

Synthesis and Biological Evaluation of Novel Benzimidazole Derivatives Bearing a Heterocyclic Ring at 4/5 Position

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A series of novel benzimidazole derivatives bearing a heterocyclic ring as oxadiazole (**21-32**), thiadiazole (**33-34**), triazole (**35-36**) were synthesized and evaluated for their activities against Coxsackie virus B3 and B6 in Vero cells. Compounds **21-26**, **31-36** with moieties of 2'-pyridyl, 3'-pyridyl and 4'-pyridyl at the 2-position and oxadiazoles, thiadiazole, or triazole substituent at the 4- or 5-position generally displayed activities against CVB3 and CVB6. Especially compound **24** (IC₅₀ = 1.08 µg/mL, SI = 61.7 against CVB3) was the promising candidate as lead compound for anti-enteroviral drug. It was observed in the incorporation of heterocyclic rings in benzimidazole at the 5-position could enhance their biological activities.

Key Words : Benzimidazole, Small-molecule inhibitors, Enterovirus, Picornavirus, Coxsackie

Introduction

Benzimidazole is a heterocyclic aromatic compound consists of benzene and imidazole rings. The most prominent benzimidazole compound in nature is *N*-ribosyl-dimethylbenzimidazole, which serves as an axial ligand for cobalt in vitamin B12.¹ Benzimidazole derivatives are structural isosteres of naturally occurring nucleotides, which allows them to interact easily with the biopolymers of the living systems. Therefore, benzimidazole derivatives have shown different therapeutic properties such as antiulcer,² antihelminthic,³ antihypertensive,⁴ anticoagulant,⁵ antiallergic,⁶ analgesic,⁷ anti-inflammatory,⁸ antimicrobial,⁹ antiviral,¹⁰ antiparasitic,¹¹ and antioxidant.¹²

Nitrogen containing heterocyclic molecules constitutes the largest portion of chemical entities, which are part of many natural products, fine chemicals, and biologically active pharmaceuticals. The oxadiazole is a five-membered nitrogen and oxygen containing heterocycle which has been commonly used as a privileged scaffold to produce various novel therapeutic molecules.¹³ 1,3,4-oxadiazoles have been found to exhibit diverse biological activities such as antimicrobial,¹⁴ anti-HIV,¹⁴ antitubercular,¹⁵ antimalarial,¹⁶ analgesic,¹⁷ anti-inflammatory,¹⁸ anticonvulsant,¹⁹ hypoglycemic.²⁰ It is reported that 1,3,4-thiadiazole derivatives possess wide spectrum insecticidal²¹ and herbicidal²² activities. 1,2,4-triazole derivatives are also known to exhibit antimicrobial,²³ antitubercular,²⁴ anticancer,²⁵ anticonvulsant,²⁶ anti-inflammatory and analgesic properties.²⁷

Enteroviruses are members of the picornavirus family, a large and diverse group of small RNA viruses characterized

by a single positive-strand genomic RNA. Enteroviruses affect millions of people worldwide each year, and cause many serious diseases as poliomyelitis, nonspecific febrile illness, aseptic meningitis, pleurodynia, myocarditis, etc. After the host cell was infected, the genome of enterovirus was translated in a cap-independent manner into a single polyprotein, and processed by virus-encoded proteases into structural capsid proteins and nonstructural proteins subsequently. Both kinds of proteins were mainly involved in the replication of virus.²⁸ Due to the special structure of benzimidazole, specific hydrogen bonded interactions could be formed with the genome, interfering the translating process. Therefore, a series of molecules were designed based on the benzimidazole system to inhibit enterovirus.

In our previous work,²⁹ a series of 2-pyridyl-1*H*-benzimidazole-4-carboxamide derivatives were synthesized and their biological activities were tested against CVA16, CVB3, CVB6 and EV71. In this manuscript, we designed and synthesized 16 novel benzimidazole derivatives (Fig. 1). It was believed that their biological activities would be enhanced by incorporating nitrogen containing heterocyclic molecules with benzimidazoles. Inhibitory activities of these benzimidazole derivatives were tested against CVB3 and CVB6. As

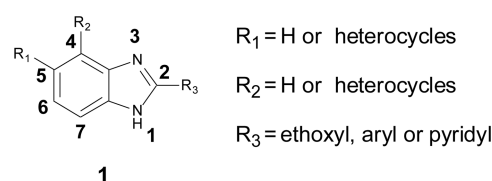


Figure 1. General structure of synthesized compounds.

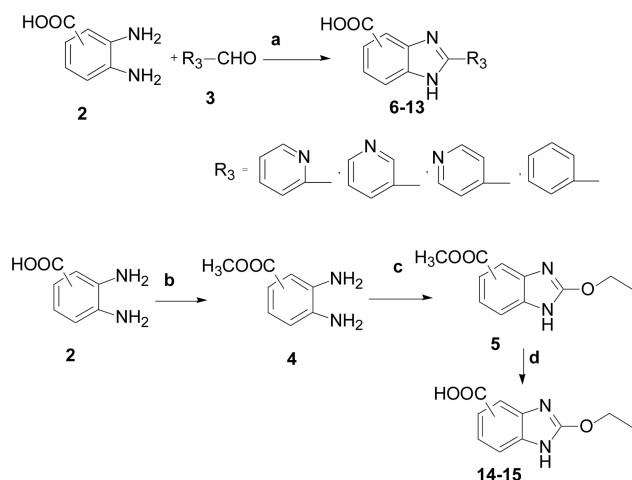
there are currently no drugs against these viruses, ribavirin (RBV)³⁰ was selected as a positive control. These benzimidazole derivatives were found to exhibit good inhibitory activities against two kinds of enteroviruses.

