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ORIGINAL PAPER

An efficient synthesis of benzimidazoles via a microwave technique and evaluation of their biological activities

Bahittin Kahveci · Emre Menteşe · Musa Özil · Serdar Ülker · Murat Ertürk

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Abstract A simple and practical protocol was developed for the synthesis of benzimidazoles. The protocol uses iminoester hydrochloride which is very useful in the reaction with 4,5-dichloro-1,2-phenylenediamine under microwave irradiation leading to the products with good yields and in short reaction times. This method can be used as a general technique for synthesizing benzimidazoles. The synthesized compounds were evaluated for their biological properties such as anti-lipase, antiviral, and antitumor activities. Five benzimidazol-1-acetic acid hydrazides showed slight antiviral activity at 25 μg/cm^3 concentration despite their low toxicity. Substituted 2-benzylbenzimidazoles were active against adenocarcinoma (CT26) and melanoma (B16F10) cancer cell lines at concentrations below 10 μg/cm^3. Six of the compounds showed anti-lipase activities at various concentrations; the IC_{50} value of one compound was 0.35 μg/cm^3, which is similar activity to that of orlistat (0.32 μg/cm^3).

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Introduction

Benzimidazoles are an important group of heterocyclic compounds in the field of medicinal chemistry because they frequently have interesting biological and pharmacological properties. For example, in 1944, Wayne Walley, one of the leading exponents of antimetabolite therapy, noted the resemblance of benzimidazole to adenine and speculated that it might act as an adenine antimetabolite (Scheme 1). He even demonstrated that it could inhibit the growth of bacteria and fungi and that this could be reversed by either adenine or guanine [1]. Obesity is widely recognized as a major public health problem which is caused by an imbalance between energy intake and expenditure. Obesity can cause different serious diseases, including hypertension, hyperlipidemia, arteriosclerosis, and type II diabetes [2]. Pancreatic lipase plays a key role for fat digestion. Moreover, pancreatic lipase inhibitors, such as orlistat, are used as therapeutic agents for curing obesity [3].

Benzimidazole structures are classified under several ATC groups [4]. Benzimidazole drugs (e.g., anthelmintics, albendazole, thiabendazole, and the proton pump inhibitor omeprazole) represent substances used in both human and veterinary medicine (Scheme 2) [5–13].

1-Benzyl-5,6-dichloro-1*H*-benzimidazole-2-amine is as a potent inhibitor of viral RNA synthesis (Scheme 3) [14]. Human cytomegalovirus (HCMV) is an important pathogen in immunocompromised conditions, such as bone marrow and organ transplant patients and individuals with AIDS [15].

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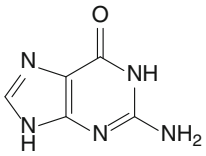
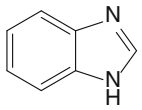
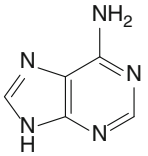


Guanine

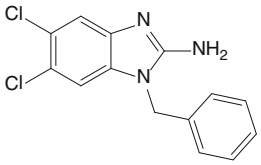


Benzimidazole

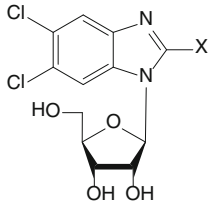


Adenine

Scheme 1



Viral RNA synthesis inhibitor



Benzimidazole ribonucleosides

Scheme 3

In the literature, there are many synthetic routes that are common to the preparation of benzimidazoles. However, many of these procedures are associated with several drawbacks such as expensive reagents, harsh reaction conditions, extended reaction times, the occurrence of side products, unsatisfactory yields, and complicated experimental procedures. Electron deficiency of the starting material in terms of the nature of the substituents on the aromatic ring can affect the success of these reactions. In general, when 4,5-disubstituted-1,2-phenylenediamine with electron-withdrawing groups, such as chloro, are used the yield and purity of the product are significantly worse, reaction times are long, and catalysts are required [16–31]. Therefore, there is a need for a new synthetic route for benzimidazoles which is short and economical.

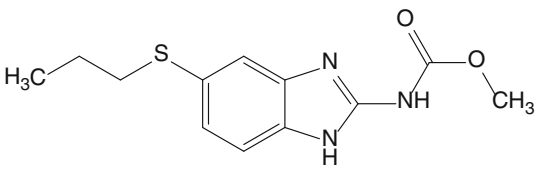
In this study we developed a novel and practical method for the synthesis of 2-substituted benzimidazole derivatives. This method can provide a convenient way to synthesize potentially bioactive benzimidazoles. The structures of new compounds were confirmed by FT-IR, ^{1}H NMR, ^{13}C NMR spectroscopy, and mass spectrometry. All the synthesized compounds were screened for their biological activities. The synthetic path of the target compounds is shown in Scheme 4.

Results and discussion

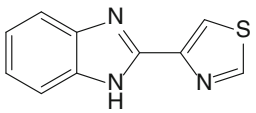
This is the first report on the synthesis of benzimidazoles **2a**–**2f** from iminoester hydrochlorides **1a**–**1f** [32, 33] both in methanol under microwave irradiation and on a solid support using microwave irradiation in the absence of organic solvents, which make the procedure a clean, efficient, and cheap method to afford various useful heterocyclic compounds. The new heterocyclic compounds **2a**–**2f** were also obtained by using conventional heating in methanol [34].

