Original article

EFFECTS OF SOME MICHAEL TYPE ADDITION PRODUCTS ON VARIOUS CYTOKINES

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Abstract

The aim of this research in to investigate the anticytokine activities of the 2-[(2-nitro-1-phenylpropyl)thio]benzoic acid (1), 2-[(2-nitro-1-phenylethyl)thiomethyl]benzimidazole (2) and 2-[(2-nitro-1-phenylpropyl)thiomethyl]benzimidazole (3) derivatives in human primary cells and cell lines. Cytokines are messengers for the regulation of the inflammatory cascades with Tumor Necrosis Factor-α (TNF-α), Interleukin (IL-1β, IL-2, IL-4 and IL-8), Gamma Interferon (IFN-γ) working synergistically.

In this study which is performed in cell assay, inhibition capacity of compound 1, 2 and 3 derivatives againts TNF-α, IL-β, IL-8, IL-2, IL-4 and IFN-γ production by human whole blood have been measured . The test results were shown that 1, 2 and 3 derivatives have dose-dependent inhibitions on the release of IL-1β, IL-8 and TNF-α in lipopolysaccharide (LPS) stimulated human whole blood and IL-2, IL-4 and IFN-γ in phorbolacetate (PHA) stimulated in human whole blood.

Key words: *Anticytokine agent, Thiobenzoic acid, Thiomethylbenzimidazole, TNF-α*

Bazı Michael Tipi Katım Ürünlerinin Değişik Sitokinler Üzerinde Etkileri

Bu çalışmanın amacı 2-[(2-nitro-1-fenilpropil)tiyo]benzoik asit (1), 2-[(2-nitro-1-feniletil)tiyometil] benzimidazol (2) ve 2-[(2-nitro-1-fenilpropil)tiyometil]benzimidazol (3) türevlerinin antisitokin aktivitelerinin insan primer hücrelerinde ve hücre hatlarında araştırılmasıdır. Sitokinler inflamatuar basamakların oluşumunda Tümör Nekrozis Faktör-α (TNF-α), İnterlökin (IL-1β, IL-2, IL-4 ve IL-8), Gama interferon (IFN-γ) ile beraber çalışan habercilerdir. Hücre kültüründe yapılan bu çalışmada bileşik 1, 2 ve 3 türevlerinin insan tam kanı tarafından üretilen TNF-α, IL-β, IL-8, IL-2, IL-4 ve IFN-γ üzerindeki inhibisyon kapasiteleri ölçülmüştür. Test sonuçları 1, 2 ve 3 türevlerinin lipopolisakkarit (LPS) ile uyarılmış insan tüm kanında IL-1β, IL-8 ve TNF-α ve forbolasetat (PHA) ile uyarılmış insan tüm kanında IL-2, IL-4 ve IFN-γ salınımında doza bağımlı inhibisyona sahip olduklarını göstermiştir.

Anahtar kelimeler: *Antisitokin ajanlar, Tiyobenzoik asit, Tiyometilbenzimidazol, TNF-α*

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INTRODUCTION

Chronic inflammatory diseases such as rheumatoid arthritis (RA) and inflammatory bowel diseases affecting millions people worldwide (1). It is now clear that inflammatory cytokines, such as interleukine-1(IL-1) and tumor necrosis factor-α (TNF-α) play an important role in these diseases (2). There is over expression of this cytokine at both protein and RNA levels in immune-mediated inflammatory diseases including RA and other inflammatory arthritides (3), Crohn's disease (4), multiple sclerosis (5), ankylosing spondylitis (6, 7), sistemic lupus erythematosus (8, 9), insulin-dependent diabetes mellitus (10, 11), psoriasis (12) and autoimmune myocarditis (13,14). The inhibition of TNF-α and IL-1 presents a useful therapeutic strategy to suppress the inflammation and prevent joint damage caused by RA, as shown by the newer biologic therapies for RA (etanercept, infliximab, and adalimumab and anakinra) that target these cytokines (15). Part of the impetus to develop new treatments has been a growing dissatisfaction with the currently available ones (16).

Traditional drugs treat only disease symptoms, rather than their root causes and typically slow but do not prevent disease progression (17). For example, nonsteroidal anti-inflammatory drugs (NSAIDs) are the most common treatment for RA patients, but these compounds do little or nothing to prevent ongoing destruction of cartilage and bone (18-20). The more powerful disease modifying antirheumatic drugs, such as methorexate and corticosteroids, may help ameliorate the symptoms RA, but rarely induce sustained remission and can have toxicities that prevent their long-term use (16, 21-24). Therefore inhibition of the over production of inflammatory mediators is an important therapeutic goal for anti-inflammatory drug development.

