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Research Article

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Design, Synthesis and Biological Evaluation of Novel Ureidobezimadazole hybrid as Potent TNF-α and IL-6 Inhibitor, and Antimicrobial agents

Sandeep V. Shinde[a], Rajesh H. Tale[b]*, Atish H. Rodg[b], Ashish D. Raote[b], Kalpana M. Patil[c] and Rajendra P. Pawar[c]

[a]*Dept of Chem. P. N. College, Nanded (MS), India*
[b]*Organic, Dendrimer and Medicinal Chemistry Laboratory, School of Chemical Sciences, Swami Ramanand Teerth Marathwada University, (SRTM) Nanded, India*
[c]*P. G. Dept of Chem. Deogiri College, Aurangabad(MS) India*

ABSTRACT

*A new series of 2-(3-Arylureido) benzimidazole derivatives (5a-j) were synthesized via sequential oxidative cyclisation of o-phenelyne diamine **1**and 4-nitrobenzaldehyde **2**, reduction followed by the reaction of resulting amine **3**with different arylisocynates **4**. All the synthesized compounds were screened for their in-vitro pro-inflammatory cytokines TNF-α and IL-6 inhibition and antimicrobial activity (antibacterial and antifungal). The compounds 5c, 5e and 5g found to be potent TNF-α and IL-6 inhibitor as compared to the standard dexamethasone but at the MIC of 10 µM, while the compounds 5d found to be moderately active as compared to standard dexamethasone. The remaining compounds were found to have low, very low or no activity at all at the MIC of 10 µM. The antimicrobial activity data revealed that compounds 5b, 5f, and 5g found to be potent antibacterial and antifungal agents. Notably, the compounds 5f and 5g, exhibited 1.5-2.5 fold antibacterial and antifungal activity to that of control drugs Micanazole and fluconazole almost against all the gram positive and gram negative bacteria and fungi and thus found to be more potent even than the standard drugs.*

INTRODUCTION

Small organic molecules can be powerful tools for impacting biology and medicine, functioning as both therapeutics and as probes that help to illuminate the macromolecules regulating biological processes.[1] Despite of advances on many fronts such as efficiency of synthetic chemists to generate huge libraries incorporating thousands of compounds, the efforts to make critical discoveries pertinent to disease remains a slow and, arguably, very often serendipitous one.[2] Since the inception of the concept of "privileged structure or Scaffold" by Evans[3] in late 1980s, "privileged-scaffold" based drug discovery has emerged as a powerful tool in medicinal chemistry. Such a "privileged scaffolds," molecular frameworks are seemingly capable of serving as ligands for a diverse array of receptors. Though Evan et al. originally put forward this concept based on benzodiazepine nucleus, which is mainly due to its ability as peppidomimic,[4] subsequent work over the past several decades from both academic and industrial groups has revealed that there are many such additional scaffolds. Consequently, this concept has attracted much attention of medicinal chemists as viable starting points in exploration, design and synthesis of novel therapeutic agents. However, still the restricted availability of "privileged structures" yet limits the scope of this approach in drug discovery to great extent.

To circumvent the problem associated with scarcity of privileged scaffold, recently generation of the library for high throughout screening for lead discovery by making the hybrid (as heterocyclic conjugates) of existing "privileged structures" has emerged as an attractive alternative. Based on this strategy, the groups of Hirschmann have actively pursued the design and development of new privileged scaffolds.[5] For example; they reported the attachment of genetically encoded and uncoded aminoacid side chains to privileged structures as a promising means to produce diverse libraries of multitarget biologically active compounds.[6]

Cytokines are intercellular messengers responsible for host defense mechanisms as inflammatory, immune and hematogenic responses. Although many of them are transient, they are produced by various cells which act as urgent response mediators in cases of invasive interventions. Disruption of this biological defense mechanism and continuous excessive cytokine production contributes to pathogenesis of inflammatory diseases. One of the key pro-inflammatory cytokine, Tumor necrosis factor-α (TNF-α) is mainly produced by the activated macrophages and monocytes, which further induces the production of the several inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and granulocyte-macrophage colony-stimulating factor (GM-CSF). It is also a multitude of biological activities linked to pathology of autoimmune diseases such as rheumatoid arthritis (RA),[7] Crohn's disease,[8] systemic lupus erythematosus, [9] and multiple sclerosis,[10] septic shock,[11] and AIDS.[12] On the other hand, cytokine interleukin-6 (IL-6) (from the series of cytokine signaling pathway) contributes to the initiation and extension of the inflammatory process and considered as a central mediator in a range of inflammatory diseases but has not yet received the desired attention in drug discovery[13] TNF-α and IL-6 are thus pharmaceutically important molecular targets for the treatment of the above mentioned diseases.

