SYNTHESIS AND ANTI-INFLAMMATORY ACTIVITY EVALUATION OF SOME BENZIMIDAZOLE DERIVATIVES

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Hyper-inflammation aggravates the symptoms of both communicable and non-communicable diseases. Therefore, anti-inflammatory compounds may have wide therapeutic application. Benzimidazole is a privileged scaffold and its success in drug development is evident from the long list of benzimidazole-based drugs with wide range of applications. This study was undertaken to develop new small molecules with anti-inflammatory properties. Compounds MBPHYD, MBNHYD and MBHYDX were synthesised, purified, characterised and found to be non-toxic both *in vitro* (in 100 μM concentration for 24 h vs. 3000 Vero cells/well) and *in vivo* (at a dose of 100 mg/kg in female Wistar rats with animals observed for 48 h for any mortality). Compounds MBPHYD and MBNHYD were found to possess significant anti-inflammatory properties. Further, *in silico* analysis suggested their compliance with drug-likeness. While no toxicity was predicted, both compounds were suggested to have good oral bioavailability. Thus, results of this study may encourage further investigation to establish new anti-inflammatory benzimidazoles for application against various disease conditions.

Keywords: benzimidazole; synthesis; anti-inflammatory.

1. INTRODUCTION

Inflammation is a protective host response. This is a primary defensive mechanism against foreign and infectious agents and also helps in healing process of other disease conditions. However, unregulated inflammatory response causes more harm than benefit. Thus, hyper-inflammatory response is implicated in the progress of many disease conditions. Chronic inflammation is associated with many non-communicative disorders including neurodegeneration, cancer, diabetes, autoimmune disorders, cardiovascular disease, kidney and liver diseases [1]. Acute inflammation aggravates and increases the severity of infectious diseases including COVID-19 [2], dengue [3] and chikungunya [4]. Since hyper-inflammation is a key factor of many diseases, and molecules with anti-inflammatory effects may have multiple pharmacodynamics, it is necessary to continuously explore different scaffolds with anti-inflammatory properties for their further progress as potential bioactive agents.

Benzimidazole is a privileged scaffold. It has been widely studied against both communicable and non-communicable diseases, and many benzimidazole based drugs are in clinical use as antiulcer (omeprazole), antihelmintic (flubendazole), antihistaminic (astmizole) and antihypertensive (telmisartan) agents[5]. In recent years there have been wide efforts to develop benzimidazole based anti-inflammatory agents [6]. Thus, we thought it worthwhile to synthesize some new benzimidazole based molecules and evaluate their anti-inflammatory properties.

2. EXPERIMENTAL

2.1. Chemicals and Instrumentation

Ethylchloroacetate (Sd. Fine Chem. Pvt. ltd), 2-methyl benzimidazole (Sigma-Aldrich), sodium bicarbonate (Sigma-Aldrich), hydrazine hydrate (Sd. Fine Chem. Pvt. ltd), phenylhydrazine (Sigma-Aldrich), hydroxylamine, magnesium sulfate, ethyl acetate (Merck), acetone (Merck), dichloromethane and ethanol (Merck). The HPLC grade methanol and water were procured from Merck, India. All chemicals and reagents were analytical grade and purchased from local vendors.

