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ARTICLE · JUNE 2015

DOI: 10.1016/j.apsb.2015.05.003

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Benzimidazole derivatives: search for GI-friendly anti-inflammatory analgesic agents

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Received 9 October 2014; received in revised form 1 March 2015; accepted 6 May 2015

KEY WORDS

Benzimidazole;
Analgesic;
Gastrointestinal toxicity;
Antioxidant activity

Abstract Non-steroidal anti-inflammatory drugs (NSAIDs) have been successfully used for the alleviation of pain and inflammation in the past and continue to be used daily by millions of patients worldwide. However, gastrointestinal (GI) toxicity associated with NSAIDs is an important medical and socioeconomic problem. Local generation of various reactive oxygen species plays a significant role in the formation of gastric ulceration associated with NSAIDs therapy. Co-medication of antioxidants along with NSAIDs has been found to be beneficial in the prevention of GI injury. This paper describes the synthesis and biological evaluation of *N*-1-(phenylsulfonyl)-2-methylamino-substituted-1*H*-benzimidazole derivatives as anti-inflammatory analgesic agents with lower GI toxicity. Studies *in vitro* and *in vivo* demonstrated that the antioxidant activity of the test compounds decreased GI toxicity.

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Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

http://dx.doi.org/10.1016/j.apsb.2015.05.003

Please cite this article as: Gaba Monika, et al. Benzimidazole derivatives: search for GI-friendly anti-inflammatory analgesic agents. *Acta Pharmaceutica Sinica B* (2015), http://dx.doi.org/10.1016/j.apsb.2015.05.003

1. Introduction

NSAIDs are among the most commonly prescribed drugs worldwide. Because of their 'over-the-counter' availability, they are also consumed on non-prescription basis as well. However, their intake is frequently associated with gastrointestinal (GI) side effects, representing an important medical and socioeconomic problem[1]. These drugs can affect all segments of the GI tract and be responsible for ulceration, dyspepsia or gastric bleeding. In some cases, they can cause serious toxicity requiring hospital admission and aggressive management[2].

It is well known that local generation of reactive oxygen species plays an important role in the formation of gastric ulceration associated with NSAIDs therapy[3,4]. Thus, co-medication of NSAIDs with antioxidants could be a useful approach to overcome gastric side effects. Some very early studies have suggested the beneficial role of vitamin C and vitamin E in NSAIDs-induced GI side effects[5]. However, drug interactions with vitamin E may result in additive blood thinning effects of NSAIDs and vitamin C can raise the level of NSAIDs in the blood by increasing their stay in the body and make them less appealing as a therapeutic strategy[6]. Therefore, as an alternative approach, anti-inflammatory analgesics with potent antioxidant activity are a potential therapeutic strategy for treatment of pain and inflammatory disorders without GI side effects.

The benzimidazole moiety represents an important pharmacophore and privileged structure in medicinal chemistry. Recently, benzimidazole has emerged as pharmacophore of choice for designing anti-inflammatory analgesic molecules[7,8]. In addition, the literature shows that benzimidazole derivatives substituted at the 1 and 2 positions exhibit potent antioxidant activity[9–11]. Moreover, 1, 2-substituted benzimidazole derivatives as proton pump inhibitors have shown their gastroprotective action, irrespective of their acid suppressive action[12]. This has prompted us to explore *N*-1-(phenylsulfonyl)-2-methylamino-substituted-1*H*-benzimidazole derivatives as gastroprotective anti-inflammatory and analgesic agents. The synthesized compounds were evaluated for their anti-inflammatory, analgesic activity along with their gastroprotective mechanism of action by *in vitro* and *in vivo* assays.

