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Synthesis, antiviral and antitumor activity of 2-substituted-5-amidino-benzimidazoles

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Abstract—We have prepared a set of heterocyclic benzimidazole derivatives bearing amidino substituents at C-5 of benzimidazole ring, by introducing various heterocyclic nuclei (pyridine, *N*-methyl-pyrrole or imidazole) at C-2, and evaluated their antitumor and antiviral activities. The most pronounced antiproliferative activity was shown with compounds **6** and **9**, having imidazolinylamidino-substituent. Interestingly, all compounds show noticeable selectivity toward breast cancer cell line MCF-7. The most distinct and selective antiviral activity toward coxsackieviruses and echoviruses was observed with compounds having pyridine ring at C-2. Especially interesting was fairly strong activity of **4** and **8** toward adenoviruses, which could be considered as leads against adenoviral replication.

1. Introduction

The incorporation of an imidazole nucleus, a biologically accepted pharmacophore, in the benzimidazole molecule has made it a versatile heterocycle possessing wide spectrum of biological activity.[1] Moreover, benzimidazole derivatives are structural isosteres of naturally occurring nucleotides, which allows them to interact easily with the biopolymers of the living systems. Therefore, numerous biological activities and functions have been described: antihelminthic,[2] antifungal,[3] antiallergic, antimicrobial,[4–6] antiviral,[7] and antineoplastic activity.[8]

Antiviral properties of various benzimidazole derivatives have been reported in a variety of studies using different virus strains, such as human cytomegalovirus (HCMV),[9] human immunodeficiency virus,[10,11] and hepatitis B and C virus.[12,13] Also, amidino-substituted benzimidazoles, such as bis(5-amidino-2-benzimidazolyl)methane (BABIM), showed ability to block respiratory syncytial (RS) virus induced cell fusion.[14] In addition, introducing amidino moiety to benzimidazole ring was shown to possess potent antimicrobial[15,16] and antiprotozoal activity.[17]

Besides, as a lead structure benzimidazole has been already used as a part of a central scaffold in some metallo- and serine protease inhibitors, because of its potential in H-bonding and π–π stacking interactions with the imidazole ring of His residues essential for the activity of these enzymes.[18] Alternatively, aromatic amidine molecules have been widely studied as competitive inhibitors of the protease enzymes because amidine moieties bind to an aspartic acid residue in the specificity pocket adjacent to the active site of several serine proteases to produce competitive inhibitors.[19] Since proteases have been linked to several disease states, including thrombosis, inflammation, bronchoconstriction, as well as tumor growth and invasion,[20] they are rational targets for inhibition by drugs. We have already shown that several amidino-substituted benzimidazoles strongly in-

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hibit dipeptidiyl peptidase III,[21] while Young et al.[22] showed inhibitory activity of various amidino-benzimidazoles toward several coagulation proteases.

In spite of the abovementioned and although many new benzimidazole derivatives have been synthesized as potential antitumor agents (e.g., pyrrolo[1,2-*a*]benzimidazoles,[23] various 2-substituted benzimidazoles[7,24,25]), there is very scarce recent literature data on antitumor and antiviral potentials of amidino-substituted benzimidazoles that should combine favorable structural properties of both amidino and benzimidazole moiety. Therefore, we have prepared a set of heterocyclic benzimidazole derivatives (Fig. 1) bearing amidino substituents at position C-5 of benzimidazole ring, by introducing various heterocyclic nuclei at position C-2, such as pyridine, *N*-methyl-pyrrole or imidazole, and evaluated their antiproliferative/antitumor, as well as antiviral, activities.

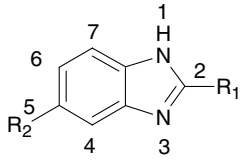


Figure 1. 2,5-Substituted benzimidazole.

2. Results and discussion

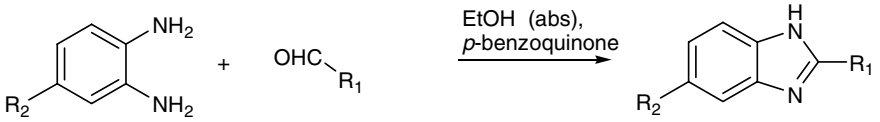
2.1. Chemistry

The synthetic pathway for preparation of the benzimidazoles is shown in Scheme 1. Compounds **1**–**9** were synthesized by condensation of corresponding aldehydes, 4(5)-imidazolecarboxaldehyde, 1-methyl-1H-pyrrole-2-carbaldehyde, and pyridine-2-carbaldehyde, appropriate 4-*N*-amidino-substituted *o*-phenylenediamines, and *p*-benzoquinone in absolute ethanol (31–91%, respectively).[26]

The corresponding 4-*N*-amidino-substituted *o*-phenylenediamines were synthesized starting from acetamidobenzonitrile which were nitrated at first place to afford 4-amino-3-nitrobenzonitrile. The nitrile group was then converted into the imidate ester, using Pinner method,[27] and the imidate ester was used directly without characterization to make the desired 4-*N*-amidino-substituted *o*-phenylenediamines by described method.[28] Corresponding aldehydes were purchased from Aldrich and used without further purification.