In the past few years our knowledge about the molecular basis of inflammation have been uncovered and now much is known about the primary role of pro-inflammatory cytokines such as IL-1 and TNF-α. In the early '90s anti-cytokine therapies have revealed to be highly effective in reducing the local and systemic inflammation in patients with RA, Crohn's Disease and psoriasis (25). The inhibition of cytokines in particular TNF-α has been successful in several clinical trials for the treatment of these diseases and conditions (26).

The clinical success of TNF-α soluble receptor etanercept (Enbrel®) and TNF-α monoclonal antibodies infliximab (Remicade®) and adalimumab (Humira®) has validated TNF as a target for the treatment of inflammatory diseases (27). As these biological drugs have shown that the lowering of pro-inflammatory cytokine levels is valid treatment for RA patients (28). Modern small-molecule approaches to the treatment of RA have targeted cell signaling systems, in particular the inhibition of the p38α mitogen-activated protein (MAP) kinase cell-signaling pathway (29). Inhibition of p38 kinase is thus an attractive approach to the treatment of both pain and inflammation in RA patients (30).

A number of small molecules lead to reduced cytokine levels *in vitro* and *in vivo* by the inhibition of key enzymes involved in the biosynthesis of TNF-α and IL-1ß. Inhibitors of the p38α MAP kinase (31) through their downstream blockage of the production of TNF-α, IL-1ß, IL-6, cyclooxygenase-2, and arachidonic acid mobilization (32) also have tremendous therapeutic potential. Over the past decade, the pursuit of p38α MAP kinase inhibitors has received an extraordinary level of attention in the medicinal chemistry (33).

Pyridylimidazoles such as the prototypic SB203580 (**4**) are significantly inhibited in a competitive manner by synthetic compound derived from the first generation anticytokine agents. But other structural series have been reported (34).

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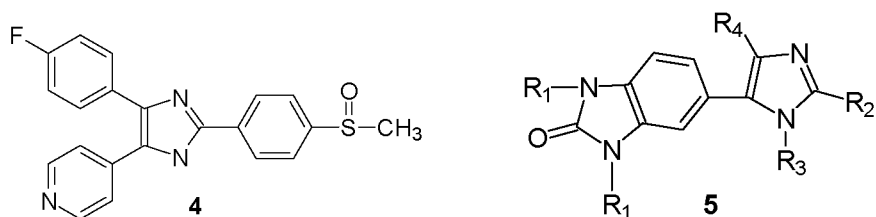
Numerous benzimidazole derivatives were prepared as small molecular anticytokine agents and drug candidates for the treatment of chronic inflammatory diseases. For example, synthesis and *in vitro* p38α inhibitor activity of novel series of benzimidazolone is described (35). Various imidazole analogues of benzimidazolone (**5**) showed potent p38α inhibitor activity comparable to that of several previously reported p38 inhibitors is observed for this novel chemotype.
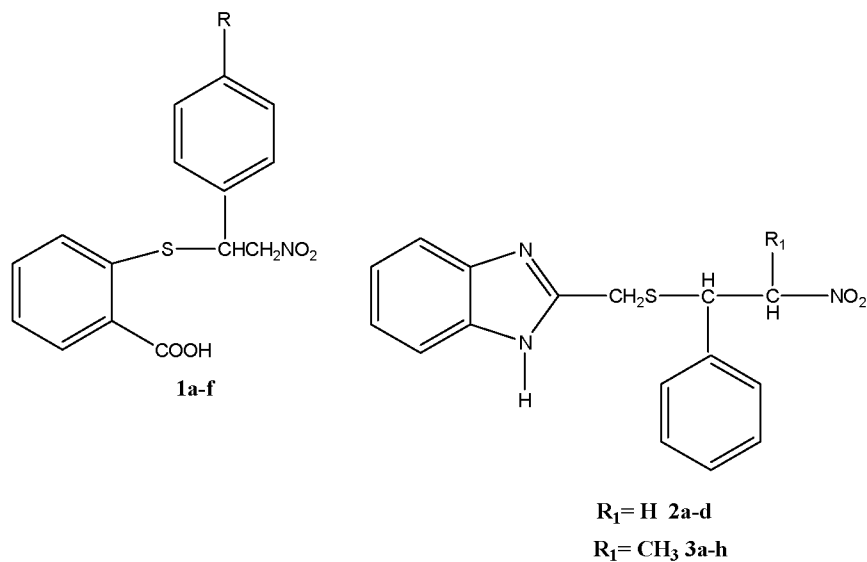
Recently, potent and selective 2-aminobenzimidazole-based p38α MAP kinase inhibitors have been reported (36). Results show that activity obtained for these aminobenzimidazoles (**6**) in inflammation and histopathology scores compares favorably with that of other advanced molecules in clinical development.
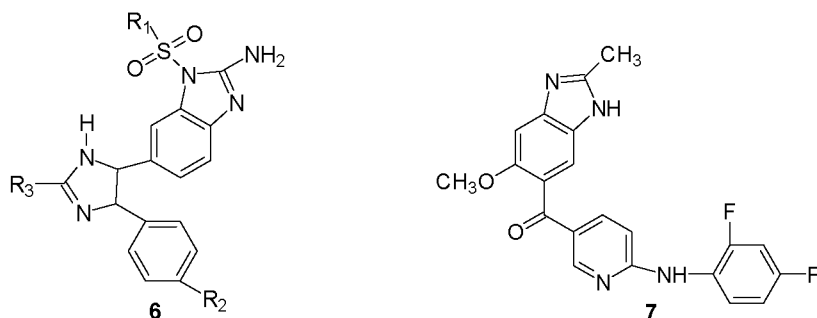
Furthermore, pyridinoylbenzimidazole derivatives were synthesized and evaluated *in vitro* as p38α inhibitors and *in vivo* several models of RA. Oral activity was found to depend upon substitution. Pyridinoyl-5-methoxybenzimidazole (**7**) derivative have been shown highest efficacy and selectivity (36).