2. Results and discussion

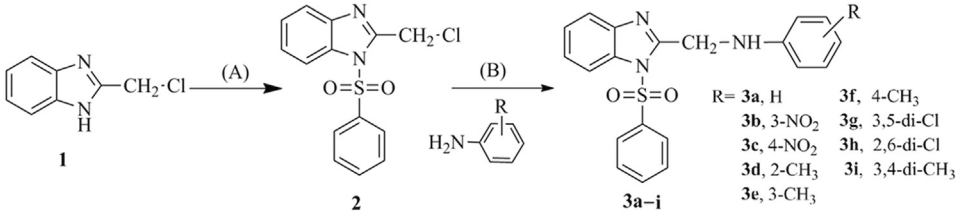
2.1. Synthesis

The present research work was carried out to search for novel benzimidazole derivatives as anti-inflammatory analgesics with low ulcerogenic potential. The sequence of the reactions employed for the synthesis of novel benzimidazole derivatives is outlined in Scheme 1. Compound **1** was prepared according to literature procedures starting from *o*-phenylenediamine[13]. Sulfonylation of compound **1** was done in the presence of dry pyridine and benzenesulfonyl chloride to give compound **2**[14]. From compound **2**, novel benzimidazole derivatives **3a–i** were synthesized by adding different substituted aryl amines. The purity of the compounds was accomplished by column chromatographic separation using silica gel as the stationary phase and chloroform: methanol (9:1) as the mobile phase.

2.2. Biological assays

The newly synthesized *N*-1-(phenylsulfonyl)-2-methylamino-substituted-1*H*-benzimidazole derivatives were screened *in vivo* in order to evaluate their pharmacological activity. The test compounds were evaluated for their anti-inflammatory activity by evaluating carrageenan-induced paw edema in rats. Compounds **3a–i** were found to exhibit encouraging anti-inflammatory activity ranging from 23.88% to 37.31% and the results are summarized in Table 1. It was observed that the tested compounds **3d**, **3e**, **3f** and **3i** showed significant reduction in edema (31.34%, 32.84%, 34.33% and 37.31%, respectively) after 3 h when administered at doses of 100 mg/kg *p.o*. Further, all the compounds were assessed for their analgesic activity by using acetic acid − induced writhing test in mice and the results are given in Table 2. The test compounds **3d**, **3e**, **3f** and **3i** demonstrated 54.03%, 52.84%, 53.55% and 57.58% protection in the number of writhes produced by acetic acid, which are comparable to the standard drug acetyl salicylic acid. These results showed a positive contribution of electron donating substituents towards anti-inflammatory as well as analgesic activity. This is in accord with published reports, which demonstrate that substitutions with electron donating groups enhance the lipophilicity of the molecule[15,16], which in-turn may be responsible for significant anti-inflammatory and analgesic activities for tested compounds **3d**, **3e**, **3f** and **3i**.

The newly synthesized compounds are designed with an aim to identify gastroprotective anti-inflammatory and analgesic agents. Oxidative stress is an important component involved in the pathophysiology of NSAIDs-induced GI ulceration. This is further supported by the finding that indomethacin administration results in increased reactive oxygen species production in the gastric mucosa, followed by gastric ulceration[17]. Chemical modification of existing NSAIDs with electron donating substitutions will lead to molecules with potent antioxidant activity, which in turn may lead to GI-safe NSAIDs. In the present study, the gastroprotective potential of the newly synthesized derivatives and the mechanism of action for better gastric tolerance were studied. The *in vitro* antioxidant potential of all the synthesized compounds was assessed by the ferric reducing antioxidant power (FRAP) assay. From the results, it is observed that compounds **3d**, **3e**, **3f** and **3i** with electron donating substituents were the most efficient compounds in the FRAP assay (Fig. 1). After the *in vitro* antioxidant activity, the test compounds were studied for translation of their *in vitro* activity to an *in vivo* effect–*i.e*., better gastric



Scheme 1 Synthetic route of compounds **3a–i**. Reagent A: (i) benzenesulfonylchloride and (ii) dry pyridine; Reagent B: (i) ethanol, potassium iodide and (ii) potassium hydroxide.

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Table 1 Anti-inflammatory activity of **3a–i** and indomethacin.

