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Antiviral activity of benzimidazole derivatives. II. Antiviral activity of 2-phenylbenzimidazole derivatives ☆

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In memory of Anna Bennicelli

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ABSTRACT

Seventy-six 2-phenylbenzimidazole derivatives were synthesized and evaluated in cell-based assays for cytotoxicity and antiviral activity against a panel of 10 RNA and DNA viruses. The most commonly affected viruses were, in decreasing order, CVB-2, BVDV, Sb-1, HSV-1, and YFV, while HIV-1 and VSV were not affected, and RSV, VV and Reo-1 were only susceptible to a few compounds. Thirty-nine compounds exhibited high activity (EC_{50} = 0.1–10 μM) against at least one virus, and four of them were outstanding for their high and selective activity against VV (**24**, EC_{50} = 0.1 μM) and BVDV (**50**, **51**, and **53** with EC_{50} = 1.5, 0.8, and 1.0 μM, respectively). The last compounds inhibited at low micromolar concentrations the NS5B RdRp of BVDV and also of HCV, the latter sharing structural similarity with the former. The considered compounds represent attractive leads for the development of antiviral agents against poxviruses, pestiviruses and even HCV, which are important human and veterinary pathogens.

1. Introduction

Benzimidazole is isosteric with indole and purine nuclei, which are present in a number of fundamental cellular components and bioactive compounds.[1] This heterocycle may represent a kind of privileged substructure, which may interact with different proteins and enzymes. Indeed, a number of important drugs used in different therapeutic areas contain the benzimidazole ring,[2] as proton pump inhibitors (omeprazole), antihypertensives (candesartan, telmisartan), antihistaminics (astemizole), antihelmintics (albendazole, mebendazole), as well as several other kinds of still investigational therapeutic agents, including antitumorals and antivirals.[3,4]

Among the antiviral benzimidazoles, an important position is held by 2-aryl/heteroarylbenzimidazole derivatives, either endowed with activity against Coxsackie Virus B3 or targeting specific hepatitis C virus enzymes. Thus, *N*-(2-fluorophenyl)-2-(2-pyridyl)benzimidazol-4-carboxamide shows an IC_{50} value of 1.69 μM against Coxsackie Virus B3,[5] while 1-cyclohexyl-2-heteroarylbenzimidazole-5-carboxylic acids and their amido derivatives inhibit the HCV NS5B polymerase at nanomolar concentrations.[6,7] Several derivatives of 1-cyclohexyl-2-phenylbenzimidazole-5-carboxylic acid behave similarly; among them, 2-{4-[4′-chloro-4-(2-oxopyrrolidin-1-yl)biphenyl-2-ylmethoxy]-2-fluorophenyl}-1-cyclohexyl-1*H*-benzimidazol-5-carboxylic acid (JTK-109) inhibits the HCV polymerase with IC_{50} = 17 nM, and the replication of subgenomic HCV RNA in a replicon cell system with EC_{50} = 0.32 μM.[8] Lastly, *N*,*N*′-bis[4-(2-benzimidazolyl)phenyl]-αω-alkandicarboxamides inhibit the HCV helicase with an IC_{50} = 0.7 μM[9] (see Fig. 1).

It is worth noting that derivatives of 2-phenylbenzimidazole-4-carboxamide and of 2-(4-acyl/aroylaminophenyl)benzimidazole, closely related to some of the above antivirals, present a growing interest as anticancer agents. Indeed, the former[10] inhibit the DNA repair enzyme (PARP) and synergistically increase the cytotoxicity of temozolamide and topotecan, while the latter[11] potently inhibit the heparanase with consequent angiogenetic and antimetastatic activity.

Our interest in the chemistry and biological activities of benzimidazole derivatives dates back to the early sixties; since then, hundreds of compounds endowed with several pharmacological activities have been described. Recently, 1-substituted-2-[(ben-

☆ For note I, see Ref. 4.

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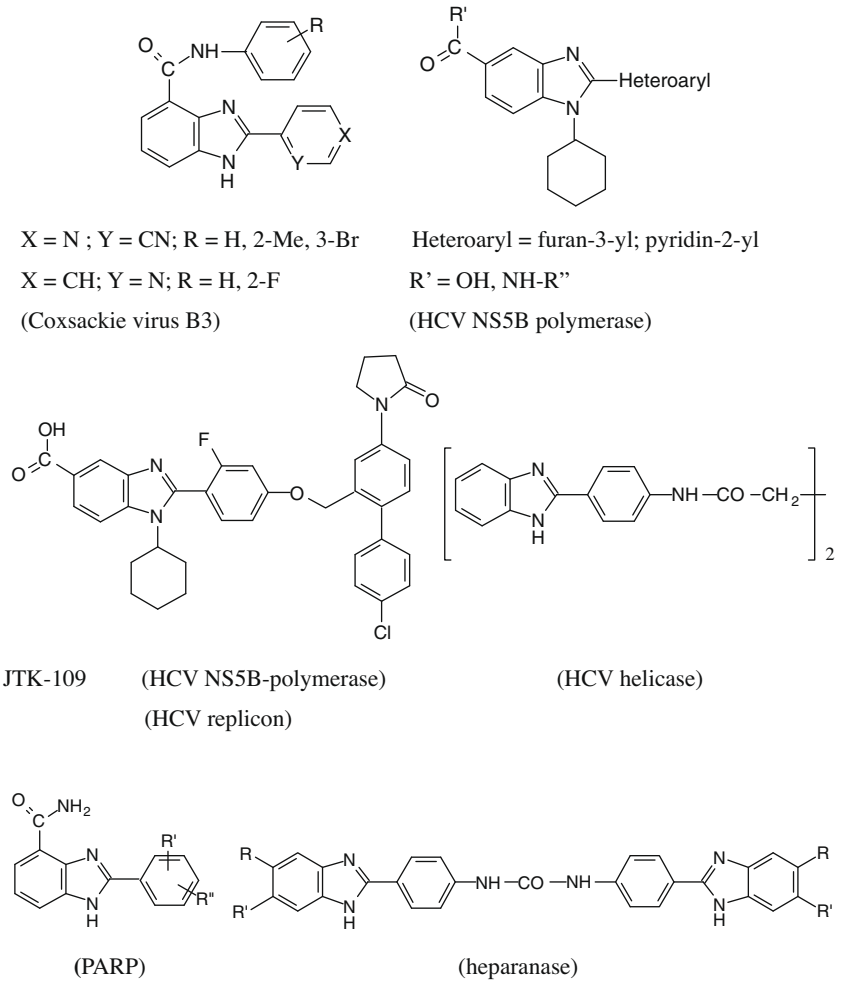


Figure 1. 2-Aryl/heteroaryl-benzimidazoles with antiviral or antitumoral activity (and corresponding target).

zotriazol-1/2-yl)methyl]benzimidazoles have demonstrated to potently inhibit the multiplication of RSV (Respiratory Syncytial Virus) in in vitro assays, with EC_{50} values as low as 20 nM.[4] Moreover, in a preliminary screening of a set of 2-phenylbenzimidazoles previously prepared with other pharmacological aims,[12] the simple 2-(4-methoxyphenyl)-5-trifluoromethylbenzimidazole proved to inhibit the multiplication of BVDV (bovine viral diarrhea virus) and CVB-2 (Coxsackie virus type B2), with EC_{50} values of 1 μM and 7 μM, respectively.

Basing ourselves on this, we found it interesting to investigate the antiviral activity of a larger number of 2-phenylbenzimidazole derivatives. On the whole, 76 compounds, incorporating several kinds of functionalities, have been evaluated in cell-based assays for cytotoxicity and antiviral activity against a panel of ten RNA and DNA viruses (see Fig. 2), in order to select hits active against individual viruses to be developed later on. In several compounds a nitro group is incorporated, in position 5 of the benzimidazole nucleus or in the 2-phenyl substituent, assuming that it could improve the binding on viral proteins through hydrogen bonds formation, and/or its electron withdrawing properties, which may result in π-interactions between the electron deficient ring of the drug and the electron rich ring of an aromatic aminoacid.

Indeed, despite some potential for toxicity, the nitro group displays fundamental role in the pharmacophore of several classes of chemotherapeutic agents, and of CNS, cardiovascular, anti-inflammatory and anti-androgen drugs.

2. Chemistry

Fifteen of the 76 tested compounds (**1**–**4**, **9**, **20**, **24**, **25**, **29**, **30**, **33**, **34**, **50**–**52**) were already known, and were prepared according to the references indicated in Supplementary data.

The remaining 61 novel compounds were synthesized according to Schemes 1–5. Three different approaches were followed for the benzimidazole ring closure reaction, namely:

- Method **a**:[13] the appropriate diamino compounds and aldehydes were reacted in the presence of $FeCl_3/O_2$; in one case (**13**), adding chloranil was found useful.
- Method **b**:[14] the diamino compounds were condensed with the aldehyde–sodium bisulfite addition compounds, which were prepared extemporaneously as indicated by Shriner and Land.[15]
- Method **c**: compound **7** was obtained by Phillips cyclization[16] of the amido compound formed by reaction of the diamine and acyl chloride.

