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A Class of Small Molecules that Inhibit TNFα-Induced Survival and Death Pathways via Prevention of Interactions between TNFαRI, TRADD, and RIP1

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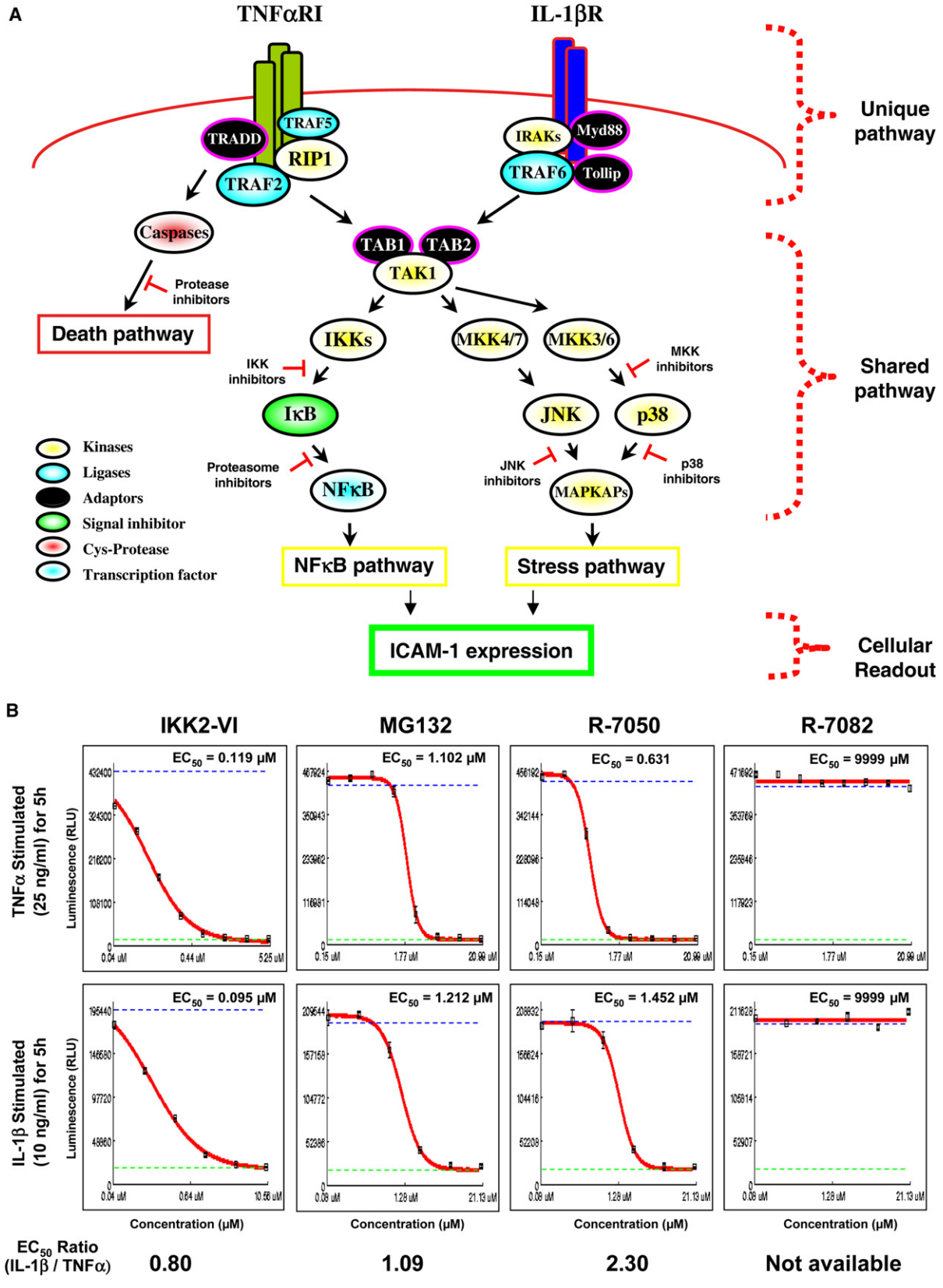
SUMMARY

Small-molecule library screening to find compounds that inhibit TNFα-induced, but not interleukin 1 β (IL-1 β)-induced, intercellular adhesion molecule 1 (ICAM-1) expression in lung epithelial cells identified a class of triazoloquinoxalines. These compounds not only inhibited the TNFα-induced nuclear factor κB (NFκB) survival pathway but also blocked death-pathway activation. Such dual activity makes them unique against other known NFκB-pathway inhibitors that inhibit only a subset of TNFα signals leading to increased TNFα-induced cytotoxicity. Interestingly, these compounds inhibited association of TNFα receptor (TNFαR) I with TNFαR-associated death domain protein (TRADD) and receptor interacting protein 1 (RIP1), the initial intracellular signaling event following TNFα stimulation. Further study showed that they blocked ligand-dependent internalization of the TNFα-TNFαR complex, thereby inhibiting most of the TNFα-induced cellular responses. Thus, compounds with a triazoloquinoxaline scaffold could be a valuable tool to investigate small molecule-based anti-TNFα therapies.

INTRODUCTION

TNFα is one of the earliest cytokines to be secreted into the site of inflammation in various cell types [1, 2]. TNFα exerts its multiple functions through two structurally related cell-surface receptors, p55 (type 1 receptor; TNFαRI, CD120a) and p75 (type 2 receptor; TNFαRII, CD120b) [3]. Whereas TNFα receptor (TNFαR) I is known to mediate the majority of TNFα-induced cellular events, TNFαRII appears to have unique and overlapping roles, although this remains the subject of ongoing research [4]. Upon ligand binding, TNFαRI recruits at least two death domain-containing proteins, TNFαR-associated death domain protein (TRADD) and receptor interacting protein 1 (RIP1), as well as E3 ubiquitin ligases, TNF receptor-associated factor (TRAF) 2, and TRAF5, to form a large signal-initiating complex [5]. TRAF2 and TRAF5 are functionally redundant molecules and play a vital role in bridging the TNFαRI complex eventually to trigger nuclear factor κB (NFκB) and stress pathways, both of which are important regulators of cell survival and gene expression [2, 6]. In contrast, TRADD associates with Fas-associated death domain (FADD) in order to send a signal to the caspase-dependent death pathway [7]. The balance between survival and death pathways appears to determine cell fate and is influenced by the cellular context as well as coexisting extracellular signals. From the perspective of inflammation, activation of the NFκB pathway is considered to be a key step, as NFκB-mediated transcription strongly amplifies the magnitude of inflammation through production of proinflammatory cytokines, chemokines, and cell-adhesion molecules [8]. As a result, tissue-infiltrating cells (e.g., neutrophils and macrophages) are attracted to the site of inflammation and cause tissue damage. Knockout or pharmacological inhibition of TNFα by neutralizing antibodies or soluble receptors has been shown to greatly diminish the progression of inflammation in various animal models as well as in human diseases, highlighting TNFα as a good drug target [9].