2.2. Biological results

2.2.1. Antiproliferative activity. The tested compounds showed different antiproliferative effect on the presented



No.	R_1	R_2
1		
2		
3		
4		
5		
6		
7		
8		
9		

Scheme 1. Synthesis of 2,5-substituted benzimidazoles.

panel cell lines (Table 1 and Fig. 2). The activity of compounds bearing amidino substituents at position C-5 varies with introducing different heterocyclic substituents at position C-2. For instance, all compounds having imidazole moiety (**1**, **2**, and **3**) showed in general modest antiproliferative activity and no cytotoxicity toward normal cells (WI 38). Interestingly, the compounds **1** and **3** have already been described to have inhibitory properties toward dipeptidyl peptidase.[21] Furthermore, replacement of imidazole moiety by heterocyclic substituents with one heteroatom (*N*-methyl-pyrrole and pyridine) resulted in much more pronounced activity, but also cytotoxicity to normal cells. For example, compounds **6** and **9** (Fig. 2), both having imidazolinyl-amidino-substituent, showed strong growth inhibitory and slight cytotoxic activity toward all cell lines, while

Table 1. In vitro inhibition of the growth of tumor cell lines and normal human fibroblasts (WI 38)

Compound	IC_{50}[a] (μM)					
	H 460	HeLa	MiaPaCa-2	SW 620	MCF-7	WI 38
1	>100	>100	34 ± 49	>100	7.2 ± 3	>100
2	>100	>100	>100	>100	26 ± 26	>100
3	>100	>100	>100	>100	⩾100	>100
4	>100	70 ± 27	>100	84 ± 3	12 ± 3	>100
5	>100	>100	>100	>100	20 ± 8	>100
6	38 ± 7	20 ± 0.9	11.5 ± 0.1	15 ± 2	5 ± 1	15.6 ± 1.9
7	>100	82 ± 17	>100	>100	⩾100	59.4 ± 42.7
8	>100	>100	79 ± 18	>100	50 ± 23	20.4 ± 9.9
9	22.5 ± 1.4	16 ± 0.8	16 ± 0.2	19 ± 0.6	12 ± 6	12 ± 2
Cis[b]	0.3 ± 0.04	2.9 ± 0.6	5.4 ± 1.6	4 ± 1.8	12 ± 6	19 ± 20
Eto[b]	0.2 ± 0.1	2.9 ± 1	15.4 ± 14	20 ± 3.4	50 ± 30	NT[c]

[a] IC_{50}: the concentration that causes a 50% reduction of the cell growth.
[b] Cis, cisplatin; Eto, etoposide.
[c] NT, not tested.

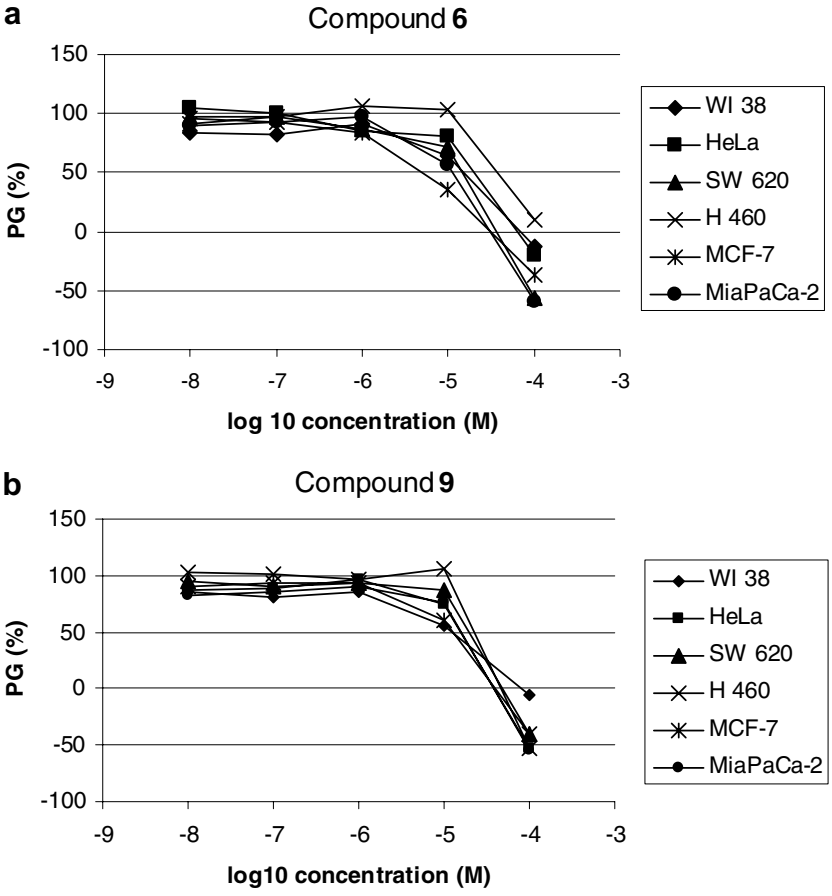


Figure 2. Dose–response profiles for compounds **6** and **9** tested on various human cell lines in vitro. The cells were treated with the compounds at different concentrations, and the percentage of growth (PG) was calculated. Each point represents a mean value of four parallel samples in three individual experiments.

N-isopropylamidino-substituted compound **8** inhibited the growth of all but H 460 cells. Interestingly, all compounds in general show noticeable selectivity toward breast cancer cell line (MCF-7), which is the most evident for carboxamidino-substituted compound **1** and *N*-isopropylamidino-substituted compound **6**.