In this new method we obtained products within short reaction times and with high yields (Table 1). In addition, the reaction was carried out catalyst-free under mild conditions. Moreover, the use of solid supports under solventless conditions is particularly important in terms of the development of green technologies.

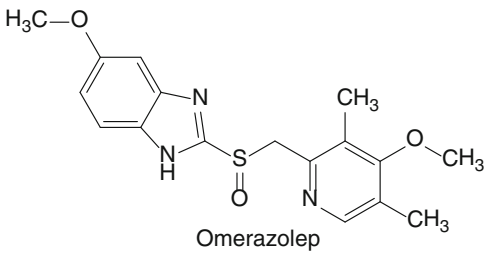
Acyl hydrazides **4a**–**4f** were synthesized as key intermediates for combinatorial benzimidazoles. Since the acyl hydrazides could serve as both a hydrogen donor and acceptor, they could be potentially more potent than the parent benzimidazoles. Acyl hydrazides **4a**–**4f** were easily



Albendazole

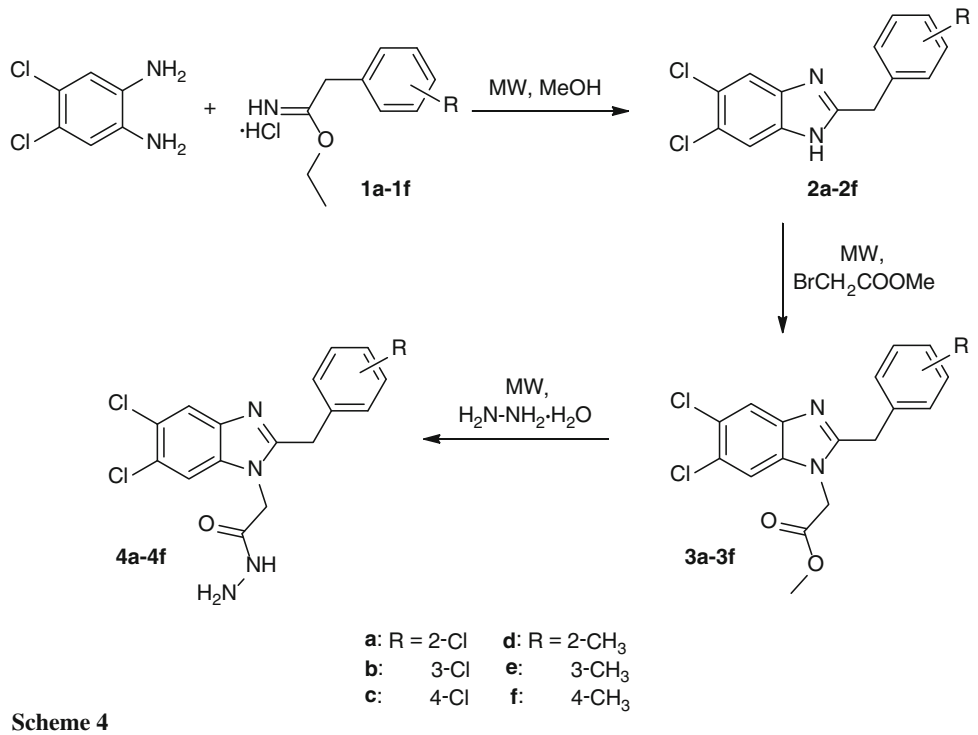


Thiabendazole



Omerazolep

Scheme 2



Scheme 4

Table 1 Comparison of yields and reaction time under microwave irradiation and conventional method for compounds **2a**–**2f**

Product	Method i		Method ii		Method iii	
	Time/min	Yield/%	Time/min	Yield/%	Time/min	Yield/%
2a	6	72	10	93	600	73
2b	7	69	12	92	600	71
2c	5	71	8	96	600	88
2d	6	68	10	87	600	62
2e	7	66	12	85	600	69
2f	6	69	7	90	600	70

Table 2 Residual lipase activity

	Residual activity/%	% SD
T+	100	±2
2d (5 mg)	39	±5
3a (5 mg)	30	±0
3b (5 mg)	10	±1
3c (3 mg)	6	±1
3f (5 mg)	19	±5
4b (5 mg)	43	±2
Orlistat (0.5 mg)	5	±2
Orlistat (0.1 mg)	13	±3

prepared in excellent yields from **2a**–**2f** by alkylation with methyl α-bromoacetate in acetone followed by a nucleophilic displacement of the methoxy group with hydrazine.

Anti-lipase activity results

All compounds were evaluated with regard to pancreatic lipase activity and **2d**, **3a**–**3c**, **3f**, and **4d** showed anti-lipase activities at various concentrations (Table 2). No inhibitory effect was detected for the other compounds. Dose-dependent pancreatic lipase activity was observed as shown in Fig. 1. Among the tested compounds, **3c** showed the best anti-lipase activity. The compound inhibited pancreatic lipase activity by 62, 92, and 96 % at concentrations of 0.625, 1.25, and 6.25 μg/cm^3, respectively. Orlistat, a known pancreatic lipase inhibitor used as an anti-obesity drug, inhibited activity by 87, 95, and 99 % at the same concentrations. IC_{50} values of orlistat and compound **3c** were calculated as 0.32 and 0.35 μg/cm^3, respectively. Orlistat is the only approved anti-obesity medication [3] but it has some side effects, such as fecal incontinence, flatulence, and steatorrhea [35, 36]. Synthesized compounds such as **3b** and **3c** can be good alternatives to orlistat.

