as albaconol (compound **5**, Fig. **3**) [41], and the diarylheptanoid, curcumin (compound **6**, Fig. **4**) [48, 49], among others. These molecules tend to show moderate to high inhibitory activity of TNF production, ranging from to 5 μM to 50 μM, often with unknown mechanisms of action. Nevertheless, in some cases a mechanism related to the inhibition of one or more intracellular signaling pathways has been demonstrated. Lanostane triterpenes such as **1**, for example, induce anti-inflammatory heme oxygenase (HO-1) expression *via* the PI3K/AKT-Nrf2 pathway [37], whereas flavonoids have been shown to modulate proinflammatory gene expression through pathways involving mostly NF-κB and MAPK signaling [50]. Instead, compound **5**, a prenylated resorcinol, is a potent inhibitor of LPS-induced TNF (almost total inhibition at 5 μg/mL), IL-1β, IL-6 and NO production in macrophages through the suppression of NF-κB activation and the upregulation of SOCS1 expression [41].

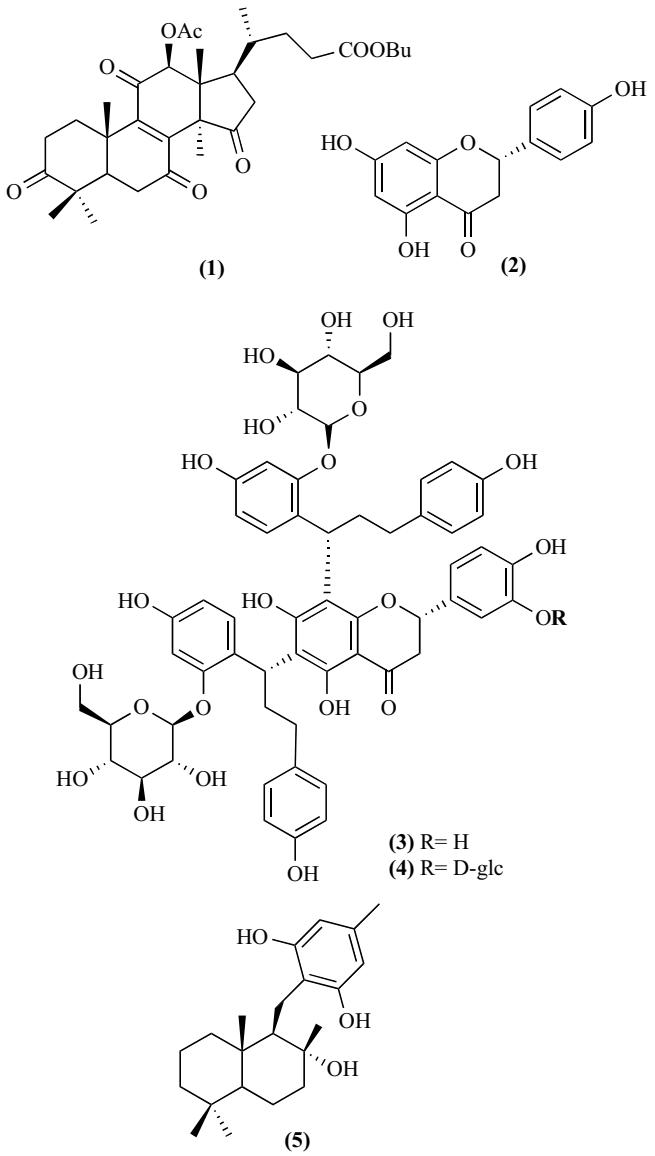


Fig. (3). Examples of the most active lanostane triterpene (**1**), flavonoids (**2**-**4**) and albaconol (**5**), some natural products that inhibit TNF production through the action on intracellular signaling pathways.

Curcumin (compound **6**, Fig. **4**), a component of turmeric (*Curcuma longa*), is probably the most extensively studied anti-TNF natural product.

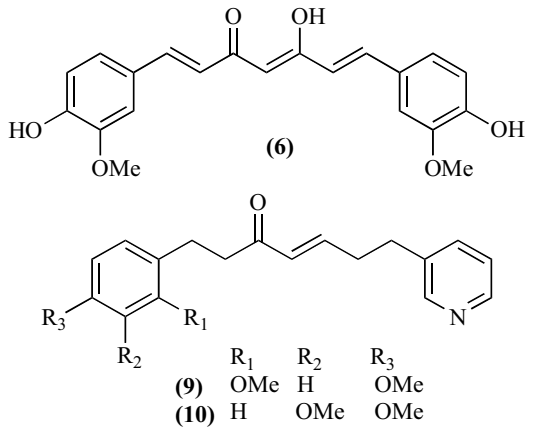


Fig. (4). Curcumin (**6**) (enol form) -the most extensively studied anti-TNF natural product- and two actives diarylheptanoid curcumin derivatives (**9** and **10**).

It has been proved that it blocks the expression of TNF that has been induced by LPS, as well as by a variety of other stimuli, including phorbol ester, palmitate and other inflammatory cytokines. TNF suppression by **6** primarily occurs at the transcriptional level. Compound **6** can mediate its effect on TNF expression by inhibiting p300/CREB-specific histone acetylation, down-modulating the activation of NF-κB, affecting the methylation of a TNF promoter, and inhibiting the expression of TLR2, TLR4 and TLR9 [51-54]. There are also numerous reports suggesting that **6** can not only block the production of TNF but also TNF-mediated cell signaling in a variety of cell types. Compound **6** can inhibit TNF-mediated NF-κB action in a variety of cell types and also down-regulate TNF-mediated expression of various cell surface adhesion molecules in endothelial cells [52, 55]. The *in vivo* anti-inflammatory activity of **6** has been extensively studied [48]. A wide variety of cell signaling pathways activated by TNF are downregulated by compound **6**, including JNK, MAPK, PI3K/Akt [48].

Chemical modifications are frequently made to the structure of natural products to improve biological activity and reduce toxicity. Recent examples are the symmetrical pyrazine-fused derivatives of sinomenine (*e.g.* compound **7**, Fig. **5**) -alkaloid extracted from the Chinese medicinal plant *Sinomenium acutum* used in traditional anti-inflammatory treatments- which have shown improved anti-TNF activity over the natural alkaloid [56]; the 4-styrylcoumarin derivative 6-chloro-2-oxo-4-[-2-(3-methoxyphenyl)ethenyl]-2H-chromene-3-carbonitrile (compound **8**, Fig. **5**) also shows good TNF inhibitory activity [57]; the TNF-inhibitory potential of two synthetic diarylheptanoids (compounds **9** and **10,** Fig. **4**) was comparable to that of curcumin (compound **6**, Fig. **4**) [38]. Other molecules with moderate anti-TNF activity include bergenin derivatives (*e.g.* compounds **11** and **12**, Fig. **5**) [46] and heterocyclic lupeol derivatives (e. g. compounds **13**-**16**, Fig. **6**) [33, 34].

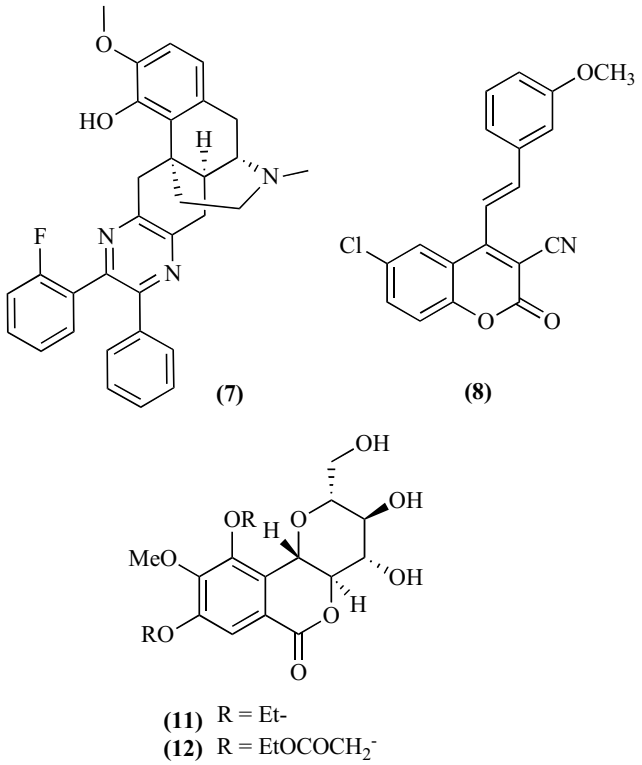


Fig. (5). Some active natural product derivatives.

Synthetic steroids, especially stigmastanes (*e.g.* compound **17**, Fig. **6**) obtained from stigmasterol, have been shown to inhibit TNF production in a dose-dependent manner, as efficiently as, or even more potently than dexamethasone, a commercial anti-inflammatory glucocorticoid [58-60]. Preliminary results indicate that the modulation of TNF secretion by one of these molecules is not associated with NF-κB blockade.