Compd.	Edema at 3 h (%, Mean ± SEM, $n=6$)	Reduction in edema (%)
Control	100.00 ± 3.59	0.00
3a	71.64 ± 4.00	28.36
3b	74.63 ± 5.50	25.37
3c	70.15 ± 2.75	29.85
3d[a]	68.66 ± 2.99	31.34[a]
3e[a]	67.16 ± 3.06	32.84[a]
3f[a]	65.67 ± 3.78	34.33[a]
3g	73.13 ± 6.30	26.87
3h	76.12 ± 5.04	23.88
3i[a]	62.69 ± 3.27	37.31[a]
Indomethacin[a]	52.23 ± 4.27	47.76[a]

[a]Statistically significant compared to control group ($P \leq 0.05$); data was analyzed by unpaired one-way ANOVA test.

Table 2 Analgesic activity of **3a–i** and acetyl salicylic acid.

Compd.	No. of writhes in 15 min (Mean ± SEM, $n=6$)	Protection (%)
Control	70.33 ± 3.01	0.00
3a	35.17 ± 2.65	50.00
3b	35.50 ± 2.55	49.53
3c	35.67 ± 3.62	49.29
3d[a]	32.33 ± 3.62	54.03[a]
3e[a]	33.17 ± 3.39	52.84[a]
3f[a]	32.67 ± 3.57	53.55[a]
3g	41.17 ± 3.01	41.47
3h	41.83 ± 1.08	40.52
3i[a]	29.83 ± 2.45	57.58[a]
Acetyl salicylic acid[a]	25.67 ± 1.45	63.51[a]

[a]Statistically significant compared to control ($P \leq 0.05$).

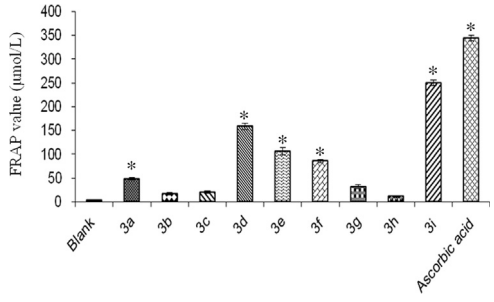

Figure 1 Ferric reducing antioxidant power of test compounds **3a–i** and ascorbic acid. "*" Statistically significant ($P \leq 0.05$) as compared to blank.

tolerance. The test compounds were further evaluated for their potential to produce GI-injury and level of thiobarbituric acid reactive substances (TBARS) in the gastric mucosa was studied as an index of oxidative stress. Oral administration of indomethacin produced gastric hemorrhagic erosions and increased the levels of TBARS in the gastric mucosa, whereas, administration of an equipotent dose of test compounds **3d**, **3e**, **3f** and **3i** produces significantly lower gastric ulceration as compared to indomethacin. The level of TBARS with the test compounds was found to be significantly lower, suggestive of lower oxidative stress, which is closely parallel to lesser gastric mucosal injury as compared to indomethacin. From the above discussion, the increased gastric tolerability of the tested compounds can be attributed to their antioxidant properties. The gastroprotective activity results of test compounds **3a–i** are depicted in Table 3.

3. Conclusions

NSAIDs-induced gastric toxicity is common and associated with serious adverse effects which affect all segments of the GI tract. Reactive oxygen species play an important role in the pathogenesis of gastric mucosal injury induced by NSAIDs such as indomethacin[18]. Co-administration of antioxidants like vitamin C and vitamin E with NSAIDs has been found by others to inhibit the pathological changes induced by NSAIDs and these combinations have shown protective action. However, unwanted drug interactions of these antioxidants with NSAIDs limit their therapeutic usefulness. Therefore, as an alternative approach, chemical derivatization of existing NSAIDs that will lead to molecules with potent antioxidant activity may be a useful approach to find safer and potent NSAIDs, provided that the molecular modifications do not abolish the anti-inflammatory analgesic activity. In the present study, we have reported a series of novel benzimidazole derivatives as anti-inflammatory analgesic agents with inherent antioxidant activity. The therapeutic utility of these derivatives as GI tolerable anti-inflammatory, analgesic agents and their mechanism of action were established by *in vitro* and *in vivo* studies. The results suggested that these derivatives might serve as interesting lead compounds and could revolutionize the future development of GI-safe anti-inflammatory analgesic agents.