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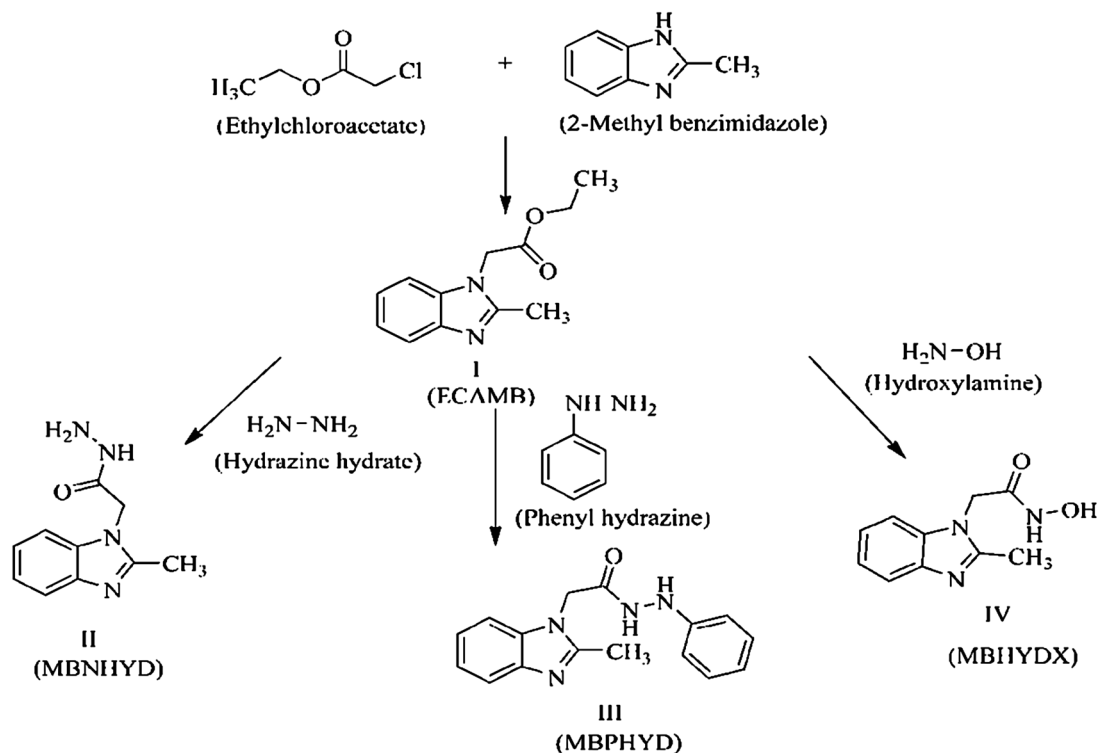* e-mail:bharatbhusans@gmail.com

Fig. 1. Synthesis of title compounds.

The chromatographic separation was performed using binary gradient HPLC system(Shimadzu, Japan)equipped with LC-20AD pump, SPD-M20A prominence diode array detector and 20 µL manual sample injection loop. The data integration was carried out by Lab Solutions software. Samples were filtered through syringe driven filter of Nylon-66 membrane-type having pore size 0.22 µm and then injected through Unisphere Aqua C18 analytical column (4.6 × 150 mm, 3 µm) by Hamilton SYR 25 µL syringe. FTIR spectra were recorded using powder samples in KBr discs (FTIR 4100, Jasco). NMR spectra were recorded on a Bruker AVANCE II 400 spectrometer using tetramethylsilane (TMS) as internal standard. Mass spectra in ESI mode were recorded using micro Agilent TOF-QII mass spectrometer.

2.2. Synthesis

In the first step, ethyl (2-methyl-1*H*-benzimidazol-1-yl) acetate (ECAMB, compound **I**, Fig. 1) was synthesised. Ethylchloroacetate (11 mmol) was dissolved in 10 mL acetone. The resulting solution was added to 2-methyl benzimidazole (9 mmol) in 10 mL acetone with continuous stirring. Sodium bicarbonate (12.5 mmol) was added to this reaction mixture with 5 mL acetone. The resulting mixture was heated under reflux and completion of the reaction was monitored by TLC with ethanol–acetone (6:4 v/v) mobile phase. After completion of the reaction, the mixture was

cooled, filtered and triply washed with acetone three times (each time). Then the mixture was concentrated under vacuum (RE100-Pro rotary evaporator, DLAB) and purified by column chromatography. The slightly reddish precipitate (ECAMB **I**, Fig. 1) with 270°C melting point was obtained in a good yield (69%) and used in the subsequent synthesis.