Similar apparent selectivity of bis-benzimidazoles to breast cancer cells has also been previously shown by Seaton et al.[29] The authors have investigated the antiproliferative activity of the methoxy and dimethylamine bis-benzimidazole derivatives against many tumor cell lines. Although these compounds are DNA minor groove binders, they do not act as classical topoisomerase I and II inhibitors and the authors presume that their activity could be a combination of various mechanisms. A series of 1-substituted-2-methyl-5-nitrobenzimidazoles have also shown cell-growth inhibitory activity to breast cancer cell line MCF-7.[30] Moreover, several compounds of benzimidazole class that have predominantly been utilized as antifungal and antihelminthic agents, such as Mebendazole,[31] oncodazole, and methyl-2-benzimidazolecarbamate (Carbendazim, FB642), have also been investigated as potential antitumor drugs. FB642 produced tumor growth inhibition of greater than 58% in five of the seven human xenograft models evaluated. Interestingly, the antitumor activity of FB642 against MCF-7 breast tumors in mice was among the highest for all tumor models studied and was also better than either paclitaxel or vinorelbine.

Consequently, the observed selectivity toward breast cancer cells should be correlated to benzimidazole, not to amidine moiety. Although different modes of action of various benzimidazoles have been described in the literature (e.g., DNA groove binding, topoisomerase I or II inhibition, interactions with microtubules, inhibition of tumor helicases), selective growth inhibitory activity toward breast cancer cells, especially MCF-7 line, has not been explained. Since MCF-7 cells are known estrogen-dependent breast cancer cell line, this selectivity could possibly be explained as antiestrogenic effect of benzimidazoles. This hypothesis should be further confirmed on other breast cancer cell lines, as well as in in vivo model.

2.2.2. Antiviral activity. Compounds **1**–**9** did not show any growth inhibitory activity against GMK cell line and were, as such, suitable for further antiviral testing on this cell line (data not shown). However, at the highest concentration compounds **6** and **9** showed cytotoxicity to HeLa cell line that was also used for antiviral testing (Table 1 and Fig. 1).

Similar to antiproliferative activity described above, it was again demonstrated that all compounds having imidazole moiety (**1**–**3**) showed very low or no antiviral activity against all tested viruses (Table 2). Compound **5** moderately and selectively inhibited the growth of echovirus 7 (EC_{50} = 23.2 μM), while **7** effectively inhibited the growth of both enteroviruses, that is, coxsackievirus B5 (EC_{50} = 1.7 μM), and echovirus 7 (EC_{50} = 3.2 μM). Furthermore, **8** efficiently inhibited the growth of adenovirus 5 (EC_{50} = 15.2 μM), coxsackievirus B5 (EC_{50} = 2.7 μM) and echovirus 7 (EC_{50} = 0.33 μM), but very poorly herpesvirus 1. Compound **9** considerably inhibited the growth of coxsackievirus B5 (EC_{50} = 4.3 μM) and echovirus 7 (EC_{50} = 0.63 μM). These results clearly demonstrate that all compounds having pyridine ring at position C-2 of an amidino-substituted benzimidazole showed selective and marked inhibitory activity toward RNA replicating enteroviruses. On the other hand, although **6** and **9** demonstrated certain antiviral effects toward herpesvirus 1 (EC_{50} = 28.5 μM and 56.7 μM, respectively), the EC_{50} concentrations were rather high and close to cytotoxic concentrations, so it was hard to discern between these effects. Interestingly, the most consistent antiviral activity was observed with compound **4** that has carboxamidine substituent at C-5 and *N*-methyl-pyrrol at C-2 against all four types of viruses (adenovirus 5; EC_{50} = 5.9 μM, herpesvirus 1; EC_{50} = 30 μM, coxsackievirus B5; EC_{50} = 3.5 μM, and echovirus 7; EC_{50} = 5 μM), while having no cytotoxic activity whatsoever.

Table 2. In vitro inhibition of the growth on respective cell lines of adenovirus 5, herpesvirus 1, coxsackievirus B5, and echovirus 7

Compound	Antiviral activity EC_{50}[a] (μM)			
	HeLa		GMK	
	Adenovirus 5	Herpesvirus 1	Coxsackievirus B5	Echovirus 7
1	NA[b]	NA	>100	>100
2	NA	>100	>100	>100
3	NA	>100	>100	>100
4	5.9 ± 10	30 ± 14.1	3.5 ± 4.9	5 ± 3.2
5	NA	NA	>100	23.2 ± 32.1
6	>100	28.5 ± 30.4	>100	>100
7	>100	>100	1.7 ± 1.6	3.2 ± 3.3
8	15.2 ± 31.4	>100	2.7 ± 4.6	0.33 ± 0.21
9	>100	56.7 ± 61.3	4.3 ± 3.1	0.63 ± 0.38

[a] EC_{50} represents the drug concentration that causes inhibition of viral growth by 50%; given values are means of at least tree independent optical evaluations of CPE for adenovirus 5 and herpesvirus 1 grown on HeLa cell lines and at least two independent MTT experiments for coxsackievirus B5 and echovirus 7 grown on GMK cell lines.

[b] NA, no antiviral activity determined.

As already mentioned, many studies confirmed antiviral activity of diverse benzimidazole derivatives, mostly against hepatitis,[12,13] herpes viruses,[32] cytomegalovirus,[9] as well as coxsackieviruses.[33,34] Our results also prove that novel modifications of amidino-benzimidazole molecules with the 2-pyridyl-substituent at position C-2 could enrich their antiviral potentials and direct the synthetic research to novel antiviral lead molecules. Furthermore, to our knowledge there have been no data reported presenting antiviral activity against adenoviruses. Therefore, compounds **4** and **8** could be promising potential antiviral agents and should be considered as leads for further synthetic structural optimization. Still, further research on understanding of the molecular mechanism of the observed antiviral effect on both DNA replicating viruses (adenovirus and herpesvirus) and the RNA replicating enteroviruses (coxsackievirus and echovirus) is needed.