Despite the clinical success of protein-based drugs against TNFα, development of small-molecule TNFα inhibitors has not been very successful [9]. Reported small-molecule TNFα antagonists include inhibitors of the processing enzyme TACE [10], disruptors of TNFα trimerization [11], and disruptors of ligand-receptor interaction [12]. There have also been notable advances in the development of small-molecule inhibitors for intracellular signaling pathways of TNFα [13–17]. However, inhibition of the NFκB pathway by small molecules may require careful evaluation with regard to potential tissue toxicities, particularly in the liver. This concern has been raised partly by phenotypes of IKKβ knockout mice or several other molecules in the NFκB pathway (e.g., RelA) in which increased apoptosis of liver cells was observed [18–21]. As inactivation of IKKβ did not affect the TNFα-induced death pathway, it is possible that liver toxicity was a result of enhanced TNFα-induced cytotoxicity. In fact, small-molecule inhibitors of IKKβ or proteasome that prevent IκB degradation and thus inhibit the NFκB pathway have been shown to increase TNFα-induced cytotoxicity [22, 23]. A stress-pathway kinase, p38 MAPK, has also been



a target of drug development by many pharmaceutical companies, yet its progress has been delayed partly due to toxicity in the liver [9]. Moreover, NFκB and stress pathways are activated not only by TNFα but also by numerous other cytokine receptors, toll-like receptors (TLRs), and immune-cell receptors [24–26]; it is therefore challenging to achieve sufficient selectivity to limit potential side effects.

Given such challenges, we initiated this study to identify small molecules that specifically inhibit all TNFα functions. As both TNFα and interleukin 1β (IL-1β) activate NFκB and stress pathways leading to the upregulation of intracellular adhesion molecule 1 (ICAM-1) expression in A549, a lung epithelial cell line, we used changes in ICAM-1 expression as a readout, and looked for compounds that inhibited TNFα-induced signaling more efficiently than IL-1β. Enzyme-linked immunosorbent assay (ELISA)-based high-throughput screening (HTS) of ~300,000 in-house compounds in such a differential cell-based assay identified a class of compounds that had a core structure of triazoloquinoxaline (TQ). Detailed mechanistic studies demonstrated that TQ compounds inhibit virtually all signaling pathways activated by TNFα without perturbing ligand-receptor interaction at potencies as low as submicromolar. We further provide evidence that TQ compounds inhibit endocytosis-dependent formation of the TNFαRI-signaling complex. Our findings show that these TQ compounds are capable of inhibiting most, if not all, TNFα functions through a unique mechanism of action.

RESULTS

Compound Screening and Hit Identification

As a number of cytokines, including TNFα and IL-1β, are known to activate NFκB and stress pathways (Figure 1A), inhibition of these pathways is unlikely to be specific toward TNFα. It was expected that numerous compounds in our library would inhibit these pathways and thus overshadow real "TNFα-specific" hits. Therefore, we employed a differential screening strategy to identify compounds that inhibited TNFα but not IL-1β-induced signals using ICAM-1 induction as a common cellular readout. ELISA-based HTS of ~300,000 compounds was carried out at 10 μM in A549 lung epithelial cells following a 5 hr incubation with TNFα and IL-1β. The coefficient of variation (CV) and the Z′ factor for our HTS assay were found to be within the acceptable range (CV = <5% and Z′ factor = 0.885). Primary hits that fulfilled the following two criteria were promoted for EC_{50} determination: (1) at least 60% reduction of ICAM-1 expression induced by TNFα, and (2) significantly lower inhibition of ICAM-1 induced by IL-1β (greater than 30% difference in percent inhibition). Among those tested for EC_{50}, compounds that reproducibly showed 1.5-fold or greater potency toward TNFα over IL-1β were nominated for further analysis including analog testing. Finally, TNFα-selective hits were confirmed for their activity in various bioassays such as production of IL-6 and IL-8, as well as a reporter assay using a 5′-transcriptional regulatory region of IL-8 (data not shown). All of the above analyses finally resulted in identification of two structurally related small-molecule compounds that had triazoloquinoxaline as a core structure (Tables 1A and 1B). Among them, R-7050, 8-chloro-4-(phenylthio)-1-(trifluoromethyl)-[1,2,4]triazolo[4,3-a] quinoxaline, emerged as a potent and fully reversible hit with greater selectivity toward TNFα. Figure 1B shows typical inhibition curves obtained for some of the active and inactive hits along with standard positive controls such as IKK2-VI and MG132. In TNFα-induced ICAM-1 expression, R-7050 inhibition potency (EC_{50} = 0.63 μM) was 2- to 3-fold greater than EC_{50} for IL-1β-induced ICAM-1 expression (1.45 μM). As shown in Tables 1A and 1B, a few more additional TQ compounds were identified through the substructure search, and we found that none of them were more potent than original hits, following EC_{50} determination.

Based on structure-activity-relationship (SAR) analysis, R-7050 contained important substituents on either side of the core heterocyclic ring structure that were critical for inhibitory activity. Also, the potency depends on the presence of chlorine substituents as well as electron-withdrawing groups such as unsubstituted phenyl, pyridine, and pyrimidine moieties. For example, the absence of chlorine (R1 substituent) and the presence of electron-donating groups such as methoxyphenyl (R3) in the case of R-7075 and R-7082 completely eliminated the inhibitory activities for these compounds. Similar observations were also made in the case of a second group of compounds derived from triazoloquinoxalines which lack the phenylthio substituent (Table 1B). Substitution of the trifluoromethyl group in both classes of triazoloquinoxalines (R2 in the case of Table 1A) was found to enhance the inhibitory activity. However, no clear effect of electron-withdrawing groups was noted, and multiple methoxy substituents were no prerequisite for increased inhibitory activity (data not shown). For further evaluation including target identification and mechanism of action studies, R-7050, R-8507, and its inactive counterpart R-7082 were selected.

Effect on TNFα-Induced NFκB and Stress-Pathway Activation

In order to investigate the mechanism/target of action of TQ compounds, we examined their effects on phosphorylation, ubiquitination, and degradation of intracellular signaling proteins. One of the major pathways activated by

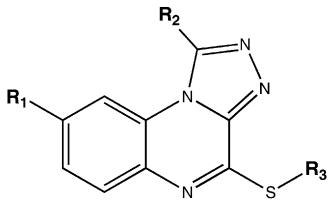
Figure 1. Activation of Key Signaling Pathways upon TNFα and/or IL-1β Stimulation and Measurement of ICAM-1 Expression as a Functional Readout

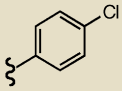
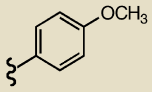
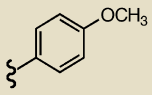
(A) Schematic of TNFα and IL-1β signaling pathways showing key members and potential sites of inhibition.

(B) Representative inhibition curves and EC_{50} values obtained for triazoloquinoxaline hits in the ELISA-based ICAM-1 induction assay. Two known inhibitors, IKK2-VI and MG132, that inhibit TNFα and IL-1β signals at equal potency were included for reference. Numbers at the bottom represent ratios of EC_{50} values. A higher number denotes greater selectivity of hits toward TNFα-induced ICAM-1 expression over IL-1β. 9999 indicates that the compound is inactive. Error bars are standard deviations of the mean of at least three replicates.