In the light of these results, we studied herein anticytokine activity of 2-[(2-nitro-1-phenylethyl)thio]benzoic acid **1**, 2-[(2-nitro-1-phenylethyl)thiomethyl]benzimidazole **2** and 2-[(2-nitro-1-phenylpropyl)thiomethyl]benzimidazole **3** derivatives that are structurally related to the small molecule cytokine inhibitors. Anticytokine activity of **1**, **2** and **3** derivatives were tested on TNF-α, IL-1ß, IL-8, IL-2, IL-4, IFN-γ production by human whole blood. Synthesis and detailed structure analysis of these compounds have been published in our previous papers (37-39). Also antimicrobial activity of compounds **1**, **2**, and **3** derivatives were investigated in recent studies (40, 41). The title compounds which their anticytokine activities have been investigated in human primary cells and cell lines in this study, are listed Table 1.

Table 1. Synthesized 2-[(2-nitro-1-phenylethyl)thio]benzoic acid **1**, 2-[(2-nitro-1-phenylethyl)thiomethyl]benzimidazole **2** and 2-[(2-nitro-1-phenylpropyl)thiomethyl]benzimidazole **3** derivatives.

R_1= H **2a-d**
R_1= CH_3 **3a-h**

Compound	R	R_1
1a	Br	
1b	Cl	
1c	CH_2CH_3	
1d	OCH_2CH_3	
1e	OCH_3	
1f	NO_2	
2a	Br	H
2b	Cl	H
2c	OCH_2CH_3	H
2d	OCH_3	H
3a	H	CH_3
3b	Br	CH_3
3c	Cl	CH_3
3d	CH_2CH_3	CH_3
3e	OCH_2CH_3	CH_3
3f	OCH_3	CH_3
3g	$N(CH_3)_2$	CH_3
3h	NO_2	CH_3

MATERIAL AND METHODS

Chemistry

The derivatives of β-nitrostyrene and β-methyl-β-nitrostyrene were synthesized according to the literature method (42, 43). The addition products of **1** derivative have been synthesized by

addition of thiosalisilic acid to the double bound of β-nitrostyrene derivatives. Another addition products of **2** and **3** derivatives have been synthesized by addition of 2-mercaptomethylbenzimidazole to the double bound of β-nitrostyrene or β-methyl-β-nitrostyrene derivatives (37-39).

In vitro cell assay methods

Cytokine inhibitory effects of compound **1**, **2** and **3** derivatives were studied by using peripheral whole blood samples obtained from healthy volunteer and commercially available Enzyme Linked Immunosorbent Assay kits (ELISA). Concentrations of IL-2, IL-4 and IFN-γ after phorbolacetate (PHA) stimulation were given in Table 2. Also concentrations and inhibition rates of IL-1β, IL-8 and TNF-α after lipopolysaccharide (LPS) stimulation were shown in Table 3. Prednisolone (0.03 μg/ml) was used as reference drug.