A wide diversity of synthetic compounds display anti-TNF activity. Thalidomide (compound **18**, Fig. **7**) was widely taken by pregnant women as an antiemetic drug for the treatment of morning sickness in the early 60s′ [61]. Despite the withdrawal of compound **18** from the market because of multiple birth defects associated with the use of this drug in early pregnancy, this drug was reintroduced and has undergone clinical

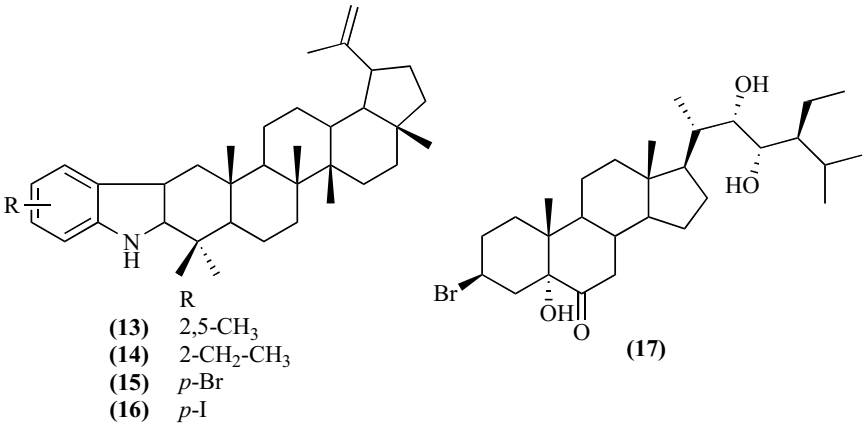


Fig. (6). Examples of the most active lupeol derivatives (**13**-**16**) and stigmasterol derivatives (**17**).

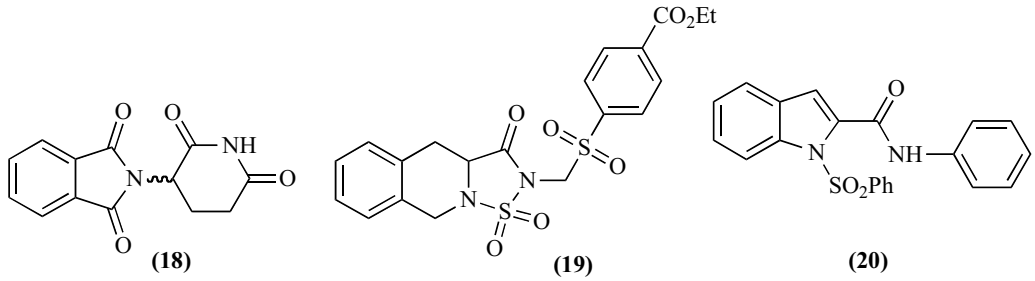


Fig. (7). Thalidomide (**18**) and its active derivatives **19** and **20**.

investigation for various immunomodulatory and anti-inflammatory applications [27, 62, 63]. Compound **18** is known to induce a wide range of immunomodulatory actions. Among thalidomide's diverse activities, it is the downregulation of TNF, mediated by TNF mRNA destabilization at its 3'-untranslated region, which leads to a reduction in the rate of synthesis of TNF protein [63, 64]. Many synthetic thalidomide derivatives such as compounds **19** and **20** (Fig. **7**) have shown moderate to potent anti-TNF activity, some being better than thalidomide itself [36, 43].

Gold compounds are currently used in the treatment of rheumatoid arthritis. Gold complexes, such as **21**-**23** (Fig. **8**) reduce TNF production by inhibiting IκB degradation, thereby blocking the NF-κB signaling pathway [45] Furthermore, gold compounds ameliorate the *in vivo* inflammatory response in the carrageenan-induced hind paw edema model. Kim *et al.* describe a synthetic compound (compound **24**, Fig. **8**) identified during a chemical library screening, that inhibits LPS-induced proinflammatory cytokine production, including TNF, both in human PBMCs and neutrophils, through the inhibition of LPS-induced ERK activity [42]. Other synthetic compounds with moderate anti-TNF activity are [1,2,4]triazolo[4,3-a]quinoxalines (compound **25**, Fig. **8**) and 4-aminopteridines (compounds **26** and **27**, Fig. **8**) [39, 40]. Based on SAR analysis, Ottosen *et al.* and Revesz *et al.* describe a series of benzoylpyridines (compounds **28** and **29**, Fig. **8**), benzophenones (compound **30**, Fig. **8**) and aminobenzophenones (compounds **31**-**35**, Fig. **8**) as potent *in vitro* p38a inhibitors [44, 65]. Benzoylpyridines and benzophenones display potent oral efficacy in rheumatoid arthritis disease models [65], while aminobenzophenones show good anti-inflammatory effects in allergic and irritative contact dermatitis models [44].

In 2007, Gururaja *et al.* [66] reported that after a high throughput screening of a library containing 300000 compounds, they identified a series of triazoloquinoxalines that inhibit TNF-induced, but not IL-1β-induced, intercellular adhesion molecule 1 (ICAM-1) expression in lung epithelial cells. These compounds not only inhibited the TNF-induced NF-κB survival pathway but also blocked death pathway activation, inhibiting the association of TNFR1 with TNFR-associated death domain protein (TRADD) and receptor interacting protein 1 (RIP1), the initial intracellular signaling event following TNF stimulation. Thus, in this novel mechanism of action, receptor internalization is limited, which prevents subsequent cellular responses after TNF binding.

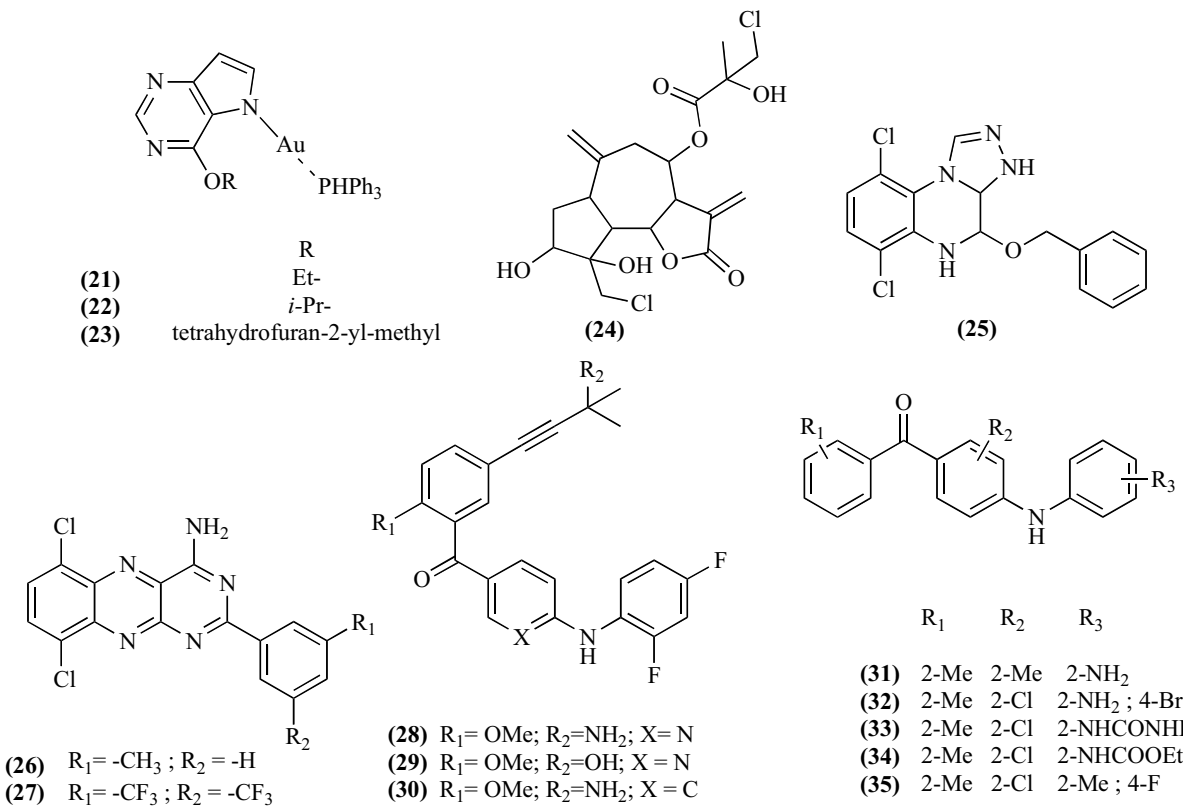
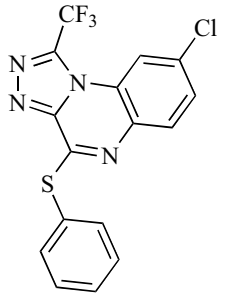


Fig. (8). Some synthetic TNF inhibitors that inhibit TNF production through the action on intracellular signaling pathways.