Antiviral activity results

All compounds tested were toxic at 100 and 25 μg/cm^3 in Vero and MDCK cells used to grow HSV-1 and influenza A virus, respectively. Only compounds **4a**, **4b**, and **4d**–**4f** showed a slight antiviral activity at 100 and 25 μg/cm^3 concentration despite their low toxicity. **3f** and **2d** were

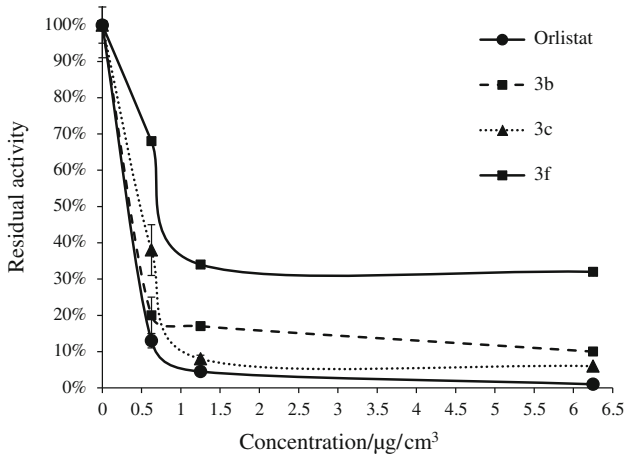


Fig. 1 Dose-dependent inhibitory effect of the compounds **3b**, **3c**, and **3f**. Orlistat was used as a positive control. All compounds were measured at final concentrations of 0.625, 1.25, and 6.25 μg/cm^3. Residual activities of compounds are expressed as the mean ± SD in triplicate

Table 3 Anti-HSV and anti-influenza A virus activity of the compounds

CN[c]	HSV % plaque reduction[a]				Anti-influenza A activity (±)[b]							
	Concentration/μg/cm^3				Concentration/μg/cm^3							
	6.25	12.5	25	50	100	50	25	12.5	6.2	3.1	1.5	0.7
2a	T[d]	T	T	T	–	–	–	–	–	–	–	–
2b	T	T	T	T	–	–	–	–	–	–	–	–
2c	T	T	T	T	–	–	–	–	–	–	–	–
2d	79 (T)	T	T	T	+	+	–	–	–	–	–	–
2e	T	T	T	T	–	–	–	–	–	–	–	–
2f	T	T	T	T	–	–	–	–	–	–	–	–
3a	0	0	0	0	–	–	–	–	–	–	–	–
3b	0	0	0	0	–	–	–	–	–	–	–	–
3c	0	0	0	0	–	–	–	–	–	–	–	–
3d	0	8 (T)	8 (T)	T	–	–	–	–	–	–	–	–
3e	0	0	0	0	–	–	–	–	–	–	–	–
3f	33 (T)	88 (T)	T	T	+	+	+	–	–	–	–	–
4a	0	0	8	41	+	+	–	–	–	–	–	–
4b	0	0	8	33	–	–	–	–	–	–	–	–
4c	0	0	8	T	+	+	–	–	–	–	–	–
4d	0	0	8	16	+	–	–	–	–	–	–	–
4e	0	0	8	33	–	–	–	–	–	–	–	–
4f	0	0	8	33	+	–	–	–	–	–	–	–

[a] Percentage of plaque reduction: [(mean number of plaques in control − mean number of plaques in test)/(mean number of plaques in control)] × 100

[b] + and − indicate 'no virus growth' and 'virus growth', respectively, as determined by hemagglutination assay using chicken erythrocytes

[c] Compound number

[d] Toxic: cell monolayer integrity is lost partially or completely

active against HSV-1 at 12.5 and 6.25 μg/cm^3 concentration albeit with some degree of associated toxicity (Table 3). No anti-influenza virus activity of the compounds was detected.

Table 4 Antitumor activity of the compounds

Compound	Tumor cell growth inhibition (GI_{50}, log μg/cm^3)	
	Cell line	
	CT26 (adenocarcinoma)	B16F10 (melanoma)
2a	0.7999	0.6654
2b	0.8162	0.6667
2c	0.7253	0.6305
2d	0.744	0.7094
2e	0.6427	0.7239
2f	0.6584	0.7474
3a	1.416	2.171
3b	1.187	4.348
3c	1.688	5.645
3d	1.258	1.296
3e	1.799	1.867
3f	0.9827	1.506
4a	1.501	1.574
4b	1.466	1.697
4c	1.248	1.506
4d	1.592	1.593
4e	1.401	1.554
4f	1.404	1.549

Antitumor activity results

The tumor cell growth inhibition results in Table 4 indicated that some of the compounds exhibited a dose-dependent inhibitory effect on adenocarcinoma (CT26) and melanoma (B16F10) cells. Six of the compounds, namely **2a**–**2f**, were active against both cancer cell lines at concentrations below 10 μg/cm^3. It was interesting to see that although compounds **3a**–**3c** at concentrations less than 100 μg/cm^3 were not active against melanoma, but they were inhibitory toward adenocarcinoma cells.