4. Experimental protocols

All chemicals were purchased from Sigma-Aldrich and Lancaster Co. as > 95% pure and used as such without further purification. The solvents, except for LR grade, were dried as per literature if necessary. The progress of chemical reactions was monitored by thin layer chromatography on pre-coated silica gel plates using the iodine chamber as a detector. Melting points of all compounds were determined using an open capillary tube method, and were uncorrected. The IR spectra were recorded on Bruker Alpha-FT-IR spectrophotometer, ^{1}H and ^{13}C NMR spectra on a Bruker Avance DPX-200 (400 MHz), mass spectra on Waters Q-TOF micro LC-MS spectrometer at ESI (+) mode and CI mode. Elemental analysis was performed on Leco CHNS-932 (Leco, St. Joseph, USA).

4.1. Chemistry

Synthesis of compound **1** and intermediate compound **2** was carried out according to procedures reported in the literature[13,14]. Novel benzimidazole derivatives **3a–i** were synthesized as follows.

4.1.1. Synthesis of N-1-(phenylsulfonyl)-2-methylamino-substituted-1H-benzimidazole derivatives *3a–i*

For the synthesis of the benzimidazole derivatives, potassium iodide (2 g, 0.012 mol) and potassium hydroxide (1 g, 0.017 mol) in dry ethanol was added to a solution of compound **2** (2 g,

Table 3 Gastroprotective activity of test compounds **3a–i**.

Compd.	Mean lesion score (mm) (Mean ± SEM)	TBARS (nmol/mL)/mg protein (Mean ± SEM)
Control	1.00 ± 0.47	0.60 ± 0.03
3a	19.67 ± 0.61	0.74 ± 0.05
3b	21.67 ± 1.58	0.76 ± 0.05
3c	20.17 ± 1.52	0.75 ± 0.04
3d[a]	16.83 ± 1.44[a]	0.68 ± 0.03[a]
3e[a]	17.00 ± 1.93[a]	0.70 ± 0.03[a]
3f[a]	16.50 ± 1.82[a]	0.67 ± 0.02[a]
3g	23.17 ± 1.32	0.80 ± 0.05
3h	24.00 ± 0.78	0.79 ± 0.03
3i[a]	13.83 ± 1.38[a]	0.64 ± 0.03[a]
Indomethacin	30.67 ± 1.85	0.82 ± 0.03

[a]Statistically significant compared to Indomethacin ($P \leq 0.05$).

0.006 mol), with different substituted aryl amines and refluxed for 12 h. The mixture was poured into ice-cold water. The precipitates formed were vacuum-filtered, washed, dried and recrystallized using hot water and ethanol.

4.1.2. *N-((1-(phenylsulfonyl)-1H-benzo[d]imidazole-2-yl)methyl) benzenamine* ***3a***

Yield: 57%; m.p. 226–228 °C; UV (ethanol) λ_{max}: 284 nm; FTIR: υ_{max} (cm^{-1}), 3389.98 (NH), 2962.86, 2920.43 (CH-aromatic), 1515.82 (C═C-aromatic), 1317.36 (CN), 1026.97, 884.30 (S═O); ^{1}H NMR ($CDCl_3$): δ 2.16 (s, 2H), 5.40 (s, NH), 6.87 (m, 4H), 6.89–7.35 (m, 5H), 7.43–7.56 (m, 5H); ^{13}C NMR ($CDCl_3$): δ 147.60, 141.54, 138.97, 137.98, 133.80, 131.11, 129.80, 128.36, 123.08, 117.21, 115.30, 113.50, 36.10; Anal. Calcd. for $C_{20}H_{17}N_3O_2S$: C, 66.10; H, 4.71; N, 11.56, Found: C, 66.01; H, 4.61; N, 11.45.