Furthermore, one of these triazoloquinoxalines, named R-7050 (compound **36**, Fig. **9**), was able to attenuate neurovascular injury and inflammation in a murine spontaneous intracerebral hemorrhage model [67], an outcome probably linked to the aforementioned mechanism.



(36)

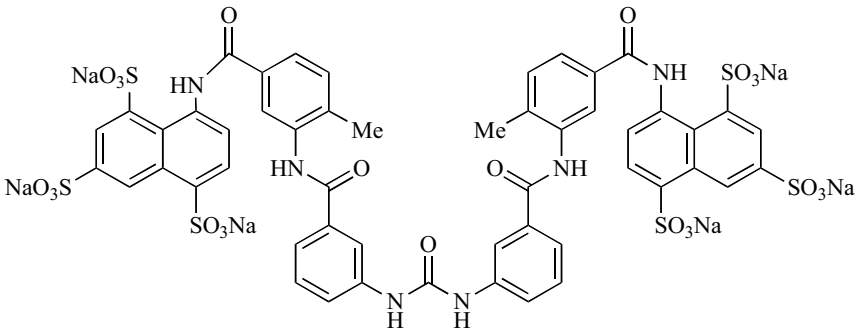
Fig. (9). Compound R-7050 (**36**) selectively inhibits the association of TNFR with intracellular adaptor molecules.

5. DIRECT INHIBITION OF TNF

As mentioned above, sTNF must form a homotrimer before it can bind to its receptors. Trimerization involves protein-protein interactions (PPIs) between the monomeric polypeptide chains. Finding small molecules able to interfere in PPIs represents a central aim in drug development [68, 69]. As protein-protein interfaces are usually flat, large and relatively featureless, for some time they were considered hard to 'drug' using small molecules, which usually prefer well-defined binding pockets. However, even in seemingly featureless protein-protein interfaces most of the binding free energy is contributed by a few residues, also called 'hot spots'. These often involve large amino acids such as tyrosine, arginine and tryptophan that bind in small pockets across the interface and contribute the major part of the binding interaction energy [70].

Some *in silico* studies suggested that protein-protein interfaces are naturally designed to exploit electrostatic and hydrophobic forces in very different ways [71, 72]. Thus, it is possible, in principle, to design small molecules able to interact with these hot spots and disrupt specific PPIs.

The first report showing that this approach was feasible for the direct inhibition of TNF action comes from a seminal work by Grazioli *et al.*, who found that suramin (compound **37**, Fig. **10**), a synthetic polysulfonated naphthyl urea used in the therapy and prophylaxis of African trypanosomiasis, was able to bind to TNF and inhibit its activity [73]. The rationale behind their study was that **37** had been known to disrupt some



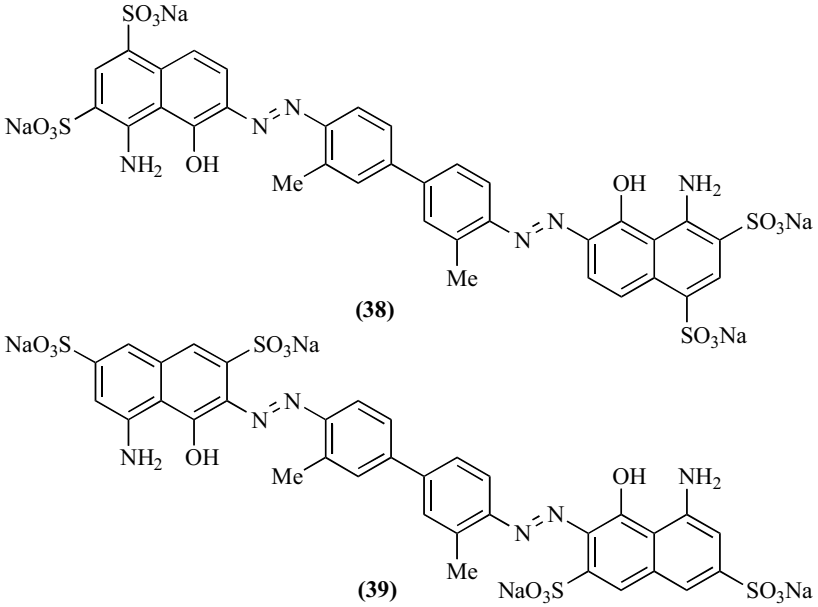
(37)

Fig. (10). Suramin (**37**) inhibits TNF action through the disruption of TNF trimerization.

protein-protein interactions by a nonspecific mechanism. In fact, they observed that compound **37** also inhibits the TNF-TNFR1 interaction, but only if this compound was previously incubated with TNF, suggesting that it might disrupt TNF trimerization. This hypothesis was confirmed in part by gel filtration chromatography [74]. When **37** was pre-incubated with TNF, both trimeric and monomeric TNF were isolated, while the filtration of untreated TNF yielded only the trimeric form. Additional experiments demonstrated that trimer formation is necessary to produce cytotoxic effects on murine LM strain fibroblasts cells, since compound **37** inhibited the TNF-TNFR interaction with a IC_{50} estimated to be between 100 and 200 μM [73].

In order to gain deeper insight into this mechanism, computational studies were performed by Mancini *et al.* to find the most likely conformation for the compound **37**/TNF complex [75]. Docking models suggested that **37** extends along the threefold axis of the TNF trimer, with one of the naphthalene rings reaching the core region, thus interacting with Tyr59, Tyr119, and Tyr151. Besides that, two of the sulfonic groups interact on the surface of TNF with Lys112 C, Arg103B, and Ser71B side chains; whereas the sulfonic groups docked inside TNF interact with the Lys98B and Tyr119C side chains. Furthermore, the symmetric nature and length of **37** might play an important role in the manner that it binds. These authors suggest that the TNF-suramin (**37**) complex may represent an intermediate step in the overall mechanism of compound **37** action leading to the dissociation of the TNF trimer.

With these results in hand, other polysulfonated compounds, such as Evans blue (compound **38**, Fig. **11**) and trypan blue (compound **39**, Fig. **11**), were



(38)

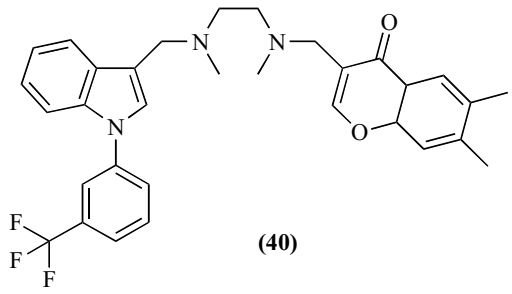
(39)

Fig. (11). Active polysulfonated compounds Evans blue (**38**) and trypan blue (**39**) share a similar binding mode to TNF-α with compound **37**.

highlighted as candidates likely to bind TNF in a similar way [75]. The capacity of these molecules to inhibit the binding of TNF with its receptor was tested *in vitro* by means of a specific immunoenzymatic assay. Compounds **38** and **39** inhibited TNF-TNFR binding with IC_{50} values of 0.75 mM and 1.00 mM, respectively, compared to suramin's IC_{50} of 0.65 mM [75] (compound **37**, Fig. **10**).

Although these polysulfonated compounds show relatively high IC_{50}, they may be of limited therapeutic interest. However, these studies on suramin (**37**) paved the way for the development of many more recent inhibitors based on the deoligomerization of TNF.

An important step forward was taken by He *et al.* in 2005, when a small molecule named SPD304 (compound **40**, Fig. **12**) was reported to inhibit the TNF-TNFR1 interaction as suramin did (compound **37**, Fig. **10**), but with a lower IC_{50} (22 μM). This led to inhibition of the TNF-promoted stimulation of NF-κB degradation in HeLa cells [76].



(40)

Fig. (12). Compound SPD304 (**40**) inhibits the TNF-TNFR1 interaction in analogously but more effectively way than **37**.

Co-crystallization showed that the compound promotes the displacement of a subunit from the TNF trimer and binds to the resulting dimer (Fig. **13**). Compound **40** has a trifluoromethylphenyl indole moiety linked to a dimethyl chromone by a dimethylamine spacer. It adopts a folded conformation when bound to the TNF dimer, establishing sixteen different contacts, including Tyr59 and Tyr119, which are known to be crucial for the stability of the TNF trimer. Interestingly, most of the contacts shown in the X-ray structure of the complex seem to be hydrophobic, with no hydrogen bonds or electrostatic interactions. Furthermore, binding of compound **40** induces a conformational change that widens the angle between the two subunits, which likely promotes the dissociation of the third unit of the original TNF trimer. Additional mass spectrometry experiments also confirmed this dissociation mechanism.