Experimental

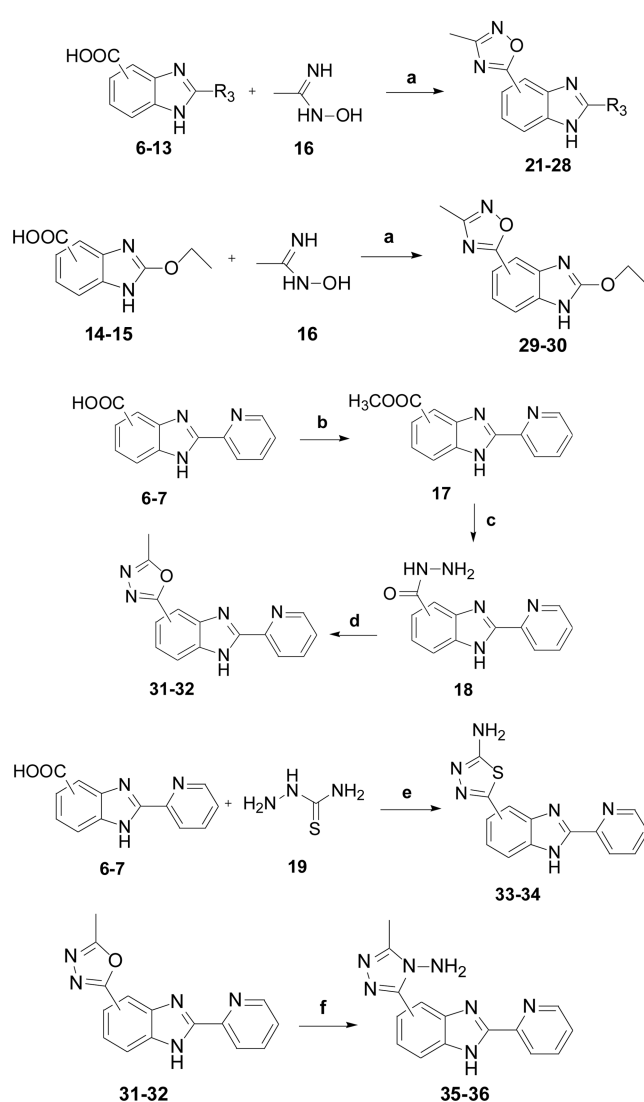
Starting from 2,3/3,4-diaminobenzoic acid (**2**), the synthetic route of the 2-substituent-1*H*-benzimidazole-4-carboxylic acids (**6-15**) is shown in Scheme 1. 2-Substituent-1*H*-benzimidazole-4-carboxylic acids (**6-13**) were obtained by condensation of 2,3/3,4-diaminobenzoic acid (**2**) with aryl and heteroaryl aldehydes in the presence of 1,4-benzoquinone. 2,3/3,4-Diaminobenzoic acid (**2**) was transformed to 2,3/3,4-diaminomethyl benzoate (**4**), which was converted into compound (**5**) by condensation of 2,3/3,4-diamino methyl benzoate with tetraethyl orthocarbonate. After the hydrolysis of the ester, the 2-ethoxyl-1*H*-benzimidazole-4/5-carboxylic acids (**14-15**) were obtained.

The synthetic route of corresponding benzimidazole derivatives bearing a heterocyclic ring at the 4-/5-position of the benzimidazole ring (**21-36**) is shown in Scheme 2. 1,2,4-Oxadiazoles (**21-30**) were obtained by the treatment of compounds (**6-15**) with *N*-hydroxyacetamide under EDC/HOBT conditions. The acid hydrazides (**18**) was treated with acetic acid in the presence of phosphorus oxychloride afforded 1,3,4-oxadiazoles (**31-32**). Compounds (**6-7**) were treated with thiosemicarbazide in the presence of phosphorous oxychloride afforded 1,3,4-thiadiazoles (**33-34**). 1,2,4-triazoles (**35-36**) were obtained by the reaction of 1,3,4-oxadiazoles (**31-32**) with hydrazine hydrate at reflux condition.

¹H NMR spectra of DMSO-*d*₆ solutions were recorded on a Bruker DPX400 spectrometer. Elemental analysis was performed on a Vario ELIII instrument within ± 0.5% of the theoretical values. The starting materials and reagents,



Scheme 1. Synthetic route of 2-substituent-1*H*-benzimidazole-4/5-carboxylic acids (**6-15**). Reagents and conditions: (a) 1,4-benzoquinone, dioxane, 80 °C; (b) SOCl₂, methanol, 0 °C-80 °C; (c) tetraethyl orthocarbonate, acetic acid, 90-100 °C; (d) ethanol/water (2:1), NaOH.



Scheme 2. Synthetic route of benzimidazole derivatives bearing a heterocyclic ring (**21-36**). Reagents and conditions: (a) EDC-HCl, HOBT, DMF or DCM, 0 °C-25 °C; (b) SOCl₂, methanol, 0 °C-reflux; (c) hydrazine hydrate, methanol, reflux; (d) acetic acid, POCl₃, 80 °C; (e) thiosemicarbazide, POCl₃, 80 °C; (f) hydrazine hydrate, reflux.

purchased from commercial suppliers, were used without further purification. All final compounds had a purity of > 95% as assessed by analytical HPLC. HPLC analyses were conducted on Shimadzu Prominence LC-20A system using YMC-PACK ODS-A 150 × 4.6 nm, 5 μm column with UV 220 nm and 245 nm detection. The mobile phase consisted of acetonitrile-methanol-water (45:45:10) with flow rate of 1 mL/min.

General Procedure A: Synthesis of 2-Substituent-1*H*-benzimidazole-4/5-carboxylic acids (Compounds 6-13). Appropriate aldehyde **3** (1.5 mmol) was added to the solution of 2,3/3,4-diaminobenzoic acid **2** (1.0 mmol) in dioxane (10 mL). 1,4-Benzoquinone was added and the solution was heated to 80 °C and stirred for about 6-9 h. Then, the solution was cooled to room temperature. The separated solid crystals were filtered, washed with ethanol

and ether, dried. The products were used in the next step without further purification.