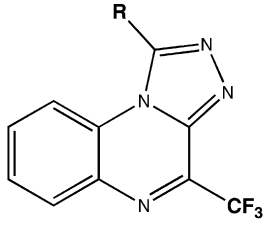
Table 1. List of Select Triazoloquinoxaline Analogs and Their Anti-ICAM-1 Expression Activity

A



Compound ID	R_1	R_2	R_3	Molecular Weight	ICAM-1 ELISA EC_{50} (μM) TNFα	IL-1β	IL-1β/TNFα
R-7050	Cl	CF3		380.78	0.631	1.452	2.30
R-7078	H	CF3		380.78	0.721	1.612	2.23
R-7081	H	CF3		346.33	1.921	3.612	1.88
R-7080	H	CF3		347.32	4.072	7.174	1.76
R-7047	H	H		280.31	7.561	8.034	1.06
R-7075	H	H		308.36	>1000	>1000	na
R-7082	H	CF3		376.36	>1000	>1000	na

B



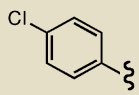
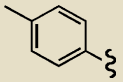
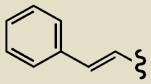
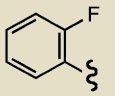
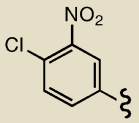
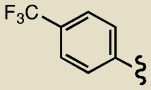
Compound ID	R	Molecular Weight	ICAM-1 ELISA EC_{50} (μM) TNFα	IL-1β	IL-1β/TNFα
R-8507		348.71	2.451	3.792	1.54

Table 1. *Continued*

Compound ID	R	Molecular Weight	ICAM-1 ELISA EC_{50} (μM) TNFα	IL-1β	IL-1β/TNFα
R-8525		328.29	5.123	8.123	1.59
R-8520		340.31	8.451	12.11	1.43
R-8515		332.26	14.23	19.83	1.39
R-8522		393.71	23.72	>1000	na
R-8510		253.18	>1000	>1000	na
R-8516		382.27	>1000	>1000	na

The anti-ICAM-1 expression activities represent an average EC_{50} value derived from ICAM-1 ELISA assays carried out in duplicate on different days separately. na, not available.

TNFα is the NFκB pathway and, in fact, the 5′-regulatory region of the ICAM-1 gene contains multiple functional NFκB binding sites [27]. Phosphorylation and ubiquitination of IκBα by IKKβ and Skp1-Cullin-F box protein ($SCF^{\beta TRCP}$), respectively, are prerequisites for NFκB to be released from its inhibition and to translocate into the nucleus to activate various genes including ICAM-1 [8, 28]. We found that the active TQ compounds R-7050 and R-8507 inhibited degradation of IκBα only when cells were stimulated with TNFα, not with IL-1β, and maintained the IκBα protein at a similar level as an IKKβ inhibitor (IKK2-VI) (Figure 2A). In contrast, an inactive analog of TQ compounds (R-7082) had no effect even at 10 μM, and IκBα was rapidly degraded upon stimulation (data not shown). In a similar concentration range, active TQ compounds were able to inhibit phosphorylation and ubiquitination of IκBα in a TNFα-specific manner (Figure 2B). Consistently, translocation of NFκB into the nucleus which occurs following IκBα degradation was blocked by the active TQ compound R-7050 (Figure 2C). These results indicate that TQ compounds inhibit the NFκB pathway and that the target of action is most likely to be upstream of IKKβ.

We next examined the effect of TQ compounds on the phosphorylation status of stress-pathway kinases. A panel of phosphoblot analysis shown in Figure 2D indicated that R-7050 inhibited phosphorylation of both the c-Jun N-terminal kinase (JNK) pathway (MKK4, JNKs, and ATF2) and p38 pathway (MKK3/6, p38, and MAPKAP2), although to different degrees. It was noted that phosphorylation of JNK-pathway molecules was relatively more sensitive to R-7050-mediated inhibition than to p38-pathway inhibition. This phenotype is reminiscent of those in TRAF2 knockout mice or in cells transfected with TRAF2 siRNA [29, 30] in which activation of JNK by TNFα was more affected than p38 activation, implying that the target of TQ compounds is either TRAF2 itself or further upstream in the TNFα signaling pathway.

Effect on TNFα-Induced Apoptosis Pathway

It is known that there are at least two types of TNFα-responsive cells: one is resistant to TNFα-induced death mainly due to efficient activation of the NFκB pathway and the other is relatively more sensitive and is killed by TNFα, which is further enhanced in the presence of protein- or RNA-synthesis inhibitors including cycloheximide or actinomycin-D (Act-D). Such sensitive cells, such as ME180 and BT-20, have been used for the study of TNFα-induced cytotoxicity and caspase activation [31]. Hence, we took advantage of ME180 cells to address whether TQ compounds could block TNFα-induced death-pathway activation.

When treated with increasing concentrations of TNFα alone, ME180 cells showed a significant, yet not striking, downward trend in cell survival (Figure 3A). In the presence of either Act-D or NFκB-pathway inhibitors IKK2-VI or MG132, TNFα-induced cytotoxicity was greatly enhanced (Figure 3A). In clear contrast, active TQ compounds (R-7050, R-8507, and R-7078) prevented the downward trend in cell survival in the presence of TNFα, although addition of

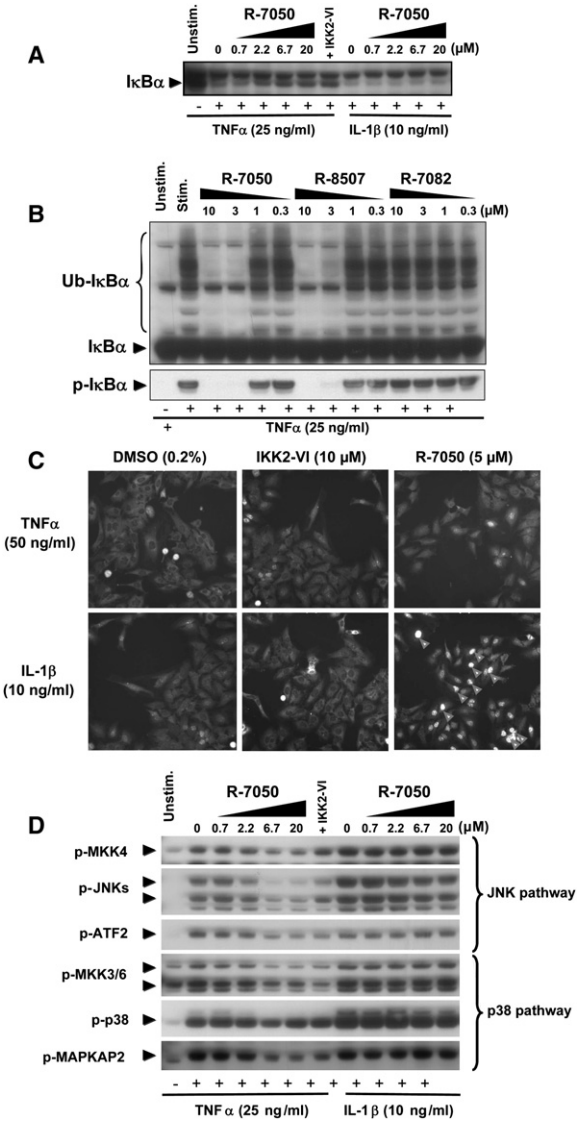


Figure 2. Effect of TQ Compounds on NFκB- and Stress-Pathway Activation

(A) Western blot analysis of IκBα degradation when cells were stimulated with TNFα or IL-1β for 20 min in the presence or absence of various concentrations of R-7050. Lower bands are IκBα-specific signals. R-7050 shows clear TNFα-specific inhibition of IκBα degradation in a dose-dependent manner.

(B) IκBα phosphorylation and ubiquitination in cells stimulated with TNFα for 5 min in the presence of various concentrations of active (R-7050 and R-8507) or inactive (R-7082) compound. Whereas active compounds inhibited both phosphorylation and ubiquitination of IκBα, an inactive compound did not. Protein levels of IκBα did not change at this time point (5 min), indicating that a decrease in phosphorylation and ubiquitination of IκBα was not a result of protein degradation. Different anti-IκBα antibodies were used in (A) (monoclonal) and (B) (polyclonal), because a ubiquitinated form of IκBα was detected better with the polyclonal antibody.