The presence of the 3-substituted indole moiety in **40** renders it potentially bioactivable by cytochrome P450s, with the capacity to form toxic electrophilic intermediates, a drawback for possible clinical applications. This might explain why no further studies with this compound have been reported. Nevertheless, the validation of compound **40** mode of action, which spurred the search for molecules that bind with the lower oligomeric states of TNF to prevent formation of the bioactive trimer, inspired others in their research.

While studying the antiproliferative activity of a series of novel biscoumarin derivatives on hepatocellular carcinoma cells, Keerthy *et al.* [77] predicted that these compounds bind to the TNF dimer *via* hydrophobic contacts, similar to SPD304 (compound **40**), which was further confirmed with molecular dynamics. Due to the lack of distinctive anchor points, no further attempts were made to rationalize binding affinities and particular ligand-protein interactions in detail. Interestingly, the most active compound, named BIHC (compound **41**, Fig. **14**), had the lowest

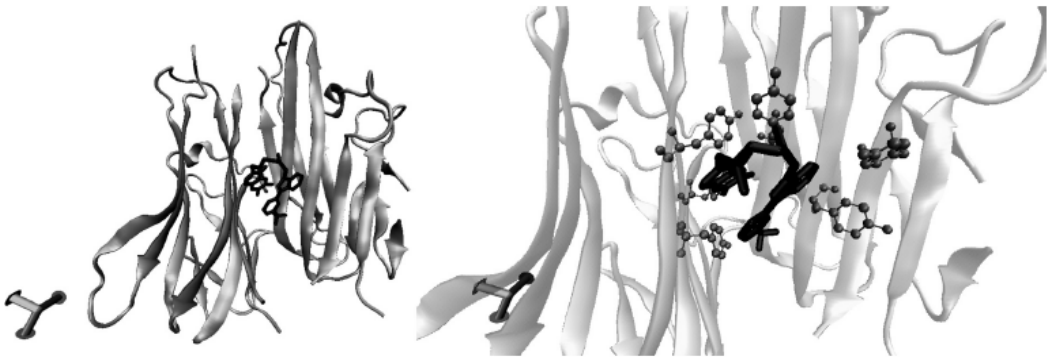
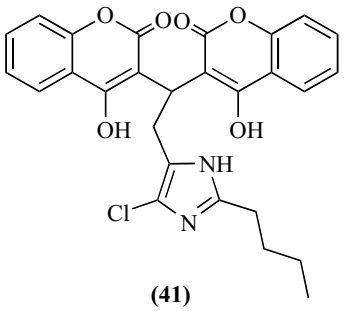


Fig. (13). Conformation adopted by compound **40** (left) that allows it to interact with TYR59 and TYR119, crucial residues for the stability of the TNF trimer. These interactions (hydrophobic) are shown in the right. (Structure from Protein Data Bank (PDB) entry: 2AZ5 [76]).

strain energy of the series, suggesting that conformational aspects could be essential for small molecule binding to TNF.



(41)

Fig. (14). Structure of BIHC (**41**) significantly reduces the local production of TNF by direct binding to the TNF dimer.

The *in silico* result, *i.e.* the binding between **41** and TNF, was validated experimentally by two methods [77]: firstly, compound **41** was incubated *in vitro* with recombinant TNF and its inhibitory effect was quantified using an anti-TNF antibody. It was found that this compound inhibited this binding in a dose-dependent manner between 5 to 40 μM, showing an IC_{50} value of 16.5 μM.

Then, the interaction between compound **41** and TNF was analyzed *via* surface plasmon resonance (SPR). The N-terminal of TNF was immobilized on a streptavidin coated sensor chip. The injection of different concentrations of compound **41** onto the surface of the sensor provided data for the estimation of the dissociation equilibrium constant (K_d), which was estimated to be 21.4 μM, confirming **41** affinity towards TNF.

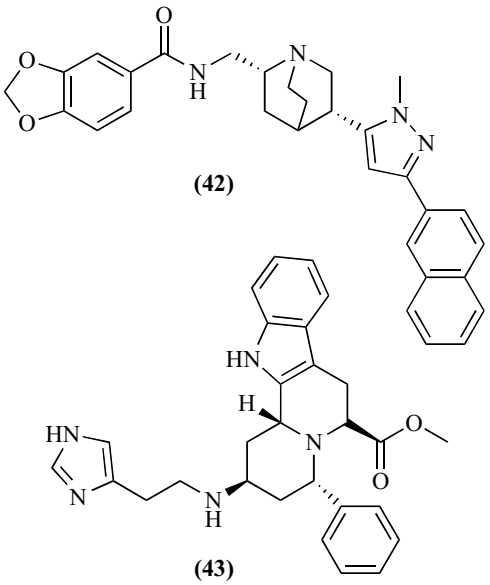
To corroborate the hypothesis that TNF is compound **41** target, these authors proved that **41**/TNF binding leads to the direct inhibition of several TNF-mediated biological effects [77]. For example, upon **41** application to HCC cells, the compound downregulates the expression of the cell cycle regulator cyclin D1 and the anti-apoptotic proteins Mcl-1, survivin, and Bcl-2 D1 in a time-dependent manner. Compound **41** also inhibits the phosphorylation and translocation of p65 NFκB.

The most interesting results came from the *in vivo* studies: **41** significantly reduces the local production of TNF in the peritoneal cavities of thioglycollate broth-treated mice. Upon administration of LPS, following compound **41** treatment, TNF release was reduced from 0.42 ng/mL (control) to 0.26 ng/mL (38% of inhibition at 0.5 mg/kg of **41**), 0.22 ng/mL (46% of inhibition at 1.5 mg/kg of **41**) and 0.19 ng/mL (55% of inhibition at 5 mg/kg of **41**). Heparin, the positive control, lowered TNF release from 0.42 to 0.12 ng/mL, corresponding to a 70% reduction at 10 mg/kg dose [77]. These results also support the hypothesis of direct binding between compound **41** and TNF.

Finally, **41** efficacy as a TNF inhibitor was validated in a murine inflammatory bowel disease model [77]. To date, this work represents the most comprehensive proof-of-concept that small molecule disruption of the TNF trimer and subsequent TNF inhibition can lead to an effective anti-inflammatory drug, at least in an animal model.

Another possible approach is based on the virtual screening of compound libraries for molecules of both natural and synthetic origin able to form complexes with the dimeric form of TNF.

Following this strategy, Chan *et al.* conducted a high-throughput virtual screening based on the TNF dimer-SPD304 (**40**) structure. From a natural product (or natural product-like) compound library containing 20,000 compounds, they selected sixteen compounds that mimic compound **40** binding [78]. Among them, compounds **42** and **43** emerged as the most promising candidates by means of an ELISA test. Their IC_{50} against the TNF-TNFR1 interaction were 50 and 10 μM, respectively.



(42)

(43)

Fig. (15). Structures of compounds **42** and **43**-selected compounds from a natural product library by virtual screening-inhibits the TNF-TNFR1 interaction.

Docking shows that the natural product-like pyrazole **42** binds to the TNF dimer with the quinuclidine core occupying a hydrophobic pocket, whereas dioxo-

lane oxygen forms a hydrogen bond with the backbone nitrogen of Gly121B. There are some additional hydrophobic contacts between the ring system and Leu120A, Gly121A and Lys122A. On the other hand, the indoloquinolizidine moiety in compound **43** forms a hydrogen bond with Tyr151B through the imidizole and makes hydrophobic contacts with Leu120A, Gly121A and Gly122A [78].

To validate the inhibitory action of both compounds, this group investigated whether these compounds were able to inhibit TNF signaling in human cells [78]. Therefore, TNF-induced NF-κB signaling in HepG2 cells was assessed and surprisingly compound **43** was less active than **42** (IC_{50} values of approximately >30 and 5 μM, respectively) in this assay.

In a similar work, Choi *et al.* [79] used the same dimer crystal structure as their starting point. They filtered a library containing 460,000 compounds using Lipinski's rules and selected about 260,000 molecules to run a virtual screening. Taking into account the role of the hydrophobic contacts in PPIs, they also modified the scoring functions with an accurate solvation model. They then selected 81 compounds that were further screened for their ability to inhibit TNF activity in a cell-based assay. From this screening, the three most active compounds **44**-**46** (Fig. **16**) (71, 64 and 54% inhibition at 10 μM, respectively) shared a pyrimidine-2,4,6-trione core. A detailed docking study shows that the compounds bind to Tyr59, Tyr119 and Tyr159 through hydrophobic interactions with this common moiety.