General Procedure B: Synthesis of 2-ethoxyl-1*H*-benzimidazole-4/5-carboxylic acids (Compounds 14-15).

Synthesis of 2,3/3,4-Diamino Methyl Benzoate (Compound 4): 1 mL SOCl₂ was added to anhydrous methanol (10 mL) dropwise below 0 °C. 2,3/3,4-diaminobenzoic acid **2** (0.15 g, 1.0 mmol) was added and the mixture refluxed for 4 h. The solvent was evaporated under reduced pressure and the residue was washed with saturated NaHCO₃, filtered and dried.

Synthesis of 2-Ethoxyl-1*H*-benzimidazole-4/5-carboxylic acid Methyl Ester (Compound 5): A mixture of 2,3/3,4-diamino methyl benzoate (0.17 g, 1.0 mmol), tetraethyl orthocarbonate (0.38 g, 2.0 mmol) and acetic acid (0.06 g, 1.0 mmol) was stirred for 4 h at 90-100 °C. Then extracted with ethyl acetate, washed with water, dried, and the solvent was removed under reduced pressure.

Synthesis of 2-Ethoxyl-1*H*-benzimidazole-4/5-carboxylic acid (Compounds 14-15): 2-Ethoxyl-1*H*-benzimidazole-4/5-carboxylic acid methyl ester (0.22 g, 1.0 mmol) was added to a solution of ethanol:water = 2:1 (10 mL). Then NaOH (0.08 g, 2.0 mmol) was added and stirred for 2 h at room temperature. The ethanol was removed under reduced pressure and the residue was washed with dilute hydrochloric acid (1 mol/L), then extracted with ethyl acetate, washed with water, dried, and the solvent was removed under reduced pressure.

General Procedure C: Synthesis of 4/5-(3-Methyl-1,2,4-oxadiazol-5-yl)-2-substituent-1*H*-benzimidazoles (Compounds 21-30). A mixture of appropriate 2-substituent-1*H*-benzimidazole-4/5-carboxylic acid (1 mmol), EDC·HCl (0.37 g, 2.0 mmol) and HOBt (0.27 g, 2.0 mmol) in DMF or DCM (10 mL) was stirred for 1 h at room temperature. Then *N*-hydroxyacetamide (1.05 mmol) was added and stirred for 24 h. 20 mL water was added and the precipitate was filtered, dried. The products were purified on silica gel column.

General Procedure D: Synthesis of 4/5-(3-Methyl-1,3,4-oxadiazol-5-yl)-2-substituent-1*H*-benzimidazoles (Compounds 31-32).

Synthesis of 2-Substituent-1*H*-benzimidazole-4/5-carboxylic acid Methyl Ester (Compounds 17): 1 mL SOCl₂ was added to anhydrous methanol (10 mL) by dropwise at below 0 °C. Then 2-substituent-1*H*-benzimidazole-4/5-carboxylic acid (1.0 mmol) was added and the mixture refluxed for 4 h. The solvent was evaporated and the residue was washed with saturated NaHCO₃, filtered and dried.

Synthesis of 2-Substituent-1*H*-benzimidazole-4/5-carboxylic acid Hydrazide (Compounds 18): 2-Substituent-1*H*-benzimidazole-4/5-carboxylic acid methyl ester (1.0 mmol) and hydrazine hydrate (2.0 mmol) was refluxed in anhydrous methanol (10 mL) for 12 h. The mixture was cooled to room temperature and the separated solid crystals were filtered, washed with cold water, dried.

Synthesis of 4/5-(3-Methyl-1,3,4-oxadiazol-5-yl)-2-substituent-1*H*-benzimidazoles (Compounds 31-32): A mix-

ture of 2-substituent-1*H*-benzimidazole-4/5-carboxylic acid hydrazide (1.0 mmol) and acetic acid (1.0 mmol) in POCl₃ (10 mL) was stirred for 12 h at 80 °C. Then the reaction mixture was cooled to room temperature and gently poured into the ice water. The separated solid crystals were filtered, washed with saturated NaHCO₃, dried. The products were purified on silica gel column.

General Procedure E: Synthesis of 4/5-(3-Methyl-1,3,4-thiadiazole-5-yl)-2-substituent-1*H*-benzimidazoles (Compounds 33-34). A mixture of 2-substituent-1*H*-benzimidazole-4/5-carboxylic acid (1.0 mmol) and thiosemicarbazide (1.05 mmol) in POCl₃ (10 mL) was stirred for 12 h at 80 °C. Then the reaction mixture was cooled to room temperature and gently poured into the ice water. The separated solid crystals were filtered, washed with saturated NaHCO₃, dried. The products were purified on silica gel column.

General Procedure F: Synthesis of 4/5-(3-Methyl-1,2,4-triazole-5-yl)-2-substituent-1*H*-benzimidazoles (Compounds 35-36). A mixture of 4/5-(3-methyl-1,3,4-oxadiazol-5-yl)-2-substituent-1*H*-benzimidazole (**31-32**) (1.0 mmol) and hydrazine hydrate (10 mL) was refluxed for 8 h. Then the reaction mixture was cooled to room temperature and gently poured into the ice water. The mixture was acidified with concentrated hydrochloric acid. The separated solid crystals were filtered, washed with water, dried. The products were purified on silica gel column.

2-(Pyridin-2-yl)-1*H*-benzimidazole-4-carboxylic acid (6): Compound **6** was synthesized from picolinaldehyde and 2,3-diaminobenzoic acid **2** using general procedure A as a greyish-white solid. Yield = 93%. ¹H NMR (DMSO, 400 MHz) δ 8.77-8.76 (m, 1H), 8.41 (d, *J* = 10.0 Hz, 1H), 8.07-8.03 (m, 1H), 7.98 (d, *J* = 8.0 Hz, 1H), 7.82 (d, *J* = 8.0 Hz, 1H), 7.60-7.56 (m, 1H), 7.46-7.41 (m, 1H). Anal. calcd. for C₁₃H₉N₃O₂: C, 65.27; H, 3.79; N, 17.56. Found: C, 65.56; H, 3.58; N, 17.43.