3. Conclusions

We have prepared a set of heterocyclic benzimidazole derivatives bearing amidino substituents at C-5 of benzimidazole ring, by introducing various heterocyclic nuclei at C-2. The principal aim of this study was to evaluate these compounds for their cytostatic and antiviral activities. The presented results confirm that novel 2-substituted-5-amidino-benzimidazoles have both antiviral and antitumor potentials. The imidazole moiety at position C-2 (**1**–**3**) strongly reduced both the antiproliferative and antiviral activity of benzimidazoles, while its replacement by heterocyclic substituents with one heteroatom (*N*-methyl-pyrrole and pyridine) resulted in much more pronounced activity. The most pronounced antiproliferative activity was shown with compounds **6** and **9**, having imidazolinylamidino-substituent. Interestingly, all compounds show selectivity toward breast cancer cell line (MCF-7). The most distinct and selective antiviral activity toward RNA replicating enteroviruses was observed with all compounds having pyridine ring at position C-2 of benzimidazole, which could thus be considered for further development. In contrast, carboxamidino-substituted compound **4** showed prominent activity against all four types of viruses. Especially interesting was fairly strong inhibitory activity of **4** and **8** toward the replication of adenovirus 5. Since there have been no data describing antiviral activity of benzimidazoles toward adenoviruses, these compounds could be considered as leads for further synthetic structural optimization for inhibition of adenoviral replication.

4. Experimental

4.1. Chemistry

Melting points were obtained on an Original Kofler Mikroheitztisch apparatus (Reichert, Wien) and are uncorrected. IR spectra were recorded on a Nicolet Magna 760 spectrophotometer in KBr disks. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 and Bruker AV-600 on 300 and 600, 75 and 150 MHz, respectively. All NMR spectra were measured in DMSO-d_6 solutions using TMS as an internal standard. Elemental analysis for carbon, hydrogen, and nitrogen was performed on a Perkin-Elmer 2400 elemental analyzer. Where analyses are indicated only as symbols of elements, analytical results obtained are within 0.4% of the theoretical value. Mass spectra were recorded by using electrospray ionization technique (ESI) on the Micromass Platform LCZ single quadropole mass spectrometer. All compounds were routinely checked by TLC with Merck silica gel 60F-254 glass plates.

4.2. General procedure for synthesis of compounds 1–9

Solution of 4-*N*-amidino-substituted *o*-phenylenediamines, corresponding aldehyde, and *p*-benzoquinone in equimolar amounts in absolute ethanol was refluxed for 4 h. After reaction mixture was cooled to room temperature, diethyl ether was added and the crude product was filtered off. The crude product was suspended in mixture of ethanol–diethyl ether several times until the powder was analytically pure.

4.2.1. 2-(1*H*-Imidazol-4-yl)-1H-benzimidazole-5-carboxamidine hydrochloride (1). From 4(5)-imidazolecarboxaldehyde (0.090 g, 0.94 mmol), 3,4-diaminobenzamidine (0.175 g, 0.94 mmol), and *p*-benzoquinone (0.108 g, 1.0 mmol) in absolute ethanol (5 ml) after refluxing for 4 h yielded 0.108 g, 41.1%, mp 275–277 °C. MS *m*/*z*: 227.2 (M^{+1}, –HCl); IR (cm^{-1}): 3153, 3005, 2771, 1691, 1432, 1605 1560; ^{1}H NMR (DMSO-d_6) (δ/ppm): 13.02 (br s, 1H, NH), 12.60 (br s , 1H, NH), 9.20 (br s, 4H, NH) 8.90–8.85 (m, 2H, H), 7.85 (d, 2H, J = 8.2 Hz, H_{arom}) 7.53 (s, 1H, H_{arom}); Anal. Calcd for $C_{11}H_{11}ClN_6$: C, 50.29; H, 4.22; N, 31.99. Found: C, 50.35; H, 4.33; N, 31.59.

4.2.2. 2-(1*H*-Imidazol-4-yl)-*N*-isopropyl-1H-benzimidazole-5-carboxamidine hydrochloride (2). From 4(5)-imidazolecarboxaldehyde (0.200 g, 2.1 mmol), 3,4-diamino-*N*-isopropylbenzamidine (0.476 g, 2.1 mmol), and *p*-benzoquinone (0.227 g, 2.1 mmol) in absolute ethanol (5 ml) after refluxing for 4 h yielded 0.585 g, 91%, mp 221–222 °C. MS *m*/*z*: 269.2 (M^{+1}, –HCl); IR (cm^{-1}): 3116, 2980, 2935, 1670, 1611, 1561; ^{1}H NMR (DMSO-d_6) (δ/ppm): 13.2 (br s, 1H, NH), 12.8 (br s, 1H, NH), 9.47 (br s, 1H, NH), 9.35 (br s, 1H, NH), 8.97 (br s, 1H, NH), 7.96–7.88 (m, 2H), 7.79–7.69 (br s, 1H, NH), 7.48 (s, 1H), 4.09–4.06 (m, 1H) $CH(CH_3)_2$, 1.28 (d, 6H, J = 6.30 Hz); Anal. Calcd for $C_{14}H_{17}ClN_6$: C, 55.17; H, 5.62; N, 27.57. Found: C, 55.35; H, 5.49; N, 27.35.