Non-steroidal anti-inflammatory drugs (NSADs) are therapeutically important in the treatment of rheumatic arthritis and in various types of inflammatory conditions, but their therapeutic utility has been limited due to their frequently observed gastrointestinal side effects. Thus, there is an urgent need for new targets that are required for the design and development of novel anti-inflammatory agents as an alternative to NSAIDs. [14] (Tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), the two important multifunctional proinflammatory cytokines that are involved in the pathogenesis of autoimmune, inflammatory, cardiovascular, neurodegenerative and cancer diseases through a series of cytokine signaling pathways.[15,16] IL-6 contributes to the initiation and extension of the inflammatory process and considered as a central mediator in a range of inflammatory diseases but has not received the desired attention in drug discovery. [17] TNF-α and IL-6 are thus pharmaceutically important molecular targets for the treatment of the above-mentioned diseases.

The concept of "privileged medicinal structures or scaffolds," originally introduced by Merck researchers in the course of their work on benzodiazepines, has recently emerged as one of the guiding principles of modern drug discovery.[18,19] It involves the utilization of molecular frameworks with inherent potential for biological activity. Through appropriate functional group modifications, these scaffolds are capable of providing ligands for a number of functionally and structurally discrete biological receptors. In addition, compound libraries designed on the basis of such frameworks exhibit enhanced drug like properties and result in high-quality leads.

Among various Herero cylices, Benzimidazole nucleus considered to be privilege medicinal scaffold due to their broad range of biological activities such as anti-inflammatory[20], antitumor [21], antimalarial [22], analgesic [23], bronchodilatory[24], antiamoebic [25], antiviral[26], antihelminthic [27], selective inhibition of the platelet-derived growth factor receptor[28], antagonist [29], neuropeptide Y1 receptor antagonism[30], antiproliferative activity[31,32], antiarrhythmic activity [33], antiviral properties[34,35], *etc*. Consequently, in recent years simple benzimidazole derivatives or complex benzimidazole conjugates emerged as an indispensable anchor for development of new therapeutic agents.

However, relatively there are few reports in the literature on the anti-inflammatory activity evaluation, in particular, their TNF-α and IL6 inhibitory activity. Moreover, the potential of benzimidazole possessing ureiodo moiety as to their anti-inflammatory activity against the pro-inflammatory cytokines (TNF-α and IL-6) and antimicrobial agents hitherto remained untested. In recent years, we have been engaged in design, synthesis and anti-inflammatory activity and antimicrobial evaluation of novel urea and thiourea derivatives[36]. Herein, we report that novel and easily accessible benzimidazole-ureioudo conjugate found to be potent TNF-α and IL-6 inhibitors and anti-microbial agent.

EXPERIMENTAL SECTION

General Techniques

All reagent used were of analytical grade (Thomas Baker, Spectrochem). ^{1}HNMR spectra were recorded on Brucker Advance spectrometer (300MHz or 400MHz) using tetramethylsilane as internal standard. Chemical shifts are reported in ppm (δ) relative to the solvent peak, Mass spectra were recorded on either GCMS (focus GC with TSQ II mass analyzer and thermoelectro) with auto sampler/direct injection (EI/CI) or LCMS (APCI/ESI; Buker daltanoics Micro TOFQ). HPLC purity was checked using Water Alliances or Dionex Ultima 3000 HPLC system. All purications were done by recrystallization (Methylene Dichloride / petroleum ether 9:1). Ethyl acetate and petroleum ether were used as mobile phase for TLC (Merck Kiesel 60 F254, 0.2mm thickness sheet)

Biological Assay

Proinflammatory cytokine production by lipopolysaccharide (LPS) in THP-1 cells was measured according to the method described by Hwang et al. (Hwang et al, 1993) during assay, THP-1 cells were cultured in RPMI 1640 culture medium (Gibco BRL, Pasley, UK) containing 100 U/mL penicillin and 100 mg/mL streptomycin containing 10% fetal bovine serum (FBS, JRH). Cells were differentiated with phorbol myristate acetate (PMA, Sigma). Following cell plating, the test compounds in 0.5% DMSO were added to each well separately and the plate was incubated for 30 min at 37^0C. Finally, LPS (E. coli 0127:B8, Sigma Chemical Co. St. Louis, MO) was added, at a final concentration of 1 µg/mL in each well. Plates were further incubated at 37^0C for 24h in 5% CO_2. After incubation, supernants were harvested and assayed for TNF-α and IL-6 inhibition by ELISA as described by the manufacturer (BD Biosciences).