2-(Pyridin-2-yl)-1*H*-benzimidazole-5-carboxylic acid (7): Compound **7** was synthesized from picolinaldehyde and 3,4-diaminobenzoic acid **2** using general procedure A as a white solid. Yield = 93%. ¹H NMR (DMSO, 400 MHz) δ 8.76-8.75 (d, *J* = 8.0 Hz, 1H), 8.35 (d, *J* = 8.0 Hz, 1H), 8.24 (s, 1H), 8.04-7.99 (m, 1H), 7.88-7.81 (m, 1H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.56-7.54 (m, 1H). Anal. calcd. for C₁₃H₉N₃O₂: C, 65.27; H, 3.79; N, 17.56. Found: C, 65.46; H, 3.42; N, 17.38.

2-(Pyridin-3-yl)-1*H*-benzimidazole-4-carboxylic acid (8): Compound **8** was synthesized from nicotinaldehyde and 2,3-diaminobenzoic acid **2** using general procedure A as a greyish-white solid. Yield = 95%. ¹H NMR (DMSO, 400 MHz) δ 9.45 (s, 1H), 8.75 (d, *J* = 6.0 Hz, 1H), 8.35 (d, *J* = 8.0 Hz, 1H), 8.07-8.03 (m, 1H), 7.96-7.95 (m, 1H), 7.58-7.53 (m, 1H), 7.33-7.28 (m, 1H). Anal. calcd. for C₁₃H₉N₃O₂: C, 65.27; H, 3.79; N, 17.56. Found: C, 65.45; H, 3.49; N, 17.53.

2-(Pyridin-3-yl)-1*H*-benzimidazole-5-carboxylic acid (9): Compound **9** was synthesized from nicotinaldehyde and 3,4-diaminobenzoic acid **2** using general procedure A as a white solid. Yield = 92%. ¹H NMR (DMSO, 400 MHz) δ 9.35-9.34 (m, 1H), 8.71-8.70 (m, 1H), 8.52-8.49 (m, 1H), 8.28 (s, 1H), 7.95-7.92 (m, 1H), 7.80 (d, *J* = 8.0 Hz, 1H), 7.62-7.59

(m, 1H). Anal. calcd. for C₁₃H₉N₃O₂: C, 65.27; H, 3.79; N, 17.56. Found: C, 64.95; H, 3.88; N, 17.73.

2-(Pyridin-4-yl)-1H-benzimidazole-4-carboxylic acid (10): Compound **10** was synthesized from isonicotinaldehyde and 2,3-diaminobenzoic acid **2** using general procedure A as a grayish-white solid. Yield = 95%. ¹H NMR (DMSO, 400 MHz) δ 8.76 (d, *J* = 6.0 Hz, 2H), 8.01-7.85 (m, 2H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.53-7.36 (m, 1H), 7.14 (d, *J* = 8.0 Hz, 1H). Anal. calcd. for C₁₃H₉N₃O₂: C, 65.27; H, 3.79; N, 17.56. Found: C, 65.04; H, 3.63; N, 17.68.

2-(Pyridin-4-yl)-1H-benzimidazole-5-carboxylic acid (11): Compound **11** was synthesized from isonicotinaldehyde and 3,4-diaminobenzoic acid **2** using general procedure A as a white solid. Yield = 93%. ¹H NMR (DMSO, 400 MHz) δ 8.76 (d, *J* = 6.0 Hz, 2H), 8.10-8.08 (m, 2H), 7.97-7.87 (m, 1H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.54-7.37 (m, 1H). Anal. calcd. for C₁₃H₉N₃O₂: C, 65.27; H, 3.79; N, 17.56. Found: C, 65.56; H, 3.48; N, 17.85.

2-Phenyl-1H-benzimidazole-4-carboxylic acid (12): Compound **12** was synthesized from benzaldehyde and 2,3-diaminobenzoic acid **2** using general procedure A as a slightly yellow solid. Yield = 83%. ¹H NMR (DMSO, 400 MHz) δ 8.10-8.08 (m, 2H), 7.62-7.58 (m, 1H), 7.50-7.46 (m, 2H), 6.51 (d, *J* = 8.0 Hz, 2H). Anal. calcd. for C₁₄H₁₀N₂O₂: C, 70.58; H, 4.23; N, 11.76. Found: C, 70.86; H, 4.57; N, 12.09.

2-Phenyl-1H-benzimidazole-5-carboxylic acid (13): Compound **13** was synthesized from benzaldehyde and 3,4-diaminobenzoic acid **2** using general procedure A as a slightly yellow solid. Yield = 85%. ¹H NMR (DMSO, 400 MHz) δ 8.09-8.07 (m, 2H), 7.64-7.59 (m, 1H), 7.51-7.47 (m, 2H), 6.50 (d, *J* = 8.0 Hz, 2H). Anal. calcd. for C₁₄H₁₀N₂O₂: C, 70.58; H, 4.23; N, 11.76. Found: C, 70.16; H, 4.38; N, 11.97.

2-Ethoxyl-1H-benzimidazole-4-carboxylic acid (14): Compound **14** was synthesized from tetraethyl orthocarbonate and 2,3-diaminobenzoic acid **2** using general procedure B as a greyish-white solid. Yield = 87%. ¹H NMR (DMSO, 400 MHz) δ 7.59-7.58 (m, 1H), 7.57-7.56 (d, *J* = 6.0 Hz, 1H), 7.13-7.10 (m, 1H), 4.52-4.47 (m, 2H), 1.38-1.35 (m, 3H). Anal. calcd. for C₁₀H₀N₂O₃: C, 58.25; H, 4.89; N, 13.59. Found: C, 58.57; H, 4.63; N, 13.88.