The required diamino compounds **F**, with Z = H, were commercially available (R = R′ = H; R = H, R′ = NO_2; R = R′ = Cl), or prepared according to the literature (R = H, R′ = CF_3[17] and $COCH_3$[18]), as were those with R = H, R′ = CF_3, and Z = Me[19] or cyclohexyl.[20]

The diamino compound with Z = 1-adamantyl, R = H, and R′ = CF_3 was unknown and was prepared by reacting 2-nitro-4-(trifluoromethyl)chlorobenzene with 1-adamantylamine, followed by the catalytic reduction of the nitro group (see Scheme 1).

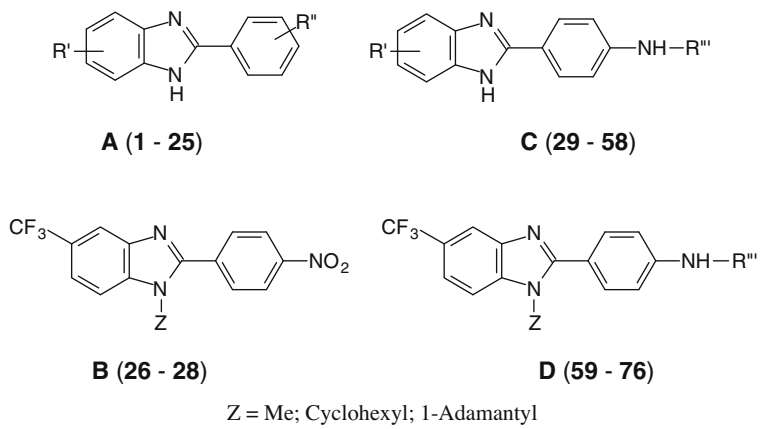
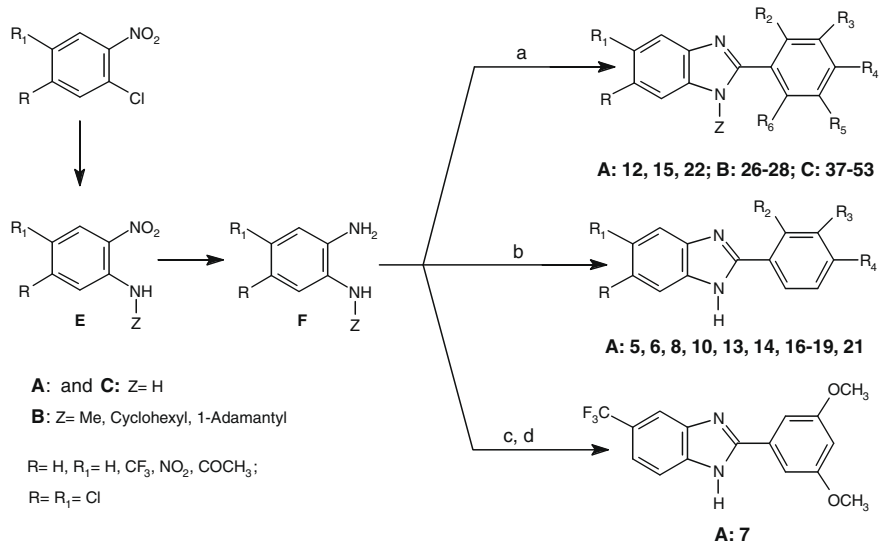
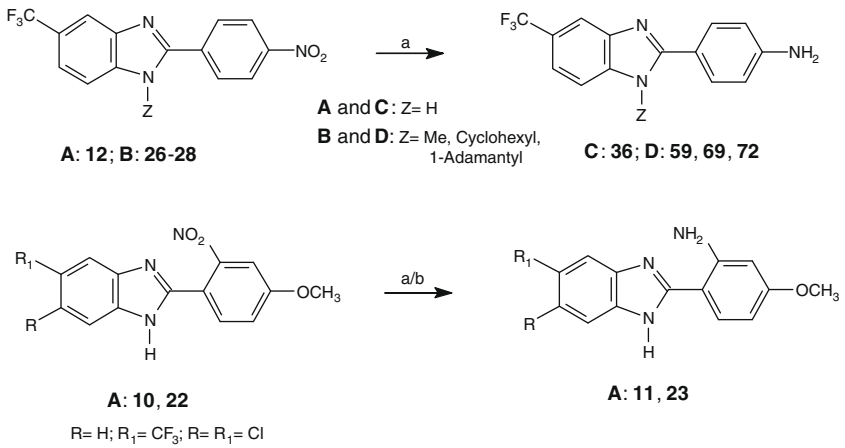


Figure 2. Structures of the investigated 2-phenylbenzimidazole derivatives.



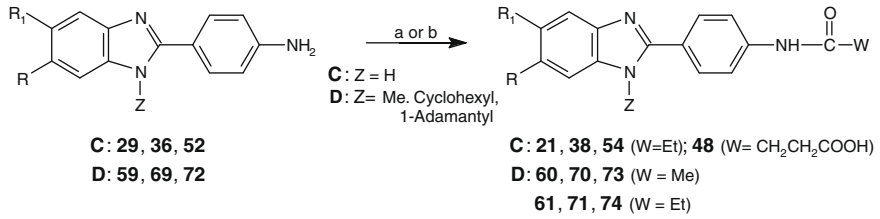
Scheme 1. Reagents and conditions: (a) arylaldehyde, MeCN, $FeCl_3/O_2$, reflux; (b) arylaldehyde–sodium bisulfite, EtOH, 3 h reflux; (c) aroylchloride, dioxane, 7 h reflux; (d) 4 N HCl, 4 h reflux.



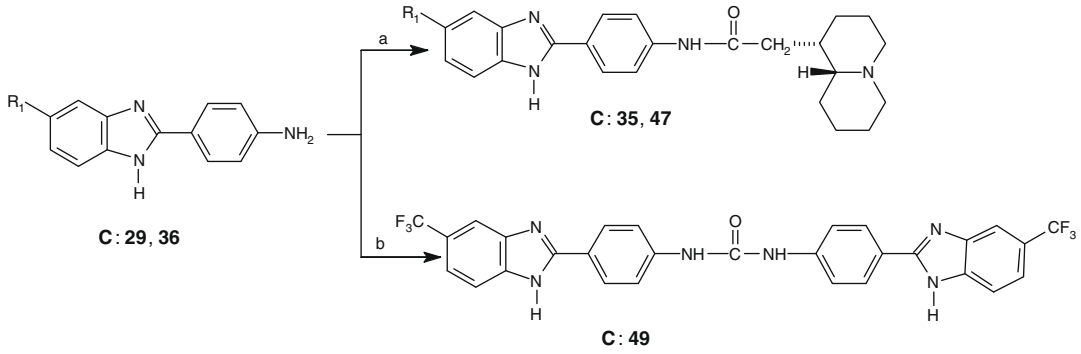
Scheme 2. Reagents and conditions: (a) H_2/Pd–C, EtOH, rt; (b) for R = R1 = Cl, $SnCl_2$ + concd HCl, EtOH, 1 h reflux.

The nitro-substituted benzimidazole derivatives were converted into the corresponding amino compounds by catalytic hydrogenation. However, compound **22** was better reduced by using $SnCl_2$ and concentrated HCl, thus avoiding the hydrogenolysis of chlorine on the aromatic ring (see Scheme 2).

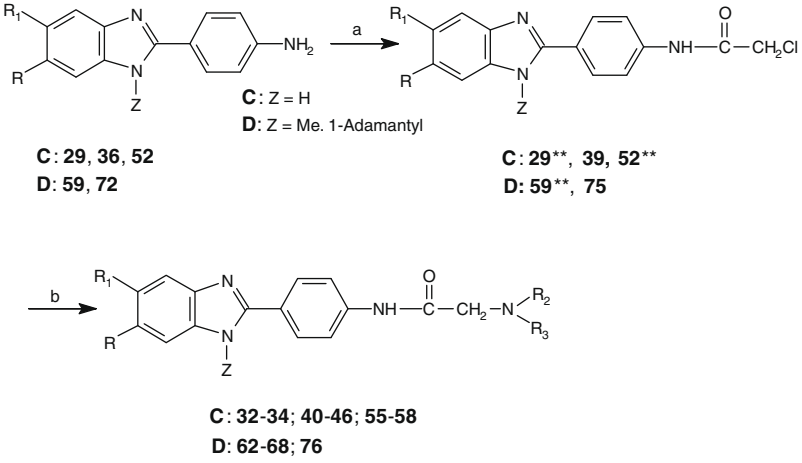
The 2-(4-aminophenyl)benzimidazoles were acylated with the appropriate anhydride (acetic, propionic, succinic, see Scheme 3) or acylchloride (see Scheme 4). It is worth noting that the treatment of 2-(4-aminophenyl)-5-trifluoromethylbenzimidazole with ethyl chloroformate, instead of the expected ethyl carbamate, gave



Scheme 3. Reagents and conditions: (a) Ac_2O or Pr_2O, benzene, 2 h reflux; (b) succinic anhydride, benzene, 18 h reflux.



Scheme 4. Reagents and conditions: (a) homolupinanoyl chloride hydrochloride, Et_3N, $CHCl_3$, 6 h reflux; (b) diphosgene or EtOCOCl, benzene, 5 h reflux.