(C) Image-based NFκB translocation assay data derived from HeLa cells stimulated with TNFα or IL-1β for 30 min in the presence or absence of R-7050 (5 μM) or IKK2-VI (10 μM), and intracellular NFκB was detected with a rabbit anti-NFκB p65/Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody pair. Note that TQ compounds specifically inhibited TNFα-induced NFκB translocation to the nucleus, whereas IKK2-VI inhibited both.

(D) Phosphoblot analysis of stress-pathway kinases following TNFα or IL-1β stimulation (20 min). R-7050 inhibited phosphorylation of these kinases in a TNFα-specific manner. JNK-pathway members appeared to be more sensitive to TQ compounds than p38 MAPK-pathway members.

these compounds alone reduced celltiter-glo reading to some extent (Figure 3B). There was no clear effect by R-7082, an inactive TQ compound analog. Apparent reduction in celltiter-glo reading by R-7050/R-7082/R-8507 alone is probably due to their mild cytostatic effect; however, it is unlikely that the anti-TNFα activity of these compounds is an indirect consequence of the cytostatic effect, because TNFα-induced cytotoxicity usually does not require cell proliferation. In agreement with these results, activation of caspase3/7 by TNFα was reduced by active TQ compounds but not by the inactive analog R-7082 (Figures 3E–3G). Furthermore, neither was Fas- nor C6-ceramide-induced cell death inhibited by TQ compounds (Figures 3C and 3D). These stimuli are known to directly activate the FADD-dependent or downstream caspase pathway independently of TRADD, eliminating the possibility that the target of TQ compounds is the FADD- or caspase-dependent pathway. These results, along with data shown in Figures 2 and 3, suggested that the mechanism of action for TQ compounds was the interference of signaling events proximal to TNFαRI.

TQ Compounds Inhibit Interaction between TNFαRI, RIP1, and TRADD

The initial event after ligand-induced TNFαRI trimerization is recruitment of a death domain (DD)-containing adaptor molecule, TRADD. TRADD in turn recruits at least two other signaling molecules, RIP1 and TRAF2, to form a large molecular weight complex, prompting us to speculate that the formation of this complex was interfered with by TQ compounds. We therefore addressed this question by conducting an immunoprecipitation experiment using an anti-TNFαRI antibody. Lysates from A549 cells treated with TNFα for 20 min in the presence or absence of TQ compounds were immunoprecipitated with an anti-TNFαRI antibody and the presence of TRADD and RIP1 in immunoprecipitates was examined by western blot analysis. Clearly, TNFαRI failed to associate with either TRADD or RIP1 in the presence of active TQ compounds (R-7050 and R-8507), whereas the complex remained intact when an inactive TQ compound (R-7082) was used (Figure 4A). The range of effective concentrations was similar to those observed in other assays, suggesting that the prevention of complex formation was indeed a cause of inhibition of downstream pathways.

TQ Compounds Block Internalization of the TNFα Ligand-Receptor Complex

Although formation of the TNFαRI-TRADD-RIP1 complex was inhibited by TQ compounds, analyses of crystal structures of TNFαRI and other DD-containing proteins including TRADD demonstrated that such an interaction is

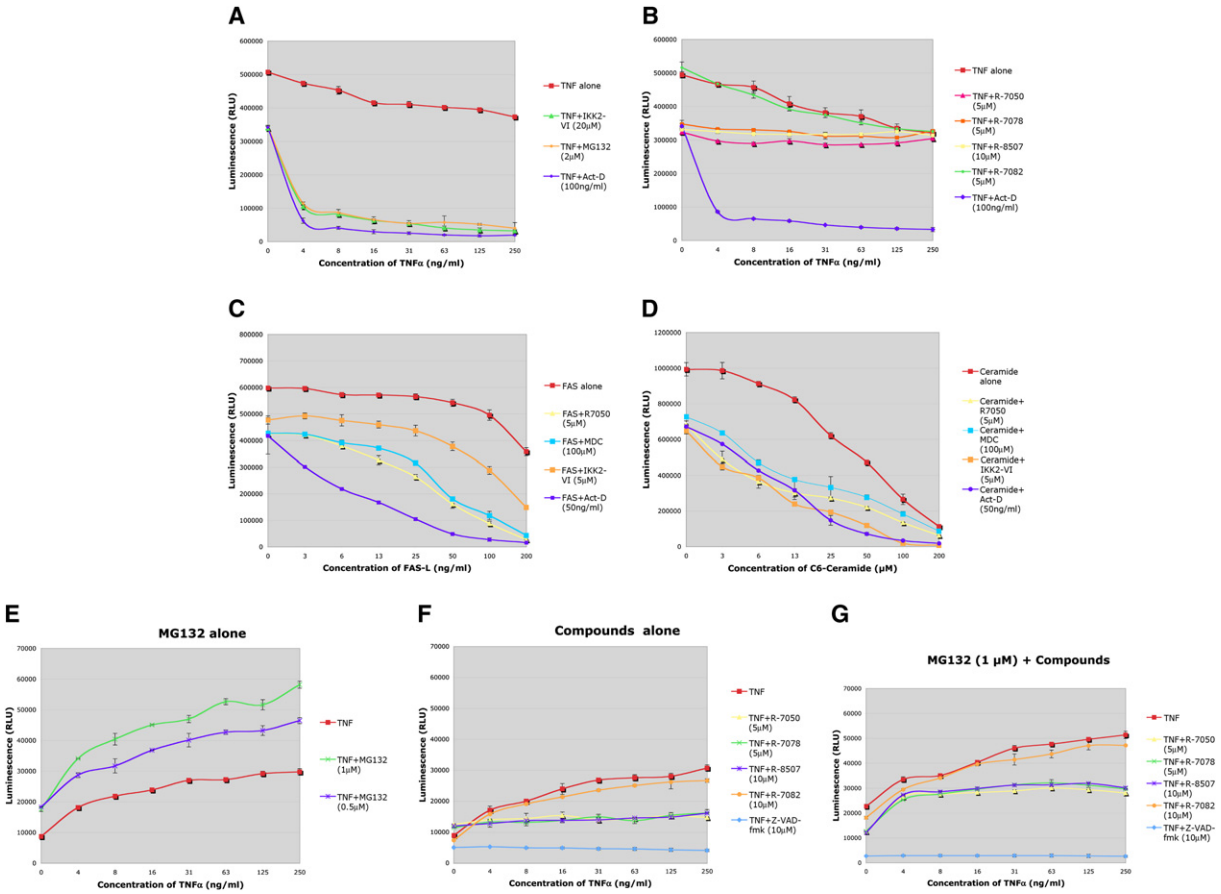


Figure 3. Effect of TQ Compounds on Death-Pathway Activation

(A–D) Inhibition of TNFα-induced cytotoxicity by TQ compounds. TNFα-sensitive ME180 cells were treated with increasing concentrations of TNFα (A and B), Fas- (C), or C6-ceramide (D) for 24 hr in the presence of various inhibitors: IKK2-VI and MG132 (NFκB-pathway inhibitors), Actinomycin D (an RNA-synthesis inhibitor), MDC (an endocytosis inhibitor), or TQ compounds identified in this study. TQ compounds inhibited TNFα-induced, but not Fas- or C6-ceramide-induced, cytotoxicity. Error bars are standard deviations of the mean of at least three replicates.