2-Ethoxyl-1H-benzimidazole-5-carboxylic acid (15): Compound **15** was synthesized from tetraethyl orthocarbonate and 3,4-diaminobenzoic acid **2** using general procedure B as a greyish-white solid. Yield = 85%. ¹H NMR (DMSO, 400 MHz) δ 7.87-7.81 (m, 1H), 7.70-7.67 (m, 1H), 7.38-7.32 (m, 1H), 4.51-4.46 (m, 2H), 1.38-1.35 (m, 3H). Anal. calcd. for C, 58.25; H, 4.89; N, 13.59. Found: C, 58.65; H, 4.53; N, 13.35.

4-(3-Methyl-1,2,4-oxadiazol-5-yl)-2-(pyridin-2-yl)-1H-benzimidazole (21): Compound **21** was synthesized from 2-(pyridin-2-yl)-1H-benzimidazole-4-carboxylic acid **6** and *N*-hydroxyacetamide using general procedure C as a slightly yellow solid. Yield = 65%. mp 169-171 °C. ¹H NMR (DMSO, 400 MHz) δ 8.77-8.76 (d, *J* = 6.0 Hz, 1H), 8.42-8.40 (d, *J* = 8.0 Hz, 1H), 8.07-8.03 (m, 1H), 7.99-7.97 (m, 1H), 7.83-

7.81 (d, *J* = 8.0 Hz, 1H), 7.60-7.55 (m, 1H), 7.48-7.41 (m, 1H), 2.46 (s, 3H). Anal. calcd. for C, 64.97; H, 4.00; N, 25.26. Found: C, 64.68; H, 3.64; N, 25.73.

5-(3-Methyl-1,2,4-oxadiazol-5-yl)-2-(pyridin-2-yl)-1H-benzimidazole (22): Compound **22** was synthesized from 2-(pyridin-2-yl)-1H-benzimidazole-5-carboxylic acid **7** and *N*-hydroxyacetamide using general procedure C as a white solid. Yield = 63%. mp 239-241 °C. ¹H NMR (DMSO, 400 MHz) δ 8.76-8.75 (d, 1H), 8.36-8.34 (d, *J* = 8.0 Hz, 1H), 8.04-8.01 (m, 1H), 7.95-7.94 (m, 1H), 7.54-7.60 (m, 1H), 7.06-7.08 (d, *J* = 8.0 Hz, 1H), 6.80-6.82 (m, 1H), 2.40 (s, 3H). Anal. calcd. for C, 64.97; H, 4.00; N, 25.26. Found: C, 64.78; H, 4.45; N, 25.65.

4-(3-Methyl-1,2,4-oxadiazol-5-yl)-2-(pyridin-3-yl)-1H-benzimidazole (23): Compound **23** was synthesized from 2-(pyridin-3-yl)-1H-benzimidazole-4-carboxylic acid **8** and *N*-hydroxyacetamide using general procedure C as a pink solid. Yield = 68%. mp 197-199 °C. ¹H NMR (DMSO, 400 MHz) δ 8.77-8.76 (d, *J* = 6.0 Hz, 1H), 8.42-8.34 (m, 1H), 8.08-8.04 (m, 2H), 7.83-7.81 (d, *J* = 8.0 Hz, 1H), 7.58-7.55 (m, 1H), 7.45-7.41 (m, 1H), 2.46 (s, 3H). Anal. calcd. for C, 64.97; H, 4.00; N, 25.26. Found: C, 64.57; H, 3.75; N, 25.68.

5-(3-Methyl-1,2,4-oxadiazol-5-yl)-2-(pyridin-3-yl)-1H-benzimidazole (24): Compound **24** was synthesized from 2-(pyridin-3-yl)-1H-benzimidazole-5-carboxylic acid **9** and *N*-hydroxyacetamide using general procedure C as a brown solid. Yield = 62%. mp 173-175 °C. ¹H NMR (DMSO, 400 MHz) δ 9.35-9.34 (d, *J* = 6.0 Hz, 1H), 8.71-8.70 (m, 1H), 8.52-8.49 (m, 1H), 8.28 (s, 1H), 7.95-7.93 (m, 1H), 7.81-7.79 (d, *J* = 8.0 Hz, 1H), 7.62-7.57 (m, 1H), 2.40 (s, 3H). Anal. calcd. for C, 64.97; H, 4.00; N, 25.26. Found: C, 64.58; H, 3.65; N, 25.58.

4-(3-Methyl-1,2,4-oxadiazol-5-yl)-2-(pyridin-4-yl)-1H-benzimidazole (25): Compound **25** was synthesized from 2-(pyridin-4-yl)-1H-benzimidazole-4-carboxylic acid **10** and *N*-hydroxyacetamide using general procedure C as a slightly yellow solid. Yield = 65%. mp 181-183 °C. ¹H NMR (DMSO, 400 MHz) δ 8.79-8.75 (d, *J* = 16.0 Hz, 2H), 8.01-7.85 (m, 2H), 7.70-7.68 (d, *J* = 8.0 Hz, 1H), 7.53-7.36 (m, 1H), 7.14-7.13 (d, *J* = 6.0 Hz, 1H), 1.91 (s, 3H). Anal. calcd. for C, 64.97; H, 4.00; N, 25.26. Found: C, 64.66; H, 3.89; N, 25.44.