4.2.3. 5-(4,5-Dihydro-1H-imidazol-2-yl)-2-(1-H-imidazol-4-yl)-1H- benzimidazole hydrochloride (3). From 4(5)-imidazolecarboxaldehyde (0.100 g, 1.0 mmol), 4-(4,5-dihydro-1H-imidazol-2-yl)-benzene-1,2-diamine (0.212 g, 1.0 mmol), and *p*-benzoquinone (0.108 g, 1.0 mmol) in absolute ethanol (5 ml) after refluxing for 4 h yielded 0.09 g, 31.2%, mp >290 °C. MS *m*/*z*: 253.2 (M^{+1}, –HCl); IR (cm^{-1}): 3381, 3103, 2968, 2854, 1629, 1608, 1509; ^{1}H NMR (DMSO-d_6) (δ/ppm): 13.10 (br s, 1H,

NH), 12.80 (br s, 1H, NH), 10.47 (br s, 2H, NH), 8.16–8.13 (m, 2H, H), 7.86 (d, 1H, $J = 8.4$ Hz, H), 7.72 (d, 1H, $J = 8.4$ Hz), 7.62 (s, 1H, H), 3.96 (s, 4H, CH_2); Anal. Calcd for $C_{13}H_{13}ClN_6$: C, 54.08; H, 4.54; N, 29.11. Found: C, 54.35; H, 4.23; N 29.49.

4.2.4. 2-(1-Methyl-1H-pyrrol-2-yl)-1H-benzimidazole-5-carboxamidine hydrochloride (4). From 1-methyl-1H-pyrrole-2-carbaldehyde (0.212 g, 1.9 mmol), 3,4-diaminobenzamidine (0.354 g, 1.9 mmol), and *p*-benzoquinone (0.21 g, 1.9 mmol) in absolute ethanol (5 ml) after refluxing for 4 h yielded 0.41 g, 77.4%, mp 205–207 °C. MS *m/z*: 239 (M^{+1}, –HCl); IR (cm^{-1}): 3356, 3090, 2991, 1651, 1609, 1562; ^{1}H NMR (DMSO-d_6) (δ/ppm): 13.08 (br s, 1H, NH), 9.23 (br s, 4H, NH), 8.16 (s, 1H), 7.91–7.79 (m, 2H), 7.66–7.61 (m, 3H), 3.8 (s, 3H, CH_3); Anal. Calcd for $C_{13}H_{14}ClN_5$: C, 56.63; H, 5.12; N, 25.40. Found: C, 56.54; H, 5.32; N, 25.33.

4.2.5. *N*-Isopropyl-2-(1-methyl-1H-pyrrol-2-yl)-1H-benzimidazole-5-carboxamidine hydrochloride (5). From 1-methyl-1H-pyrrole-2-carbaldehyde (0.109 g, 1.0 mmol), 3,4-diamino-*N*-isopropylbenzamidine (0.228 g, 1.0 mmol), and *p*-benzoquinone (0.108 g, 1.0 mmol) in absolute ethanol (5 ml) after refluxing for 4 h yielded 0.195 g, 61.3%, mp 212–213 °C. MS *m/z*: 282.3 (M^{+1}, –HCl); IR (cm^{-1}): 3344, 3111, 2980, 1666, 1613, 1504; ^{1}H NMR (DMSO-d_6) (δ/ppm): 13.01 (br s, 1H, NH), 9.49 (br s, 1H, NH), 9.37 (br s, 1H, NH), 9.02 (br s, 1H, NH), 8.00–7.86 (m, 3H), 7.66–7.64 (m, 2H), 7.50 (d, 1H, $J = 8.1$ Hz), 4.16–4.07 (m, 1H), 3.50 (s, 3H, CH_3), 1.29 (d, 6H, $J = 6.36$ Hz),; ^{13}C NMR (DMSO-d_6) (δ/ppm): 163.03 (s), 150.75 (s), 143.06 (s), 132.53 (s), 124.47 (s), 122.50 (s), 122.06 (d, 2C), 118.24 (d), 116.09 (d, 2C), 112.09 (d), 45.42 (d) 38.15 (q), 21.79 (q 2C); Anal. Calcd for $C_{16}H_{20}ClN_5$: C, 60.47; H, 6.34; N, 22.04. Found: C, 60.55; H, 6.52; N, 21.96.

4.2.6. 5-(4,5-Dihydro-1H-imidazol-2-yl)-2-(1-methyl-1H-pyrrol-2-yl)-1H-benzimidazole-5-carboxamidine hydrochloride (6). From 1-methyl-1H-pyrrole-2-carbaldehyde (0.109 g, 1.0 mmol), 4-(4,5-dihydro-1H-imidazol-2-yl)-benzene-1,2-diamine (0.212 g, 1.0 mmol), and *p*-benzoquinone (0.108 g, 1.0 mmol) in absolute ethanol (5 ml) after refluxing for 4 h yielded 0.101 g, 33.9%, mp >290 °C. MS *m/z*: 266.3 (M^{+1}, –HCl); IR (cm^{-1}): 3390, 3094, 2976, 1606, 1582; ^{1}H NMR (DMSO-d_6) (δ/ppm): 13.26 (br s, 1H, NH), 10.2 (br s, 2H, NH), 8.03 (s, 1H), 7.81–7.78 (m, 1H), 7.75 (d, 1H, $J = 8.34$ Hz), 7.49 (d, 1H, $J = 8.34$ Hz), 7.46–7.43 (m, 2H), 4.11 (s, 4H, CH_2), 4.0 (s, 3H, CH_3); Anal. Calcd for $C_{15}H_{16}ClN_5$: C, 59.70; H, 5.34; N, 23.21. Found: C, 59.73; H, 5.21; N, 23.32.