Conclusion

The present synthetic methods are rapid, inexpensive, and efficient routes for the synthesis of 2-substituted benzimidazole derivatives. These new methods can be efficiently used to synthesize many new benzimidazole derivatives which have a broad spectrum of biological activities. Synthesized compounds such as **3b** and **3c** could be good alternatives to orlistat. The IC_{50} value of **3c** was 0.35 μg/cm^3, which is similar to that of orlistat (0.32 μg/cm^3). Compounds **2a**–**2f** were active against adenocarcinoma (CT26) and melanoma (B16F10) cancer cell lines at concentrations below 10 μg/cm^3.

Experimental

Melting points were determined in open capillaries on a Büchi digital melting point apparatus. IR spectra were recorded in KBr pellets on a Perkin-Elmer 100 FTIR spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were measured on a Varian 200 spectrometer using DMSO-d_6 as solvent and TMS as internal standard. Chemical shifts are given in parts per million, coupling constants J in Hz. Mass spectra (MS) were determined in H-ESI mode on a Thermo Quantum Mars. Elemental analyses were performed on a Carlo Erba 1106 CHN analyzer, and the results agreed favorably with calculated values. Starting materials were obtained from Fluka or Aldrich. A monomode CEM-Discover Microwave was used in the standard configuration as delivered, including proprietary software. All experiments were carried out in microwave process vials (30 cm^3) with temperature control by an infrared detection temperature sensor. The temperature was computer monitored and maintained constant by a discrete modulation of delivered microwave power. After completion of the reaction, the vial was cooled to 60 °C by air jet cooling.

*General procedure for the synthesis of 5,6-dichloro-2-(substituted benzyl)-1H-benzimidazoles **2a–2f** under microwave irradiation (method i)*

A mixture of 4,5-dichloro-1,2-phenylenediamine (0.010 mol), iminoester hydrochlorides **1a–1f** (0.013 mol), and 9 g acidic alumina oxide was taken in a 20-cm^3 round-bottom flask and solubilized in 10 cm^3 dichloromethane. The solvent was completely evaporated under reduced pressure. The mixture was microwave-irradiated at 80 °C for 2 × 3 min (hold time) at 60 W. After the completion of the reaction (monitored by TLC, ethyl acetate/hexane 3:1), the mixture was extracted with ethanol (3 × 15 cm^3) and poured into water. The precipitate was filtered and recrystallized from ethanol/water (1:3) to give pure **2a–2f**.

*General procedure for the synthesis of 5,6-dichloro-2-(substituted benzyl)-1H-benzimidazoles **2a–2f** under microwave irradiation (method ii)*

A mixture of 4,5-dichloro-1,2-phenylenediamine (0.010 mol) and iminoester hydrochlorides **1a–1f** (0.013 mol) in 15 cm^3 dry methanol was irradiated in closed vessels with pressure control at 65 °C for 10 min (hold time) at 300 W maximum power. After the completion of the reaction (monitored by TLC, ethyl acetate/hexane 3:1), the mixture was poured into water. The precipitate was collected by filtration and recrystallized from ethanol/water (1:3) to give pure **2a–2f**.

*General procedure for the synthesis of 5,6-dichloro-2-(substituted benzyl)-1H-benzimidazoles **2a–2f**, conventional method (method iii)*

A mixture of 4,5-dichloro-1,2-phenylenediamine (0.010 mol) and iminoester hydrochlorides **1a–1f** (0.013 mol) in 30 cm^3 dry methanol was taken in a round-bottom flask. The solution was stirred for 10 h at room temperature. After the completion of the reaction (monitored by TLC, ethyl acetate/hexane 3:1), the mixture was poured into water. The precipitate was collected by filtration and recrystallized from ethanol/water (1:3) to give pure **2a–2f**.

5,6-Dichloro-2-(2-chlorobenzyl)-1H-benzimidazole (**2a**, $C_{14}H_9Cl_3N_2$)
M.p.: 237–238 °C; IR (KBr): $\bar{\nu}$ = 3,422 (NH), 3,071, 2,914, 1,629, 1,574, 1,287, 765, 744, 536 cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6): δ = 4.31 (s, 2H, CH_2), 7.28–7.77 (m, 6H, Ph), 12.63 (s, 1H, NH + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): δ = 32.66 (CH_2), 119.60, 123.80, 124.46, 127.29, 128.75, 129.24, 131.50, 133.23, 134.48 (Ar–C), 155.18 (C=N) ppm; ESI–MS (70 eV): m/z = 311 ([M + H]$^+$).

5,6-Dichloro-2-(3-chlorobenzyl)-1H-benzimidazole (**2b**, $C_{14}H_9Cl_3N_2$)
M.p.: 202–203 °C; IR (KBr):$\bar{\nu}$ = 3,430 (NH), 3,004, 2,932, 1,619, 1,599, 1,286, 1,097, 862, 796 cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6): δ = 4.25 (s, 2H, CH_2), 7.31–7.82 (m, 6H, Ph), 12.68 (s, 1H, NH + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): δ = 34.09 (CH_2), 115.97, 123.93, 126.69, 127.60, 128.66, 130.33, 132.97, 138.15, 139.21 (Ar–C), 155.81 (C=N) ppm; ESI–MS (70 eV): m/z = 311 ([M + H]$^+$).