Peripheral whole-blood cultures

One ml of whole blood collected from the healthy volunteer was collected in a heparinized (20 U/ml) tube. 0.1 ml of the heparinized blood was transferred in a 24-well multicluster plate and added 1.0 ml RPMI-1640 medium into each well and incubated at 37 °C for 24 h in the absence or presence of 3 μg/ml of 0.01 μl LPS or 10 μg/ml PHA. The culture supernatants were then mixed with PBS/0.05% thiomersal at the ratio of 1:2 and were assayed using the spesific ELISA method for TNF-α, IL-1ß, IL-8, IL-2, IL-4 and IFN-γ (Otsuka Pharmaceutical Co., Ltd, Japan).

Measurement of the effects on cytokine biosynthesis

The activities of TNF-α, IL-1ß, IL-8, IL-2, IL-4 and IFN-γ were investigated in peripheral whole blood collected from healthy volunteers as previously described by Yeşilada et al (44). Briefly, heparinized peripheral whole bloods collected from healthy volunteers were stimulated with either 10 μg/ml PHA for IL-2, IL-4 and IFN-γ or bacterial LPS for IL-1β, IL-8 and TNF-α and incubated in the presence of compound **1**, **2** and **3** derivatives and references compound prednisolone. The cultured supernatants were obtained and concentrations of the cytokines produced from macrophages or lymphocytes were directly determined by a commercially available ELISA. Prednisolone was used as the reference compound in 0.03 μg/ml concentration. 0.1% of dimethyl sulfoxyde (DMSO) was used to dissolve the test compound **1**, **2** and **3** derivatives. Effects of PHA or LPS stimulation, DMSO and reference compound on TNF-α, IL-1ß, IL-8, IL-2, IL-4 and IFN-γ are given in Tables 2 and 3.

Evaluation of results

Releasing activity of a compound on a cytokine is represented as (-) numericals, while inhibitory effect is shown with direct numeric values as described before (45).

RESULTS AND DISCUSSION

As it can be seen in Table 2, **1a**, **1b**, **1d**, **1e**, **3c**, **3d**, ve **3h** have been found more active inhibitors than prednisolone against IL-2 release after PHA stimulation. Rest of the synthesized compounds were shown significant inhibitor activity IL-2 release. Also compound **3b** was exhibited excellent IL-4 release inhibition after PHA stimulation. All of the other compounds were exerted moderate inhibition on IL-4 release. Only compound **1f** have been shown slight inhibition on IFN-γ release after PHA stimulation but inhibitor activity of this compound is superior than prednisolon.

Table 2. Effect of 2-[(2-Nitro-1-phenylethyl)thio]benzoic acid **1**, 2-[(2-nitro-1-phenylethyl) thiomethyl]benzimidazole **2** and 2-[(2-nitro-1-phenylpropyl)thiomethyl]benzimidazole **3** derivatives on various cytokines production in whole blood samples after PHA stimulation