Experimental Procedure

a) **Synthesis of 2-(4-nitrophenyl)-1*H*-benzo[*d*]imidazole (3)**

To a mixture of benzene-1,2-diamine **1**(1 equiv.) and 4-nitrobenzaldehyde **2**(1 equiv.) in toluene (10 ml), molecular I_2 (0.02 equiv.) and the resultant mixture was heated at 70^0C for 8 h. After completion of reaction, toluene was evaporated and to the residue was added $Na_2S_2O_3$ solution to remove iodine and extracted with ethyl acetate (3*10 ml). The combined organic extract was washed with brine and concentrated to get crude product which was then purified by crystallization (hot ethanol) to obtain analytically pure title compound as Yellow solid. Yield: 61%

b) **Synthesis of 4-(1*H*-benzo[*d*]imidazol-2-yl) aniline (4)**

Procedure: To a solution of 2-(4-nitrophenyl)-1H-benzo[d]imidazole **(3)** (1 equiv.) in ethyl acetate, $SnCl_2$ (2 equiv.) was added and the resultant reaction mixture was heated at 45-50 ^{0}C for 8 h. After completion of reaction, the solvent was evaporated under vaccu and residue was poured in 10 % aq. $NaHCO_3$ and pH was maintained beweeen 8-9. The mixture was then extracted with ethyl acetate (3 *10) and combined organic layer was washed with brine and water and and concentrated to get product with sufficient purity and used as such for next step. Yield: 76%;

c) **General procedure for the Synthesis of (4-(1*H*-benzo[*d*]imidazol-2-yl)phenyl)aryl)urea (5a-j)**

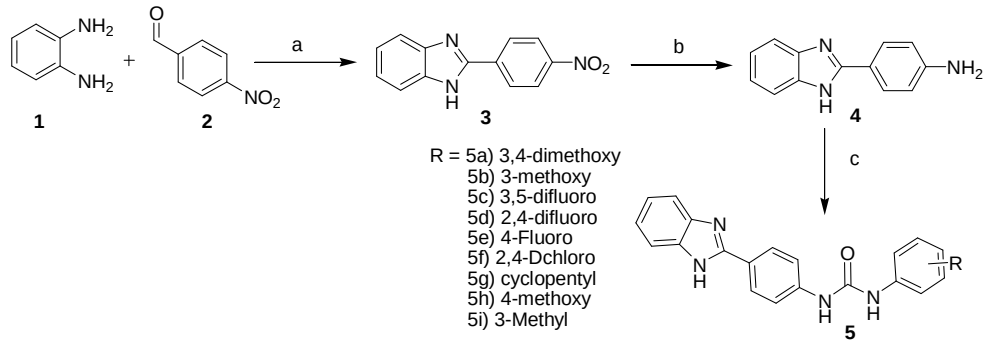
To a solution of 4-(1*H*-benzo[*d*]imidazol-2-yl) aniline **(4)** (1 equiv) in THF, appropriate arylisocyanate (1.2 eq.) was added and the resultant mixture was stirred at room temperature for 8-10 hr. After completion of reaction, the reaction mixture was poured into water and extracted with ethyl acetate (3*10 ml). The combined organic extract was washed with brine and then with water and concentrated under vaccum to get crude product. The column chromatography of crude product (EtOAc / Hexane, 7:3) afforded the title compounds in good to high yields

Having secured a series of the novel benzimidazole urea derivatives, next in order to search for the potent compounds from these newly synthesized ureiodbenzimadazole derivatives, compounds, 5a-j were evaluated for their in vitro anti-inflammatory activity by measuring Proinflammatory cytokine production by lipopolysaccharide (LPS) in THP-1 cells and antibacterial and antifungal activity against various Gram-positive, Gram-negative bacteria and fungal strains using agar well diffusion method.

RESULTS AND DISCUSSION

Our synthetic strategy for the synthesis of aryl urea derivatives bearing benzimidazole moiety is outline in scheme-1. The 4-amino benzimidazole **4** forms the key precursor for the synthesis of desired compounds. The oxidative cyclisation of *o*-phenelyne dimaine **1** and 4-nitrobenzaldehyde **2** catalyzed by molecular iodine in toluene at 70 ^{0}C followed by the reduction of nitro analogue **3** using $SnCl_2$ yielded the yielded amine amine **4** in high yields. The

reaction of 4 with different arylisocyanates in THF at room temperature afforded the corresponding ureiodobenzimadazoles (**5a-j)** in good to high yields under mild conditions. The purity of the newly synthesized compounds was checked by TLC and HPLC. The ^{1}HNMR and Mass spectral data was found to be consistent with structures of the newly synthesized benzimidazole derivatives.