Scheme 5. Reagents and conditions: (a) chloroacetyl chloride, Et_3N, benzene, 6 h reflux; (b) aminocompound (ratio 2:1), benzene (or EtOH), 18 h reflux (for **55**–**57** no solvent, but excess of the aminocompound). (The *N*-chloroacetylderivatives **29****, **52**** and **59**** were not included in the antiviral screening.)

the same urea derivative that was obtained in higher yield by the action of trichloromethyl chloroformate (diphosgene). The homolupinanoyl chloride hydrochloride, required for the synthesis of **35** and **47**, was prepared as previously described.[21,22]

Finally, in order to obtain the 2-[4-(aminoacetyl)amino]phenylbenzimidazoles of structure **C** and **D** (see Tables 3 and 4), the suitable amines were reacted with the 2-[4-(chloroacetylamino)phenyl] benzimidazoles (see Scheme 5); some of the latter (**29****, **52****, and **59****) were purposely prepared in order to obtain compounds **32**–**34**, **55**–**58**, and **62**–**68**, but were not considered for antiviral activity screening.

The known 2-[4-(chloroacetylamino)phenyl]benzimidazole (**29****)[23] was prepared by acylation of 2-(4-aminophenyl)benzimidazole which, in turn, was obtained according to the literature.[24,25]

The structures of all novel compounds were supported by elemental analyses and ^{1}H NMR spectra that are fully consistent with the described structures.

3. Results and discussion

3.1. Biological activity—general considerations

The 2-phenylbenzimidazole derivatives synthesized in this work were evaluated for antiviral activity against ten RNA and DNA viruses. Among single-stranded, positive RNA viruses (ssRNA$^+$), we considered a *Retrovirus* (Human Immunodeficiency Virus type 1, HIV-1) two *Picornaviruses* (Coxsackie Virus type-2, CVB-2 and Poliovirus type-1, Sabin strain, Sb-1), a *Flavivirus* (Yellow Fever Virus, YFV) and a *Pestivirus* (Bovine Viral Diarrhea Virus, BVDV). Among single-stranded, negative RNA viruses (ssRNA$^-$) a *Paramyxoviridae* (Respiratory Syncytial Virus, RSV) and a *Rhabdoviridae* (Vesicular Stomatitis Virus, VSV) were selected as representatives. Among double-stranded RNA (dsRNA) viruses, a *Reoviridae* family member (Reo-1) was included. Finally, two representatives of DNA virus families were also included:

Table 1
Number of active compounds on susceptible viruses and range of their EC_{50}

Virus[a]	No. of active over 76 tested compounds[b]	No. of active compounds (range of EC_{50}, μM)				
Reo-1[c]	2		1 (7)			1 (85)
VV[d]	5	1 (0.1)	3 (4–8)			1 (87)
RSV[e]	6		4 (7–10)	1 (20)	1 (40)	
YFV[f]	10		5 (7–8)	3 (12–20)	1 (46)	1 (100)
HSV-1[d]	12		1 (7)	5 (12–28)	3 (38–49)	3 (51–60)
Sb-1[f]	17	1 (2)		8 (14–30)	6 (35–50)	2 (89–90)
BVDV[f]	31	13 (0.8–3)	9 (4–10)	3 (15–16)	3 (42–47)	3 (60–90)
CVB-2[f]	41	5 (1.5–3)	11 (4–10)	19 (11–30)	4 (32–35)	2 (87–100)

[a] HIV-1 and VSV were unaffected by all tested compounds.
[b] Compounds with EC_{50} >100 μM or higher than CC_{50} for host cells are considered inactive. Twenty over seventy-six compounds were not able to inhibit the multiplication of any virus.
[c] Double stranded RNA virus.
[d] DNA virus.
[e] Single stranded RNA^- virus.
[f] Single stranded RNA^+ virus.

Herpes Simplex Virus type-1, HSV-1 (*Herpesviridae*) and Vaccinia Virus, VV (*Poxviridae*).

AZT (3′-azido-thymidine), NM 108 (2′-*C*-methyl-guanosine), NM 176 (2′-*C*-ethynyl-cytidine), Ribavirin, NM 299 (6-azauridine), M 5255 (mycophenolic acid), and ACG (acyclovir) were used as reference inhibitors of $ssRNA^+$, $ssRNA^-$, and DNA viruses, respectively.

Fifty-six of the 76 tested compounds exhibited antiviral activity against one or more viruses; in particular, 17 compounds exhibited a selective activity against a single virus, while 20, 11, 7, and 1 molecules were active against two, three, four, and six viruses, respectively. None of the active compounds inhibited the replication of HIV-1 and VSV, but an increasing number of molecules exhibited antiviral activity against, in the order: Reo-1 (2), VV (5), RSV (6), YFV (10), HSV-1 (12), Sb-1 (17), BVDV (31), and CVB-2 (41) (see Table 1).

Thirty-nine of 56 compounds (70%) showed an $EC_{50} \leqslant 10$ μM against at least one virus, and a subset of 17 had at least one EC_{50} value in the range 0.1–3 μM.

Twenty compounds [12/55 1-unsubstituted (**A**–**C**) and 8/21 1-substituted (**B**–**D**) derivatives] were not able to inhibit the replication of any virus at concentrations up to the corresponding CC_{50} for their host cells, or up to the highest concentration tested (100 μM).

Cytotoxicity and antiviral activities of all the newly synthesized and reference compounds are reported in Tables2–4. In each Table, those viruses for which none or only one active compound has been found are not listed, the relevant results being reported as footnotes.

3.2. Cytotoxicity on host cells

The title compounds showed different degrees of cytotoxicity against the confluent cell monolayers (in stationary growth) used to support the replication of the different viruses.

The exponentially growing lymphoblastoid human cells (MT-4) used to grow HIV-1 were found to be the most susceptible to toxicity. The nonhuman host cell lines exhibited a progressively reduced sensitivity in the order: MDBK > BHV > Vero-76. Only 21% of compounds showed no toxicity (CC_{50} >100 μM) against MT-4, versus ~70% on Vero-76 cells; on the contrary 58% of compounds exhibited a $CC_{50} \leqslant 30$ μM for MT-4 compared with only 13% in the case of Vero-76 cells. Moreover, 9% of compounds had $CC_{50} \leqslant 3$ μM (with a lowest value of 0.3 μM) for MT-4 cells. The BHK cell line was somewhat more susceptible to toxicity than Vero-76 cell line, while MDBK was only slightly less susceptible than MT-4 cell line.

The high cytotoxicity of so many compounds against the MT-4 human cells decreases their interest as antiviral agents even if, in several cases, they exert a strong activity as viral replication inhibitors and only a moderate or no toxicity on the specific host cells. This observation is well exemplified by the cases of compounds **3** and **4**. The first is active against six viruses with EC_{50} values in the range 5–25 μM. While its CC_{50} for the corresponding host cell lines is >100 μM, the CC_{50} for the human cell line MT-4 is only 17 μM. Moreover, compound **4** exhibited high activity (EC_{50} = 1 μM) against BVDV and CC_{50} >100 μM for the host cells MDBK; however, the CC_{50} for MT-4 was as low as 2 μM. Thus, it will be of fundamental importance to identify the determining factors of toxicity on human cells in order to improve the drug-likeness of these benzimidazole derivatives.

The very high cytotoxicity observed in some of these compounds may warrant their evaluation as possible antiproliferative agents. Even compound **49**, which is devoid of toxicity on three host cell lines, may be interesting for cancer therapy, since several analogous 1,3-bis[4-(benzimidazol-2-yl)phenyl]ureas have shown to strongly inhibit heparanase, exhibiting anti metastasis efficacy.[11]

In any case, it must be observed that the structure–toxicity relationships appear rather intriguing. For example, the introduction of a nitro group, contrary to common expectation, gives rise to toxicity on the host cells only in a minority of cases, mainly when other groups are simultaneously present. Moreover, the introduction of the same 1-adamantyl residue at position 1 of the benzimidazole ring of compounds **39** (CC_{50} = 5 μM) and **46** (CC_{50} = 0.9 μM) produces opposite effects; in the first case, a 16-fold increase of cytotoxicity is observed (**75**: CC_{50} = 0.3 μM), while in the second case the cytotoxicity is drastically reduced (**76**; CC_{50} >100 μM).

3.3. Antiviral activity: structure–activity relationships

On the whole, the considered 2-phenyl benzimidazole derivatives appear particularly active against CVB-2 and BVDV viruses (although interesting levels of activity against other viruses have also been observed, though more randomly distributed).