Compound	Volunteers	Inhibitory rates (%)					
		IL-2/PHA		IL-4/PHA		IFN-γ/PHA	
		pg/ml	%	pg/ml	%	pg/ml	%
Stimulation -	V1/ V2			574/410		20/20	
Stimulation +	V1/ V2	57/181		694/720		6318/6614	
DMSO	V1/ V2	40/41	0.0/0.0	746/845	0.0/0.0	6830/6432	0.00.0
Prednisolone	V1/ V2	61/138	-51.1/-1.6	835/665	-11.9/21.3	5677/6545	16.9/-1.8
1a	V1/ V2	86/123	113.6/9.6	484/475	35.1/43.8	6095/5253	10.8/18.3
1b	V1/ V2	84/121	108.8/11.2	410/453	45.0/46.4	6611/5872	3.2/8.7
1c	V1/ V2	51/132	-27.5/2.8	470/400	37.0/52.6	6331/6349	7.3/1.3
1d	V1/ V2	69/169	-71.0/-24.6	475/267	36.4/68.4	6624/5887	3.0/8.5
1e	V1/ V2	68/129	-69.4/5.2	416/430	44.3/49.1	6276/5858	8.1/8.9
1f	V1/ V2	53/72	-32.6/44.7	374/367	49.8/56.5	5331/5598	21.7/13.0
2a	V1/ V2	51/108	-27.5/20.3	433/482	41.9/42.9	6491/6181	5.0/3.9
2b	V1/ V2	42/87	-5.2/36.0	527/583	29.3/31.1	6582/6163	3.6/4.2
2c	V1/ V2	50/112	-24.1/17.5	525/539	29.6/36.3	6337/6175	7.2/4.0
2d	V1/ V2	42/63	-5.2/54.0	530/629	29.0/25.6	6485/6275	5.1/2.4
3a	V1/ V2	59/62	-46.0/54.3	508/747	31.9/11.6	6540/6099	4.2/5.2
3b	V1/ V2	61/67.0	-52.7/50.5	228/682	91.5/19.3	6654/6073	2.6/5.6
3c	V1/ V2	77/101	-92.4/25.8	680/672	8.9/20.5	6889/6569	-0.9/-2.1
3d	V1/ V2	71/79	-76.4/41.9	546/658	26.1/22.2	6857/6710	-0.4/-4.3
3e	V1/ V2	41/39	-1.7/71.3	544/595	27.1/29.6	7144/6435	-4.6/0.0
3f	V1/ V2	47/69	-15.5/48.9	590/428	20.9/49.4	6959/6432	-1.9/0.0
3g	V1/ V2	45/74	-12.1/45.4	443/383	40.6/54.7	6292/6157	7.9/4.3
3h	V1/ V2	66/64	-64.4/52.8	574/502	23.0/40.6	6670/6486	2.3/-0.8

As it seen Table 3 compound **1f** was exhibited potent inhibition (95.2/59.8 %) against TNF-α release after PHA stimulation. Also **1e**, **2a**, **2b**, **3h** were shown significant inhibition on TNF-α release. Furthermore, **3a**, **3b** and **3f** have been found considerably inhibitor against IL-1β after LPS stimulation. On the contrary of these results; **1**, **2** and **3** derivatives are completely inactive on IL-8 after LPS stimulation. As a result of some structural requirements of **1**, **2** and **3** derivatives can be existed on cytokine inhibitor activity. Further pharmacological investigations of these compounds and the structural optimization are in progress.

Table 3. Effect of 2-[(2-Nitro-1-phenylethyl)thio]benzoic acid **1**, 2-[(2-nitro-1-phenylethyl) thiomethyl] benzimidazole **2** and 2-[(2-nitro-1-phenylpropyl)thiomethyl]benzimidazole **3** derivatives on various cytokines production in whole blood samples after LPS stimulation

Compound	Volunteers	Inhibitory rates (%)					
		IL-1β/LPS		IL-8/LPS		TNF-α/LPS	
		pg/ml	%	pg/ml	%	pg/ml	%
Stimulation -	V1/V2			574/410		20/20	
Stimulation +	V1/V2	57/181		694/720		6318/6614	
DMSO	V1/V2	40/136	0.0/0.0	746/845	0.0/0.0	6830/6432	0.00.0
Prednisolone	V1/V2	2169/6303	26.6/11.3	4479/6233	-24.2/-7.3	526/550	28.6/44.0
1a	V1/V2	3046/3041	-3.1/57.2	5045/6488	-39.8/-11.7	459/446	37.7/54.5
1b	V1/V2	3010/5482	-1.9/22.9	6023/5833	-66.9/-0.4	519/769	29.5/21.6
1c	V1/V2	3452/6439	-16.9/9.4	4644/6221	-28.7/-7.1	585/1177	20.6/-20.0
1d	V1/V2	3146/4430	-4.5/37.7	4686/5525	-29.9/4.9	550/971	25.4/1.1
1e	V1/V2	4293/7030	-45.3/1.1	5152/6133	-42.8/-5.5	302/486	59.0/50.5
1f	V1/V2	3637/3442	-9.5/51.6	5233/5536	-44.2/4.7	35/394	95.2/59.8
2a	V1/V2	2421/4465	18.1/37.2	5283/5373	-46.4/7.5	411/401	69.0/58.1
2b	V1/V2	2439/5327	17.4/25.1	5101/5844	-41.4/-0.6	239/403	67.5/58.9
2c	V1/V2	2437/5884	17.5/17.2	5118/5645	-41.9/2.8	456/752	38.0/23.4
2d	V1/V2	2421/7735	18.1/-8.8	4983/6277	-38.1/-8.0	551/664	25.1/32.3
3a	V1/V2	1565/2815	47.0/60.4	5266/5999	-46.0/-3.2	935/1770	-27.0/-80.4
3b	V1/V2	1421/6324	51.9/11.0	5089/6499	-41.1/-11.8	543/257	26.2/97.6
3c	V1/V2	3772/8309	-4.5/-16.9	5464/5711	-51.5/1.7	142/1264	-44.4/-28.8
3d	V1/V2	2662/7480	9.9/-5.2	5394/6032	-49.5/-3.8	720/663	2.2/32.4
3e	V1/V2	6380/1893	10.2/35.9	5389/4917	-42.1/-36.3	1471/923	-49.9/-25.3
3f	V1/V2	1283/4490	56.6/36.8	5347/5481	-48.2/5.7	831/1911	-12.8/-94.7
3g	V1/V2	2818/6306	4.6/11.3	5084/6587	-40.9/-13.4	982/922	-33.4/6.0
3h	V1/V2	3132/8005	-4.6.9/-12.6	5446/5988	-51.0/-3.1	599/1089	58.4/-11.0

