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A novel class of potent NF-κB signaling inhibitors

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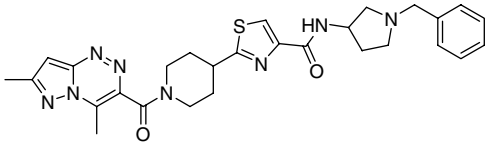
Abstract—A novel class of NF-κB pathway signaling inhibitors was discovered by virtual screening, medicinal chemistry, and QSAR analysis. An initial set of compounds inhibited NF-κB signaling in a whole cell reporter gene assay in the micro-molar range. Activity was improved step by step by medicinal chemistry to yield nano-molar signaling inhibitors.

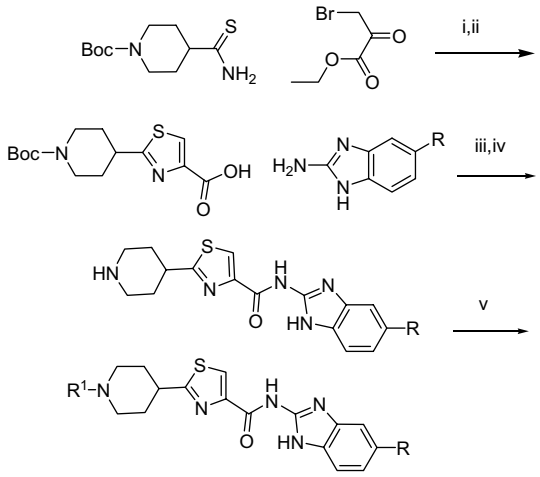
The important role of the nuclear factor κB (NF-κB) signaling pathway in cancer, immunology, and inflammation is well established.[1] For example, in antigen stimulated lymphocytes, NF-κB controls the expression of multiple genes essential for survival, proliferation, and effector function.[2,3] Additionally, some viruses take advantage of the NF-κB signaling pathway of the host cell for their own replication.[4,5] The approach of targeting host pathways for anti-viral therapy holds the promise of a low potential for the development of resistant strains. Finally, it has been reported that NF-κB down-regulation can sensitize cells to the anti-viral effects of interferon.[6,7]

The mechanism of action of some known drugs or folk medicines is presumed to function via the inhibition of NF-κB signaling pathway.[8] In general, the NF-κB signaling pathway consists of a complex set of biochemical principles, such as receptors, protein adapters, protein kinases and phosphoprotein binding proteins, DNA binding proteins, and transporter proteins. A series of compounds targeting various enzymes in this pathway have been developed.[8,9] Other compounds, such as proteasome inhibitors, were shown to interfere with the NF-κB signaling pathway by inhibiting degradation of IκB.[10]



Compound ***1***. Proteasome IC_{50} 0.58 μM, NF-κB inhibition 30% at 20 μM, PBMC proliferation IC_{50} 37 μM.

Virtual screening for proteasome inhibitors[11] resulted in the discovery of compounds, for example, compound **1**, with inhibitory activity for the proteasome,[12] NF-κB signaling,[13] and PBMC proliferation.[14]



Scheme 1. Reagents and conditions: (i) DMF, heat; (ii) NaOH; (iii) HBTU, DIEPA, DMF, 75 °C; HCl, Dioxan, DCM, 2 h; R^1-Cl, DIEPA, 80 °C, 3 h, DMF.

Keywords: NF-κB; Nuclear factor κB; Signaling; Inhibitor; Anti-viral; Anti-inflammatory.
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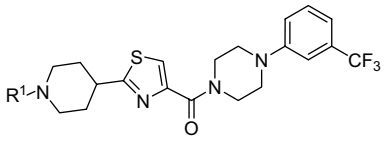
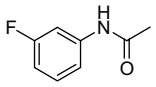
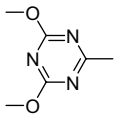
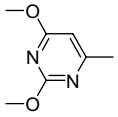
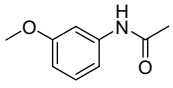
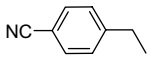
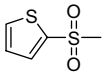
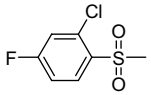
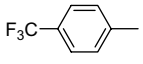
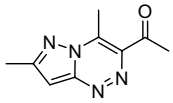
Accordingly, the piperidinyl-thiazole scaffold was explored by medicinal chemistry. The synthesis proceeded as published in a patent (see Scheme 1).[15] This procedure allows the synthesis of analogs modified at the N- and C-terminal of the molecule.[16] All building blocks were from commercial sources. Some of the 2-aminobenzimidazoles were synthesized from the appropriate commercial *o*-phenylenediamine by the reaction with cyanogen-bromide.[17] Heterocycles for the N-terminal modification were from commercial source. The heterocycle for compound **22** was synthesized according to Ref. 18 and the heterocycle for compound **11** according to Ref. 19.