Scheme 1. Reagents and conditions: (a) I_2, tolune, 70^{0}C 8 h; (b)$SnCl_2$, EtOAc, 40 ^{0}C, 8 h; (c) substituted isocynate, THF, r.t., 10-12hours.

Scheme-1

The results of biological activity of novel ureiodbenzimidazole derivatives have been presented in Tables **1–3.** The interesting trend was observed as to the effect of substituent present on terminal ring of urea moiety on various activities was observe red. It was found that the lipophilicity as well as nature of the substituent affecting the biological activity of the synthesized analogues. Thus, from the TNF-α and IL-6 inhibitory activity data (Table 1), it is observed that a majority of the analogues of this series found to be active as IL-6 inhibitor while very few exhibited TNF-α inhibitory activity. As can be seen from Table 1, compounds 5c, and 5e exhibited the good (82% and 80%) TNF-α and IL-6 (96% and 91%) inhibitory activity as compared to the standard dexamethasone but at higher concentration (10 µM) and found to be moderately potent anti-inflammatory agents.

Table 1. Anti-inflammatory activity data of novel benzimidazole derivatives

Compounds	% Inhibition at 10 µM	
	TNF-α	IL-6
4	0	0
5a	18	21
5b	21	26
5c	**82**	**96**
5d	55	68
5e	**80**	**91**
5f	30	31
5g	**64**	**78**
5h	11	28
5i	28	36
5j	12	16
Dexamethasone	74	81

Compounds 5d and 5g exhibited moderate activity (64-78% inhibition) while other compounds exhibited low inhibitory activities at same level of concentration. It is also observed that the two-potent compounds in this series

5c and 5e present no or very low anti-microbial activity, could indicate low toxicity associated and should be considered as ideal anti-inflammatory agents.

Table-2. Antibacterial activity data of novel benzimidazole derivatives

Compounds	Gram-positive		Gram-negative	
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Salmonella typhimurium*
4	55	45	50	50
5a	65	60	40	55
5b	10	10	15	10
5c	70	85	60	90
5d	60	85	85	70
5e	75	80	90	90
5f	25	10	10	15
5g	65	65	60	75
5h	10	25	25	35
5i	40	25	35	35
5j	20	10	10	10
Ciprofloxacin	20	25	20	25

Table-3 Antifungal activity data of novel benzimidazole derivatives

Compounds	*Candida albicans*	*Aspergillus niger*	*Fusarium solani*	*Aspergillus flavus*
4	40	35	35	55
5a	50	45	40	60
5b	20	10	10	15
5c	60	75	55	50
5d	75	90	75	70
5e	60	80	60	65
5f	15	10	15	15
5g	55	50	50	75
5h	10	10	15	10
5i	25	30	25	35
5j	25	15	15	10
Miconazole	25	20	15	20

It is observed that the position of the substituent on terminal benzene ring of urea moiety has profound effect on the activity. The 2, 3 and 4 positions on the terminal benzene ring are the favourable site for the higher potency. Evidently, the compound 5c (82-96% inhibition) with methyl at 3 positions, respectively, exhibiting highest TNF-a and IL-6 inhibitory activity, while presence of methyl at 4-position and methyl at 3 position with other substituents like Cl compounds 5f and 5h (30-31% and 11-28% inhibition) exhibits moderate TNF-alpha or low IL-6 inhibitory activity. The compound 5e with Cl group at 2- and 4-position of terminal benzene ring showed better TNF-α (80% at 10 µM) and IL-6 (91% at 10 µM) inhibitory activity. Interestingly, unsubstituted analogue, compound 5e was found to be moderate TNF- α or IL-6 inhibitor (64-78% inhibition). However, other substituents than described above on the terminal benzene ring found to have detrimental effect on inhibition as it exhibits no TNF-α activity or very low IL-6 inhibitory activity.

The antibacterial activity data is represented in Table 2. As shown in our results, some analogues of this series were found to have even more potency than the standard drug ciprofloxacin while some of them have comparable potency. The compounds 5b, 5h and 5j bearing OMe-, methyl and F- group at 2 and 4-position found to be most potent followed by compounds 5f and 5i bearing Cl- and F- at 2- or 4-position. Thus compounds bearing substituent such as OMe-, methyl and F- group at 2 and 4-position of the terminal benzene ring of urea part found to have higher potency than the compounds bearing such a group or other group at 3 positions.