Hydrazine hydrate (0.015 mol) was added dropwise to stirring solution of 0.005 mol ECAMB (**I**) in 20 mL of absolute ethanol and the mixture was refluxed for 2 h and left overnight. The reaction was monitored by TLC (in ethanol–acetone, 6:4). Then, the mixture was concentrated under reduced pressure and mixed with 1:1 aqueous brine. Then it was triply extracted with dichloromethane (15 mL each times) and once with ethyl acetate (15 mL). Both organic phases were combined and dried with magnesium sulfate. After that, it was concentrated under vacuum (RE100-Pro rotary evaporator, DLAB) and kept overnight. The slight reddish crude precipitate was purified by column chromatography (ethanol–toluene, 60:40 v/v) and recrystallized from ethanol to obtain 2-(2-methyl-1*H*-benzimidazol-1-yl) acetohydrazide (MBNHYD, compound **II**, Fig. 1) with good yield (64%), melting point of 315°C; FTIR (ν, cm^{-1}): 3328.53(NH str.), 3047. 94 (NH str.), 1619.91 (NH bend.), 1681.62 (C=O str.), 2846.42 (CH str.) 2983. 34 (Ar CH str.);^{1}H NMR (δ, ppm): 8.1 (s, NH), 7.2-7.5 (M-ArH), 4.6 (s, CH$_2$), 2.5(s,CH$_3$); MS (ESI) *m/z*: 205.12(M$^+$+1) for C$_{10}$H$_{12}$N$_4$O.
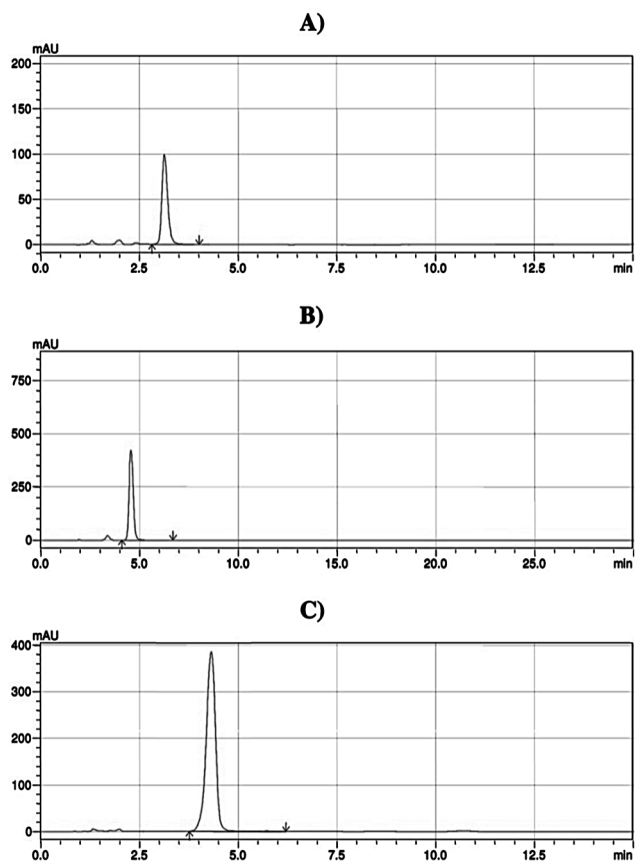
Fig. 2. HPLC patterns of (A) MBNHYD at 242nm, (B) MBPHYD at 247nm, and(C) MBHYDX at 246nm.

Synthesis of 2-(2-methyl-1*H*-benzimidazol-1-yl)-*N'*-phenylacetohydrazide (MBPHYD) was carried out in a similar manner with phenyl hydrazine (0.015mol). The slight reddish precipitate was purified by column chromatography (ethanol: toluene, 60:40 v/v) and recrystallized from ethanol to obtain MBPHYD (compound **III**, Fig. 1) with a yield of 60%, having a melting point of 305°C; FTIR (ν, cm^{-1}): 3598.52 (NH str.), 3327.57 (NH str.), 1619.2 (NH bend.), 1656.55 (C=O str.), 2846.42 (CH str.) 2947.66 (Ar CH str.). ^{1}H NMR (δ, ppm): 8.1 (s, NH), 4.2 (s, NH), 7.2-7.8 (M-ArH), 4.5 (s, CH$_2$), 2.5 (s,CH$_3$); MS (ESI) *m/z*: 281.25(M$^+$+1) for C$_{16}$H$_{16}$N$_4$O.