Compounds of structure **A** and **B** (see Table 2), bearing a variety of substituents (although mainly NO_2 and OCH_3) on the 2-phenyl residue, active against CVB-2 (16 over 28 compounds = 57%) are also characterized by a large spectrum of activity, with 3–5 (11–18%) compounds active against each of the following viruses: Sb-1, YFV (10.7% each), BVDV, VV (14.3% each), HSV-1 and RSV (18% each) and only one against Reo-1 virus. It is interesting to observe that the activity against VV is confined to the 2-(4-nitrophenyl) derivatives, while no activity against BVDV was found among 2-(*x*-nitrophenyl)benzimidazoles, with the exception of compound **27** (Table 2). On the other hand, the 2-(4-aminophenyl) and

Table 2
Cytotoxicity against MT-4, MDBK, BHK and Vero-76 cell lines and antiviral activity of 2-phenylbenzimidazole derivatives of structure **A** (**1**–**25**) and **B** (**26**–**28**)

Compd[a]	R′	R″	Z	MT-4 CC_{50}[b]	MDBK CC_{50}[c]	BVDV EC_{50}[d]	BHK CC_{50}[e]	YFV EC_{50}[f]	Vero-76 CC_{50}[g]	CVB-2 EC_{50}[h]	Sb-1 EC_{50}[i]	HSV-1 EC_{50}[j]	RSV EC_{50}[k]	VV EC_{50}[l]
1	H	2-NO_2		60	>100	>100	>100	>100	>100	>100	>100	>100	40	>100
2	H	2,4-DiNO_2		7	20	>20	>100	>100	>100	40	>100	25	>100	>100
3[m]	5-CF_3	4-OH		17	51	>51	>100	12	>100	5	24	25	20	>100
4	5-CF_3	4-OCH_3		2	>100	1	>100	>100	15	7	>15	>15	>15	>15
5	5-CF_3	3-OCH_3		19	28	>28	90	>90	>100	4	>100	12	7	>100
6	5-CF_3	2,4-DiOCH_3		>100	>100	>100	>100	>100	>100	32	>100	>100	>100	>100
7	5-CF_3	3,5-DiOCH_3		19	>100	>100	>100	>100	>100	3	>100	>100	10	>100
8	5-CF_3	2,3,4-TriOCH_3		93	>100	>100	32	>32	70	16	42	>70	>70	>70
9	5-CF_3	3,4,5-TriOCH_3		4	18	>18	31	>31	29	11	>29	>29	>29	>29
10	5-CF_3	2-NO_2,4-OCH_3		85	55	>55	100	>100	20	>20	>20	>20	>20	>20
11	5-CF_3	2-NH_2,4-OCH_3		5	4	>4	⩾100	20	50	6	18	>50	>50	>50
12	5-CF_3	4-NO_2		6	6	>6	18	>18	45	12	>45	>45	>45	4
13	5-CF_3	2,4-DiNO_2		9	16	>16	8	>8	25	9	>25	>25	>25	>25
14	5-CF_3	4-F		22	19	>19	25	>25	60	13	>60	>60	>60	8
15	5-CF_3	2,6-DiF		100	⩾100	>100	⩾100	>100	25	>25	>25	>25	>25	>25
16	5-NO_2	2,4-DiOCH_3		>100	>100	45	>100	>100	>100	>100	>100	>100	>100	>100
17	5-NO_2	2,3,4-TriOCH_3		>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
18	5-$COCH_3$	2,4-DiOCH_3		>100	>100	42	>100	>100	>100	>100	>100	>100	>100	>100
19	5-$COCH_3$	2,3,4-TriOCH_3		>100	>100	>100	>100	>100	>100	>100	>100	40	>100	>100
20	5,6-DiCl	4-OH		12	11	>11	29	>29	18	>18	>18	>18	7	>18
21	5,6-DiCl	4-OCH_3		1	2.5	>2.5	18	>18	30	10	>30	20	>30	>30
22	5,6-DiCl	2-NO_2,4-OCH_3		2	28	>28	80	>80	80	>80	>80	>80	>80	>80
23	5,6-DiCl	2-NH_2,4-OCH_3		3	6.5	>6.5	>100	>100	75	>75	>75	>75	>75	>75
24	5,6-DiCl	4-NO_2		17	56	>56	>100	>100	⩾100	>100	>100	>100	>100	0.1
25	5,6-DiCl	2,6-DiF		17	43	>43	>100	>100	>100	30	>100	>100	>100	>100
26	5-CF_3	4-NO_2	Me	⩾100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
27	5-CF_3	4-NO_2	Ch[n]	49	68	60	>100	>100	>100	45	>100	>100	>100	87
28	5-CF_3	4-NO_2	Ad[o]	⩾100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
NM 108				>100	>100	1.7	90	1.8	>100	20	>100	>100	>100	>100
NM 299				2	>100	>100	>100	26	20	>20	>20	>20	1.2	11
ACG				>100	>100	>100	>100	>100	>100	>100	>100	3	>100	>100
Ribavirin				31	>100	7	>100	>100	>100	>100	>100	>100	7	>100
M 5255				0.2	42	>42	>100	>100	⩾13	>13	>13	>13	0.6	1.8
NM 176				⩾100	>100	38	>100	>100	>100	27	23	>100	>100	>100

CC_{50} and EC_{50} (μM).

[a] None of these compounds inhibited the multiplication of HIV-1 and VSV viruses.

[b] Compound concentration (μM) required to reduce the viability of mock-infected MT-4 ($CD4^+$ human T cells containing an integrated HJLV-1 genome) cells by 50%, as determined by the MTT method.

[c] Compound concentration (μM) required to reduce the viability of mock-infected MDBK (Bovine normal kidney) cells by 50%, as determined by the MTT method.

[d] Compound concentration (μM) required to achieve 50% protection of MDBK cells from BVDV (Bovine Viral Diarrhea Virus) induced cytopathogenicity, as determined by the MTT method.

[e] Compound concentration (μM) required to reduce the viability of mock-infected BHK (Hamster normal kidney fibroblast) monolayers by 50%, as determined by the MTT method.

[f] Compound concentration (μM) required to achieve 50% protection of BHK cells (kidney fibroblast) from YFV (Yellow Fever Virus) induced cytopathogenicity, as determined by the MTT method.

[g] Compound concentration (μM) required to reduce the viability of mock-infected VERO-76 (Monkey normal kidney) monolayers by 50%.

[h] Compound concentration (μM) required to reduce the plaque number of CVB-2 (Coxsackie Virus B2) by 50% in VERO-76 monolayers.

[i] Compound concentration (μM) required to reduce the plaque number of Sb-1 (Poliovirus type-1, Sabin strain) by 50% in VERO-76 monolayers.

[j] Compound concentration (μM) required to reduce the plaque number of HSV-1 (Herpes Simplex Virus, type-1) by 50% in VERO-76 monolayers.

[k] Compound concentration (μM) required to reduce the plaque number of RSV (Respiratory Syncytial Virus) by 50% in VERO-76 monolayers.

[l] Compound concentration (μM) required to reduce the plaque number of VV (Vaccinia Virus) by 50% in VERO-76 monolayers.

[m] Active also on Reo-1 virus (EC_{50} = 7 μM; NM 108: EC_{50} = 2.4 μM).

[n] Ch = cyclohexyl.

[o] Ad = 1-adamantyl.

2-(4-acylaminophenyl) derivatives (Table 3) have predominant activity against BVDV (66.7%) and CVB-2 (43.3%), with the two activities simultaneously present in 36.7% of cases. These compounds exhibit additional activity against Sb-1 (23%), HSV-1 (17%), and YFV (13%), while RSV, VV and Reo-1 viruses are affected by only one compound each.

The introduction of an aliphatic/cycloaliphatic residue at position 1 of the 2-(4-amino/acylaminophenyl)-5-trifluoromethylbenzimidazoles results in different effects, depending on its chemical nature (methyl, cyclohexyl, or 1-adamantyl), as well as on the presence and the nature of the acyl residue on the 4-amino group.

Taking into consideration all the compounds of structure **D** (see Table 4), an inverted trend with respect to that exhibited by the 1-unsubstituted compounds, is observed, with one-third of compounds sharing the activity on BVDV and CVB-2, and another third being active only on CVB-2. Indeed, comparing compounds with the same substitution pattern, the 1-substitution tends, the only exception being compound **66**, to reduce or suppress the activity against BVDV, while only occasionally does it interfere negatively with the activity versus CVB-2. Ultimately, it may even convert inactive into active compounds, as observed in the cases of **62**, **63**, **66**, **68**, and **76**. All these compounds and the corresponding unsubstituted compounds **41**, **42**, **45**, and **46** bear a basic head on the acyl moiety, and the onset of activity may be the consequence of balancing the excessive hydrophilicity due to the protonable nitrogen of the chain. Similar improvement of activity or conversion to activity of the inactive basic compounds is also observed for Sb-1 virus (**59**, **60**, **62**, **63**, **68**, and **71**). However the

Table 3
Cytotoxicity and antiviral activity of 2-(4-aminophenyl)- and 2-(4-acylaminophenyl)-5-trifluoromethyl benzimidazole derivatives of structure **C** (**29**–**58**)