4.2.7. 2-Pyridin-2-yl-1H-benzimidazole-5-carboxamidine hydrochloride (7). From pyridine-2-carbaldehyde (0.025 g, 0.23 mmol), 3,4-diaminobenzamidine (0.040 g, 0.22 mmol), and *p*-benzoquinone (0.025 g, 0.23 mmol) in absolute ethanol (5 ml) after refluxing for 4 h yielded 0.041 g, 65.1%, mp >290 °C. MS *m/z*: 238.0 (M^{+1}, –HCl); IR (cm^{-1}): 3021, 1629, 1595, 1560, 1535; ^{1}H NMR (DMSO-d_6) (δ/ppm): 13.70 (br s, 1H, NH), 9.40 (br s, 4H, NH), 8.79 (d, 1H, $J = 4.4$ Hz), 8.38 (d, 1H, $J = 7.9$ Hz), 8.28 (s, 1H), 8.06 (dd, 1H, $J = 7.6$ Hz), 7.71-7.58 (m, 3H); Anal. Calcd for ($C_{13}H_{12}ClN_5$): C, 57.04; H, 4.42; N, 25.59. Found: C, 56.99; H, 4.56; N 25.34.

4.2.8. *N*-Isopropyl-2-pyridin-2-yl-1H-benzimidazole-5-carboxamidine hydrochloride (8). From pyridine-2-carbaldehyde (0.107 g, 1.0 mmol), 3,4-diamino-*N*-isopropylbenzamidine (0.228 g, 1.0 mmol), and *p*-benzoquinone (0.108 g, 1.0 mmol) in absolute ethanol (5 ml) after refluxing for 4 h yielded 0.202 g, 64.1%, mp 259–261 °C. MS *m/z*: 280.1 (M^{+1}, –HCl); ^{1}H NMR (DMSO-d_6) (δ/ppm): 13.70 (br s, 1H, NH), 9.40 (br s, 4H, NH), 8.79 (d, 1H, $J = 4.4$ Hz), 8.38 (d, 1H, $J = 7.8$ Hz), 8.28 (s, 1H), 8.06 (dd, 1H, $J = 7.8$ Hz), 7.71–7.58 (m, 3H), 4.14 (m, 1H), 1.30 (d, 6H, $J = 6.3$ Hz); ^{13}C NMR (DMSO-d_6) (δ/ppm): 166.45 (s), 150.09 (d), 148.14 (s), 143.78 (s), 139.11 (s), 138.31 (d), 134.96 (s), 123.28 (d), 122.39 (d), 122.25 (s), 121.89 (d), 116.09 (d), 113.03 (d); Anal. Calcd for $C_{16}H_{18}ClN_5$: C, 60.85; H, 5.75; N, 22,18. Found: C, 60.90; H, 5.61; N 22.01.

4.2.9. 5-(4,5-Dihydro-1H-imidazol-2-yl)-2-pyridin-2-yl-1H-benzimidazole-5-carboxamidine hydrochloride (9). From pyridine-2-carbaldehyde (0.095 g, 1.0 mmol), 4-(4,5-dihydro-1H-imidazol-2-yl)-benzene-1,2-diamine (0.212 g, 1.0 mmol), and *p*-benzoquinone (0.108 g, 1.0 mmol) in absolute ethanol (5 ml) after refluxing for 4 h yielded 0.156 g, 52.2%, mp >290 °C. MS *m/z*: 264.2 (M^{+1}, –HCl); IR (cm^{-1}): 3045, 2980, 1629,1595,1560; ^{1}H NMR (DMSO-d_6) (δ/ppm): 13.80 (br s, 1H, NH), 10.6 (br s, 2H, NH), 8.79 (d, 1H, $J = 3.96$ Hz), 8.48 (s, 1H), 8.38 (d, 1H, $J = 7.8$ Hz), 8.06 (dd, 1H, $J = 7.66$ Hz), 7.93 (d, 1H, $J = 8.43$ Hz), 7.75 (d, 1H, $J = 8.52$ Hz), 7.60 (dd, 1H, $J = 6.48$ Hz), 4.01 (s, 4H, CH_2); Anal. Calcd for $C_{15}H_{14}ClN_5$: C, 60.10; H, 4.71; N, 23.36. Found: C, 59.98; H, 4.80; N, 23.15.