5,6-Dichloro-2-(4-chlorobenzyl)-1H-benzimidazole (**2c**)
M.p.: 215–216 °C (Ref. [29] 214–215 °C)

5,6-Dichloro-2-(2-methylbenzyl)-1H-benzimidazole (**2d**, $C_{15}H_{12}Cl_2N_2$)
M.p.: 192–196 °C; IR (KBr): $\bar{\nu}$ = 3,427 (NH), 3,027, 2,977, 1,628, 1,528, 1,296, 1,096, 867, 750, 698 cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.67 (s, 3H, CH_3), 4.34 (s, 2H, CH_2), 7.20–7.83 (m, 6H, Ph), 12.53 (NH, s, 1H + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): δ = 20.15 (CH_3), 39.20 (CH_2), 112.35, 119.50, 123.44, 123.92, 126.63, 127.20, 128.46, 133.82, 142.62, 142.98 (Ar–C), 160.17 (C=N) ppm; ESI–MS (70 eV): m/z = 291 ([M + H]$^+$).

5,6-Dichloro-2-(3-methylbenzyl)-1H-benzimidazole (**2e**, $C_{15}H_{12}Cl_2N_2$)
M.p.: 197–198 °C; IR (KBr): $\bar{\nu}$ = 3,435 (NH), 3,067, 2,923, 1,609, 1,580, 1,283, 862, 803, 756 cm^{-1}; ^{1}H NMR

(200 MHz, DMSO-d_6): $\delta = 2.10$ (s, 3H, CH_3), 4.11 (s, 2H, CH_2), 7.10–7.76 (m, 6H, Ph), 12.58 (s, 1H, NH + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): $\delta = 20.85$ (CH_3), 34.66 (CH_2), 115.88, 119.42, 123.66, 125.79, 127.21, 128.34, 129.31, 133.32, 135.90, 136.78, 137.54 (Ar–C), 156.48 (C=N) ppm; ESI–MS (70 eV): $m/z = 291$ ($[M + H]^+$).

5,6-Dichloro-2-(4-methylbenzyl)-1H-benzimidazole (**2f**, $C_{15}H_{12}Cl_2N_2$)
M.p.: 185–186 °C; IR (KBr): $\bar{\nu} = 3{,}430$ (NH), 3,004, 2,920, 1,625, 1,550, 1,288, 864, 768, 522 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): $\delta = 2.23$ (s, 3H, CH_3), 4.10 (s, 2H, CH_2), 7.10–7.71 (m, 6H, Ph), 12.54 (s, 1H, NH + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): $\delta = 21.30$ (CH_3), 35.09 (CH_2), 111.58, 119.30, 124.35, 129.39, 129.76, 134.62, 136.42, 141.90 (Ar–C), 157.45 (C=N) ppm; ESI–MS (70 eV): $m/z = 291$ ($[M + H]^+$).

*General procedure for the synthesis of methyl 5,6-dichloro-2-(substituted benzyl)-1H-benzimidazol-1-acetates **3a–3f***

A mixture of compounds **2a–2f** (0.01 mol), methyl α-bromoacetate (0.01 mol), and K_2CO_3 (0.025 mol) in 10 cm^3 acetone was irradiated in closed vessels with pressure control at 85 °C for 7 min (hold time) at 300 W maximum power. After the completion of the reaction (monitored by TLC, ethyl acetate/hexane 3:1), the mixture was poured into water. The precipitate was collected by filtration and recrystallized from acetone/water (1:3) to give pure **3a–3f**.

Methyl 5,6-dichloro-2-(2-chlorobenzyl)-1H-benzimidazol-1-acetate (**3a**, $C_{17}H_{13}Cl_3N_2O_2$)
M.p.: 155–156 °C; yield: 92 %; IR (KBr): $\bar{\nu} = 3{,}063$, 2,943, 1,738 (C=O), 1,618, 1,570, 1,250 (C–O), 1,096, 758 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): $\delta = 3.55$ (s, 3H, OCH_3), 4.32 (s, 2H, CH_2), 5.27 (s, 2H, NCH_2), 7.29–7.96 (m, 6H, Ph) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): $\delta = 31.61$ (CH_2), 45.36 (NCH_2), 53.14 (OCH_3), 112.85, 120.58, 124.97, 125.32, 127.98, 129.51, 129.97, 132.41, 134.13, 134.60, 136.00, 142.30 (Ar–C), 156.37 (C=N), 168.78 (C=O) ppm; ESI–MS (70 eV): $m/z = 383$ ($[M + H]^+$).

Methyl 5,6-dichloro-2-(3-chlorobenzyl)-1H-benzimidazol-1-acetate (**3b**, $C_{17}H_{13}Cl_3N_2O_2$)
M.p.: 187–188 °C; yield: 95 %; IR (KBr): $\bar{\nu} = 3{,}065$, 2,952, 1,731 (C=O), 1,596, 1,231 (C–O), 1,094, 702 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): $\delta = 3.54$ (s, 3H, OCH_3), 4.27 (s, 2H, CH_2), 5.25 (s, 2H, N-CH_2), 7.23–7.91 (m, 6H, Ph) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): $\delta = 32.00$ (CH_2), 44.49 (NCH_2), 52.12 (OCH_3), 112.09, 119.72, 124.18, 124.53, 126.53, 128.66, 130.07, 132.78, 135.11, 138.14, 141.43 (Ar–C), 155.90 (C=N), 167.74 (C=O) ppm; ESI–MS (70 eV): $m/z = 383$ ($[M + H]^+$).