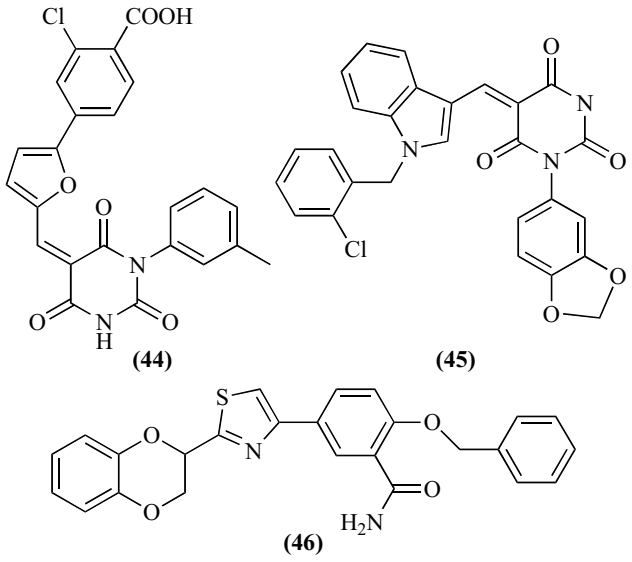


Fig. (16). Structures of compounds **44**-**46**, selected by virtual screening, and then, by their ability to inhibit TNF activity in a cell-based assay.

Additionally, the top scoring molecule **44** also interacts by hydrogen bonds between the two oxygen atoms of the terminal carboxylate with Lys98B and the backbone nitrogen of Tyr119B, suggesting that its higher affinity for the TNF dimer might account for its higher activity. Unfortunately, the authors did not assess the IC_{50} values, which prevents comparison with compounds found by other researchers.

Taking into account the success of this approach, Leung *et al.* [80] started with the structure of the TNF dimer/SDP304 (**40**) and screened *in silico* 3,000 compounds in a library of marketed drugs that are approved by the US Food and Drug Administration. This screening was based in the concept of "drug repurposing", *i.e.* the reoptimization of existing drugs to evoke their secondary effects. Finding a second use for a known drug could lead to a dramatic reduction in the cost of drug development. Approved drugs have well-established pharmacokinetic and pharmacodynamic profiles, and their safety in human subjects will have already been extensively tested as part of the approval process. Seven compounds were selected in the virtual screening and evaluated in a preliminary ELISA to assess their ability to inhibit the binding of TNF to its receptor. The FDA-approved drugs darifenacin (compound **47**), a drug used in the treatment of overactive bladder [81], and ezetimibe (compound **48**), a potent inhibitor of cholesterol absorption in the intestine [82], emerged as the most active compounds (IC_{50}´s about 100 μM, Fig. **17**).

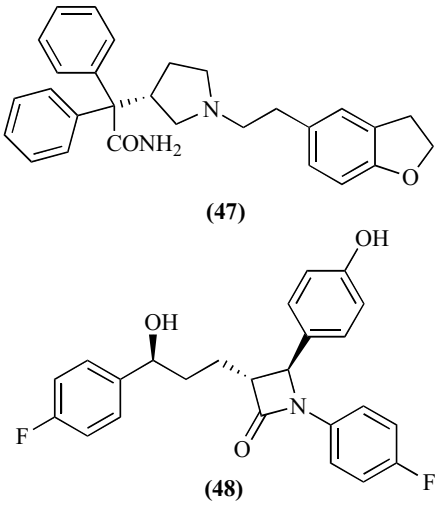


Fig. (17). Darifenacin (**47**) and ezetimibe (**48**), marketed drugs approved by the US Food and Drug Administration that inhibit TNF.

Docking studies showed that both **47** and **48** are large enough to contact both TNF monomers simulta-

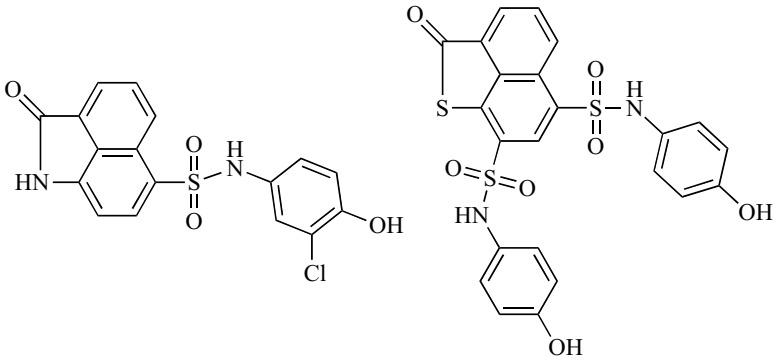
neously, which may prevent the formation of the biologically active TNF trimer complex. The 2,3-dihydrobenzofuran moiety of compound **47** interacts closely with Leu120A, Gly121A and Gly122A, while one of its phenyl rings is in close proximity to the corresponding residues of the other subunit in what are mostly hydrophobic interactions. In addition, a hydrogen-bonding interaction appears between the amide nitrogen of **47** and the side chain hydroxy group of Tyr151. Similar hydrophobic interactions are present in the model for compound **48** which seems to be more closely bound to the TNF subunit A. Compound **48** also forms a hydrogen bond with the side chain of Tyr151 *via* its aliphatic hydroxy group.

Finally, and in order to validate these findings, the authors evaluated the ability of compounds **47** and **48** to inhibit TNF-induced NF-κB signaling in HepG2 human cells. Both compounds were able to inhibit this signaling pathway with IC_{50} values of 35 and 40 μM, respectively. The authors hypothesized that the higher potency observed in the cell-based assay compared to the binding assay might imply that these compounds reduce TNF activity through other mechanisms than inhibition by direct binding with TNF.

Recently, Shen *et al.* [83] used the same strategy; starting with a commercial database containing 200,000 compounds, they performed a virtual screening that selected compounds able to bind to the SPD304 (**40**) binding pocket on the surface of the TNF dimer. In the preliminary screening, each compound was docked to restrict it to the most stable conformation, *i.e.* a rigid conformation. About 6,200 compounds were selected according to their dock score, and a second evaluation allowing multiple conformations, reduced the set to the 101 most promising compounds.

An *in vitro* competitive binding assay based on surface plasmon resonance was developed to test whether the computationally selected compounds could indeed reduce TNF-receptor binding. Initially, all compounds were tested at a final concentration of 100 μM. 10 of the 101 compounds reduced the TNF receptor binding signal. These 10 candidates were selected for a cell-based assay looking for suppression of the NF-κB pathway in HEK293T cells activated with TNF, in which compound **49** (Fig. **18**), an aromatic sulfonamide, showed an IC_{50} of 42.3 μM. Compound **49** was subjected to SAR studies to verify the pharmacophore required for TNF binding. After a search in the SciFinder database, 15 analogs with different substituents were subjected to docking studies and tested by both the SPR competitive assay and cell assay. Among them, compound **50** (Fig. **18**) showed an IC_{50} of 14.0 μM, more potent than that of SPD304 (**40**) (IC_{50} = 20.3 μM).

A detailed docking on compound **49** suggested that the two aromatic rings can form π-π stacking interactions with multiple aromatic side chains on the surface of the TNF dimer pocket. Additionally, the polar groups on the molecule can form hydrogen bonds with the polar residues in the binding pocket. As previously reported by other researchers, the binding pocket on the inner surface of the TNF dimer primarily consists of tyrosine and leucine residues, making hydrophobic interactions the primary means of small molecule binding. Molecular recognition *via* hydrophobic interactions are rather unspecific, thus, the introduction of suitable polar groups may enhance binding specificity. Furthermore, the presence of polar groups will increase water solubility of the compounds. This is the first work that attempted to optimize the properties of a hit compound found by virtual screening. Furthermore, a



(49) **(50)**

Fig. (18). Compound **49** - selected first by virtual screening, second by an *in vitro* competitive binding assay and finally by a cell-based assay looking for suppression of the NF-κB pathway in HEK293T cells activated with TNF and its analog **50**, found by SAR studies.

pharmacophore model was proposed which may be useful for the development of new inhibitors.

In the examples discussed above, it is clear that hydrophobic interactions play a central role in the binding of each compound to the TNF dimer and, therefore, play a similarly key role in the subsequent inhibition of the formation of the biologically active trimeric form. With this rationale, Leung *et al.* decided to test the octahedral iridium complex **51** (Fig. **19**) as a direct inhibitor of TNF [84]. First, molecular modeling analysis of the interaction between the two enantiomers (*i.e.* **51** and **51***) with TNF was performed, using, as usual, the X-ray cocrystal structure of the TNF dimer bound to SPD304 (**40**).