4.1.3. *3-Nitro-N-((1-(phenylsulfonyl)-1H-benzo[d]imidazol-2-yl) methyl)benzenamine* ***3b***

Yield: 59%; m.p. 228–230 °C; UV (ethanol) λ_{max}: 255 nm; FTIR: υ_{max} (cm^{-1}), 3426.30 (NH), 3067.84, 3202.91 (CH-aromatic), 1616.81 (C═C-aromatic), 1083.75, 1004.62, 809.84, 866.70 (S═O), 1513.57 (NO_2), 1339.08 (CN); ^{1}H NMR (DMSO-d_6): δ 2.10 (s, 2H), 3.43 (s, NH), 7.17–7.25 (m, 5H), 7.28–7.34 (m, 2H), 7.43–7.44 (m, 2H), 7.60–7.62 (m, 3H), 8.01 (s, H,); ^{13}C NMR (DMSO-d_6): δ 149.20, 148.51, 141.50, 138.97, 137.98, 133.80, 131.11, 130.50, 129.80, 128.36, 123.08, 119.60, 115.30, 109.51, 107.62, 36.10; Anal. Calcd. for $C_{20}H_{16}N_4O_4S$: C, 58.81; H, 3.95; N, 13.72, Found: C, 58.72; H, 3.80; N, 13.60.

4.1.4. *4-Nitro-N-((1-(phenylsulfonyl)-1H-benzo[d]imidazol-2-yl) methyl)benzenamine* ***3c***

Yield: 52%; m.p. 202–204 °C; UV (ethanol) λ_{max}: 399 nm; FTIR: υ_{max} (cm^{-1}), 3426.16 (NH), 2964.01 (CH-aromatic), 1513.57 (C═C-aromatic), 1339.08 (CN), 1513.57 (CH_2), 1122.76, 886.70, 809.84 (S═O), 1513.57, 1339.08 (NO_2); ^{1}H NMR (DMSO-d_6): δ 2.08 (s, 2H), 3.44 (s, NH), 6.57 (d, 2H, J=9.6 Hz), 7.17–7.29 (m, 5H), 7.57 (d, 2H, J=6.9 Hz), 7.74–7.76 (m, 2H), 7.86 (d, 2H, J=8.2 Hz); ^{13}C NMR (DMSO-d_6): δ 153.70, 141.50, 138.97, 137.98, 136.89, 133.80, 131.11, 129.80, 128.36, 123.08, 121.91, 115.30, 114.40, 36.10.

4.1.5. *2-Methyl-N-((1-(phenylsulfonyl)-1H-benzo[d]imidazole-2-yl)methyl)benzenamine* ***3d***

Yield: 50%; m.p. 219–220 °C; UV (ethanol) λ_{max}: 284 nm; FTIR: υ_{max} (cm^{-1}), 3316.29 (NH), 3061.51, 3027.81 (CH-aromatic), 1500.49 (C═C-aromatic), 1372.19, 1625.37 (CN), 1445.79, 2918.85 (CH_3), 1076.39, 1028.90, 873.24 (S═O); ^{1}H NMR ($CDCl_3$): δ 2.25 (s, 3H), 2.56 (s, 2H), 5.4 (s, NH), 6.84–6.86 (m, 2H), 6.95 (d, 2H, J=7.2 Hz), 7.05–7.08 (m, 4H), 7.19–7.31 (m, 5H); ^{13}C NMR ($CDCl_3$): δ 146.51, 141.54, 138.97, 137.98, 133.80, 131.11, 129.80, 128.36, 126.21, 123.08, 117.21, 115.03, 113.40, 36.41, 15.50; Anal. Calcd. for $C_{21}H_{19}N_3O_2S$: C, 66.82; H, 5.07; N, 11.13, Found: C, 66.70; H, 5.15; N, 11.01.