Methyl 5,6-dichloro-2-(4-chlorobenzyl)-1H-benzimidazol-1-acetate (**3c**, $C_{17}H_{13}Cl_3N_2O_2$)
M.p.: 154–155 °C; yield: 92 %; IR (KBr): $\bar{\nu} = 3{,}050$, 2,956, 1,737 (C=O), 1,614, 1,227 (C–O), 761 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): $\delta = 3.53$ (s, 3H, OCH_3), 4.25 (s, 2H, CH_2), 5.24 (s, 2H, NCH_2), 7.24–7.92 (m, 6H, Ph) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): $\delta = 31.90$ (CH_2), 44.54 (NCH_2), 52.18 (OCH_3), 112.10, 119.71, 124.23, 124.60, 128.23, 130.78, 134.68, 135.18, 141.45 (Ar–C), 156.15 (C=N), 167.79 (C=O) ppm; ESI–MS (70 eV): $m/z = 383$ ($[M + H]^+$).

Methyl 5,6-dichloro-2-(2-methylbenzyl)-1H-benzimidazol-1-acetate (**3d**, $C_{18}H_{16}Cl_2N_2O_2$)
M.p.: 117–118 °C; yield: 91 %; IR (KBr): $\bar{\nu} = 3{,}027$, 2,921, 1,741 (C=O), 1,621, 1,590, 1,223 (C–O), 1,099, 702 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): $\delta = 2.20$ (s, 3H, CH_3), 3.63 (s, 3H, OCH_3), 4.32 (s, 2H, CH_2), 5.30 (s, 2H, NCH_2), 7.29–7.97 (m, 6H, Ph) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): $\delta = 21.48$ (CH_3), 36.80 (CH_2), 44.24 (NCH_2), 51.94 (OCH_3), 112.06, 119.85, 124.03, 124.48, 126.63, 127.28, 128.45, 135.33, 141.30, 141.78 (Ar–C), 159.46 (C=N), 167.34 (C=O) ppm; ESI–MS (70 eV): $m/z = 363$ ($[M + H]^+$).

Methyl 5,6-dichloro-2-(3-methylbenzyl)-1H-benzimidazol-1-acetate (**3e**, $C_{18}H_{16}Cl_2N_2O_2$)
M.p.: 165–166 °C; yield: 92 %; IR (KBr): $\bar{\nu} = 3{,}040$, 2,949, 1,738 (C=O), 1,607, 1,512, 1,227 (C–O), 1,094, 763 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): $\delta = 2.22$ (s, 3H, CH_3), 3.49 (s, 3H, OCH_3), 4.21 (s, 2H, CH_2), 5.20 (s, 2H, NCH_2), 7.00–7.91 (m, 6H, Ph) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): $\delta = 21.62$ (CH_3), 38.93 (CH_2), 45.42 (NCH_2), 52.90 (OCH_3), 112.91, 120.52, 124.96, 125.30, 126.70, 128.20, 129.10, 130.17, 136.10, 136.30, 138.25, 142.35 (Ar–C), 157.30 (C=N), 168.54 (C=O) ppm; ESI–MS (70 eV): $m/z = 363$ ($[M + H]^+$).

Methyl 5,6-dichloro-2-(4-methylbenzyl)-1H-benzimidazol-1-acetate (**3f**, $C_{18}H_{16}Cl_2N_2O_2$)
M.p.: 126-127 °C; yield: 96 %; IR (KBr): $\bar{\nu} = 3{,}043$, 2,946, 1,734 (C=O), 1,615, 1,512, 1,223 (C–O), 1,094, 769 cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): $\delta = 2.23$ (s, 3H, CH_3), 3.49 (s, 3H, OCH_3), 4.19 (s, 2H, CH_2), 5.19 (s, 2H, NCH_2), 7.08–7.90 (m, 6H, Ph) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): $\delta = 20.50$ (CH_3), 38.08 (CH_2), 44.54 (NCH_2), 52.15 (OCH_3), 112.06, 119.66, 123.62, 124.10, 124.42, 128.64, 128.87, 132.48, 133.76, 135.24, 135.65, 141.49 (Ar–C), 156.63 (C=N), 167.72 (C=O) ppm; ESI–MS (70 eV): $m/z = 363$ ($[M + H]^+$).

*General procedure for the synthesis of 5,6-dichloro-2-(substituted benzyl)-1H-benzimidazole-1-acetic acid hydrazides **4a–4f***

A mixture of compounds **3a**–**3f** (0.01 mol) and hydrazine hydrate (0.01 mol) in 10 cm^3 absolute ethanol was irradiated in closed vessels with pressure control at 120 °C for 5 min (hold time) at 300 W maximum power. After the completion of the reaction (monitored by TLC, ethyl acetate/hexane 3:1), the mixture was cooled to room temperature. The precipitate was washed with ethanol and dried over $CaCl_2$ to give pure **4a**–**4f**.