(E–G) Effect of TQ compounds on TNFα-induced caspase3/7 activation. Test compounds were added to ME180 cells stimulated with TNFα in the presence (E and G) or absence (F) of MG132 for 24 hr, and caspase3/7 activity in cell lysates was measured by a caspase3/7 assay kit. A cell-permeable caspase inhibitor, Z-VAD-fmk, was used as a positive control. TQ compounds inhibited TNFα-induced caspase3/7 activation regardless of the presence or absence of MG132, although the level of inhibition was lower than that of Z-VAD-fmk. Error bars are standard deviations of the mean of at least three replicates.

highly rigid and their conformational spaces are extremely limited [32, 33]. Interestingly, it has been suggested that formation of the functional TNFαRI-signaling complex requires its internalization upon ligand binding via the classical clathrin-dependent receptor-mediated endocytotic pathway [34]. Therefore, we investigated the effect of TQ compounds on internalization of the TNFα-TNFαR complex by an indirect immunofluorescence staining method [35]. Biotinylated TNFα was added to the culture in the presence or absence of a TQ compound (R-7050) or monodansylcadaverine (MDC), and the cellular distribution of the ligand-receptor complex was analyzed using a fluorescence microscope after staining with fluorescein isothiocyanate (FITC)-avidin. In the absence of an inhibitor, TNFα binding on the cell surface at 4°C showed a uniform distribution of the ligand-receptor complex throughout the plasma membrane (Figure 5A). Upon shifting the temperature to 37°C, the fluorescence intensity became more condensed and perinuclear staining due to colocalization at lysosomal compartments became more prominent during the period of incubation up to 1 hr. This appeared to be the typical process of ligand-induced TNFαR internalization, and the kinetics of intracellular staining is in good agreement with previous observations [36, 37]. In the presence of R-7050, translocation of the ligand-receptor complex to perinuclear and lysosomal compartments was efficiently prevented and the staining remained uniform on the plasma membrane similar to that in unstimulated cells. Similarly, MDC prevented internalization of the ligand-receptor complex. Although this assay does not distinguish between TNFαRI and TNFαRII, it is clear that TNFα was retained on the plasma membrane in the presence of a TQ compound (R-7050) regardless of receptor subtypes.

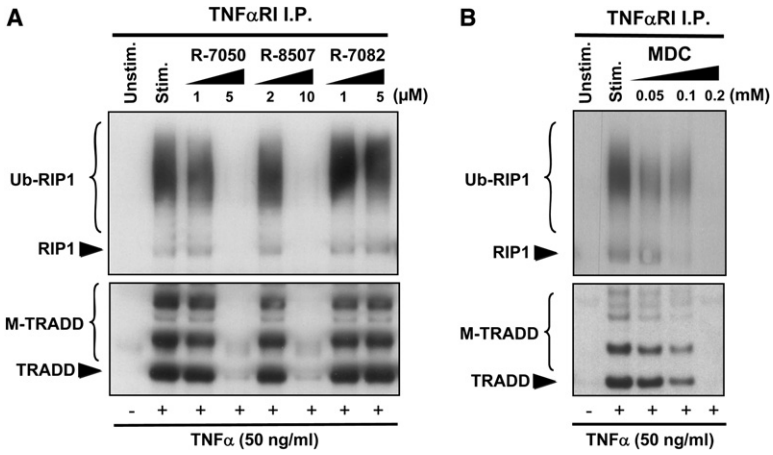


Figure 4. TQ Compounds Prevent Formation of the TNFαRI-TRADD-RIP1 Complex

(A) TQ compounds prevent formation of the TNFαRI-TRADD-RIP1 complex. A549 cells were stimulated with TNFα in the presence of active (R-7050 and R-8507) or inactive (R-7082) compound for 20 min. TNFαRI was immunoprecipitated and the presence of TRADD and RIP1 in the immune complex was assayed by western blots. TNFα-induced association of TRADD and RIP1, preferably in its ubiquitinated form, with TNFαRI was inhibited by active, but not by inactive, compounds.

(B) MDC, a known endocytosis inhibitor, also inhibited TNFα-induced association between TNFαRI, TRADD, and RIP1. Upper bands in the TRADD blots are presumably multimerized forms of TRADD (M-TRADD).

Finally, we addressed whether known internalization inhibitors were capable of inhibiting TNFα-mediated signals specifically over IL-1β signals. We carried out a differential ICAM-1 assay for two of known inhibitors for endocytosis; one is a clathrin-dependent endocytosis inhibitor, MDC [35], and the other is a nonspecific endocytosis inhibitor, phenylarsenic oxide (PAO) [38] (Table 2). As expected, MDC showed TNFα-skewed activity with an IL-1β/TNFα EC_{50} ratio of 2.26, whereas PAO had a marginal 1.68 ratio that was still higher than those obtained for IKK2-VI or MG132 that inhibited TNFα- and IL-1β-induced ICAM-1 with similar potency (a ratio of 0.9:1.2). As it has been reported that a subset of TNFα-induced signaling requires localization of TNFαRI into a lipid raft, a membrane microsubdomain enriched in cholesterol and sphingolipid [39, 40], we tested a disruptor of lipid rafts, methyl-β-cyclodextrin (MCD), and found that there was not much difference in EC_{50} between TNFα- and IL-1β-mediated ICAM-1 production (Table 2). To further assess the selectivity of TQ compounds against other receptor systems, we examined the effect of R-7050 on phosphorylation of JNK and AKT used as surrogate assays induced by EGF [41], IGF-1 [42], insulin [43], and lysophosphatidic acid (LPA) [44] to indirectly monitor ligand-induced receptor internalization. Figures 5B and 5C show that a TQ compound (R-7050) exhibited inhibitory effects only in TNFα and LPA receptor systems, but not in IL-1βR, InsulinR, IGFR, or EGFR. In contrast, MDC inhibited endocytosis of most of the receptor systems tested. Taken together, we conclude that inhibition of internalization of the TNFα-TNFαR complex was, at least in part, the mechanism of action of TQ compounds.

DISCUSSION

Our high-throughput screening aimed at TNFα inhibitors identified a class of small molecules that contained a core structure of TQ. These compounds inhibited TNFα-induced ICAM-1 expression more efficiently than IL-1β-induced ICAM-1 in the same cell type (A549, human lung epithelial cells). It has also been reported that TQ compounds inhibit cytokine-induced ICAM-1 expression [45]; however, there has been no study on cytokine selectivity or detailed mechanism of action for these compounds. Our results described below demonstrated that TQ compounds specifically inhibit TNFα-induced signaling pathways and subsequent biological functions by preventing the formation of the TNFαRI-signaling complex. During this study, we also found that many of the compounds that inhibited TNFα- and IL-1β-induced ICAM-1 at a similar potency had NFκB-pathway inhibitory activity (data not shown). This was not surprising, because both TNFα and IL-1β are known to activate the NFκB pathway in A549 cells. Thus, our simple differential screening system appeared to be robust in pulling out TNFα-selective hits from our ~300,000-compound library. By a similar differential screening approach, we previously isolated specific inhibitors for the spleen tyrosine kinase (Syk) [46, 47]. These compounds are currently being evaluated in multiple clinical trials for treating immunological disorders and cancer. Accordingly, our cell-based differential screening is efficient in identifying selective compounds with reasonable potency for drug development.