From a series of C-terminal modifications substituted phenyl-piperazine analogs were found to have superior activity (Table 1, compounds **2**–**10**). In this set of compounds low micro-molar activities in the NF-κB reporter gene assay were obtained. But, in spite of an effort with many analogs we were not able to reach lower than micro-molar activity with this series. The structure–activity relationships seen in the cell-based NF-κB reporter gene assay, however, could not be explained consistently by proteasome inhibition. For example, some compounds showed considerably higher activities in the reporter gene assay compared to the proteasome enzyme assay (e.g., compound **15**: NF-κB EC_{50} 0.3 μM, proteasome $IC_{50} > 3$ μM) while other compounds were inactive in the NF-κB assay but active in the proteasome assay (e.g., compound **9**: NF-κB $EC_{50} \sim 20$μM, proteasome IC_{50} 0.48 μM). Additionally, even some compounds (e.g., compound **13**) with activity in the proteasome enzyme assay did not show activity in a secondary proteasome assay, which uses native proteasome extracted from cells.[20] Due to the particular importance of the NF-κB signaling pathway further efforts were directed to optimize the piperidinyl-thiazole scaffold with respect to inhibition of this pathway.

Table 1. Phenyl-piperazine analogs



Compound	R^1	NF-κB EC_{50} (μM)
2		3.4
3		4.7
4		5.0
5		7.2
6		8.5
7		~20
8		~20
9		~20
10		>20

In order to guide the optimization of the compounds a QSAR model was used. The QSAR model of the NF-κB activities was built using 28 MOE descriptors and ridge regression ($\lambda = 1$, 20 effective degrees of freedom). This model was incrementally refined as more experimental data became available and finally reached a correlation coefficient of $R^2 = 0.84$, cross-validated $Q^2 = 0.58$, and a root mean square error of prediction (RMSEP) of 0.34 log units as validated with a leave-multiple-out bootstrapping procedure. This is close to the experimental error (SD) of 0.2 log units as estimated from multiple measurements of two selected compounds ($n = 6$ and $n = 7$). Compounds that showed a negative effect on cell viability were not considered in this model (Fig. 1).

Selection of building blocks with respect to availability and predicted NF-κB activity resulted in a series of compounds, which is depicted in Table 2 with compounds

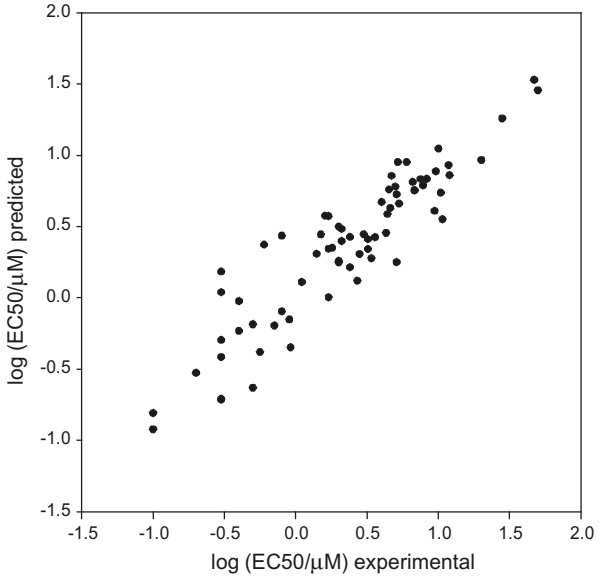
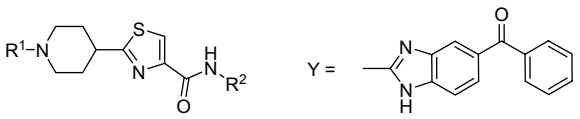


Figure 1. QSAR of NF-κB activity of 71 compounds as determined in the reporter gene assay.

Table 2. Amino-benzimidazole analogs



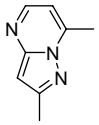
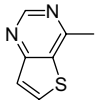
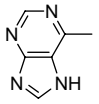
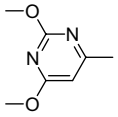
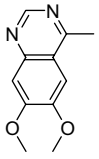
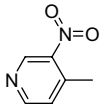
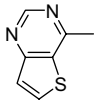
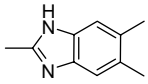
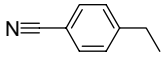
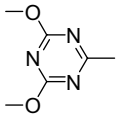
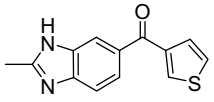
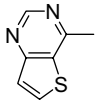
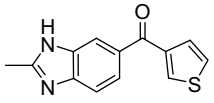
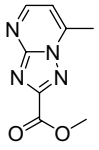
Compound	R^1	R^2	NF-κB EC_{50} (μM)
11		Y	0.1
12		Y	0.1
13		Y	0.2
14		Y	0.3
15		Y	0.3
16		Y	0.3
17		Y	0.3
18			0.3
19		Y	0.3
20			0.4
21			0.5
22		Y	0.5

Table 2 (*continued*)