4.3. Biological tests

4.3.1. Proliferation assays. The HeLa (cervical carcinoma), MiaPaCa-2 (pancreatic carcinoma), SW 620 (colon carcinoma), MCF-7 (breast carcinoma), H 460 (lung carcinoma), and WI 38 (normal fibroblasts) cells were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. The cell lines were inoculated onto a series of 96-well microtiter plates on day 0, at 1×10^4 to 3×10^4 cells/ml, depending on the doubling times of specific cell line. Test agents were then added in five, 10-fold dilutions (10^{-8}–10^{-4} M) and incubated for a further 72 h. Working dilutions were freshly prepared on the day of testing. The solvent (DMSO) was also tested for eventual inhibitory activity by adjusting its concentration to be the same as in working concentrations. After 72 h of incubation the cell-growth rate was evaluated by performing the MTT assay, which detects dehydrogenase activity in viable cells, as described previously.[35,36] The absorbance

(OD, optical density) was measured on a microplate reader at 570 nm. The percentage of growth (PG) of the cell lines was calculated according to one or the other of the following two expressions:

$$\text{If}\,(\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}}) \geqslant 0\ \text{then}$$
$$\text{PG} = 100 \times (\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}})/(\text{mean OD}_{\text{ctrl}} - \text{mean OD}_{\text{tzero}}).$$
$$\text{If}\,(\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}}) < 0\ \text{then}$$
$$\text{PG} = 100 \times (\text{mean OD}_{\text{test}} - \text{mean OD}_{\text{tzero}})/\text{OD}_{\text{tzero}}.$$

where:

Mean OD_{tzero} = the average of optical density measurements before exposure of cells to the test compound.

Mean OD_{test} = the average of optical density measurements after the desired period of time.

Mean OD_{ctrl} = the average of optical density measurements after the desired period of time with no exposure of cells to the test compound.

Each test point was performed in quadruplicate in three individual experiments. The results are expressed as IC_{50}, which is the concentration necessary for 50% of inhibition. The IC_{50} values for each compound are calculated from dose–response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the reference value (i.e., 50%). If, however, for a given cell line all of the tested concentrations produce PGs exceeding the respective reference level of effect (e.g., PG value of 50), then the highest tested concentration is assigned as the default value, which is preceded by a '>' sign. Each result is a mean value from three separate experiments.

4.3.2. Antiviral activity assays

4.3.2.1. Cell lines. HeLa (human cervical carcinoma) and GMK (green monkey kidney) cell lines were used. Monolayer cell cultures were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.

4.3.2.2. Virus strains. Adenovirus 5 (ATCC VR-5) and herpesvirus 1 (ATCC VR-1545) were grown on HeLa cells, while coxsackie B5 (ATCC VR-185) and echovirus 7 (ATCC VR-1047) were grown on GMK cells in DMEM supplemented with 2% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C

4.3.2.3. Antiviral assay. HeLa cells were seeded at 10^5 cells/mL on 24-well microtiter plates, while GMK cells were seeded on 96-well microtiter plates at 10^5 cells/mL. Different concentrations of tested compounds (serial dilution from 10^{-4} to 10^{-10} M) were added to 1-day-old confluent cell monolayers immediately after that were infected with either adenovirus, herpesvirus or enteroviruses (coxsackievirus and echovirus) at 10 CCID50 (1 CCID50 corresponds to the viral stock dilution that is infective for 50% of the cell cultures) in DMEM supplemented in 2% FS. The inhibition of the cytopathic effect (CPE) was followed by an optical microscope 24 h after infection for enteroviruses (coxsackievirus and echovirus) and 48 h for adenoviruses and herpesviruses.[37] Furthermore, CPE of enteroviruses (cell lysis) was evaluated by MTT test.[38] The results were shown as the percentage of CPE inhibition compared to CPE without compounds on each plate and were statistically analyzed on a personal computer. The concentration values that inhibit 50% of viral CPE (EC_{50}) were calculated using the linear regression model.

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References and notes

1. Rastogi, R.; Sharma, S. *Synthesis* **1983**, 861.
2. Mavrova, A. Ts.; Anichina, K. K.; Vuchev, D. I.; Tsenov, J. A.; Kondeva, M. S.; Micheva, M. K. *Bioorg. Med. Chem.* **2005**, *13*, 5550.
3. Göker, H.; Kus, C.; Boykin, D. W.; Yildiz, S.; Altanlar, N. *Bioorg. Med. Chem.* **2002**, *10*, 2589.
4. Göker, H.; Özden, S.; Yıldız, S.; Boykin, D. W. *Eur. J. Med. Chem.* **2005**, *40*, 1062.
5. Andrzejewska, M.; Yépez-Mulia, L.; Cedillo-Rivera, R.; Tapia, A.; Vilpo, L.; Vilpo, J.; Kazimierczuk, Z. *Eur. J. Med. Chem.* **2002**, *37*, 973.
6. Özden, S.; Atabey, D.; Yıldız, S.; Göker, H. *Bioorg. Med. Chem.* **2005**, *13*, 1587.
7. Ramla, M. M.; Omar, M. A.; El-Khamry, A.-M. M.; El-Diwani, H. I. *Bioorg. Med. Chem.* **2006**, *14*, 7324.
8. Boiani, M.; Gonzalez, M. *Mini Rev. Med. Chem.* **2005**, *5*, 409.
9. Evers, D. L.; Komazin, G.; Ptak, R. G.; Shin, D.; Emmer, B. T.; Towsend, L. B.; Drach, J. C. *Antimicrob. Agents Chemother.* **2004**, *48*, 3918.
10. Middleton, T.; Lim, H. B.; Montgomery, D.; Rockway, T.; Tang, H.; Cheng, X.; Lu, L.; Mo, H.; Kohlbrenner, W. E.; Molla, A.; Kati, W. M. *Antiviral Res.* **2004**, *64*, 35–45.
11. Karen, K. Biron. *Antiviral Res.* **2006**, *71*, 154.
12. Li, Y. F.; Wang, G. F.; He, P. L.; Huang, W. G.; Zhu, F. H.; Gao, H. Y.; Tang, W.; Luo, Y.; Feng, C. L.; Shi, L. P.; Ren, Y. D.; Lu, W.; Zuo, J. P. *J. Med. Chem.* **2006**, *49*, 4790.
13. Hirashima, S.; Suzuki, T.; Ishida, T.; Noji, S.; Yata, S.; Ando, I.; Komatsu, M.; Ikeda, S.; Hashimoto, H. *J. Med. Chem.* **2006**, *49*, 4721.
14. Tidwell, R. R.; Geratz, J. D.; Dubovi, E. J. *J. Med. Chem.* **1983**, *26*, 294.
15. Göker, H.; Boykin, D. W.; Yildiz, S. *Bioorg. Med. Chem.* **2005**, *13*, 1707.
16. Weidner-Wells, M. A.; Ohemeng, K. A.; Nguyen, V. N.; Fraga-Spano, S.; Macielag, M. J.; Werblood, H. M.; Foleno, B. D.; Webb, G. C.; Barrett, J. F.; Hlasta, D. J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1545.