5-(3-Methyl-1,2,4-oxadiazol-5-yl)-2-(pyridin-4-yl)-1H-benzimidazole (26): Compound **26** was synthesized from 2-(pyridin-4-yl)-1H-benzimidazole-5-carboxylic acid **11** and *N*-hydroxyacetamide using general procedure C as a brown solid. Yield = 60%. mp 163-165 °C. ¹H NMR (DMSO, 400 MHz) δ 8.79-8.77 (d, *J* = 6.0 Hz, 2H), 8.10-8.08 (m, 2H), 7.97-7.87 (m, 1H), 7.70-7.68 (d, *J* = 8.0 Hz, 1H), 7.54-7.37 (m, 1H), 3.87 (s, 3H). Anal. calcd. for C, 64.97; H, 4.00; N, 25.26. Found: C, 64.76; H, 3.69; N, 25.64.

4-(3-Methyl-1,2,4-oxadiazol-5-yl)-2-phenyl-1H-benzimidazole (27): Compound **27** was synthesized from 2-phenyl-1H-benzimidazole-4-carboxylic acid **12** and *N*-hydroxyacetamide using general procedure C as a greyish-white solid. Yield = 68%. mp 121-123 °C. ¹H NMR (DMSO, 400 MHz) δ 8.10-8.08 (m, 2H), 7.62-7.58 (m, 1H), 7.50-7.46 (m,

2H), 6.51 (s, 2H), 1.81 (s, 3H). Anal. calcd. for C, 69.55; H, 4.38; N, 20.28. Found: C, 69.86; H, 4.69; N, 20.58.

5-(3-Methyl-1,2,4-oxadiazol-5-yl)-2-phenyl-1H-benzimidazole (28): Compound **28** was synthesized from 2-phenyl-1H-benzimidazole-5-carboxylic acid **13** and *N*-hydroxyacetamide using general procedure C as a yellow solid. Yield = 65%. mp 115-117 °C. ¹H NMR (DMSO, 400 MHz) δ 8.09-8.07 (m, 2H), 7.64-7.59 (m, 1H), 7.51-7.47 (m, 2H), 6.50 (s, 2H), 1.79 (s, 3H). Anal. calcd. for C, 69.55; H, 4.38; N, 20.28. Found: C, 69.79; H, 4.76; N, 20.65.

2-Ethoxyl-4-(3-methyl-1,2,4-oxadiazol-5-yl)-1H-benzimidazole (29): Compound **29** was synthesized from 2-ethoxyl-1H-benzimidazole-4-carboxylic acid **14** and *N*-hydroxyacetamide using general procedure C as a slightly yellow solid. Yield = 75%. mp 114-116 °C. ¹H NMR (DMSO, 400 MHz) δ 7.66-7.65 (d, *J* = 6.0 Hz, 1H), 7.49-7.48 (d, *J* = 6.0 Hz, 1H), 7.16-7.12 (m, 1H), 4.48-4.42 (m, 2H), 1.84 (s, 3H), 1.41-1.38 (m, 3H). Anal. calcd. for C, 59.01; H, 4.95; N, 22.94. Found: C, 59.34; H, 4.63; N, 22.66.

2-Ethoxyl-5-(3-methyl-1,2,4-oxadiazol-5-yl)-1H-benzimidazole (30): Compound **30** was synthesized from 2-ethoxyl-1H-benzimidazole-5-carboxylic acid **15** and *N*-hydroxyacetamide using general procedure C as a white solid. Yield = 78%. mp 106-108 °C. ¹H NMR (DMSO, 400 MHz) δ 7.92 (s, 1H), 7.60-7.58 (d, *J* = 8.0 Hz, 1H), 7.41 (s, 1H), 4.51-4.45 (m, 2H), 1.42 (s, 3H), 1.23-1.18 (m, 3H). Anal. calcd. for C, 59.01; H, 4.95; N, 22.94. Found: C, 59.48; H, 4.55; N, 22.63.

4-(3-Methyl-1,3,4-oxadiazol-5-yl)-2-(pyridin-2-yl)-1H-benzimidazole (31): Compound **31** was synthesized from 2-(pyridin-2-yl)-1H-benzimidazole-4-carboxylic acid **6** using general procedure D as a white solid. Yield = 65%. mp 229-231 °C. ¹H NMR (DMSO, 400 MHz) δ 8.77-8.75 (m, 1H), 8.38-8.36 (d, *J* = 8.0 Hz, 1H), 8.05-8.00 (m, 1H), 7.86-7.84 (d, *J* = 8.0 Hz, 1H), 7.82-7.80 (m, 1H), 7.57-7.54 (m, 1H), 7.43-7.39 (m, 1H), 2.65 (s, 3H). Anal. calcd. for C, 64.97; H, 4.00; N, 25.26. Found: C, 64.53; H, 4.48; N, 25.70.

5-(3-Methyl-1,3,4-oxadiazol-5-yl)-2-(pyridin-2-yl)-1H-benzimidazole (32): Compound **32** was synthesized from 2-(pyridin-2-yl)-1H-benzimidazole-5-carboxylic acid **7** using general procedure D as a slightly yellow solid. Yield = 63%. mp 295-297 °C. ¹H NMR (DMSO, 400 MHz) δ 8.76-8.75 (d, *J* = 6.0 Hz, 1H), 8.36-8.34 (d, *J* = 8.0 Hz, 1H), 8.23-8.19 (m, 1H), 8.04-7.99 (m, 1H), 7.88-7.80 (m, 1H), 7.70-7.68 (d, *J* = 8.0 Hz, 1H), 7.57-7.53 (m, 1H), 2.58 (s, 3H). Anal. calcd. for C, 64.97; H, 4.00; N, 25.26. Found: C, 64.64; H, 4.38; N, 25.47.

4-(3-Methyl-1,3,4-thiadiazole-5-yl)-2-(pyridin-2-yl)-1H-benzimidazole (33): Compound **33** was synthesized from 2-(pyridin-2-yl)-1H-benzimidazole-4-carboxylic acid **6** and thiosemicarbazide using general procedure E as a dark yellow solid. Yield = 60%. mp 268-270 °C. ¹H NMR (DMSO, 400 MHz) δ 8.76-8.75 (d, *J* = 6.0 Hz, 1H), 8.37-8.35 (d, *J* = 8.0 Hz, 1H), 8.07-8.03 (m, 1H), 7.96-7.95 (m, 1H), 7.58-7.53 (m, 1H), 7.33-7.28 (m, 2H). Anal. calcd. for C, 57.13; H, 3.42; N, 28.55. Found: C, 57.45; H, 3.74; N, 28.83.