REFERENCES

1. **Taylor, C.P., Feldmann, M.,** Rheumatoid arthritis: pathogenic mechanisms and therapeutic targets, *Drug Discov., Today*, 1, 289-295, **2004**.

2. **Dobler, R.M.,** Design and novel synthesis of aryl-heteroaryl-imidazole MAP kinase inhibitors, *Tetrahedron Lett.*, 44, 7115-7117, **2003**.

3. **Larche, M. J., Sacre, S. M,. Foxwell, B. M.,** Pathogenic role of TNFα in rheumatoid arthritis, *Drug Discov., Today*, 2, 367-375, **2005**.

4. **Gustot, T., Lemmers, A., Louis, E., Nicaise, C., Quertinmont, E., Belaiche, J.,** Profile of soluble cytokine receptors in Crohn's disease, *Gut*, 54, 488-495, **2007**.

5. **Cannella, B., Raine, S. C.,** Multiple Sclerosis: Cytokine receptorson oligodendrocytes predict innate regulation. *Ann. Neurol.*, 55, 46-57, **2004**.

6. **Jr. Davis, J. C.,** Understanding the role of tumor necrosis factor inhibition in ankylosing spondylitis, *Semin, Arthritis R*heu., 34, 668-77, **2005**.

7. **Zeerleder, S., Hack, C. E., Caliezi, C., Van Mierlo, G., Eerenberg-Belmer, A., Wolbink, A.,** Activated cytotoxic T cells and NK cells in severe and septic shock and their role in multiple organ dysfunctions, *Clin. Immunol.*, 116:158-165, **2005**.

8. **Sjowall, C., Ernerudh, J., Bengtsson, A. A., Sturfelt, G., Skogh, T.,** Reduced anti-TNFα autoantibody levels coincide with flare in systemic lupus erythematosus, *J. Autoimmun.*, 2, 315-323, **2004**.

9. **Mocellin, S., Marincola, F., Rossi, C. R., Nitti, D., Lise, M.,** The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle, *Cytokine Growth F. R.*, 15, 61-76, **2004**.

10. **Kuczynski, S., Winiarska, H., Abramczy, M., Szczawińska, K., Wierusz-Wysocka, B., Dworacka, M.,** IL-15 is elevated in serum patients with type 1 diabetes mellitus, *Diabetes Res. Clin. Pr.*, 69, 231-236, **2005**.

11. **Harsch, I. A., Brzozowski, T., Bazela, K., Konturek, S. J., Kukharsky, V., Pawlik, T.,** Impaired gastric ulcer healing in diabetic rats: role of heat shock protein, growth factors, prostaglandins and proinflammatory cytokines, *Eur. J. Pharmacol.*, 481, 249-260, **2003**.

12. **Cvetkovic, I., Stosic-Grujicic, S.,** Neutralization of macrophage migration inhibitory factor-novel approach for the treatment of immunoinflammatory disorders, *Int. Immunopharmacol.*, 6, 1527-1534, **2006**.

13. **Okura, Y., Yamamoto, T., Goto, S., Inomato, T., Hirono, S., Hanawa, H.,** Characterization of cytokine and INOS mRNA expression in situ during the course of experimental autoimmune miyocarditis in rats, *J. Mol. Cell Cardiol.*, 29, 491-502, **1997**.