17. Ismail, M. A.; Batista-Parra, A.; Miao, Y.; Wilson, W. D.; Wenzler, T.; Brun, R.; Boykin, D. W. *Bioorg. Med. Chem.* **2005**, *13*, 6718.
18. Sahli, S.; Stump, B.; Welti, T.; Blum-Kaelin, D.; Aebi, J. D.; Oefner, C.; Böhm, H.-J.; Diederich, F. *ChemBioChem* **2004**, *5*, 996.
19. Paul, J. J.; Kircus, S. R.; Sorrell, T. N.; Ropp, P. A.; Thorp, H. H. *Inorg. Chem.* **2006**, *45*, 5126.
20. Šimaga, Š.; Babić, D.; Osmak, M.; Šprem, M.; Abramić, M. *Gynecol. Oncol.* **2003**, *91*, 194.
21. Agić, D.; Hranjec, M.; Jajčanin, N.; Starčević, K.; Karminski-Zamola, G.; Abramić, M. *Bioorg. Chem.* **2007**, *35*, 153.
22. Young, W. B.; Sprengeler, P.; Shrader, W. D.; Li, Y.; Rai, R.; Verner, E.; Jenkins, T.; Fatheree, P.; Kolesnikov, A.; Janc, J. W.; Cregar, L.; Elrod, K.; Katz, B. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 710.
23. Skibo, E. B.; Islam, I.; Heileman, M. J.; Schulz, W. G. *J. Med. Chem.* **1994**, *37*, 78.
24. Hao, D.; Rizzo, J. D.; Stringer, S.; Moore, R. V.; Marty, J.; Dexter, D. L.; Mangold, G. L.; Camden, J. B.; Von Hoff, D. D.; Weitman, S. D. *Invest. New Drugs* **2002**, *20*, 261.
25. Denny, W. A.; Rewcastle, G. W.; Baguley, B. C. *J. Med. Chem.* **1990**, *33*, 814.
26. Starcevic, K.; Boykin, D. W.; Karminski-Zamola, G. *Heterocycl. Commun.* **2002**, *8*, 222.
27. Roger, R.; Neilsen, D. *Chem. Rev.* **1961**, *61*, 179.
28. Hranjec, M.; Starcevic, K.; Zamola, B.; Mutak, S.; Đerek, M.; Karminski-Zamola, G. *J. Antibiot.* **2002**, *55*, 308.
29. Seaton, A.; Higgins, C.; Mann, J.; Baron, A.; Bailly, C.; Neidle, S.; Van den Berg, H. *Eur. J. Cancer* **2003**, *39*, 2548.
30. Ramla, M. M.; Omar, M. A.; El-Khamry, A.-M. M.; El-Diwani, H. I. *Bioorg. Med. Chem.* **2006**, *14*, 7324.
31. Mukhopadhyay, T.; Sasaki, J.; Ramesh, R.; Roth, J. A. *Clin. Cancer Res.* **2002**, *8*, 2963.
32. Williams, S. L.; Hartline, C. B.; Kushner, N. L.; Harden, E. A.; Bidanset, D. J.; Drach, J. C.; Townsend, L. B.; Underwood, M. R.; Biron, K. K.; Kern, E. R. *Antimicrob. Agents Chemother.* **2003**, *47*, 2186.
33. Castelli, M.; Malagoli, M.; Lupo, L.; Riccomi, T. R.; Casolari, C.; Cermelli, C.; Zanca, A.; Baggio, G. *Pharmacol. Toxicol.* **2001**, *88*, 67.
34. Cheng, J.; Xie, J.; Luo, X. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 267.
35. Jarak, I.; Kralj, M.; Šuman, L.; Pavlović, G.; Dogan Koružnjak, J.; Piantanida, I.; Žinić, M.; Pavelić, K.; Karminski-Zamola, G. *J. Med. Chem.* **2005**, *48*, 2346.
36. Starčević, K.; Kralj, M.; Piantanida, I.; Šuman, L.; Pavelić, K.; Karminski-Zamola, G. *Eur. J. Med. Chem.* **2006**, *41*, 925.
37. Barbaric, M.; Kraljević, S.; Grce, M.; Zorc, B. *Acta Pharm.* **2003**, *53*, 175.
38. Smee, D. F.; Morrison, A. C.; Dale, L.; Sidwell, B. W.; Sidwell, R. W. *J. Virol. Methods* **2002**, *106*, 71.