Detailed biological profiling studies revealed that TQ compounds selectively inhibited most, if not all, of the signaling pathways induced by TNFα that are involved in cell survival and gene expression (NFκB and stress pathways) as well as caspase-dependent death pathways. It is a striking property, because many of the reported inhibitors for TNFα are able to inhibit only a subset of signaling pathways. For example, inhibitors for IKKs inhibit phosphorylation and degradation of IκBα and subsequent NFκB translocation, DNA binding, and transcriptional activation [48]. However, these inhibitors do not inhibit TNFα-induced death-pathway activation and rather enhance its cytotoxicity, correlating with phenotypes of IKKβ knockout mice. In these mice, massive apoptosis was observed particularly in the liver [18–20] as well as in the skin of the conditional knockout model [49]. Although such defects in the liver were observed mainly during development, conditional inactivation of the NFκB pathway in adult hepatocytes resulted in enhanced

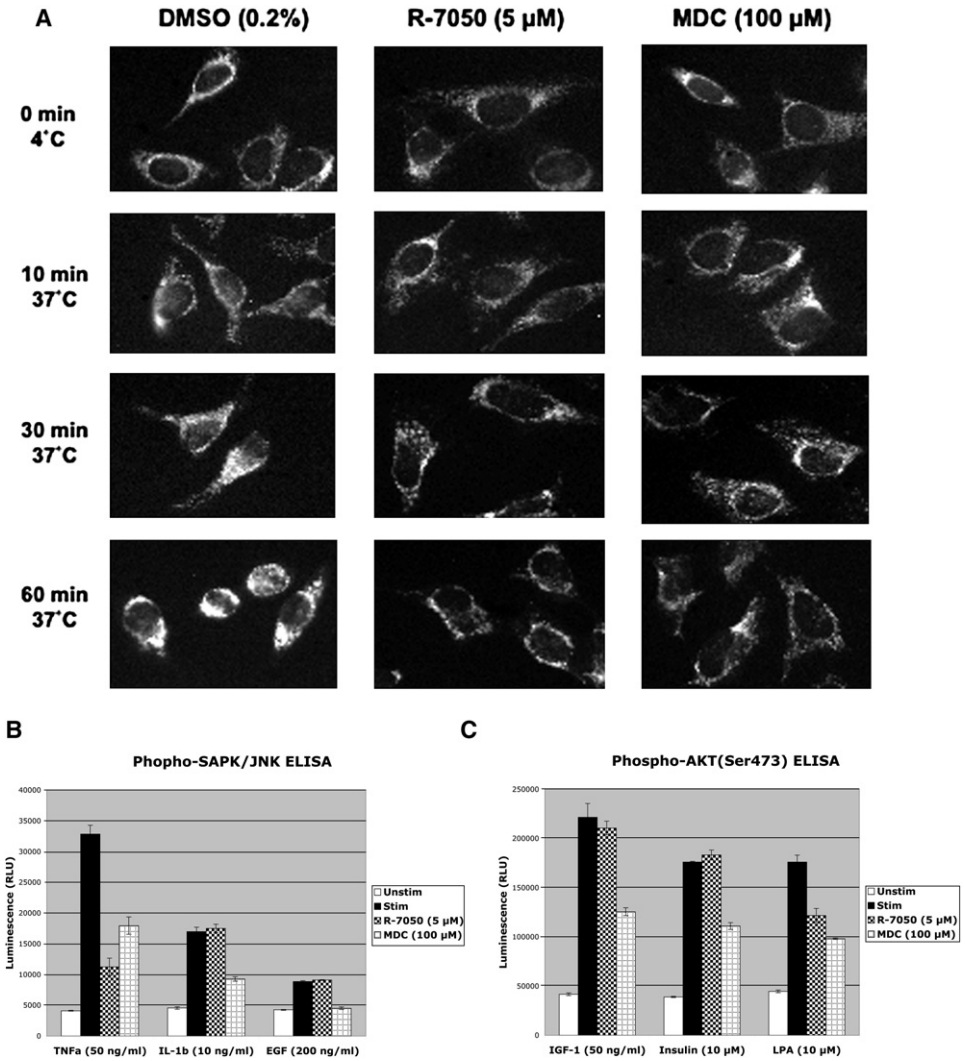


Figure 5. Effect of TQ Compounds on Internalization of the TNFα Ligand-Receptor Complex and Its Selectivity toward Other Nonrelated Ligand-Receptor Internalization Activities

(A) Subcellular localization of the TNFα-TNFαR complex. A549 cells stimulated with biotinylated TNFα were fixed at indicated time points and stained with FITC-labeled avidin. Both R-7050 and MDC inhibited TNFα-induced internalization of the TNFα-TNFαR complex.

(B and C) Selectivity of a TQ compound (R-7050) and a known receptor internalization inhibitor (MDC) over other nonrelated ligand-receptor internalization-mediated signaling events measured by phospho-JNK ELISA (B) and phospho-AKT ELISA (C) as a cellular functional readout. A549 cells seeded onto a six-well plate were serum starved overnight and preincubated with R-7050 (5 μM) and MDC (100 μM) for 1 hr at 37°C before ligand stimulation for 10 min. Cell-lysate preparation and phospho-JNK/AKT sandwich ELISA was carried out as described in the assay kits (Cell Signaling Technology). Error bars are standard deviations of the mean of at least three replicates.

apoptosis upon TNFα administration in vivo [50]. This property of these compounds can be problematic when they are used systemically for human immunological diseases that often require long-term treatment. The liver toxicity appears to be not only limited to IKK inhibition, because knockout models for JNK and SEK1/MKK4, stress-pathway kinases, also showed increased cell death in liver cells [51]. In this context, simultaneous inhibition of both death and survival pathways seems to be more desirable. Our compounds inhibited caspase activation and subsequent cell death in addition to NFκB and stress pathways. Besides the selectivity over IL-1β inhibition, TQ compounds prevented cell death induced by TNFα, but not by Fas- or C6-ceramide, extending their selectivity over other death-inducing signals. This is another desirable feature of this class of compounds, because nonspecific inhibition of cell death in vivo could result in hyperplasia, neoplastic transformation, or generation of self-reactive immune cells [52].

The fact that TQ compounds inhibited most of the TNFα signaling led us to consider that the target of action for TQ compounds could be in the proximity of TNFαRI or TRADD. It has been suggested that TRADD is the point where survival and death pathways are bifurcated through

Table 2. EC_{50} Values and Ratios in the ICAM-1 Induction Assay for Known Inhibitors of Endocytosis (MDC and PAO) or Lipid Rafts (MCD)

Compound (ID)	TNFα EC_{50} (μM)	IL-1β EC_{50} (μM)	Ratio IL-1β/TNFα
Triazoloquinoxaline (R-7050)	0.631	1.45	2.30
Monodansylcadaverine (MDC)	61.52	139	2.26
Phenylarsenic oxide (PAO)	0.47	0.79	1.68
Methyl-β-cyclodextrin (MCD)	1923	2482	1.29

EC_{50} = effective concentration for 50% inhibition.
A detailed assay protocol is described in Experimental Procedures.

association with at least two proteins, FADD and TRAF2, on distinct regions of TRADD [1, 7]. FADD associates with a C-terminal death domain of TRADD and transmits a signal to the downstream death pathway. Deletion of the FADD-associating region on TRADD or pharmacological inhibition of the downstream caspase pathway interferes with TNFα-induced cytotoxicity, whereas activation of the NFκB or stress pathways was relatively unaffected [7]. In clear contrast, TRAF2 associates with a non-DD region on the N-terminal half of TRADD, independently of FADD, and leads to activation of the NFκB and stress pathways [5, 7]. Meanwhile, TNFα-induced cell death was greatly enhanced in TRAF2 and TRAF2/TRAF5 double knockout mice, suggesting that only a survival pathway was perturbed by deletion of TRAF2 [29]. Moreover, inactivation of other molecules associating with the TNFαRI complex or in downstream pathways such as RIP1, TAK1, IKKβ, and RelA has been shown to increase sensitivity to TNFα-induced cell death [1, 3]. On the other hand, knockout of TNFαRI and expression of dominant-negative TRADD inhibited both survival and death pathways following TNFα challenge [34, 37]. Because the TQ compounds did not inhibit ligand-receptor interaction at all (data not shown), the most likely target for TQ compounds is in close proximity to the TNFαRI complex.