Compd[a]	R′	R‴	MT-4 CC_{50}[b]	MDBK CC_{50}[c]	BVDV EC_{50}[d]	BHK-21 CC_{50}[e]	YFV EC_{50}[f]	Vero-76 CC_{50}[g]	CVB-2 EC_{50}[h]	Sb-1 EC_{50}[i]	HSV-1 EC_{50}[j]
29[k]	H	H	81	>100	16	>100	>100	>100	19	>100	>100
30	H	CH_3CO	100	>100	90	>100	>100	>100	87	>100	>100
31	H	CH_3CH_2CO	35	>100	47	⩾100	>100	>100	9	35	>100
32	H	$(CH_2)_4N–CH_2CO$	40	70	10	55	19	>100	11	90	>100
33	H	$(CH_2)_5N–CH_2CO$	31	18	7	31	>31	>100	17	>100	>100
34	H	$O(CH_2\ CH_2)_2N–CH_2CO$	100	77	>77	>100	>100	>100	>100	>100	>100
35	H	Homolupinanoyl	18	36	10	28	8	>100	⩾100	>100	60
36	5-CF_3	H	62	82	15	100	>100	>100	17	47	>100
37	5-CF_3	CH_3CO	19	50	1.3	53	>53	50	3	16	7
38	5-CF_3	CH_3CH_2CO	⩾100	>100	>100	>100	>100	>100	4	>100	>100
39	5-CF_3	$ClCH_2CO$	5	17	>17	22	>22	35	24	>35	>35
40	5-CF_3	1-Adamantyl-NH–CH_2CO	15	23	>23	18	>18	45	>45	>45	45
41	5-CF_3	$(C_2H_5)_2N–CH_2CO$	8	13	2	23	7	24	>24	>24	>24
42	5-CF_3	$(CH_2)_4N–CH_2CO$	18	15	3	29	7	>100	>100	>100	>100
43	5-CF_3	$(CH_2)_5N–CH_2CO$	6	6	2	12	⩾12	31	9	>31	>31
44	5-CF_3	$O(CH_2\ CH_2)_2N–CH_2CO$	>100	>100	>100	86	>86	>100	>100	>100	>100
45	5-CF_3	$S(CH_2CH_2)_2N–CH_2CO$	6	>100	>100	>100	>100	>100	>100	>100	>100
46	5-CF_3	$C_6H_5N(CH_2CH_2)_2N–CH_2CO$	0.9	32	>32	>100	>100	>100	>100	>100	>100
47	5-CF_3	Homolupinanoyl	4	12	2	15	>15	40	>40	14	>40
48	5-CF_3	$HOOC(CH_2)_2CO$	>100	>100	>100	>100	>100	>100	>100	>100	>100
49	5-CF_3	4-(5-CF_3-benzimidazol-2-yl)phenylcarbamoyl	>100	>100	>100	17	>17	>100	>100	>100	>100
50	5-NO_2	H	100	79	1.5	>100	>100	>100	>100	>100	>100
51	5-NO_2	CH_3CO	>100	>100	0.8	>100	>100	>100	>100	>100	>100
52[l]	5,6-DiCl	H	17	7.5	1	30	>30	⩾100	11	>100	28
53	5,6-DiCl	CH_3CO	>100	58	1	>100	>100	>100	>100	>100	>100
54	5,6-DiCl	$(CH_2)_4N–CH_2CO$	8	16	2.5	64	>64	>100	1.5	25	>100
55	5,6-DiCl	$(CH_2)_5N–CH_2CO$	54	45	2	39	>39	>100	>100	>100	38
56	5,6-DiCl	$O(CH_2CH_2)_2N–CH_2CO$	18	16	2.4	51	>51	⩾100	13	>100	>100
57	5,6-DiCl	$O(CH_2CH_2)_2N–CH_2CO$	>100	>100	>100	>100	>100	>100	>100	>100	>100
58[m]	5,6-DiCl	$CH_3N(CH_2CH_2)_2N–CH_2CO$	11	90	4	41	>41	>100	>100	89	51
NM 108			>100	>100	1.7	90	1.8	>100	20	>100	>100
6-Azauridine			2	>100	>100	>100	26	20	>20	>20	>20
ACG			>100	>100	>100	>100	>100	>100	>100	>100	3
Ribavirin			31	>100	7	>100	>100	>100	>100	>100	>100
NM 176			⩾100	>100	>100	>100	>100	>100	27	23	>100

CC_{50} and EC_{50} (μM).
[b–j] For the meaning see Table 2.
[a] None of these compounds inhibited the multiplication of HIV-1 and VSV viruses.
[k] Active also on Reo-1 virus (EC_{50} = 85 μM; NM-108: EC_{50} = 2.4 μM).
[l] Active also on RSV virus (EC_{50} = 10 μM; 6-azauridine: EC_{50} = 1.2 μM).
[m] Active also on VV virus (EC_{50} = 6 μM; M 5255: EC_{50} = 1.8 μM).

1-substitution has variable effects on activity against HSV-1 and YFV, and suppresses that on RSV, VV, and Reo-1 viruses.

Of the 17 most active compounds with a EC_{50} <3 μM, only three belong to group **A**, while all remaining molecules are 2-(4-amino or 4-acylaminophenyl)benzimidazoles, either 1-unsubstituted (12; Table 3) or 1-methyl substituted (2; see Table 4).

On the other hand, selective activity against a single virus is more commonly found among compounds of groups **A** and **B** (10/28 = 35.7%; see Table 2) than among compounds of groups **C** and **D** (7/48 = 14.6%; see Tables 3 and 4).

Combined high activity and selectivity are shown by compounds **51**, **53**, and **50** against BVDV (EC_{50} = 0.8–1.5 μM), and by compound **24** with respect to VV (EC_{50} = 0.1 μM). Moreover, these compounds are well tolerated by the proper host cell lines (MDBK, Vero-76), and even more by the human cell line MT-4, and compare favorably with their reference compounds NM 108 (EC_{50} = 1.7 μM) and M 5255 (EC_{50} = 1.8 μM).

Some interest also deserves compound **38**. Its selective activity against CVB-2 (EC_{50} = 4 μM) compares well with that of the reference compound NM 108 (EC_{50} = 20 μM). Several other compounds exhibit a high, even if not selective, activity against BVDV or CVB-2 viruses (EC_{50} ⩽ 4 μM), and only moderate toxicity on the relevant host cells, but their selectivity versus the human MT-4 cell line was rather low. Taking into account the EC_{50} and the selectivity index (S.I.) values, the best of these compounds (with S.I. >4) are collected in Table 5.

As previously observed, eight compounds exhibit a large spectrum of activity regarding 4–6 viruses, most commonly BVDV, CVB-2, Sb-1, HSV-1, and YFV. The degree of activity is generally high, with EC_{50} in the range 1–20 μM for two thirds of the molecular set; however, toxicity versus the human cell line MT-4 (CC_{50} <20 μM) is frequently found.

In order to determine the possible step(s) which is inhibited by our compounds during the BVDV replication cycle, a time of addition experiment was performed with the most potent compound **51**, that was added at different time points after infection of MDBK cell cultures with the relevant virus. Compound **51** retained its inhibitory activity when even added no later than 6 h after infection (see Fig. 3). The reference compound NM 108 (2′-*C*-methylguanosine) is a nucleoside analog that interacts with the viral polymerase (up to 4 h pi) either as competitive inhibitor or as substrate, preventing further chain elongation. The comparison of the two time of addition curves pointed to a post viral entry step, before the budding stage (approximately after 13 h pi), in the BVDV replication cycle as possible antiviral target of our compound series.

Selective compounds **50**, **51**, and **53**, endowed with the highest activity against BVDV and the lowest toxicity for both the host cells

Table 4
Cytotoxicity and antiviral activity of 2-(4-aminophenyl)- and 2-(4-acylaminophenyl)-5-trifluoromethyl-1-substituted benzimidazoles derivatives of structure **D** (**59**–**76**)

Compd[a]	Z	R‴	MT-4 CC_{50}[b]	MDBK CC_{50}[c]	BVDV EC_{50}[d]	BHK CC_{50}[e]	YFV EC_{50}[f]	Vero-76 CC_{50}[g]	CVB-2 EC_{50}[h]	Sb-1 EC_{50}[i]	HSV-1 EC_{50}[j]
59	CH_3	H	20	74	69	85	>85	>100	14	20	>100
60	CH_3	CH_3CO	15	46	>46	>100	>100	>100	2.5	14	49
61	CH_3	CH_3CH_2CO	8	>100	>100	>100	>100	>100	>100	>100	>100
62	CH_3	$(C_2H_5)_2N–CH_2CO$	9	57	10	16	7	90	21	35	>90
63	CH_3	$(CH_2)_4N–CH_2CO$	16	65	7	27	>27	>100	13	50	51
64	CH_3	$(CH_2)_5N–CH_2CO$	9	100	6	>100	>100	>100	32	>100	>100
65	CH_3	$O(CH_2CH_2)_2N–CH_2CO$	⩾100	>100	>100	>100	>100	>100	>100	>100	>100
66	CH_3	$S(CH_2CH_2)_2N–CH_2CO$	10	>100	8	>100	46	>100	22	>100	>100
67	CH_3	$CH_3N(CH_2CH_2)_2N–CH_2CO$	22	44	15	16	7	>100	19	41	>100
68	CH_3	$C_6H_5N(CH_2CH_2)_2N–CH_2CO$	1.5	1.4	>1.4	100	>100	30	3	2	>30
69	Cyclohexyl	H	44	>100	>100	>100	>100	>100	>100	>100	>100
70	Cyclohexyl	CH_3CO	24	17	>17	15	>15	30	9	>30	>30
71	Cyclohexyl	CH_3CH_2CO	17	18	>18	15	>15	>100	7	30	>100
72	1-Adamantyl	H	19	19	7	16	>16	60	11	>60	>60
73	1-Adamantyl	CH_3CO	28	82	>82	>100	>100	>100	>100	>100	>100
74	1-Adamantyl	CH_3CH_2CO	86	>100	>100	>100	>100	>100	>100	>100	>100
75	1-Adamantyl	$ClCH_2CO$	0.3	18	>18	51	>51	>100	>100	>100	>100
76	1-Adamantyl	$C_6H_5N(CH_2CH_2)_2N–CH_2CO$	>100	>100	>100	>100	>100	>100	27	>100	>100
NM 108			>100	>100	1.7	90	1.8	>100	20	>100	>100
6-Azauridine			2	>100	>100	>100	26	20	>20	>20	>20
ACG			>100	>100	>100	>100	>100	>100	>100	>100	3
Ribavirin			31	>100	7	>100	>100	>100	>100	>100	>100
NM 176			⩾100	>100	38	>100	>100	>100	27	23	>100

CC_{50} and EC_{50} (μM).
[b–j] For the meaning see Table 2.
[a] None of these compounds inhibited the multiplication of HIV-1, Reo-1, RSV, VSV, and VV viruses.