5-(3-Methyl-1,3,4-thiadiazole-5-yl)-2-(pyridin-2-yl)-1H-

benzimidazole (34): Compound **34** was synthesized from 2-(pyridin-2-yl)-1H-benzimidazole-5-carboxylic acid **7** and thiosemicarbazide using general procedure E as a pink solid. Yield = 63%. mp 294-296 °C. ¹H NMR (DMSO, 400 MHz) δ 8.76-8.75 (d, *J* = 6.0 Hz, 1H), 8.36-8.34 (d, *J* = 8.0 Hz, 1H), 8.23-8.19 (d, *J* = 16.0 Hz, 1H), 8.04-7.99 (m, 1H), 7.88-7.81 (m, 1H), 7.70-7.68 (d, *J* = 8.0 Hz, 1H), 7.57-7.55 (m, 1H). Anal. calcd. for C, 57.13; H, 3.42; N, 28.55. Found: C, 57.58; H, 3.86; N, 28.94.

4-(3-Methyl-1,2,4-triazole-5-yl)-2-(pyridin-2-yl)-1H-benzimidazole (35): Compound **35** was synthesized from 4-(3-methyl-1,3,4-oxadiazol-5-yl)-2-(pyridin-2-yl)-1H-benzimidazole **31** and hydrazine hydrate using general procedure F as a white powder. Yield = 60%. mp 245-247 °C. ¹H NMR (DMSO, 400 MHz) δ 8.77-8.76 (d, *J* = 6.0 Hz, 1H), 8.44-8.42 (d, *J* = 8.0 Hz, 1H), 8.07-8.03 (m, 1H), 7.90-7.88 (d, *J* = 8.0 Hz, 1H), 7.72-7.70 (d, *J* = 8.0 Hz, 1H), 7.59-7.56 (m, 1H), 7.39-7.35 (m, 1H), 4.75 (s, 3H). Anal. calcd. for C, 61.84; H, 4.50; N, 33.66. Found: C, 61.48; H, 4.88; N, 33.25.

































5-(3-Methyl-1,2,4-triazole-5-yl)-2-(pyridin-2-yl)-1H-benzimidazole (36): Compound **36** was synthesized from 5-(3-methyl-1,3,4-oxadiazol-5-yl)-2-(pyridin-2-yl)-1H-benzimidazole **32** and hydrazine hydrate using general procedure F as a white powder. Yield = 61%. mp 261-263 °C. ¹H NMR (DMSO, 400 MHz) δ 9.79-9.75 (d, *J* = 16.0 Hz, 1H), 8.73-8.72 (d, *J* = 6.0 Hz, 1H), 8.34-8.32 (d, *J* = 8.0 Hz, 1H), 8.19 (s, 1H), 8.03-7.97 (m, 1H), 7.77-7.67 (m, 1H), 7.55-7.51 (m, 1H), 4.49 (s, 3H). Anal. calcd. for C, 61.84; H, 4.50; N, 33.66. Found: C, 61.77; H, 4.13; N, 33.85.

Antiviral Activity Evaluation *In vitro*. The virus strains of CVB3 and CVB6 were provided by American Type Culture Collection (ATCC). The positive control drug, RBV was produced by Hubei Keyi Pharmaceutical Factory. The newly synthesized benzimidazole compounds (**21-36**) were dissolved in DMSO and diluted with the culture medium. Vero cells were planted in 96-well culture plates. After 24 h the plates were placed in the corresponding virus bulk for 2 h. Then the solutions of benzimidazole compounds and RBV were added in the plates and cell wells and virus wells were set simultaneously. When the cytopathic effect (CPE) of virus wells was over 4, the CPE of cell wells was observed. The concentration required to inhibit virus growth by 50% (IC₅₀) was determined by the Reed-Muench method.³¹

Results and Discussion

The anti-enterovirus activities and cytotoxicities of our compounds were evaluated in Vero cells against CVB3 and CVB6 using RBV as a positive control. Results are summarized in Table 1. The anti-enterovirus activity of each compound was expressed as the concentration of compound that caused 50% inhibition (IC₅₀) of enterovirus growth. The cytotoxicity of each compound was expressed as the concentration of compound required to kill 50% (TC₅₀) of the Vero cells. As a major pharmaceutical parameter for possible future clinical development, the selectivity index (SI) was determined as the ratio of TC₅₀ to IC₅₀. The bioactivity of

Table 1. Activity of benzimidazole derivatives against Coxsackie virus B3 and B6 in Vero cells

Compound	R ₁	R ₂	R ₃	TC ₅₀ ^a (μg/mL)		IC ₅₀ ^b (μg/mL)		SI ^c	
				Vero cells		CVB3	CVB6	CVB3	CVB6
21	H			3.99		0.47	0.64	8.5	6.2
22		H		22.22		1.43	1.88	15.5	11.8
23	H			28.63		0.82	3.18	34.9	9.0
24		H		66.67		1.08	3.25	61.7	20.5
25	H			5.14		> 2.47	> 2.47	-	-
26		H		46.22		7.41	12.83	6.2	3.6
27	H			7.41		1.92	> 2.47	3.9	-
28		H		7.41		2.47	> 2.47	3.0	-
29	H			5.14		2.47	> 2.47	2.1	-
30		H		115.47		> 66.67	66.67	-	1.7
31	H			5.14		0.27	0.64	19.0	8.0
32		H		38.49		1.43	2.47	26.9	15.6
33	H			5.14		0.82	> 2.47	6.3	-
34		H		38.49		1.43	5.63	26.9	6.8
35	H			66.67		1.88	2.47	35.5	27.0
36		H		115.47		9.75	9.75	11.8	11.8
RBV	-	-	-	2000		384.90	384.90	5.2	5.2

^aCytotoxic concentration required to inhibit Vero cell growth by 50%. ^bConcentration required to inhibit Coxsackie virus growth by 50%. ^cSelective Index values equaled to TC₅₀/IC₅₀.

each compound was evaluated by the combination of its IC_{50} and SI.