Our immunoprecipitation analysis clearly showed that formation of the TNFαRI complex was interrupted by TQ compounds. The TNFαRI-TRADD complex is formed by the interactions of DD on both TRADD and TNFαRI. As neither TNFαRI nor TRADD has been shown to have any considerable enzymatic activity, we first speculated that the mechanism of action by TQ compounds was inhibition of complex formation. However, crystal structure analysis of DD-containing proteins demonstrated that an individual DD has a very compact α helix bundle-based structure and forms an extremely rigid quaternary structure through surface-surface interaction [32, 33]. It seemed unlikely that a DD-mediated protein complex has wide enough space for a small molecule to prevent or disrupt its formation. Interestingly, it is known that at least two more physiological steps are required for TNFαRI to form a functional signaling complex, that is, receptor internalization and compartmentalization, to the plasma membrane microsubdomain called a lipid raft [39, 40]. We showed that TQ compounds were able to inhibit TNFα-induced internalization of the TNFα-TNFα receptor complex. Moreover, known internalization inhibitors MDC and PAO showed TNFα-skewed activity over IL-1β in our ICAM-1 assay, whereas the effect of a lipid raft-disrupting reagent (MCD) was negligible. These results suggest that the mechanism of action for TQ compounds was prevention of ligand-dependent internalization of TNFαRI, which is a prerequisite for recruitment of TRADD. It is known that there are multiple mechanisms for endocytosis, and that each mechanism is differentially involved in various receptor systems [53]. Our data suggest that a TQ compound (R-7050) has a clear selectivity toward TNFα and to a slightly weaker extent toward the LPA receptor which belongs to a GPCR class over IL-1β, tyrosine kinase, or Fas receptor systems. Therefore, TQ compounds specifically inhibit a certain mechanism(s) of endocytosis that is shared with LPA rather than inhibiting global endocytotic processes, although the exact target is unknown.

Furthermore, TQ compounds have also been studied in relation to their capability to modulate subclasses of adenosine receptors (ARs) [54]. Because adenosine is known to participate in regulation of immune systems, typically through cAMP- and/or Ca^{2+}-dependent signaling pathways [55], agents that modulate cAMP (Forskolin and Rolipram) or Ca^{2+} influx (Ionomycin) did not affect TNFα/IL-1β-induced IκB degradation or ICAM-1 expression in our system (data not shown). Therefore, we believe that activity of TQ compounds on TNFα functions is novel and is not mediated by modulation of ARs that also belong to a GPCR class.

In conclusion, we demonstrate a class of small-molecule TNFα antagonists that block most TNFα functions by inhibition of intracellular events rather than inhibiting ligand trimerization or ligand-receptor interaction. Detailed mechanistic studies indicated that these compounds impaired the formation of signal-initiating complexes that consisted of TNFαRI, TRADD, and RIP1 through inhibition of ligand-induced receptor endocytosis, which is required for signaling. Thus, our cell-based screening strategy allowed us to find inhibitors that broadly inhibited TNFα-signaling events, and in so doing, to identify a critical link in a highly complex signaling network that is often redundant. This strategy also uncovered potential modulators

of cytokine receptor and plasma membrane dynamics, which are otherwise difficult to obtain in a cell-free system.

SIGNIFICANCE

The success of protein-based therapy against TNFα for rheumatoid arthritis and some other immunological diseases has provided proof of concept for the use of anti-TNFα therapy in humans. However, there are several intrinsic problems with these protein-based drugs, such as a lack of oral availability, potential antigenicity, and low tissue-penetrating capabilities. To circumvent such problems, the quest for small-molecule inhibitors is increasingly growing, with anticipation to replace protein-based drugs or, at least, to give physicians and their patients more options for treatment. In this study, we present a class of compounds that inhibit both survival (NFκB and stress pathways) and death pathways induced by TNFα, mimicking the property of anti-TNFα antibodies or soluble TNFα receptors that block all of the biological functions of TNFα. This activity of compounds is unique, because many other small-molecule inhibitors, such as IKKβ, proteasome, and caspase inhibitors, block only a subset of TNFα-induced signaling pathways. The mechanism of action of our compounds may not confer perfect selectivity for TNFα, because several other receptor classes also require internalization for signaling. However, they were at least selective over IL-1β-induced signals as well as over Fas- and C6-ceramide-induced death signaling pathways besides nonrelated tyrosine kinase receptor systems. Although further work is necessary to understand more precisely the mechanism of action and selectivity over other signals, our observations provide an important tool to explore the possibility of inhibiting TNFα signaling with small molecules.

EXPERIMENTAL PROCEDURES

Materials

The following materials were obtained from the indicated sources: human insulin, monodansylcadaverine (MDC), phenylarsenic oxide (PAO), methyl-β-cyclodextrin (MCD), actinomycin-D (Act-D), lysophosphatidic acid (LPA), and a proteasome inhibitor (MG132) were purchased from Sigma (St. Louis, MO, USA). Human TNFα (catalog number 300-01A), human IL-1β (200-01B), human EGF (100-15), human IGF-1 (100-11), and human FAS-L (310-03) were from Peprotech (Rocky Hill, NJ, USA). IKK-β kinase inhibitor IKK2-VI (401483) and caspase inhibitor Z-VAD-fmk (219007) were purchased from Calbiochem (La Jolla, CA, USA). C_6-ceramide (ALX-303-003-M005) was from Axxora, LLC (San Diego, CA, USA). Mouse anti-CD54 (MHCD5400-4) was from Caltag Laboratories (Burlingame, CA, USA); rabbit anti-IκBα (sc-371) and rabbit anti-NFκB p65 (sc-109) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); human phospho-SAPK/JNK ELISA kit (7325), human phospho-AKT ELISA kit (7252), mouse anti-IκBα (9247), mouse anti-phospho-IκBα (Ser32/36) (9246), rabbit anti-phospho-MKK4 (Ser257/Thr261) (9156), rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) (9251), rabbit anti-phospho-ATF2 (Thr69/71) (9225), rabbit anti-phospho-MKK3/6 (Ser189/207) (9231), rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) (9215), rabbit anti-phospho-MAPKAP2 (Thr334) (3047), and rabbit anti-phospho-IKKα (Ser180)/IKKβ (Ser181) (2681) were from Cell Signaling Technology (Boston, MA, USA); mouse anti-TRADD (610572) and mouse anti-RIP1 (610458) were obtained from BD Biosciences Pharmingen (San Diego, CA, USA); goat anti-sTNFR1 (AB-225-PB) was from R&D Systems (Minneapolis, MN, USA). HRP-conjugated anti-mouse and anti-rabbit IgG were from Jackson Laboratory (Bar Harbor, ME, USA); HRP-conjugated anti-mouse IgG2a (02017E) was obtained from BD Biosciences Pharmingen; and Alexa Fluor 488-conjugated goat anti-rabbit IgG (A-11008) was from Molecular Probes (Eugene, OR, USA). All human tumor cell lines used in this study were from the American Type Culture Collection (Manassas, VA, USA).