14. **Gong, X., Feng, H., Zhang, S., Yu, Y., Li, J., Wang, J.,** Increased expression of CCR5 in experimental autoimmune miyocarditis reduced severity induced by anti-CCR5 monoclonal antibody, *J. Mol. Cell Cardiol.*, 42, 781-791, **2007**.

15. **Tamayo, N., Liao, L., Goldberg, M., Powers, D., Tudor, Y.Y., Yu, V.,** Design and synthesis of potent pyridazine inhibitors of p38 MAP kinase, *Bioorg. Med. Chem. Lett.*, 15, 2409-2413, **2005**.

16. **Chang, J., Kavanaugh, A.,** Novel therapies for rheumatoid arthritis, *Pathophysiol.*, 12, 217-225, **2005**.

17. **Henry, J. R., Rupert, K. C., Dodd, J. H., Turchi, I. J., Wadsworth, S. A., Cavender, D. E.,** 6-Amino-2-(4-fluorophenyl)-4-methoxy-3-(4-pyridyl)-1*H*-pyrolo[2,3-b]pyridine RWJ 68354): A potent and selective p38 kinase inhibitor, *J. Med .Chem.*, 41, 4196-4198, **1998**.

18. **Schiff, M. H., Whelton, A.,** Renal toxicity associated with disease-modifying antirheumatic drugs used for the treatment of rheumatoid arthritis, *Semin. Arthritis Rheu.*, 30, 196-208, **2000**.

19.	**Raskin, J. B.,** Gastrointestinal effects of nonsteroidal anti-inflammatory therapy, *Am. J. Med.* 106, 3-12, **1999**.

20.	**Chavez, M. L., DeKorte, C. J.,** Valdecoxib: a review, *Clin. Ther.*, 25:817-851, **2003**.

21.	**Schiff, M. H., Whelton, A.,** Renal toxicity associated with disease-modifying antirheumatic drugs used for the treatment of rheumatoid arthritis, *Semin. Arthritis and Rheu.*, 30,196-208, **2000**.

22.	**Van Ede, A. E., Laan, R. F., Blom, H. J., Abreu, R. A., Putte,	L. B.,** Methotrexate in rheumatoid arthritis: An update with focus on mechanisms involved in toxicity, *Semin. Arthritis and Rheu.*, 27,277-292, **1998**.

23.	**Sewell, K., Schein, J. R.,** Osteoporosis therapies for rheumatoid arthritis patients: minimizing gastrointestinal side effects, *Semin. Arthritis and Rheu.*, 30, 288-297, **2001**.

24.	**Anaya, J. M., Diethelm, L., Ortiz, L. A., Gutierrez, M., Citera, G., Welsh, R. A.,** Pulmonary involvement in rheumatoid arthritis, *Semin. Arthritis and Rheu.*, 24: 242-254, **1995**.

25.	**Tincani, A., Andreoli, L., Bazzani, C., Bosiso, D., Sozzani, S.,** Inflammatory molecules: a target for treatment of systemic autoimmune diseases, *Autoimmun Rev.*, 7(1), 1-7, **2007**.

26.	**Townes, J. A., Golebiowski, A., Clark, M. P., Laufersweiler, M. J., Brugel, T. A., Sabat, M.,** The development of new bicyclic pyrazole-based cytokine synthesis inhibitors, *Bioorg. Med. Chem. Lett.*, 14, 4945-4948, **2004**.

27.	 **Lombart, H. G., Feyfant, E., Joseph-McCarthy, D., Huang, A., Lovering. F., Sun, L. H.,** Design and synthesis of 3,3-piperidine hydroxamate analogs as selective TACE inhibitors, *Bioorg. Med. Chem. Lett.*, 17, 4333-4337, **2007**.

28.	**Chen, J. J., Dewdney, N., Lin, X., Martin, R. L., Walker, K.,, Huang, J.,** Design and synthesis of orally active inhibitors of TNF synthesis as anti-rheumatoid arthritis drugs, *Bioorg. Med. Chem. Lett.*, 13,3951-3954, **2003**.

29.	**Brown, D. S., Belfield, A. J., Brown, G. R., Campbell, D., Foubister, A., Masters, D. J.,** A novel series of p38 MAP kinase inhibitors for the potential treatment of rheumatoid arthritis, *Bioorg. Med. Chem. Lett.*, 14, 5383-5387, **2004.**

30.	**Laufer, S., Greim, C., Bertsche, T.,** An in-vitro screening assay for the detection of inhibitors of proinflammatory cytokine synthesis: a useful tool for the development of new antiarthritic and disease modifying drugs, *Osteoarthr. Cartilage*, 10,961-967, **2002**.