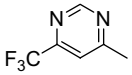
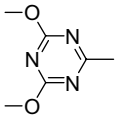
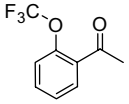
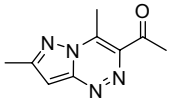
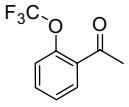
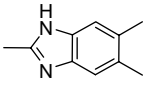
Compound	R^1	R^2	NF-κB EC_{50} (μM)
23		Y	0.5
24		Y	0.8
25		Y	0.8
26		Y	0.9
27			3.0

Table 3. Inhibition of human PBMC proliferation

Compound	IC_{50} (μM)
3	15
12	3.6
13	2.2
22	2.5

11–**27**. The replacement of the phenyl-piperazino moiety by an amino-benzimidazole was tolerated but did not improve activity considerably (compound **27**). On the average the substitution of the amino-benzimidazole by a phenylketo residue resulted in a substantial improvement. (e.g., compound **25** vs **27**, compound **13** vs **18**). At the N-terminus of the compounds different aromatic substituents are tolerated without effecting activity, however heterocycles similar to adenine mimics did increase potency. Finally, potent inhibitors of the NF-κB signaling pathway were identified.

To solidify the assumption that these compounds have immunosuppressive activity we tested a subset of compounds in a PBMC proliferation assay. Due to the phytohemagglutinin stimulus used in the assay the measured effect can be attributed mainly to T-cells. A significant suppressive effect was detected (Table 3).[14]

In conclusion, we have discovered a novel class of potent NF-κB signaling inhibitors.[21] Such compounds have the potential to be useful for the treatment of inflammatory and autoimmune diseases, for anti-viral therapy and interferon co-therapy, and could lead to a novel concept of host cell pathway modulation toward an anti-viral therapy.

Acknowledgments

We thank Stefan Strobl and Daniel Vitt for helpful discussions and support, and Roland Baumgartner for measuring the proteasome enzyme data.

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11. Virtual screening: proteasome X-ray structure (PDB ID 1IRU) was used for virtual screening due to the sequence identity within the chymotryptic subpocket of the bovine proteasome located on the L and M chain and the human proteasome X and HC5 chains. Protein–ligand docking was used for virtual screening with the binding pocket defined around the catalytically active threonine (THR1) containing the subpockets S1, S2, and S3. Virtual high-throughput screening was done using the docking engine of 4SCan/ProPose (Seifert, M. H.; Wolf, K.; Vitt, D. *DDT Biosilico* **2003**, *1*, 143.; Seifert, M. H.; Schmitt, F.; Herz,

T.; Kramer, B. *J. Mol. Model.* **2004**, *10*, 342) on 4SC's database of five million chemical compounds. The 5000 best docking hits were submitted to filtering: a distance cutoff of 10 Å to THR1 was applied, molecular properties (molecular weight, topological polar surface area, number of rotational bonds) were filtered, unwanted functional groups were omitted, and finally a visual inspection of the docked structures yielded a ranking list. A representative set of 236 compounds was selected for purchasing and biochemical testing.

12. 20S proteasome enzyme assay: (Stein, R. L.; Melandri, F.; Dick, L. *Biochemistry* **1996**, *35*, 3899.) compounds were characterized by monitoring the inhibitory effect on the chymotryptic activity of the human 20S proteasome (Biomol). The assay was performed using a Tecan Ultra plate reader and the fluorogenic substrate Suc-LLVY-AMC (Bachem). In a black 96-well polypropylene plate, 2 μl of compound, dissolved in 100% DMSO, was mixed with 50 μl substrate solution (25 mM HEPES, pH 7.5, 0.5 mM EDTA, and 75 μM Suc-LLVY-AMC). Reaction was initiated by adding 150 μl proteasome solution (1.3 μg 20S proteasome in 25 mM HEPES, pH 7.5, 0.5 mM EDTA, and 0.033% (w/v) SDS, pre-incubated for 10 min at room temperature). Substrate hydrolysis was followed by fluorescence spectroscopy (excitation wavelength: 360 nm; emission wavelength: 465 nm) for 20 min at 30 °C and signals were calculated as relative fluorescence units (RFU) per minute. For the determination of the IC_{50} values (concentration of inhibitor required for 50% inhibition) eight inhibitor concentrations were applied. Data points were recorded in triplicate on single measurement day. To obtain dose–response curves and IC_{50} values data were fitted to a four parameter logistic function using SigmaPlot.
13. NF-κB reporter gene assay: The NF-κB reporter gene assay was prepared with A549-NF-κB-SEAP cell line (CCS cell culture service, Hamburg, Germany) according to manufacturer's instructions. In short, A549 cells stably transfected with pNF-κB-SEAP reporter gene plasmid were plated at 2×10^4/well and allowed to attach overnight. The cells were subsequently incubated for 5 h with described compounds at 100, 30, 10, 3, 1, 0.3, 0.1, and 0 μM and then stimulated with 10 ng/ml TNF-α for 22 h. The supernatant of the cell was analyzed for SEAP activity using a chemiluminescent SEAP reporter gene assay (Roche, Mannheim, Germany), and a cell viability assay was prepared using a CellTiter-BluTM Cell Viability Assay (Promega, Mannheim, Germany). For each concentration of the compound four replicates were measured.
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