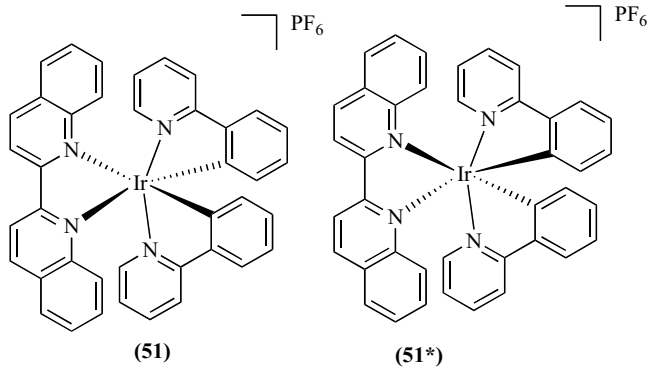


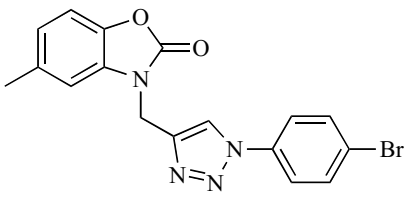
Fig. (19). The active octahedral iridium complexes $[Ir(ppy)_2(biq)]PF_6$ **51** and **51***, its enantiomer, which activities are in the low micromolar range in a cell-based luciferase assay.

The main hypothesis was that the extended aromatic biq ligand (2,2'-biquinoline ligand) could form favorable interactions with the hydrophobic binding interface of TNF. Docking studies showed that the biq ligand of **51** does indeed make contact with the β-strand of TNF's A subunit, while the two ppy moieties interact with the β-strand from subunit B inside the binding pocket. In contrast, compound **51***, the enantiomer, seems to occupy the same binding pocket but with a different binding position. The biq ligand and one of the **51*** ppy ligands (bidentate 2-phenylpyridinato ligand) contacts the β-strands of both TNF dimer subunits. As with the previously described inhibitors, the interaction between the TNF dimer with these iridium complexes seems to be predominantly hydrophobic, with no hydrogen bonding or ionic interactions contributing to the binding.

The IC_{50} against the TNF-TNFR was determined using an ELISA. The authors found values of 20 and 25 μM for complexes **51** and **51*** respectively, potencies comparable with that of SPD304 (**40**) (IC_{50} =ca. 22 μM). Interestingly, the racemic mixture of both complexes was equally potent as the enantiopure complexes. Inhibitory activity was further assessed in a cell based assay, where TNF was preincubated with both **51**, **51*** and the racemate before being applied to HepG2 cells that had been transfected with a luciferase gene activated by the NF-κB signaling pathway. Enantiomers showed comparable activities against TNF-induced NF-κB signaling, with approximate IC_{50} values of 2.5 and 4 μM, respectively, and both were slightly more potent than SPD304 (**40**) (IC_{50} =10 μM). As before, the racemic mixture was as active as the enantiopure complexes.

Due to the non-specific nature of the predicted interactions of these complexes with TNF, it is highly possible that compounds **51** and **51*** were not selective inhibitors. This issue was not addressed by the authors. Nevertheless, their work is an interesting example of the use of organometallic complexes as promising scaffolds for the development of bioactive molecules.

In recent years some research groups have used the SPD304 (**40**) mechanism of action proposed by He *et al.* as a basis for their work on new TNF inhibitors. For example, Haider *et al.* synthesized a small library of 1,2,3-triazole based benzoxazolinones [85] and found that some had significant *in vivo* anti-inflammatory activity on rats in a carrageenan-induced hind paw edema model. With this result in hand, a plausible mechanism of action was sought. When tested *in vitro* at 1 μg/mL (about 2.6 μM) compound **52** (Fig. **20**) had a 51% inhibitory effect on the TNF production by LPS-stimulated macrophages. To explain this finding, a docking model of **52** with the TNF dimer was done. It was observed that the compound **52** is oriented in the binding site in such a way to allow two π-π interactions of the two benzene rings with Tyr59A and Tyr119B. As with other inhibitors, no hydrogen bonding was observed.



(52)

Fig. (20). Compound **52**, an active 1,2,3-triazole based benzoxazolinone that has significant *in vivo* anti-inflammatory activity on rats in a carrageenan-induced hind paw edema model.

Several other works followed a similar experimental scheme to the study highlighted above. Kumar *et al.* described a multicomponent synthesis of a small library of isoindolo[2,1-a]quinazoline derivatives [86] in which they found a TNF-production inhibitor in RAW 264.7 macrophages with an IC_{50} of about 9 μM (compound **53**, Fig. **21**). This interaction between the inhibitor and TNF was supported by molecular docking, which showed strong interactions with the hydrophobic binding site.

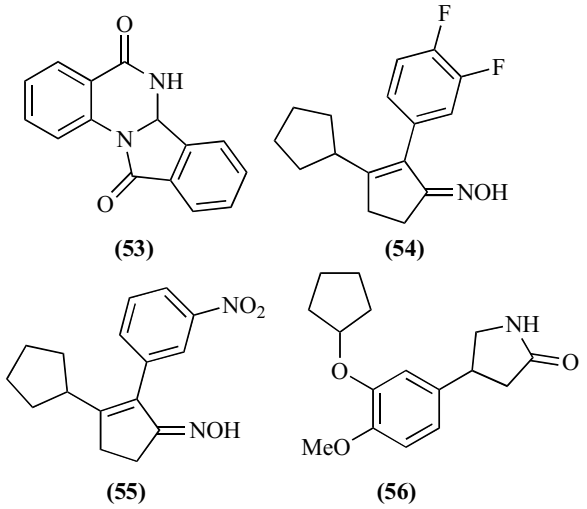


Fig. (21). The active isoindolo[2,1-a]quinazoline derivative **53**, the active oximes **54** and **55** and the positive control rolipram (**56**).

Some cyclopentenone-derived oximes were synthesized and tested by Kim *et al.* [87] for *in vitro* inhibition of TNF production in rat and human peripheral blood mononuclear cells stimulated by LPS. The oximes **54** and **55** (Fig. **21**) with 3,4-difluoro and 3-nitro substituents, respectively, showed the most potent activities, with IC_{50} values of 0.13 and 0.07 μM, both of which are more potent than rolipram (compound 56, Fig. **21**), a drug known to inhibit TNF production (with an IC_{50} of 0.2 μM) [88]. Once more, docking was performed with the TNF dimer. The active oximes made similar hydrophobic contacts to those described for SDP304 (**40**, Fig. **12**), but with an additional hydrogen bond with Tyr151, that, according to the authors, might be responsible of the higher inhibition observed.

Recently, Bhandari *et al.* [33] described the synthesis of several derivatives of oleanolic acid, a naturally occurring pentacyclic triterpene acid that is widely distributed in food and medicinal plants. The authors assessed the *in vitro* anti-inflammatory activity by measuring the inhibitory effect on nitric oxide, IL-1β and TNF production in macrophages (RAW 264.7 and J774A.1 cells). Some compounds (*e.g.* **57** and **58**, Fig. **22**) exhibit mild inhibition (27.9-51.9%) of LPS-induced TNF production when evaluated at 20 μg/mL. Docking analysis was based, as ever, with the TNF dimer/SPD304 (**40**) complex. Compound **57** showed similar interaction to that of SPD304. The carbonyl oxygen of **57** has shown a π-σ* interaction with Tyr119. Surprisingly, other active compounds did not show good docking results with the TNF dimer. The authors suggest that these analogs could be inhibiting cytokine production through another mechanism.

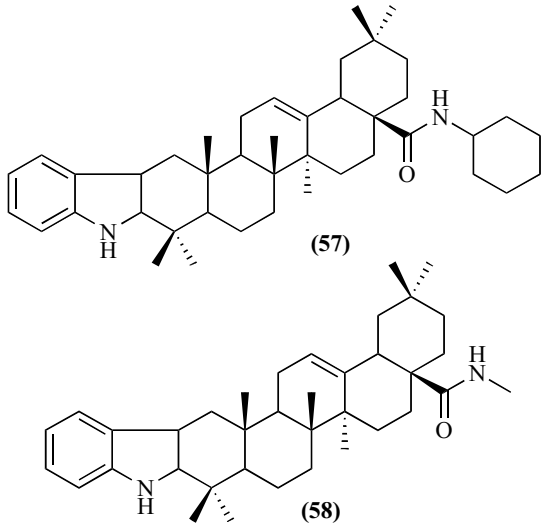


Fig. (22). Active oleanolic acid derivatives **57** and **58** that exhibit 40 and 52% inhibition of LPS-induced TNF production at 20 μg/mL.

This last example clearly indicates that, although the docking studies may help to explain the inhibitory effect observed for some of the inhibitors described, such as compound **41** (Fig. **14**), natural products-like **42** and **43** (Fig. **15**) and the marketed drugs darifenacin (**47**) and ezetimibe (**48**, Fig. **17**) other modes of action cannot be ruled out (*e.g.* modulation of relevant signaling pathways). For this reason, until the appropriate clarifying experiments are performed, they should not be considered inhibitors acting by TNF trimer disruption.