31.	**Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D. A.,** Protein kinase involved in the regulation of inflammatory cytokine biosynthesis, *Nature*, 372, 739-746, **2002**.

32.	**Borsch-Haubold, A.G., Kramer, R. M., Watson, S. P.,** Phosphorylation and activation of cytosolic phospholipase A2 by 38-kDa mitogen-activated protein kinase in collagen-stimulated human platelets, *Eur. J. Biochem.*, 245, 751-759, **1997**.

33.	**Hu, Y., Gren, N., Gavrin, L.K., Janz, K., Kaila, N., Li, H.Q.,** Inhibition of Tpl2 kinase and $TNF\alpha$ production with quinoline-3-carbonitriles for the treatment of rheumatoid arthritis. *Bioorg. Med. Chem. Lett.*, 16, 6067-6072, **2006**.

34.	 **Laufer, S.A., Liedtke, A. J.,** A concise and optimized four-step approach toward 2[aryl-]alkylsulfanyl-, 4[5]-aryl-, 5[4]-heteroaryl-substituted imidazoles using alkyl- or arylalkyl thiocyanates, *Tetrahedron Lett.*, 47, 7199-7203, **2006**.

35.	 **Dombroski, M. A., Letavic, M.A., McClure, K.F., Barberia, J.T., Carty, T. J., Cortina, S.R.,** Benzimidazolone p38 inhibitors, *Bioorg. Med. Chem. Lett.*, 14, 919-923, **2004**.

36.	**Dios, A. D., Shih, C. B. Uralde, L. D., Sanchez, C., Prado, M. D. , Cabrejas, M. M.,** Desing of potent and selective 2-aminobenzimidazole-based p38α MAP kinase inhibitors with excellent in vivo efficacy, *J. Med. Chem.*, 48, 2270-2273, **2005**.

37. **Gökçe, M., Berçin, E.,** The addition products of thisalycilic acid and β-nitrostyrenes and their NMR Studies, *J. Fac. Pharm. Gazi*, 13,153-60, **1996**.

38. **Gökçe, M., Berçin, E.,** The products of Michael type addition 2-mercaptomethylbenzimidazole on the derivatives of β-nitrostyrenes and their structure elucidation, *J. Fac. Pharm. Gazi*, 13, 33-44, **1996**.

39. **Berçin, E., Özgüçlü, S.,** The addition products of β-methyl-β-nitrostyrene derivatives with 2-mercaptomethylbenzimidazole and their NMR studies, J. *Fac. Pharm. Gazi*, 13,133-142, **1996**.

40. **Gökçe, M., Utku, S., Berçin, E., Özçelik, B., Karaoğlu, T., Noyanalpan, N.,** Synthesis and in vitro antimicrobial and cytotoxicity evaluation of 2-[(2-nitro-1-phenylalkyl)thio]benzoic acid derivatives, *Turkish J. Chem.*, 29, 207-217, **2005**.

41. **Utku, S., Gökçe, M., Özçelik, B., Berçin, E.,** Evaluation of antimicrobial activity of 2-[(2-nitro-1-phenylalkyl)thiomethyl]benzimidazole derivatives, *T. J. Pharm. Science*, 5(2), 107-115, **2008**.

42. **Thiele, J.,** Condensation des nitromethans mit aromatischen aldehyden, *Chem. Ber.*, 32, 1293-7, **1899**.

43. **Koremura, M., Oku, H., Shono, T., Nakanishi, T.,** Synthesis of β-alkyl-β-nitrostyrene derivatives and their antimicrobial and insecticidal activities, *Takamine Kenkyusho Nempo*, 13, 198-204, **1961**.

44. **Yeşilada, E., Ustün, O., Sezik, E., Takaishi, Y., Ono, Y.. Honda, G.,** Inhibitory effects of Turkish folk remedies on inflammatory cytokines: interleukin-1α, interleukin-1β and tumor necrosis factor α, *J. Ethnopharmacol.*, 58, 59-73, **1997**.

45. **Yeşilada, E., Taninaka, H., Takaishi, Y., Honda, G., Sezik, E., Momota, H.,** In vitro inhibitory effects of Daphne Oleoides ssp. Oleoides on inflammmatory cytokines and activity-guided isolation of active constituents, *Cytokine*, 13, 359-64, **2001**.