Finally, *N*-hydroxy-2-(2-methyl-1*H*-benzimidazol-1-yl) acetamide (MBHYDX) was also prepared following the above method by using hydroxylamine (0.015 mol.) The slight reddish crude precipitate was purified by column chromatography (ethanol–toluene, 80:20 v/v) and recrystallized from ethanol to obtain MBHYDX (compound **IV**, Fig. 1) in a yield of 63% with a melting point of 295°C; FTIR (ν, cm^{-1}): 3581.16 (OH str.), 3174.26 (NH str.), 1615.09 (NH bend.), 1706.69 (C=O str.), 3084.58 (Ar CH str.); ^{1}H NMR (δ, ppm): 8.2 (s, NH), 7.2 – 7.7 (M-ArH), 4.5 (s, CH$_2$), 2.5 (s,CH$_3$). MS (ESI) *m/z*: 206.11 (M$^+$+1) for C$_{10}$H$_{11}$N$_3$O$_2$.

2.3. Chromatographic Analysis

Various trials were performed to develop chromatographic conditions for the best resolution and check the purity of MBNHYD, MBPHYD and MBHYDX. The separation of MBNHYD, MBPHYD and MBHYDX was carried out isocratically at a flow rate of 1.0 mL/min using water–methanol mixture as mobile phase in different ratios. The mobile phase was filtered through a millipore vacuum filter system equipped with 0.45 μm membrane filter and degassed through in an ultrasonic bath. The mobile phase ratio for MBPHYD, MBHYDX was water: methanol (50:50 v/v) and for MBNHYD (30:70 v/v). The analytes were monitored at 242, 247, and 246 nm for MBNHYD, MBPHYD and MBHYDX, respectively. All the experiments were performed at ambient temperature. The retention times for MBNHYD, MBPHYD and MBHYDX were 3.133, 4.565 and 4.317 min, respectively. The purity of MBNHYD, MBPHYD and MBHYDX was 92%, 95% and 96% respectively. The resulting chromatograms are shown in Fig. 2.

2.4. Toxicity Assay:

For *in vitro* cytotoxicity assay, approximately 3000 Vero cells/well were seeded in 96 well plates (Corning) at 90% confluency, the cells were treated with test compounds (100 μM) for 24 h at 37°C in CO$_2$ incubator. The metabolically active cell percentage was compared with the control and the cellular cytotoxicity was determined. This assay was performed in three independent runs. Then, the acute oral toxicity (*in vivo*) assay was done at a dose of 100 mg/kg in female Wistar rats. The animals were fasted overnight before the treatment with the compounds. As per the OECD guidelines (no. 425) the animals were observed for 48 h for any mortality.

2.5. Anti-Inflammatory Activity Tested by Carrageenan-Induced Hind Paw Edema in Rats

The protocol for this test was approved by the Institutional Animal Ethical Committee, School of Pharmaceutical Sciences, Siksha 'O' Anusandhan Deemed to be University(Regd. No. 1171/C/08/CPCSEA) with protocol No. IAEC/ SPS/ SOA/ 12/ 2019. The study was carried out following established methods and protocols[7, 8]. Briefly, five groups of Wistar rats (*n*=6) were orally treated with vehicle, ibuprofen, MBNHYD, MBPHYD and MBHYDX (20 mg/kg) 1 h before carrageenan injection. About 0.1 mL of 1% carrageenan solution was injected into the subplantar region of left hind paw. The swelling was subsequently measured at preset time intervals (1, 2, 3, and 4h) using a digital plethysmometer (INCO-Nivique). Reduction in the paw displacement volume in test groups was compared with the carrageenan control groups by the XLSTAT software using one way ANOVA. The percentage paw edema inhibition was calculated using the following formula:

$$\text{Inhibition of paw edema (\%)} = \frac{(E_C - E_T)}{E_C} \times 100,$$

where E_C is the edema thickness in the toxic control group and E_T is the edema thickness in drug-treated group.