Explicitly, 2- or 4-position proved to be the favorable site for high antibacterial activity. The high potency of 5b, 5h and 5j may be attributed to the presence of lipophilic or H-bond acceptor type groups such as F-, Cl-, and OCH_3 at 2- or 4-position.

Rest of the compounds having different substituent on terminal benzene ring of urea showed moderate or no activity with respect to standard drug against the test strains. No activity was observed in case of compounds 4 up to

concentration of 200 µg/mL against some bacteria and fungi. It is clear from our results (Table 2 vs Table 3) that the SAR of benzimidazole urea derivatives for antibacterial activity strongly correlates with their SAR of antifungal activity. Again the position 2 and 4 of terminal benzene ring found to be the favorable sites for high activity. The compounds **5b, 5h** and **5j** found to be 2.5-fold more potent than the standard drug Micanazole while **5f, 5i** and **5j** exhibited comparable antifungal activity. Similar to the antibacterial activity trend, the remaining compounds had no major effect on the antifungal activity up to concentration of 200 µg/mL.

Analytical data

1-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-3-(4-methoxyphenyl)urea (5b)

White solid, Yield: 64%; ^{1}HNMR (DMSO, 300MHz): 9.2 (s, 1H), 8.02 (s, 1H), 7.83-7.82 (m, 1H), 7.69-7.53 (m, 2H), 7.51-7.44 (m, 4H), 7.42-7.12 (m, 6H), 3.78 (s, 3H). M/z- 359.1(M+1), HPLC- 98.55%

1-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-3-(m-tolyl)urea (5c)

White solid Yield: 73%; 1HNMR (DMSO, 300MHz): 9.45 (s, 1H), 8.45 (s, 1H), 7.82-7.76 (m, 3H), 7.53-7.51 (m, 2H), 7.43-7.42 (m, 5H), 7.23-7.21 (m, 2H), 2.31 (s, 3H), M/z- 343.2(M+1), HPLC- 97.46%

1-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-3-(2,4-difluorophenyl)urea (5d)

White solid Yield: 61%; 1HNMR (DMSO, 300MHz): 9.51(s, 1H), 8.07-8.05 (s, 1H), 7.83-7.82 (m, 1H), 7.69-7.42 (m, 7H), 7.39-7.12 (m, 3H), M/z- 365.1(M+1), HPLC- 97.43%

1-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-3-(2,4-dichlorophenyl)urea (5e)

Off white solid Yield: 68%; 1HNMR (DMSO, 300MHz): 9.69 (s, 1H), 8.58 (s, 1H), 8.46 (s, 1H), 8.25-8.23 (m, 1H), 7.86-7.81 (m, 3H), 7.65 (m, 1H), 7.56-7.42 (m, 4H), M/z- 397.1 (M+1), HPLC- 98.55%

1-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-3-(2-chloro-3-methylphenyl)urea (5f)

Off white solid Yield: 76%; 1HNMR (DMSO, 300MHz): 9.45 (s, 1H), 8.48 (s, 1H), 7.82-7.76 (m, 3H), 7.53-7.51 (m, 2H), 7.43-7.42 (m, 3H), 7.23-7.22 (m, 2H), 2.45 (s, 3H), M/z- 397.1 (M+1), HPLC- 98.96%

1-(4-(1H-benzo[d]imidazol-2-yl)phenyl)-3-phenylurea (5g)

Off white solid Yield: 56%; 1HNMR (DMSO, 300MHz): 9.56 (s, 1H), 8.07-8.05 (m, 1H), 7.83-7.47 (m, 6H), 7.44-7.12 (m, 5H), M/z- 329.1(M+1), HPLC- 98.33%

CONCLUSION

In conclusion, the structurally diversed (3-arylureiodo)benzimidazole derivatives were synthesis and screened in order to search for the potent TNF-α and IL-6 inhibitory, antibacterial and antifungal activity. The present study lead to the discovery of compounds 5c, 5e and 5g as potent TNF-α and IL-6 inhibitors and 5b, 5f, 5g, as antimicrobial agents. In view of the simple, practical and diversity oriented synthetic approach coupled with good number of candidates exhibiting TNF-α and IL-6 inhibitory, and antimicrobial activity, the present study could be potentially useful in medicinal chemistry.

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