On the other hand, there are some research groups that have reported the discovery of small molecules that were experimentally proven to be direct inhibitors of TNF, although no docking studies were performed to gain deeper insight their mode of action.

For example, Lian *et al.* [89] described the synthesis of a library of bicyclic peptides that were designed to maximize molecule surface area, and therefore, their ability to interact with a flat protein surface. They chose trimesic acid as a rigid, planar, small molecule

scaffold to which a peptide sequence of 6-10 random residues was attached, seeking to bias the resulting bicyclic peptides toward an overall planar (as opposed to globular) shape. The synthesis was performed in solid phase, and a theoretical diversity of about 10^7 compounds was achieved. The library was screened against recombinant TNF labeled with biotin or a fluorescent dye molecule.

The peptides, bound to the resin, were incubated with biotinylated TNF and streptavidin-coated magnetic particles. The magnetic beads (~400 beads) were isolated from the library by magnetic sorting. After some additional screening steps, intended to reduce the occurrence of false positive and negatives, two peptides, anticachexins C1 and C2, were isolated (Compounds **59** and **60**, respectively. Fig. **23**). These compounds were resynthesized with a fluorescein isothiocyanate label to detect TNF binding and assayed against TNF by fluorescent anisotropy analysis. Compound **59** and **60** bound to TNF with K_d values of 0.45 and 1.6 μM, respectively. However, an experiment revealed that compound **60** was not a specific ligand of TNF-α and showed substantial binding to other proteins.

To test whether **59** inhibits the interaction between TNF and TNFR, biotinylated TNF was placed onto microtiter plate and was incubated in the presence of varying concentrations of compound **59**. The amount of TNFR bound to each well was quantified by ELISA, showing that **59** inhibited the TNF−TNFR1 interaction in a concentration-dependent manner, with an IC_{50} value of 3.1 μM.

Finally, compound **59** was shown to protect WEHI-13VAR fibroblasts against TNF-induced cell death *in vitro*, in a concentration-dependent manner. In this assay, TNFα exhibited an LD_{50} value of 0.46 ng/mL in the absence of the TNF inhibitor; in presence of 50 μM of compound **59**, the LD_{50} value shifted to 1.8 ng/mL.

After a phytochemical screening, Hu *et al.* have reported that the dimeric sesquiterpene lactone Japonicone A (compound **61**, Fig. **24**) antagonizes TNF action by directly disrupting the interaction between TNF and its receptor [90]. Using SPR, it was determined that this natural product, isolated from the herb *Inula japonica*, is able to bind to TNF but not to TNFR1 (Kd = 7 μM). Nevertheless, compound **61** inhibited the TNF-TNFR1 interaction when measured by ELISA. Interestingly, TNF-TNFR2 binding was only slightly inhibited, leading to a selective inhibition profile.

If **61** antagonizes TNF function by disrupting TNF-TNFR1 interaction, it should be able to inhibit all of the related signaling pathways. In fact, compound **61** was able to inhibit the TNF-triggered, TNFR1-mediated, proinflammatory NF-κB signaling pathway along with the expression of genes activated also by TNF, such as MCP-1, ICAM-1 and VCAM-1 in bEnd.3 cells. Interestingly, no effect was observed when the NF-κB pathway was activated *via* IL-1, supporting the idea that this compound specifically alters the TNF-stimulated cell signaling. Furthermore, **61** also reduced inflammation and liver damage in a TNF/D-GalN-induced hepatitis model in mice. This is the first report of a small molecule that selectively disrupts the TNF-TNFR1 receptor, which validates this approach.

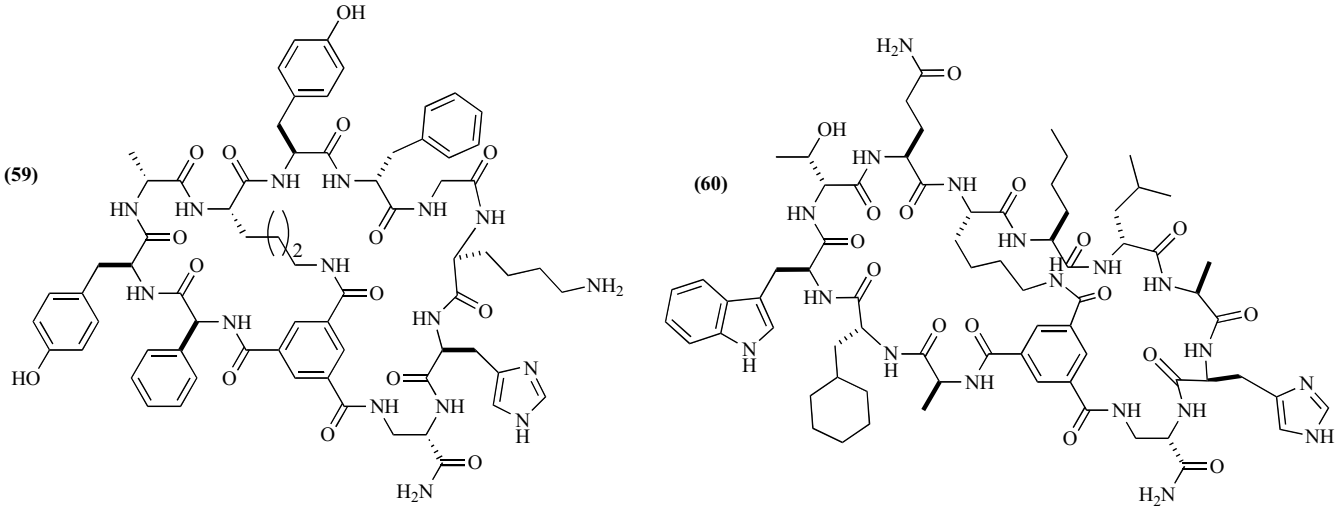
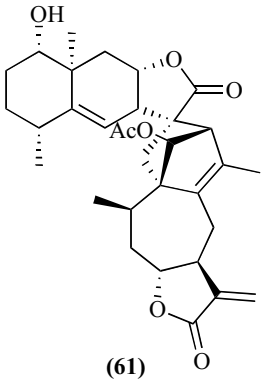


Fig. (23). Active bicyclic peptides **59** and **60**, potent antagonist that inhibits the TNFα−TNFα receptor interaction (*in vitro*). **59**, also, protects cells from TNFα-induced cell death.

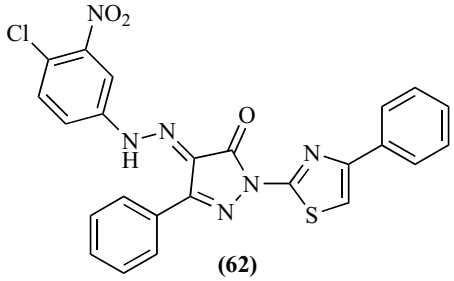


(61)

Fig. (24). The active dimeric sesquiterpene Japonicone A (**61**), a natural compound that directly binds to TNF-α rather than TNFR1 as determined by surface plasmon resonance.

In a recent work, Ma *et al.* [91] discovered a molecule named C87 (compound **62**, Fig. **25**), using a different approach: although the crystal structure of the TNFα-TNFR1 complex has not been described, they chose a seven-amino acid peptide in the loop 2/domain 2 of TNFR1 (amino acids 77-83) that has been identified as a potential key site for TNF-TNFR1 interactions [92]. The space of simulation was restricted to the aforementioned loop in a virtual screening of a 90,000 compound library aiming to identify compounds that closely mimic the spatial structure of the initial template. Compound **62** was identified and chosen as a candidate for further study. Initial experiments with L929 cells showed that the IC_{50} of compound **62** on TNF-mediated cytotoxicity was about 9 μM. The effects of **62** on TNF-triggered signal transduction pathways were evaluated. Specifically, they tested the capacity of this compound to block the activation of caspase-3 and caspase-8, which are widely used readouts for TNF signaling, and found that compound **62** completely blocked TNF-induced activation of these proteins. Furthermore, **62** prevented the degradation of IκBα, and downregulated the expression of TNF, IL-1α, and MIP-2 genes in L929 cells treated with TNF.

With these *in vitro* results in hand, a murine hepatitis model was used to explore the potential anti-inflammatory effect of **62**. The TNF inhibitor etanercept or compound **62** was injected into BALB/c mice, and LPS/GalN were subsequently used to induce inflammation. Blood and liver tissues were collected for further analysis. Etanercept application led to survival of 95% of the animals, while compound **62** delayed the incidence of death and increased the survival rate by two fold when compared with the vehicle control (58.5% *versus* 20.8%). Histopathological analysis showed that treatment with **62** or etanercept both significantly reduced the liver damage and emergence of hemorrhage compared to the severe liver damage and wide hemorrhagic necrosis seen with the LPS/GalN alone.