3. RESULTS AND DISCUSSION

3.1. Synthesis

2-Methyl benzimidazole was taken as the starting material and its reaction with ethyl chloroacetate yielded the intermediate compound ethyl (2-methyl-1*H*-benzimidazol-1-yl) acetate (compound **I**, Fig. 1). This is an efficient method, and our result (69% yield) agreed with previously reported data [9 – 11]. Due to the good yield, recrystallized product **I** was used in subsequent synthesis without further purification and analysis. Compound I is a good template for amide formation [10, 11]. Thus, with little modifications of prior methods [9 – 11], hydrazine hydrate and phenylhydrazine were reacted with this group to yield MBNHYD and MBPHYD. Both these compounds were purified by column chromatography using ethanol– toluene (60:40 v/v) as the mobile phase and recrystallized from ethanol. Further, the purity was checked by HPLC and found to be more than 90% (Fig. 2). MBNHYD showed a retention time of 3.133 min at a flow rate of 1.0 mL/min with a mobile phase of water–methanol (30:70 v/v) and a purity better than 92% (Fig. 2A). Similarly, MBPHYD yield was found to be >95% (Fig. 2B) pure and obtained at a retention time of 4.565 min by using a mobile phase of water–methanol (50:50 v/v) at a flow rate of 1.0 mL/min. The higher retention time of MBPHYD suggests its better lipophilicity. This can be attributed to the additional phenyl ring in this compound. Compound **I** was also used to synthesize the hydroxamic acid derivative (MBHYDX) with little modifications of the earlier method[11]. It was purified by column chromatography using ethanol–toluene (80:20 v/v) mobile phase and recrystallized from ethanol. Under similar conditions, its purity was assessed to be more than 96 % (Fig. 2C) with a retention time of 4.317 min. The proposed structures were supported by the FTIR, NMR and mass spectrometry (MS) data.

3.2. Toxicity

Toxicity of the synthesized compounds was evaluated *in vitro* and *in vivo*. The *in vitro* cytotoxicity was tested on Vero cells. These cells derived from monkeys are one of the most common mammalian continuous cell lines used for *in vitro* studies[12] in which test drugs should preserve the viability of Vero cells. All test compounds were found to not adversely affect the viability of Vero cells, with minimum viability of 95%. This result suggests the safety of compounds that was further supported by the *in vivo* studies for acute oral toxicity. As per the OECD-425 guidelines, the acute oral toxicity is demonstrated by the observation of mortality during 24 h in rats following treatment with a single dose of test
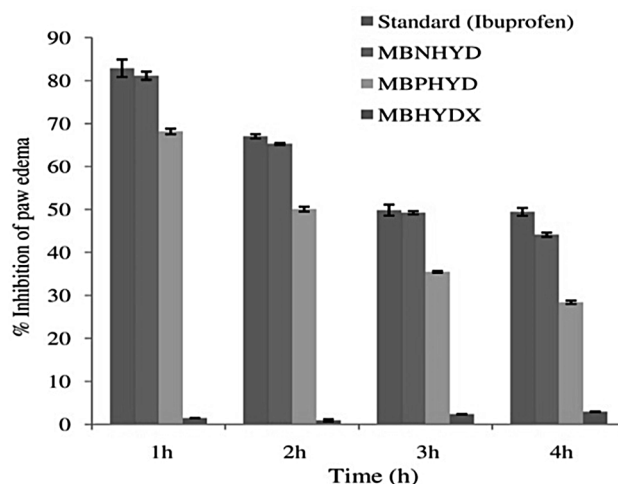
Fig. 3. Change in paw edema (acute inflammation) volume for standard drug (ibuprofen), MBNHYD, MBPHYD, and MBHYDX at a dose of 20 mg/ kg bw.

compounds. All test compounds at a dose of 100 mg/kg did not show mortality. Further, there was no any sign of morbidity within 14 days upon the administration of compounds. Thus, both *in vitro* cytotoxicity and *in vivo* oral toxicity studies indicated the safety of tested compounds and suggest their suitability for further evaluation.