4.1.6. *3-Methyl-N-((1-(phenylsulfonyl)-1H-benzo[d]imidazol-2-yl)methyl)benzenamine* ***3e***

Yield: 53%; m.p. 213–215 °C; UV (ethanol) λ_{max}: 285 nm; FTIR: υ_{max} (cm^{-1}), 3393.60 (NH), 3058.78, 2958.81 (CH-aromatic), 1515.98 (C═C-aromatic), 1485.07, 1644.97 (CN), 1397.04 (CH_3), 1074.59 (S═O); ^{1}H NMR ($CDCl_3$): δ 2.21 (3H, s), 2.53 (2H, s), 5.1 (1NH, s), 6.01 (1H, s), 6.74 (3H, m), 7.04–7.08 (2H, m), 7.20–7.31 (5H, m), 7.44–7.51 (2H, d, J=6.8 Hz); ^{13}C NMR ($CDCl_3$): δ 147.60, 141.54, 139.20, 138.97, 137.98, 133.80, 131.11, 129.80, 128.36, 123.01, 117.21, 115.30, 113.50, 110.51, 36.10, 24.31.

4.1.7. *4-Methyl-N-((1-(phenylsulfonyl)-1H-benzo[d]imidazol-2-yl)methyl)benzenamine* ***3f***

Yield: 50%; m.p. 221–223 °C; UV (ethanol) λ_{max}: 223 nm; FTIR: υ_{max} (cm^{-1}), 3421.34 (NH), 3003.79, 3056.78 (CH-aromatic), 1646.67 (C═C-aromatic), 1083.11, 992.80 (S═O), 2867.18 (CH_3); ^{1}H NMR ($CDCl_3$): δ 2.21 (s, 3H), 2.48 (s, 2H), 4.50 (s, NH), 6.60 (d, 2H, J=8.5 Hz), 6.93 (d, 2H, J=8.0 Hz), 7.20–7.30 (m, 5 H), 7.46–7.52 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 144.60, 141.54, 138.97, 137.98, 133.80, 131.11, 129.80, 128.36, 126.80, 123.08, 115.30, 113.50, 36.10, 24.31; Exact Mass: 377 Mol. Wt.: 377 *m/z*: 377 (16.0%), 378 (17.0%), 379 (24.0%), 376 (12.0%), 362 (8.0%).

4.1.8. *3,5-Dichloro-N-((1-(phenylsulfonyl)-1H-benzo[d]imidazol-2-yl)methyl)benzenamine* ***3g***

Yield: 49%; m.p. 240–242 °C; UV (ethanol) λ_{max}: 347 nm; FTIR: υ_{max} (cm^{-1}), 3306.58 (NH), 3068.25, 2959.21 (CH-aromatic), 1596.31 (C═C-aromatic), 1303.17 (CN), 1449.09 (CH), 1074.99, 1027.58 (S═O), 797.04 (CCl); ^{1}H NMR ($CDCl_3$): δ 2.77 (s, 2H), 4.57 (s, NH), 6.49 (d, 2H, J=1.76 Hz), 6.67 (s, H), 7.17–7.31 (m, 5H), 7.47–7.50 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 150.40, 141.54, 138.97, 137.98, 133.80, 131.11, 129.80, 128.36, 123.08, 118.80, 115.30, 112.01, 36.10; Anal. Calcd. for $C_{20}H_{15}Cl_2N_3O_2S$: C, 55.56; H, 3.50; N, 9.72, Found: C, 55.45; H, 3.59; N, 9.60.

4.1.9. *2,6-Dichloro-N-((1-(phenylsulfonyl)-1H-benzo[d]imidazol-2-yl)methyl)benzenamine* ***3 h***