(62)

Fig. (25). Structure of compound C87 (**62**), identified as a potential inhibitor for the TNF-TNFR1 interactions by virtual screening, and then validated *in vitro* and in cell-based assays.

In order to validate the mechanism of action, direct compound **62**-TNF binding was measured using SPR; a K_d of 110 nM was found. To further test whether the direct binding between **62** and TNF was able to eliminate interaction between TNF and its receptors, a competition ELISA was used. As expected, the anti-TNF antibody successfully abolished the interactions between TNF and TNFR, providing a validation for this assay. Surprisingly, compound **62** failed to block TNF binding to either TNFR1 or TNFR2 in the competitive assay. The authors speculated that instead of preventing TNF-TNFR interactions, the direct binding of **62** with TNF might somehow disrupt the function of the TNF-TNFR complex, thus leading to the strong inhibition of TNF-induced signaling observed by compound **62** by an alternative, undiscovered, mechanism.

6. INHIBITION OF THE TNF-TNFR INTERACTION

Another possible strategy for the inhibition of TNF action is to direct antagonize the TNF receptors. Unfortunately, the inhibition of both TNFR1 and TNFR2 signaling can cause serious side effects, such as an increased risk of infection [26, 93, 94]. Furthermore, selectively inhibiting TNFR1-mediated signaling, while preserving TNFR2 signaling may reduce inflammation and maintain the host immune response to pathogens [95]. A variety of compounds blocking TNFR1 have been proposed in the literature, including monoclonal antibodies [96] and antagonistic TNF mutants specific to TNFR1 [97]. In addition to these inhibitory strategies, RNAi [98] and antisenseoligos

[99] have been used successfully to downregulate TNFR1 and reduce pathology *in vivo* [100]. TNFR can also be disabled by inducing allosteric modulation of an identified loop in the receptor involved in TNF binding [101]. However, disruption of the TNF-TNFR interaction with a molecule-based antagonist that appropriately engages this receptor is difficult, due ligand-receptor avidity, which is related to the large contact surface area [102]. Carter *et al.* disclose four classes of small molecules (compounds **63**-**66**, Fig. **26**) that are potent inhibitors of the TNF-TNFR1 interaction. Indeed, detailed biochemical studies revealed that these compounds are actually reversible inhibitors that covalently modify TNFR1 in a light-dependent fashion [103]. The compounds, which contain a rhodanine heterocycle, were found by screening their own library of chemical compounds. Then, to obtain the most potent compounds, several analogs were prepared focusing on the heterocyclic group. The most potent antagonist synthesized in these efforts was IW927 (compound **63**, Fig. **26**), which exhibited an IC_{50} value of 50 nM for inhibiting TNF-TNFR1 binding. This compound exhibited >2000-fold selectivity for TNFR1 relative to TNFR2 or CD40, and was not cytotoxic at concentrations up to 100 μM. Due to the fact that molecules like these are readily excited/isomerized photochemically, testing the interaction of these compounds with TNFR1 in the absence of light was necessary. Indeed, when the binding assays were performed in the dark, the potencies of all of the compounds tested were attenuated 50-fold to >1000-fold (IC_{50} values >30 μM). But three of them still displayed a measurable binding affinity in the absence of light: RQ989 (compound **64**, 47 μM), 5B981 (compound **65**, 34 μM), and IV703 (compound **66**, 50 μM), which were sufficient for site-specific covalent modification of TNFR1 in the presence of light, thereby leading to the apparent high-affinity binding.

Crystallography studies were performed with compound **66** due to its superior solubility in aqueous media relative to the other inhibitors, even though it was not the most potent antagonist. Although the **66**-TNFR1 complex does not appear to induce large structural changes in the receptor, the x-ray crystallography clearly revealed the attachment of compound **66**'s nitrophenyl ring to the backbone nitrogen of Ala62 of TNFR1. This covalent binding may disrupt TNFα binding by blocking some of the interactions between the exposed Tyr-containing β-turn of the native ligand and the region near the Ala62 of TNFR1, a known hot spot.

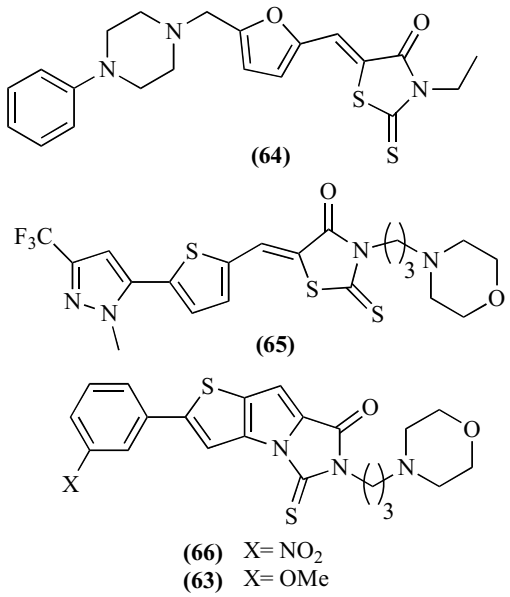


Fig. (26). ''Photochemically enhanced'' inhibitors that covalently modify TNFR1 in a light-dependent fashion.

In conclusion, Carter *et al.* discovered four N-alkyl 5-ar ylalkylidene-2-thioxo-1,3-thiazolidin-4-one photochemically enhanced inhibitors that bind reversibly to TNFR1 with weak affinity (ca. 40-100 μM) and then covalently modify TNFR1 through a photochemical reaction. Given that the compounds are reversible (albeit weak) inhibitors of the TNF-TNFR1 interaction in light-excluded conditions, they are interesting lead compounds, especially considering their capacity for receptor selectivity.

CONCLUDING REMARKS

Extensive research during the past century has revealed that inflammation plays a major role in most chronic diseases, but it is only within the past few decades that the mechanisms by which inflammation is mediated at the molecular level have become apparent. Among the many processes, it is clear now that TNF plays a role in tissue destruction and the remodeling associated with inflammatory diseases. In fact, TNF dysregulation has been linked to a wide variety of pathologies, including cancer, obesity, cardiovascular, pulmonary, metabolic and autoimmune diseases.

Much effort has been devoted to the discovery of small molecules able to suppress TNF production and a plethora of compounds, both natural and synthetic, have been described. Nevertheless, most are non-selective and inhibit intracellular signaling pathways that also affect the production of other cytokines, increasing the complexity of the response. Besides, considering that TNF facilitates many homeostatic proc-

esses within the Central Nervous System, it will be critical to consider the effects brain penetrating small molecules can have on the CNS and whether modifications will need to be made in order to keep them out of the brain when treating non-CNS diseases. Furthermore, blocking peripheral TNF can influence TNF signaling in the brain as these messages are transmitted to the CNS through a variety of mechanisms [104].

Notwithstanding these limitations, promising results have been obtained in some cases. Curcumin (compound **6**), for example, emerged as a safe, orally bioavailable TNF blocker. As of publication, the evidence suggests that compound **6** may be an alternative treatment for all of the diseases for which TNF blockers are currently being used. [48]

The ideal therapy will be based on the selective inhibition of TNF action, an approach that has seen a number of advances in recent years. Most have been aimed at the disruption of the TNF trimer. The availability of the crystal structure of the TNF dimer bound to the inhibitor SPD304 (**40**) has been an important step in this direction. Although no selective interactions seem to be operating at the TNF dimer/SPD304 (**40**) binding site, which is a common situation when PPIs are the target of small molecules, recent progress in this area supports the feasibility of this approach [69]. The anticipated availability of crystal structures of TNF bound to additional inhibitors may prove the existence of alternative, targetable hot spots and would be an important contribution to the field. In this context, this review has highlighted the effectiveness of computational studies, such as virtual screening and docking, which are crucial tools for the development of this kind of inhibitor. Contrary of their current, widespread use, docking studies should ideally be used to support experimental data that confirm the direct interaction with TNF. When this data is not available, further molecular dynamics studies of the proposed complex should be performed to support the complex stability.

Moreover, it is very likely that in the near future the crystal structure of TNF bound to TNFR1 will be available, which may prove invaluable for the development, through rational design, of a potent inhibitor acting at this level.

In conclusion, the success of injectable, protein-based therapies directed against TNF has been remarkable. However, questions remain concerning the long-term use of these drugs. The recent advances in the understanding of TNF biology at molecular level, along with the availability of reliable computational methods and screening techniques has given rise to the presumption that novel, small molecule-based oral therapies for chronic inflammatory diseases are not far in the future.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIALS

A table with additional information (sources, biological data) for all of the compounds mentioned in the text can be found.

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Received: February 12, 2015 **Revised: July 08, 2015** **Accepted: July 28, 2015**