Table 5
Most potent antiviral compounds against BVDV, CVB-2 and VV, in order of decreasing selectivity index (S.I.) versus human MT-4 cell line (S.I. >4)

Virus	Compd	EC_{50} (μM)	S.I. for proper host cell lines	S.I. for MT-4 cell line	Other susceptible viruses
BVDV	**51**	0.8	>125	>125	Selective
	53	1.0	58	>100	Selective
	50	1.5	53	67	Selective
	55	2.0	23	27	HSV-1
	52	1.0	7.5	17	CVB-2, HSV-1
	37	1.3	39	15	CVB-2, Sb-1, HSV-1
	56	2.4	6.7	7.5	CVBV-2
	42	3.0	5.0	6.0	YFV
	41	2.0	6.5	4.0	YFV
CVB-2	**38**	4.0	>25	⩾25	Selective
	7	3.0	>33	6.3	RSV
	37	3.0	17	6.3	BVDV, Sb-1, HSV-1
	60	2.5	>40	6.0	Sb-1, HSV-1
	54	1.5	>67	5.3	BVDV, Sb-1
	5	4.0	>25	4.8	HSV-1, RSV
VV	**24**	0.1	⩾1000	170	Selective

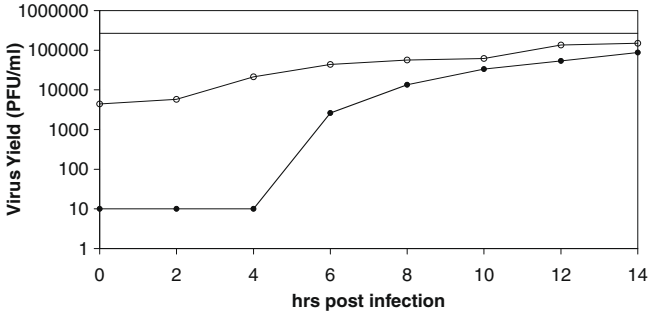


Figure 3. Effect of time of (drug)-addition on antiviral activity of **51** (open circles). The same test was performed using the nucleoside analog NM 108 (filled circles) for comparison. Infected control: continuous line.

MDBK and the human cells MT-4, were also assayed against the surface binding site of NS5B RNA-dependent RNA polymerase (RdRp) of BVDV. The enzymatic inhibition assays showed that these compounds inhibit the RNA polymerase at micromolar concentrations, in a dose-dependent way (see Table 6), indicating that the RdRp is indeed their target. Since BVDV is often used as a surrogate model to screen antiviral agents against HCV, the three compounds were also assayed against HCV1b polymerase and found to inhibit this enzyme with similar IC_{50} (see Table 6).

The molecular target of our compounds has been further supported by the generation of compounds **50**, **51**, and **53** BVDV resistant mutants,[26] observing that while NS3 protease is not affected, the NS5B RdRp was modified by the three compounds even if with some differences. Functional mapping and sequence analysis of resistant cDNAs revealed a single (A392 and N264D mutations for **50** and **51**, respectively) or a double amino acid substitution (N264D, I261M mutations for **53**) in the NS5B polymerase. These regions of NS5B are highly conserved among pestiviruses suggesting that these sites play an important role in the function of the

Table 6
Comparison of antiviral activity (EC_{50}) versus BVDV, inhibitory activity (IC_{50}) on NS5B RdRp of BVDV and HCV viruses, and activity in HCV replicon assay

Compd	Anti-BVDV activity			IC_{50} (μM) on NS5B RdRp		HCV replicon assay	
	CC_{50} (μM)	EC_{50} (μM)	S.I.	BVDV	HCV	CC_{50} (μM)	EC_{50} (μM)
50	79	1.5	53	18	33		
51	>100	0.8	>125	12	30	>90	38
53	58	1	58	3	5	>75	32

enzyme; mutational analysis of this region abolished the RNA synthesis in vitro, suggesting that this sequence is important for replicase activity. Furthermore, docking studies upon pharmacophoric constraints and mutational data were carried out,[26] and the binding affinity of all active compounds for the BVDV RdRp were estimated with excellent agreement between in silico and in vitro data.

Finally compounds **51** and **53** were also evaluated in the HCV subgenomic replicon system, confirming activity against HCV, even if lower than that against BVDV (see Table 6).

4. Conclusions

Seventy-six 2-phenylbenzimidazole derivatives have been synthesized and assayed for antiviral activity against a panel of 10 RNA and DNA viruses. Fifty-six of the tested compounds exhibited antiviral activity against one or more viruses, and 39 of them showed $EC_{50} \leqslant 10$ μM against at least one virus. A subset of 17 compounds (mainly 2-(4-aminophenyl)- or 2-(4-acylaminophenyl)benzimidazoles had at least one EC_{50} value in the range 0.1–3 μM.

The considered 2-phenylbenzimidazole derivatives resulted particularly effective against BVDV (31/56) and CVB-2 (41/56) viruses, but interesting levels of activity against other viruses (Sb-1, HSV-1, YFV, RSV, VV, and Reo-1) have been also observed.

Despite the high antiviral activity and good selectivity index for the proper host cell lines, the value of some compounds results somewhat impaired by their cytotoxicity for the human MT-4 cell line. Nevertheless, taking into account the EC_{50} and S.I. values, several compounds appear as very interesting.

Compound **24** [5,6-dichloro-2-(4-nitrophenyl)benzimidazole] exhibited a high and selective activity against VV (EC_{50} = 0.1 μM), resulting 18 and 110 times more potent than the reference drugs mycophenolic acid and 6-azauridine (EC_{50} = 1.8 μM and 11 μM, respectively). Moreover, these reference drugs are much more toxic than **24** versus the proper host cell line (Vero-76) and the human MT-4 cell line.

Even if the natural smallpox infection has been declared eradicated by WHO, some concern is raised by the possible terroristic use of the virus, thus the acquisition of protective agents, possibly acting by oral route, should be fostered. At present the only antiviral agent considered for this use by the U.S. Centers for Disease Control and Prevention (CDC) is the costly cidofovir (CDV), by iv infusion, which, however, according to De Clercq,[27] could be reformulated as lipid prodrug for oral use.

A potent and specific inhibitor of orthopoxvirus replication has recently been discovered[28] (ST-246: 4-trifluoromethyl-*N*-(3,3a,4,4a,5,5a,6,6a,-octahydro-1,3-dioxo-4,6-ethenocycloprop[*f*] isoindol-2(1*H*)-yl)benzamide), which was effective when administered orally in mice. However poxvirus strains that are resistant to CDV or ST-246 have been generated in cell culture.[29]

More recently, through the high-throughput screening of about 50,000 compounds, 16 hit compounds have been identified and found to block viral infection with low cytotoxicity.[30] Comparing to our results, it is observed that their EC_{50} for plaque reduction was in the range 10–150 μM.

Thus compound **24** still represents an interesting hit for the development of anti-poxvirus agents, and provided that it will not exhibit unduly toxicity in vivo, may in itself deserve further investigation for in vivo efficacy against poxvirus infections, and for identification of its mechanism of action.

On the other hand, simple compounds such as **50**, **51**, and **53** exhibited a high and selective activity against BVDV with EC_{50} in the range 0.8–1.5 μM, thus comparing favorably with the reference drug NM 108 (EC_{50} = 1.7 μM). BVDV is the prototype of pestiviruses which are responsible of severe epidemic outbreaks in livestock with high mortality, and the availability of effective and inexpensive anti-pestivirus drugs is of importance to relieve such an heavy economic burden.