Yield: 50%; m.p. 217–219 °C; UV (ethanol) λ_{max}: 295 nm; FTIR: υ_{max} (cm^{-1}), 3427.64 (NH), 3045.47 (CH-aromatic), 1570.04, 1520.59 (C═C-aromatic), 1261.91, 1216.04 (CN), 1013.51, 891.73, 804.88, 1148.14 (S═O), 732.30 (CCl); ^{1}H NMR ($CDCl_3$): δ 2.23 (s, 2H), 4.44 (s, NH), 6.59–6.63 (t, 1H, J=8 Hz), 7.15–7.33 (m, 9H), 7.49 (d, 2H, J=10.2 Hz); ^{13}C NMR ($CDCl_3$): δ 144.31, 141.54, 138.97, 137.98, 133.80, 131.11, 129.80, 128.36, 127.87, 123.08, 120.01, 115.30, 35.10. Exact Mass: 431 Mol. Wt.: 431 *m/z*: 431 (1.0%), 432 (10.0%), 433 (4.0%), 434 (2.0%).

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*4.1.10. 3,4-Dimethyl-N-((1-(phenylsulfonyl)-1H-benzo[d]imidazol-2-yl)methyl)benzenamine **3i***

Yield: 58%; m.p. 239–240 °C; UV (ethanol) λ_{max}: 235 nm; FTIR: υ_{max} (cm^{-1}), 3393.69 (NH), 3058.10, 3008.30 (CH-aromatic), 1515.17 (C═C-aromatic), 1216.47, 1339.76 (CN), 2959.72 (CH_3), 995.76 (S═O); ^{1}H NMR ($CDCl_3$): δ 2.11 (s, 6H,), 2.46 (s, 2H), 3.09 (s, NH), 6.95–6.99 (m, 3H), 7.20–7.32 (m, 4H), 7.44–7.91 (m, 5H); ^{13}C NMR ($CDCl_3$): δ 144.50, 141.54, 138.97, 137.98, 133.80, 131.11, 129.80, 128.36, 125.31, 123.08, 115.30, 113.50, 110.40, 36.10, 17.8; Anal. Calcd. for $C_{22}H_{21}N_3O_2S$: C, 67.50; H, 5.41; N, 10.73, Found: C, 67.38; H, 5.30; N, 10.60.

4.2. Pharmacology

Sprague-Dawley (SD) rats of either sex weighing 180–250 g were used to study anti-inflammatory activity and ulcerogenic activity of the test compounds. Albino mice of either sex weighing 20–25 g were used for evaluation of analgesic activity. All the experimental procedures used in this study were approved by the Institutional Animal Ethical Committee (IAEC), registered under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Animals were housed individually in polypropylene cages, maintained under standard conditions of alternating 12 h light-and-dark cycles at a constant temperature (25±2 °C and 35%–60% relative humidity). Animals were fed with standard rat pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*.

4.3. Anti-inflammatory activity

The anti-inflammatory activity of the test compounds was carried out by using the carrageenan-induced rat paw edema model, employing 0.1 mL of 1.0% carrageenan solution as the phlogistic agent[19]. SD rats of either sex were randomized into vehicle control, standard and different test groups of six rats each. The 2% sodium carboxy methylcellulose (CMC) served as vehicle control and indomethacin was used as the standard drug at the dose level of 50 mg/kg body weight. The test compounds were administered *p.o.* as a suspension in 2% sodium CMC, at a dose level of 100 mg/kg body weight, 30 min before the injection of the phlogistic agent. The paw edema volume was measured with the help of a plethysmograph by the mercury displacement method at 0 h (immediately after injection) and 3 h (post injection of carrageenan). The edema (%) is shown in Table 1. The percent anti-inflammatory activity was calculated according to the formula as given below:

$$\text{Edema (\%)} = 100 - \left(1 - V_t/V_c\right) \times 100 \quad (1)$$

$$\text{Reduction in edema (\%)} = \left(1 - V_t/V_c\right) \times 100 \quad (2)$$

V_t and V_c is the edema volume in drug-treated and control groups, respectively.