High-Throughput Screening

Compounds were dissolved in DMSO at a final stock concentration of 10 mM and stored at –80°C. Compound library screening was performed using a fully integrated, programmable robotic liquid handling system (Biomek FX, Beckman Coulter, Fullerton, CA, USA) with an integrated plate reader (Accent Fluoroscan, Ramsey, MN, USA) and environmentally controlled plate carousel set at 37°C and 5% CO_2. A549 cells (1×10^4/well) were seeded overnight into 96-well white flat clear-bottomed plates (Costar, Cambridge, MA, USA) in 100 μl of culture medium. The next day, 100 μl aliquots from the ~300,000-compound library prepared in the same culture medium were added at a final concentration of 10 μM in 0.2% (v/v) DMSO. Cells were preincubated with compounds for 1 hr prior to a 4 hr stimulation with either TNFα or IL-1β (1 ng/ml final concentration). At the end of the stimulation period, cells were stained for ICAM-1 using mouse anti-human CD54 monoclonal antibody (Caltag Laboratories) and HRP-conjugated anti-mouse IgG2a (BD Biosciences Pharmingen) added together at final dilutions of 1:1,000 and 1:10,000, respectively. Following 1 hr of incubation at 37°C, plates were washed four times using 300 μl PBS and developed using supersignal ELISA pico chemiluminescent substrate (Pierce, Rockford, IL, USA). For EC_{50} determination, cells were stimulated with either TNFα (25 ng/ml) or IL-1β (10 ng/ml) only for 4 hr to avoid secondary stimulation by other cytokines produced by the cells.

Image-Based NFκB Nuclear Translocation Assay

One day before the assay, HeLa cells were seeded into 96-well plates at a density of 2000 cells/well. On the day of assay, cells were preincubated with serially diluted test compounds for 1 hr and stimulated with either TNFα (25 ng/ml) or IL-1β (10 ng/ml) for 30 min at 37°C. Cells were washed twice with PBS and fixed using methanol for 1 hr at room temperature. After blocking the cells with 1% BSA in PBS-Tween 20 buffer for 1 hr, cells were treated overnight with the same buffer containing anti-NFκB p65 rabbit polyclonal antibody (1:1000 dilution) followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG staining (1:1000 dilution) for 3 hr at room temperature using a protocol described previously [56]. Cells were also costained with 4′,6-diamidino-2-phenylindole (DAPI) for 1 hr to track the nuclear morphology. Digital images were captured using a 10× objective on a Zeiss Axiovert S100 (Carl Zeiss MicroImaging, Thornwood, NY, USA) microscope equipped with a UV filter set and a Photometrics (Tucson, AZ, USA) camera.

Cell Death and Viability Assays

Cell viability was assessed using the celltiter-glo luminescent cell viability assay kit from Promega (Madison, WI, USA). TNFα-sensitive ME180 cells were preincubated with test compounds for 1 hr at 37°C and stimulated with various concentrations of TNFα for 24 hr. At the end of the incubation period, culture media were replaced with 50 μl of prediluted celltiter-glo reagent (1:1 with PBS) and relative luminescence intensity was measured using a microtiter plate reader. Two known NFκB-pathway inhibitors (IKK2-VI and MG132) and an RNA-synthesis inhibitor (Act-D) known to enhance TNFα-induced cytotoxicity were used as controls.

Caspase Assay

The effect of compounds on caspase activity was determined using the Caspase-Glo 3/7 assay kit (G8091) obtained from Promega.

ME180 cells were preincubated with test compounds for 1 hr at 37°C and stimulated with various concentrations of TNFα for 24 hr. At the end of the incubation period, culture media were replaced with 50 μl of diluted caspase-3/7 reagent (1:1 with PBS) and relative luminescence intensity was determined following the manufacturer's instructions. A proteasome inhibitor, MG132 (known to increase TNFα-induced cytotoxicity), and a general caspase inhibitor, Z-VAD-fmk, were used as controls.

Immunofluorescence Analysis of TNFα Receptor Internalization

This study was carried out as reported by Schutze et al. [35]. Briefly, HeLa cells were seeded onto 12-well tissue culture plates containing sterile circular microscope coverslips. Following preincubation of cells with a test compound or a positive control (MDC) for 1 hr at 37°C, the temperature was shifted to 4°C and biotinylated human TNFα (100 ng/ml) provided in the Fluorokine kit (NFTA0) obtained from R&D Systems was added to the culture medium. After 2 hr, cells were washed with PBS to remove unbound TNFα, and biotinylated TNFα-TNFαR complex was allowed to internalize for 10, 30, and 60 min. Cells were then washed twice with PBS and fixed using cold methanol (90%) for 30 min. Cells were again washed with PBS and blocked with Super Block solution (Pierce) containing 0.5% Triton X-100 for 2 hr at room temperature. Cells were stained with FITC-conjugated avidin (1:50 dilution) overnight in the same blocking solution at 4°C. Coverslips were flipped onto glass slides preloaded with a drop of Vecta-shield mounting media (Vector Laboratories, Burlingame, CA, USA). Localization of the ligand-receptor complex was examined under an Axiovert S100 fluorescence microscope (Carl Zeiss MicroImaging).

Immunoprecipitation of TNFαRI-Associated Signaling Proteins and Western Analysis

For immunoprecipitation of TNFαRI-associated signaling proteins, A549 cells (1×10^7 cells in a 10 cm dish) were preincubated with or without test compounds for 1 hr at 37°C and stimulated with TNFα (50 ng/ml) for 10 min. Following stimulation, cells were washed with PBS and lysed with 500 μl of RIPA buffer composed of 10 mM Tris (pH 7.2), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA, complete protease inhibitor cocktail, 2 mM sodium fluoride, and 1 mM sodium orthovanadate. Plates were then incubated for 30 min at 4°C and cell lysates were harvested by gently scrapping off the cells from plates using a cell scraper. After centrifugation (14,000 rpm, 20 min, 4°C), the supernatant was collected and the protein content was determined using a Micro-BCA protein estimation kit (Pierce). For the TNFαRI immunoprecipitation experiment, 500 μl of supernatant was precleared using protein A/G agarose beads (50 μl of a 50% slurry; Santa Cruz Biotechnology) and incubated with a mixture of goat anti-TNFαRI polyclonal antibody (5 μg) plus 50 μl of protein A/G agarose beads overnight at 4°C with constant end-to-end mixing. Immune complexes were washed three times with RIPA lysis buffer and resuspended in the same buffer (50 μl).

Proteins from total cell lysates and immunoprecipitates were boiled in 4× NuPAGE sample buffer containing lithium dodecyl sulfate/dithiothreitol and subjected to SDS-PAGE (4%–12% bis-Tris gradient gels). Proteins were electroblotted onto PVDF membranes and probed with appropriate primary antibodies followed by HRP-conjugated secondary antibodies. Blots were then developed using an enhanced chemiluminescence plus reagent (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA) and detected on Kodak Bio-MAX MR film (VWR Scientific, San Francisco, CA, USA).

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