The antiviral activities of these compounds against CVB3 and CVB6 are summarized in Table 1. As shown in Table 1, compounds **21-26**, **31-36** with moieties of 2'-pyridyl, 3'-pyridyl and 4'-pyridyl at the 2-position and oxadiazole, 1,3,4-thiadiazole or 1,2,4-triazole ring at the 4- or 5-position were found to have excellent IC_{50} s (IC_{50} s of less than 10 $\mu\text{g}/\text{ml}$ against CVB3 and IC_{50} s of less than 13 $\mu\text{g}/\text{mL}$ against CVB6), much better than RBV with IC_{50} of 384.90 $\mu\text{g}/\text{mL}$. IC_{50} s of compounds **21**, **23**, **31** and **33** were even less than 1 $\mu\text{g}/\text{mL}$. The most potent compound, **24** (IC_{50} = 1.08 $\mu\text{g}/\text{mL}$, SI = 61.7 against CVB3 and IC_{50} = 3.25 $\mu\text{g}/\text{mL}$, SI = 20.5 against CVB6) was more selective than RBV (SI = 5.2).

It was observed that TC_{50} values of compounds **22**, **24**, **26**, **30**, **32**, **34** and **36** are much higher than those of compounds **21**, **23**, **25**, **29**, **31**, **33** and **35**, which indicated compounds with a heterocyclic ring at the 5-position showed lower cytotoxicities than those compounds with a heterocyclic ring at the 4-position. Therefore, the relatively higher selectivity indices of the compounds with a heterocyclic ring at the 5-position were obtained. The selectivity index of compound **24** (SI = 61.7) indicated it is a promising candidate as lead compound for anti-enteroviral drug. The better linearity of the molecular structure of compounds with 5-heterocyclic ring could be attributed to the lower cytotoxicities than those compounds with 4-heterocyclic ring. Much lower IC_{50} values of compounds **21**, **23**, **25**, **29**, **31**, **33** and **35** showed better antiviral activities of compounds with a heterocyclic ring at the 4-position than those compounds with a heterocyclic ring at the 5-position.

Compounds **21-26** were all substituted with 1,2,4-oxadiazole at the 4- or 5-position and pyridyl at the 2-position. Compounds **23**, **24** appeared to be less toxic (TC_{50} = 28.63 $\mu\text{g}/\text{mL}$ and 66.67 $\mu\text{g}/\text{mL}$ respectively) than some of the other compounds (TC_{50} of less than 10 $\mu\text{g}/\text{mL}$). Compound **21** with 2'-pyridyl at the 2-position had best IC_{50} s (IC_{50} = 0.47 $\mu\text{g}/\text{mL}$ against CVB3 and IC_{50} = 0.64 $\mu\text{g}/\text{mL}$ against CVB6). Generally, compounds with 2'-pyridyl derivatives were more efficient than compounds 3'-pyridyl and 4'-pyridyl at the 2-position. Structurally, the main difference between compounds with 2'-pyridyl and compounds with 3'-/4'-pyridyl was that a hydrogen bond could be formed between H in position 1 of benzimidazole and N in 2'-pyridyl, while it was not achieved in 3'-pyridyl and 4'-pyridyl derivatives. Compounds of **27** to **30** with phenyl or ethoxyl at the 2-position showed higher IC_{50} values and lower selectivity indices than that of RBV with a SI of 5.2. It could be inferred that benzimidazole compounds with a phenyl or ethoxyl at the 2-position had little antiviral activities against both CVB3 and CVB6.

Compounds **32**, **34**, **35** with moieties of 2'-pyridyl at the 2-position and 1,3,4-oxadiazole, 1,3,4-thiadiazole or 1,2,4-triazole ring at the 4- or 5-position showed better IC_{50} s against both CVB3 and CVB6 (IC_{50} values ranging from 1.43 $\mu\text{g}/\text{mL}$ to 9.75 $\mu\text{g}/\text{mL}$) and higher TC_{50} (TC_{50} values ranging from 38.49 $\mu\text{g}/\text{mL}$ to 115.47 $\mu\text{g}/\text{mL}$). These struc-

ture-activity relationship suggested that benzimidazole compounds containing 1,3,4-oxadiazole, 1,3,4-thiadiazole at the 5-position and 1,2,4-triazole at the 4-position could enhance their biological activities against both CVB3 and CVB6. They hold promise as candidates for further drug anti-enteroviral development.

Conclusion

In summary, a series of novel benzimidazole derivatives based on **1** was synthesized and assessed for their anti-enterovirus activities in Vero cells. Most of the synthesized compounds were proved to be potential enterovirus inhibitors. Compounds **27** to **30** with phenyl or ethoxyl at the 2-position and 1,2,4-oxadiazole at the 4- or 5-position had little biological activities against enterovirus. Compounds **21-26**, **31-36** with moieties of 2'-pyridyl, 3'-pyridyl and 4'-pyridyl at the 2-position and oxadiazole, thiadiazole, triazole substituents at the 4- or 5-position generally displayed good activities against both CVB3 and CVB6. The most promising result was observed in the incorporation of heterocyclic rings with benzimidazoles at the 5-position could enhance their biological activities. Their antiviral activities and favorable cytotoxicity profiles make them attractive candidate compounds for further assessment *in vivo* as anti-enterovirus agents.

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