4.4. Analgesic activity

The test compounds **3a–i** were evaluated for their analgesic activity by using acetic acid−induced writhing method[20]. Albino mice of either sex (20−25 g, body weight) were randomized into vehicle control, standard and test groups of six mice each. After randomization, the control group mice were administered 2% sodium CMC, acetyl salicylic acid (standard drug) or compounds **3a–i** at a dose of 100 mg/kg orally. After 30 min of dosing, acetic acid solution (0.6% *v/v* in distilled water) was administered i.p. at a dose of 1 mL/kg. The number of writhes in each animal was recorded for 15 min. The analgesic activity was expressed as percentage of protection and the results are presented in Table 2.

$$\text{Protection (\%)} = 100 - \left(V_t/V_c\right) \times 100 \quad (3)$$

4.5. In vitro antioxidant activity

The *in vitro* antioxidant activity of compounds **3a–i** was performed by FRAP assay based on the reduction of a colorless Fe^{3+}-tripyridyltriazine complex into a blue-colored Fe^{2+}-tripyridyltriazine complex by the action of electron-donating antioxidants[21]. The working FRAP reagent was prepared by mixing 10 mL of sodium acetate buffer (pH=3.6) with 1 mL of TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) solution and 1 mL $FeCl_3 \cdot 6H_2O$ (10:1:1, *v/v/v*, respectively). The FRAP reagent was warmed to 37 °C before being used and the assay was started by adding 228 μL of FRAP reagent into a 96-well microtiter-plate, followed by ascorbic acid (200 μmol/L) as a standard and test compounds (1 mmol/L). The blank was 12 μL of methanol. The reaction was allowed to run for 30 min and absorbance (Abs) was read at 593 nm. The experiments were performed in triplicate and their mean was calculated for each compound. The absorbance change was translated into FRAP value (μmol/L) by the following formula:

$$\text{FRAP value} = [(Abs_{\text{Sample30 min}} - Abs_{\text{blank}})/(Abs_{\text{Standard30 min}} - Abs_{\text{blank}})] \times Conc_{\text{standard}} \quad (4)$$

where ferrous sulfate was used as standard (200 μmol/L).

4.6. Gastric ulcerogenic potential and gastric lipid peroxidation

NSAIDs like indomethacin induce gastric lesions in experimental animals by impairing barrier properties of the mucosa and suppression of gastroprotective prostaglandin (PGE_2 and PGI_2) synthesis. The test compounds **3a–i** were tested for their gastric ulcerogenic potential. SD rats of either sex weighing 180–250 g were divided into vehicle control, standard and different test compound groups (n=6). The test compounds and indomethacin were administered orally in 2% sodium CMC orally at the dose levels of 100 and 50 mg/kg, respectively. After 6 h, the rats were sacrificed by cervical dislocation and their stomachs were removed. The stomachs were opened along the greater curvature and examined under 3-fold magnification. The length of the longest diameter of the lesions was measured and summated to give a total lesion score (in mm) for each animal[22]. Mean count for each group was calculated and is given in Table 3.

After measuring the lesions, the mucosa of the stomachs was scraped with glass slides and the concentration of TBARS, an index of oxidative stress, was measured according to Ohkawa et al.[23]. 1,1,3,3-Tetramethoxy propane was used as an external standard and the concentration of the TBARS was expressed as (nmol/mL)/mg protein[23].

4.7. Statistical analysis

Statistical analysis of the pharmacological activity of the synthesized compounds was performed by one-way analysis of variance (ANOVA) followed by Tukey's test. The P value of less than 0.05 was considered statistically significant. All values were expressed

Please cite this article as: Gaba Monika, et al. Benzimidazole derivatives: search for GI-friendly anti-inflammatory analgesic agents. *Acta Pharmaceutica Sinica B* (2015), http://dx.doi.org/10.1016/j.apsb.2015.05.003

as mean ± SEM (standard error of the mean). SIGMASTAT, version 2.0 by Jandel Corporation was used for statistical analysis.

Acknowledgments

The author(s) would like to acknowledge Punjab Technical University, Kapurthala, Punjab, India and ASBASJSM College of Pharmacy, Bela for providing an institutional research platform and necessary facilities. The authors would like to thank Mr. Sarbjot Singh, Senior Research Scientist at Panacea Biotec Ltd. for constant support during the research work.

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