5,6-Dichloro-2-(2-chlorobenzyl)-1H-benzimidazol-1-acetic acid hydrazide (**4a**, $C_{16}H_{13}Cl_3N_4O$)
M.p.: 246–247 °C; yield: 83 %; IR (KBr): $\bar{\nu}$ = 3,325, 3,310 (NH_2), 3,212 (NH), 1,651 (C=O), 1,573 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6): δ = 4.34 (s, 2H, CH_2), 4.36 (s, 2H, NH_2 + D_2O exchangeable), 4.93 (s, 2H, NCH_2), 7.27–7.87 (m, 6H, Ph), 9.52 (s, 1H, NH + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): δ = 31.69 (CH_2), 45.44 (NCH_2), 112.61, 120.52, 124.73, 125.09, 127.97, 129.45, 129.93, 132.35, 134.12, 134.93, 135.95, 142.35 (Ar–C), 156.60 (C=N), 166.20 (C=O) ppm; ESI–MS (70 eV): m/z = 383 ($[M + H]^+$).

5,6-Dichloro-2-(3-chlorobenzyl)-1H-benzimidazol-1-acetic acid hydrazide (**4b**, $C_{16}H_{13}Cl_3N_4O$)
M.p.: 215–216 °C; yield: 82 %; IR (KBr): $\bar{\nu}$ = 3,311 (NH_2), 3,164 (NH), 1,655 (C=O), 1,598 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6): δ = 4.25 (s, 2H, CH_2), 4.39 (s, 2H, NH_2 + D_2O exchangeable), 4.89 (s, 2H, NCH_2), 7.30–7.84 (m, 6H, Ph), 9.50 (s, 1H, NH + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): δ = 32.11 (CH_2), 44.58 (NCH_2), 111.58, 119.66, 123.95, 124.24, 126.52, 127.77, 128.83, 130.06, 132.77, 135.07, 138.58, 141.53 (Ar–C), 156.26 (C=N), 165.36 (C=O) ppm; ESI–MS (70 eV): m/z = 383 ($[M + H]^+$).

5,6-Dichloro-2-(4-chlorobenzyl)-1H-benzimidazol-1-acetic acid hydrazide (**4c**, $C_{16}H_{13}Cl_3N_4O$)
M.p.: 266–267 °C; yield: 85 %; IR (KBr): $\bar{\nu}$ = 3,341, 3,317 (NH_2), 3,164 (NH), 1,655 (C=O), 1,560 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6): δ = 4.23 (s, 2H, CH_2), 4.33 (s, 2H, NH_2 + D_2O exchangeable), 4.86 (s, 2H, NCH_2), 7.28–7.82 (m, 6H, Ph), 9.48 (s, 1H, NH + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): δ = 31.95 (CH_2), 44.57 (NCH_2), 104.14, 111.84, 119.62, 123.92, 124.23, 128.18,130.85, 131.21, 135.08, 135.12, 141.52 (Ar–C), 156.43 (C=N), 165.34 (C=O) ppm; ESI–MS (70 eV): m/z = 383 ($[M + H]^+$).

5,6-Dichloro-2-(2-methylbenzyl)-1H-benzimidazol-1-acetic acid hydrazide (**4d**, $C_{17}H_{16}Cl_2N_4O$)
M.p.: 204–205 °C; yield: 79 %; IR (KBr): $\bar{\nu}$ = 3,303 (NH_2), 3,188 (NH), 1,671 (C=O), 1,542 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.65 (s, 3H, CH_3), 4.27 (s, 2H, CH_2), 4.42 (s, 2H, NH_2 + D_2O exchangeable), 4.87 (s, 2H, NCH_2), 7.19–7.93 (m, 6H, Ph), 9.41 (s, 1H, NH + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): δ = 22.14 (CH_3), 37.59 (CH_2), 44.90 (NCH_2), 112.77, 120.63, 124.48, 124.87, 127.27, 127.85, 129.10, 135.87, 142.25, 142.04 (Ar–C), 160.49 (C=N), 165.89 (C=O) ppm; ESI–MS (70 eV): m/z = 363 ($[M + H]^+$).

5,6-Dichloro-2-(3-methylbenzyl)-1H-benzimidazol-1-acetic acid hydrazide (**4e**, $C_{17}H_{16}Cl_2N_4O$)
M.p.: 235–236 °C; yield: 81 %; IR (KBr): $\bar{\nu}$ = 3,300 (NH_2), 3,163 (NH), 1,653 (C=O), 1,535 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6): δ = 2.49 (s, 3H, CH_3), 4.19 (s, 2H, CH_2), 4.32 (s, 2H, NH_2 + D_2O exchangeable), 4.84 (s, 2H, NCH_2), 7.07–7.84 (m, 6H, Ph), 9.48 (s, 1H, NH + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): δ = 21.44 (CH_3), 33.33 (CH_2), 45.20 (NCH_2), 112.50, 120.21, 124.47, 124.76, 126.51, 127.79, 128.83, 130.00, 135.84, 136.47, 138.04, 142.22 (Ar–C), 157.50 (C=N), 165.97 (C=O) ppm; ESI–MS (70 eV): m/z = 363 ($[M + H]^+$).