In the last years several potent anti-BVDV agents have been developed and shown to target the RNA-dependent RNA-polymerase (RdRp),[31–37] but the structural simplicity of compounds **50**, **51**, and **53** make them an attractive model to develop ever better anti-pestivirus agents. Moreover, BVDV is surrogate model for the evaluation of novel, urgently needed agents against HCV,[35] an emerging threat to human health worldwide. Indeed, compounds **50**, **51** and particularly **53** were found able to inhibit both the BVDV and HCV NS5B RdRp at similar concentrations and, therefore, may represent also an interesting starting point for the development of anti-HCV agents. Indeed compound **51** proved moderately active in the HCV subgenomic replicon system.

5. Experimental

5.1. General

Melting points were taken in open glass capillaries on a Büchi apparatus and were uncorrected. Purity of compounds was checked by thin-layer chromatography that was performed on silica gel plates [Merck (G F_{254})] and the spots were observed under UV light. Column chromatography (CC) was performed using basic alumina (Across). Elemental analyses were performed on a Carlo Erba EA-1110 CHNS-O instrument in the Microanalysis Laboratory of the Department of Pharmaceutical Sciences of Genoa University. The analytical results are within ±0.4% of calculated values. ^{1}H NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 on Varian Gemini-200 spectrometer; δ in ppm rel. to Me_4Si as internal standard. *J* in Hz. Results of elemental analyses, TLC and NMR spectra indicated that the purity of all compounds was $\geqslant$95%.

5.2. Intermediates

Chemicals, solvents and commercially available intermediates were purchased from Aldrich (Milan). The noncommercially available intermediates were prepared according to the literature (see: Section 2), or as follows, when not previously known.

5.2.1. *N*-(2-Nitro-4-trifluoromethylphenyl)-1-adamantaneamine

A solution of 1-adamantaneamine (20 mmol) and 4-chloro-3-nitrobenzotrifluoride (10 mmol) in DMF (3 mL) was heated with stirring (90 min; 140 °C) in a pressure tube (Aldrich). After cooling,

the residue was taken up with water, filtered and washed with water and finally crystallized from Et_2O. Yield: 88%. Mp 210–213 °C. ^1H NMR ($CDCl_3$): 1.78 (br s, 6H, CH_2, adamantane nucleus); 2.13 (br s, 6H, CH_2, adamantane nucleus); 2.25 (br s, 3H, CH_2, adamantane nucleus); 4.75 (br s, NH, collapses with D_2O); 7.29 (d, J = 9.6, 1 arom. H); 7.55 (dd, J = 9.6, 1.1, 1 arom. H); 8.53 (d, J = 10, 1 arom. H). Anal. Calcd for $C_{17}H_{19}F_3N_2O_2$: C, 59.99; H 5.63; N, 8.23. Found: C, 60.02; H, 5.70; N, 8.24.

5.2.2. *N*-(2-Amino-4-trifluoromethylphenyl)-1-adamantaneamine

A suspension of the above nitrocompound (10 mmol) in 50 mL of EtOH was hydrogenated at rt and atmospheric pressure in the presence of 10% Pd/C (0.4 g). After 2 h and 30 min the calculated volume of H_2 was absorbed. The catalyst was removed and the solvent was evaporated in vacuo, leaving an almost quantitative yield of product which was crystallized from pentane. Yield: 98%. Mp 114–116 °C (pentane). ^1H NMR ($CDCl_3$): 1.71 (br s, 6H CH_2, adamantane nucleus); 1.95 (br s, 6H, CH_2, adamantane nucleus); 2.15 (br s, 3H CH_2, adamantane nucleus); 3.58 (br s, NH_2 and NH, collapse with D_2O); 6.92–7.05 (m, 3 arom. H). Anal. Calcd for $C_{17}H_{21}F_3N_2$: C, 65.79; H, 6.82; N, 9.03. Found: C, 65.86; H, 6.46; N, 8.87.

5.3. 2-Phenylbenzimidazoles. Method 'a' of ring closure

To a solution of the suitable 1,2-phenylenediamine (5 mmol) in 15 mL of MeCN the appropriate benzaldehyde (5 mmol) dissolved in 15 mL of MeCN was added. The mixture was refluxed for 1 h with stirring, and then $FeCl_3{\cdot}6H_2O$ (14 mg) in 2 mL of MeCN was added. Bubbling O_2, the heating was maintained for 6 h with stirring. After cooling, the precipitate was filtered and washed with MeCN, leaving generally a pure product. Compounds **22** and **27** were crystallized from EtOH.

In the case of compound **15**, it was necessary to remove the solvent from the reaction mixture, and to purify the residue by CC (Al_2O_3, CH_2Cl_2). Finally the oily product was crystallized from dry Et_2O/pentane (1:1).

5.3.1. 2-(4-Nitrophenyl)-5-trifluoromethyl-1*H*-benzimidazole (12)

Yield: 46%. Mp 216–218 °C (MeCN). ^1H NMR (DMSO-d_6): 7.67 (d, J = 8.6, 1 arom. H); 7.97 (d, J = 8.6, 2 arom. H); 8.10–8.23 (m, 2 arom. H); 8.41 (d, J = 8.8, 2 arom. H); 13.51 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{14}H_8F_3N_3O_2$: C, 54.73; H, 2.62; N, 13.68. Found: C, 54.84; H, 2.29; N, 13.70.

5.3.2. 2-(2,6-Difluorophenyl)-5-trifluoromethyl-1*H*-benzimidazole (15)

Yield: 55%. Mp 166–168 °C (Et_2O/pentane). ^1H NMR (DMSO-d_6): 7.38 (t, J = 8.6, 2 arom. H); 7.50–7.96 (m, 3 arom. H); 8.06 (s, 1 arom. H); 13.35 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{14}H_7F_5N_2$: C, 56.39; H, 2.37; N, 9.39. Found: C, 56.03; H, 2.41; N, 9.36.

5.3.3. 5,6-Dichloro-2-(4-Methoxy-2-nitrophenyl)-1*H*-benzimidazole (22)

Yield: 77%. Mp 193–194 °C (EtOH). ^1H NMR ($CDCl_3$): 3.93 (s, OCH_3); 7.13 (d, J = 8.2, 1 arom. H); 7.39 (s, 1 arom. H); 7.55 (s, 2 arom. H); 7.80 (d, J = 8.2, 1 arom. H); 9.56 (s, NH of benzim., collapse with D_2O). Anal. Calcd for $C_{14}H_9Cl_2N_3O_3$: C, 49.73; H, 2.68; N, 12.43. Found: C, 49.78; H, 2.51; N, 12.36.

5.3.4. 1-Methyl-2-(4-nitrophenyl)-5-trifluoromethyl-1*H*-benzimidazole (26)

Yield: 65%. Mp 218–221 °C (MeCN). ^1H NMR (DMSO-d_6): 4.0 (s, NCH_3); 7.68 (d, J = 8.6, 1 arom. H); 7.93 (d, J = 8.8, 1 arom. H); 8.12 (dd, J = 8.8, 1.2, 1 arom. H); 8.20 (d, J = 9.0, 2 arom. H); 8.43 (d, J = 9.0, 2 arom. H). Anal. Calcd for $C_{15}H_{10}F_3N_3O_2$: C, 56.08; H, 3.14; N, 13.03. Found: C, 56.15; H, 3.24; N, 13.08.

5.3.5. 1-Cyclohexyl-2-(4-nitrophenyl)-5-trifluoromethy-1*H*-benzimidazole (27)

Yield: 62%. Mp 159–161 °C (EtOH). ^1H NMR (DMSO-d_6): 1.32–2.43 (m, 10H, cyclohexane nucleus); 4.18–4.40 (m, 1H, cyclohexane nucleus); 7.62 (d, J = 8.6, 1 arom. H); 7.95 (d, J = 8.9, 2 arom. H); 8.08–8.22 (m, 2 arom. H); 8.43 (d, J = 8.9, 2 arom. H). Anal. Calcd for $C_{20}H_{18}F_3N_3O_2$: C, 61.38; H, 5.15; N, 10.74. Found: C, 61.44; H, 4.75; N, 10.73.

5.3.6. 1-(1-Adamantyl)-2-(4-nitrophenyl)-5-trifluoromethyl-1*H*-benzimidazole (28)

Yield: 82%. Mp 266–269 °C (MeCN). ^1H NMR (DMSO-d_6): 1.57–1.80 (m, 6H, adamantane nucleus); 2.21- 2.34 (m, 9 H, adamantane nucleus); 7.56 (d, J = 8.6, 1 arom. H); 7.93 (d, J = 8.9, 2 arom. H); 8.06–8.20 (s, 1 arom. H); 8.20–8.43 (m, 1 arom. H and 8.33, d, J = 9.0, 2 arom. H superimposed). Anal. Calcd for $C_{24}H_{22}F_3N_3O_2$: C, 65.30; H, 5.02; N, 9.52. Found: C, 65.13; H, 5.37; N, 9.80.

5.3.7. 2-(4-Acetylaminophenyl)-5-trifluoromethyl-1*H*-benzimidazole (37)

Yield: 45%. Mp 281–282 °C (MeCN). ^1H NMR (DMSO-d_6): 2.12 (s, CH_3CO); 7.67 (d, J = 8.5, 1 arom. H); 7.98 (d, J = 8.8, 2 arom. H); 8.09–8.20 (m, 2 arom. H); 8.43 (d, J = 8.8, 2 arom. H); 10.15 (s, NHCO, collapses with D_2O); 13.38 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{16}H_{12}F_3N_3O$: C, 60.19; H, 3.79; N, 13.16. Found: C, 59.96; H, 4.08; N, 13.26.