3.3. Anti-Inflammatory Activity

The carrageenan-induced paw edema test is a standard method that is widely used to evaluate the anti-inflammatory activity of substances [7, 8, 13, 14]. Using this model, the anti-inflammatory activity of all three test compounds was evaluated and the data are presented in Fig. 3. The percentage paw edema inhibition was used as a quantitative parameter for the anti-inflammatory activity of test compounds and ibuprofen (20 mg/kg) was used as a positive control. Among

TABLE 1. Predicted ADME Properties of MBPHYD and MBNHYD

Property	MBPHYD	MBNHYD
Drug likeness violation	0	0
PAINS alert	0	0
Synthetic accessibility	2.74	2.17
Bioavailability score	0.55	0.55
GI absorption	High	High
BBB permeability	Yes	No
Pgp substrate	No	No
Water solubility	Moderate	High
Log P/W	2.11	0.52
Off-target probability	0.09	0.05

the test compounds, MBNHYD exhibited the highest anti-inflammatory activity comparable to that of ibuprofen at all time points of observation. MBPHYD showed relatively lower activity as compared to that of MBNHYD. Nonetheless, the anti-inflammatory effect was very significant (* $p < 0.0001$) compared to the negative control. In contrast to these findings, MBHYDX effect was found to be rather insignificant, with a low percentage inhibition of paw edema.

3.4. ADME Properties

Encouraged by the significant anti-inflammatory activity of MBNHYD and MBPHYD, their suitability for further studies and development was assessed by prediction of their drug-likeness. To analyze probable affinity for host targets (homosopiens), they were submitted to the Swiss Target Prediction tool[15]. This prediction showed low probability for binding to different host targets both for MBPHYD (0.09) and MBNHYD (0.05). This is also supported by their 0 PAINS alert scores that suggest that these compounds do not react non-specifically to multiple host targets and have potential for binding to specific targets [16].Based on the Lipinski′s rule of 5, drug-likeness is desirable for ensuring optimum oral bioavailability [17]. As both MBPHYD and MBNHYD comply with the ideal drug-like features they are likely to have good oral bioavailability. This is also supported by the prediction (Table 1) that suggests high absorption from gastrointestinal tract. Since poor water solubility is a hindrance to oral bioavailability, prediction of solubility is necessary to support bioavailability[18].While MBNHYD is very aqueous soluble, MBPHYD is moderately soluble. This is in agreement with the relatively higher lipophilicity observed for MBPHYD in the HPLC chromatogram (Fig. 2B) as compared to MBNHYD (Fig. 2A). In agreement with this observation, MBPHYD is predicted to have blood-brain-barrier (BBB) permeability. This is a desirable feature for application of anti-inflammatory compounds against central nervous system inflammation and related neurodegenerative disorders [19, 20]. Also, both compounds are not supposed to be substrates for the efflux pump (Pgp) which is desirable to ensure higher drug access at the site of action. Thus, both MBPHYD and MBNHYD exhibit desirable drug-like features while demonstrating significant anti-inflammatory activity.

4. CONCLUSION

This study reveals significant anti-inflammatory activity of novel compounds MBNHYD and MBPHYD. At a non-toxic dose, the effect of MBNHYD was found to be comparable to that of the standard drug (ibuprofen). Both

compounds exhibit good-drug likeness features and are predicted to have very low host toxicity issues. Since inflammation is associated with both communicable and non-communicable diseases, these compounds can either be used or optimised for application against them following further investigation. The MBPHYD is predicted to have brain permeability that may encourage its study for application against inflammation-induced central nervous system disorders.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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