5,6-Dichloro-2-(4-methylbenzyl)-1H-benzimidazol-1-acetic acid hydrazide (**4f**, $C_{17}H_{16}Cl_2N_4O$)
M.p.: 256–257 °C; yield: 82 %; IR (KBr): $\bar{\nu}$ = 3,324, 3,307 (NH_2), 3,173 (NH), 1,660 (C=O), 1,513 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6): δ = 2.23 (s, 3H, CH_3), 4.15 (s, 2H, CH_2), 4.48 (s, 2H, NH_2 + D_2O exchangeable), 4.83 (s, 2H, NCH_2), 7.07–7.83 (m, 6H, Ph), 9.48 (s, 1H, NH + D_2O exchangeable) ppm; ^{13}C NMR (50 MHz, DMSO-d_6): δ = 21.09 (CH_3), 32.99 (CH_2), 45.17 (NCH_2), 112.46, 120.18, 124.45, 124.73, 129.31, 129.50, 133.49, 135.84, 136.19, 142.22 (Ar–C), 157.49 (C=N), 165.97 (C=O) ppm; ESI–MS (70 eV): m/z = 363 ($[M + H]^+$).

Anti-lipase activity

The inhibitory effects of the compounds were evaluated against porcine pancreatic lipase (15 μg/cm^3). Lipase activity assays were done according to Verger et al. [37]. Microtiter plates were coated with purified TAGs of tung oil. Compounds and the lipase were incubated for 30 min at a ratio 1:2 (v/v). The microtiter plates containing purified tung oil, lipase solution, and assay buffer (10 mM Tris–

HCl buffer, pH 8.0, containing 150 mM NaCl, 6 mM $CaCl_2$, 1 mM EDTA, and 3 mg/cm^3) were recorded continuously for 40 min against only the buffer alone by using a microplate reader (SpectraMax M5, Molecular Devices) at 272 nm. The inhibitory activity of those compounds and orlistat (as positive control) against pancreatic lipase was measured at concentrations of 6.25, 1.25, and 0.625 μg/cm^3. Residual activities were calculated by comparing to control without inhibitor (T+). The assays were done in triplicate. The IC_{50} value was determined as the concentration of compound that give 50 % inhibition of maximal activity.

Antiviral activity testing

Antiviral activity of the compounds against HSV-1 (wal strain) and influenza A virus (A/PR/8, H1N1) was tested by plaque reduction and hemagglutination assays using Vero and MDCK cells, respectively, as described [38, 39]. For the anti-HSV-1 activity test, briefly monolayers of Vero cells grown in 24-well plates were infected with the virus (ca. 100 pfu/well). After incubation for 1 h to allow viral adsorption, the inoculum was aspirated and the infected cells were overlaid with 0.8 % methylcellulose in maintenance medium (minimal essential medium with 2 % fetal bovine serum) containing various concentrations of the compounds in duplicate. Controls included mock-infected wells with and without compounds. After 72 h of incubation, the cell monolayers were washed with phosphate buffer and then stained with naphthol blue black dye. The plaques were counted and the percentage of plaque reduction was calculated as follows: [(mean number of plaques in control − mean number of plaques in test)/(mean number of plaques in control)] × 100. For the anti-influenza activity test, briefly monolayers of MDCK cells were grown in 96-well plates and infected with 0.1 cm^3 of × 100 TCID50 of influenza A virus (A/PR/8, H1N1) prepared in a maintenance medium (minimal essential medium with no serum but containing 1 μg/cm^3 trypsin). After incubation for 1 h to allow viral adsorption at 37 °C, the inoculum was decanted and the infected cells were overlaid with fresh maintenance medium containing various concentrations (200, 100, 50, 25, 12.5, 6.2, 3.1, 1.5 μg/cm^3) of the compounds in triplicate. After 72 h of incubation at 37 °C and 5 % CO_2, 50 mm^3 of culture supernatant from each well was transferred into U-bottom microwell plates to detect the presence of virus by the hemagglutination assay. The result were reported as the presence (+) or absence (−) of the virus growth.

Antitumor activity testing

The test for inhibition of tumor cell growth in the presence of the compounds was performed essentially as described [40] using murine tumor cell lines CT26 (adenocarcinoma) and B16F10 (melanoma). Briefly, 1 × 10^5 viable cells from each cell line in RPMI-1640 growth medium supplemented with 10 % FBS were seeded in a 96-well plate and incubated for 24–48 h. When cells reached greater than 80 % confluence, the medium was decanted and cells were incubated with twofold dilutions (100, 50, 25, 12.5, 6.2, 3.1, and 1.5 μg/cm^3) of the test compounds prepared in 0.5 % dimethyl sulfoxide in triplicate. After 48 h of incubation at 37 °C, the treated and untreated cells (controls) were fixed by the addition of 1 % glutaraldehyde solution for 15 min, washed with deionized water, and dried in air. The cells were then stained with 0.4 % crystal violet for 30 min, then extensively washed with phosphate buffered saline and allowed to dry overnight before dissolving the retained dye with 75 % ethyl alcohol. The absorbance of developing color was determined by measuring the optical density (OD) at 570–630 nm using a multiwell spectrophotometer. The cell growth inhibiting (GI_{50}) concentration of the compounds given as μg/cm^3 in log units was calculated by GraphPad Prim 4 software. All determinations were performed in triplicate.

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