5.3.8. 2-(4-Acetylaminophenyl)-5,6-dichloro-1*H*-benzimidazole (53)

Yield: 78%. Mp >300 °C (MeCN). ^1H NMR (DMSO-d_6): 2.05 (s, CH_3CO); 7.87 (s, 2 arom. H); 7.89 (d, J = 9.8, 2 arom. H); 8.16 (d, J = 9.8, 2 arom. H); 10.11 (s, NHCO, collapses with D_2O); 12.57 (br s, NH benzim., collapses with D_2O). Anal Calcd for $C_{15}H_{11}Cl_2N_3O + 0.25H_2O$: C, 55.49; H, 3.57; N, 12.94. Found: C, 55.77; H, 3.78; N, 13.11.

5.4. 2-Phenylbenzimidazoles. Method 'b' of ring closure

To a solution of the suitable 1,2-phenylenediamine (3.5 mmol) in 18 mL of EtOH the appropriate benzaldehyde–sodium bisulfite adduct (3.5 mmol) (prepared according to the method of (Shriner and Land[14]) was added. The mixture was refluxed for 3 h with stirring. The solvent was evaporated and the residue was taken up with water and filtered, affording a residue that was crystallized from the suitable solvent.

In the case of compound **13**, chloranil (0.8 mmol) was added to the reaction mixture that was refluxed for 72 h. After cooling, a precipitate was filtered and the ethanolic solution was evaporated to dryness, leaving a solid product that was purified by CC (Al_2O_3/CH_2Cl_2).

5.4.1. 2-(3-Methoxyphenyl)-5-trifluoromethyl-1*H*-benzimidazole (5)

Yield: 25%. Mp 186–187 °C (benzene). ^1H NMR (DMSO-d_6): 3.89 (s, OCH_3); 7.07–7.20 (m, 1 arom. H); 7.42–7.89 (m, 5 arom. H); 8.05 (s, 1 arom. H); 13.38 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{15}H_{11}F_3N_2O$: C, 61.64; H, 3.79; N, 9.59. Found: C, 61.64; H, 3.79; N, 9.56.

5.4.2. 2-(2,4-Dimethoxyphenyl)-5-trifluoromethyl-1*H*-benzimidazole (6)

Yield: 87%. Mp 175–176 °C (EtOH/H_2O). ^1H NMR (DMSO-d_6): 3.86 (s, OCH_3); 4.03 (s, OCH_3); 6.65–6.76 (m, 2 arom. H); 7.41–

7.82 (m, 2 arom. H); 7.91 (d, J = 7.8, 1 arom. H); 8.23–8.35 (m, 1 arom. H); 12.28 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{16}H_{13}F_3N_2O_2$: C, 59.62; H, 4.47; N, 8.69. Found: C, 59.42; H, 4.41; N, 8.52.

5.4.3. 5-Trifluoromethyl-2-(2,3,4-trimethoxyphenyl)-1*H*-benzimidazole (8)

Yield: 73%. Mp 77–78 °C (EtOH/H_2O). ^{1}H NMR (DMSO-d_6): 3.86 (s, OCH_3); 3.91 (s, OCH_3); 4.00 (s, OCH_3); 7.04 (d, J = 9.0, 1 arom. H); 7.48 (d, J = 8.6, 1 arom. H); 7.75–8.10 (m, 3 arom. H); 12.42 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{17}H_{15}F_3N_2O_3$ + 0.25H_2O: C, 59.44; H, 4.37; N, 7.85. Found: C, 59.43; H, 4.21; N, 8.00.

5.4.4. 2-(4-Methoxy-2-nitrophenyl)-5-trifluoromethyl-1*H*-benzimidazole (10)

Yield: 42%. Mp 113–114 °C (CH_2Cl_2). ^{1}H NMR ($CDCl_3$): 3.92 (s, OCH_3); 7.18 (dd, J = 8.6, 2.7, 1 arom. H); 7.43 (d, J = 2.7, 1 arom. H); 7.54 (s, 2 arom. H); 7.66–7.83 (m, 1 arom. H); 7.89 (d, J = 8.6, 1 arom. H); 10.94 (br s, NH, collapses with D_2O). Anal. Calcd for $C_{15}H_{10}F_3N_3O_3$: C, 53.42; H, 2.99; N, 12.46. Found: C, 53.12; H, 2.92; N, 12.31.

5.4.5. 2-(2,4-Dinitrophenyl)-5-trifluoromethyl-1*H*-benzimidazole (13)

Yield: 48%. Mp 187–188 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 7.64 (d, J = 8.6, 1 arom. H); 7.90 (d, J = 8.6, 1 arom. H); 8.09 (s, 1 arom. H); 8.33 (d, J = 8.6, 1 arom. H); 8.74 (dd, J = 8.6, 2.3, 1 arom. H); 8.91 (d, J = 2.3, 1 arom. H); 13.84 (br s, NH, collapses with D_2O). Anal. Calcd for $C_{14}H_7F_3N_4O_4$: C, 47.74; H, 2.00; N, 15.91. Found: C, 47.41; H, 1.71; N, 15.97.

5.4.6. 2-(4-Fluorophenyl)-5-trifluoromethyl-1*H*-benzimidazole (14)

Yield: 53%. Mp 170–171 °C (pentane). ^{1}H NMR (DMSO-d_6): 7.34–7.60 (m, 3 arom. H); 7.78 (d, J = 8.4, 1 arom. H); 7.96 (s, 1 arom. H); 8.15–8.33 (m, 2 arom. H); 13.24 (br s, NH, collapses with D_2O). Anal. Calcd for $C_{14}H_8F_4N_2$: C, 60.01; H, 2.88; N, 10.00. Found: C, 60.14; H, 2.60; N, 9.93.

5.4.7. 2-(2,4-Dimethoxyphenyl)-5-nitro-1*H*-benzimidazole (16)

Yield: 75%. Mp 268–270 °C (EtOH/H_2O). ^{1}H NMR (DMSO-d_6): 3.90 (s, OCH_3); 4.06 (s, OCH_3); 6.72–6.84 (m, 2 arom. H); 7.75 (d, J = 9.0, 1 arom. H); 8.08 (dd, J = 8.0, 2.7, 1 arom. H); 8.30 (d, J = 8.6, 1 arom. H); 8.48 (s, 1 arom. H); 12.48 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{15}H_{13}N_3O_4$ + 0.25H_2O: C, 59.30; H, 4.47; N, 13.83. Found: C, 59.64; H, 4.37; N, 13.86.

5.4.8. 5-Nitro-2-(4-trimethoxyphenyl)-1*H*-benzimidazole (17)

Yield: 76%. Mp 174–175 °C (EtOH/H_2O). ^{1}H NMR (DMSO-d_6): 3.86 (s, OCH_3); 3.92 (s, OCH_3); 4.00 (s, OCH_3); 7.04 (d, J = 8.8, 1 arom. H); 7.97 (d, J = 8.6, 1 arom. H); 7.98–8.20 (m, 2 arom. H); 8.51 (s, 1 arom. H); 12.63 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{16}H_{15}N_3O_5$: C, 58.36; H, 4.59; N, 12.76. Found: C, 58.12; H, 4.61; N, 12.85.

5.4.9. 5-Acetyl-2-(2,4-dimethoxyphenyl)-1*H*-benzimidazole (18)

Yield: 75%. Mp 162–165 °C (EtOH/H_2O). ^{1}H NMR (DMSO-d_6): 2.64 (s, CH_3CO); 3.87 (s, OCH_3); 4.04 (s, OCH_3); 6.69–6.90 (m, 2 arom. H); 7.68–7.88 (m, 2 arom. H); 8.18–8.34 (m, 2 arom. H); 10.22 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{17}H_{16}N_2O_3$ + 0.25H_2O: C, 67.87; H, 5.52; N, 9.31. Found: C, 67.68; H, 5.35; N, 9.07.

5.4.10. 5-Acetyl-2-(2,3,4-trimethoxyphenyl)-1*H*-benzimidazole (19)

Yield: 64%. Mp 106–109 °C (EtOH/H_2O). ^{1}H NMR (DMSO-d_6): 2.63 (s, CH_3CO); 3.82 (s, OCH_3); 3.86 (s, OCH_3); 3.92 (s, OCH_3); 7.00 (d, J = 8.8, 1 arom. H); 7.66 (d, J = 8.0, 1 arom. H); 7.82–8.30 (m, 3 arom. H); 12.35 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{18}H_{18}N_2O_4$: C, 66.24; H, 5.56; N, 8.58. Found: C, 66.11; H, 5.59; N, 8.47.

5.4.11. 5,6-Dichloro-2-(4-methoxyphenyl)-1*H*-benzimidazole (21)

Yield: 55%. Mp 218–220 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 3.98 (s, OCH_3); 6.93 (d, J = 8.0, 2 arom. H); 7.80 (s, 2 arom. H); 7.99 (d, J = 8.0, 2 arom. H); 13.00 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{14}H_{10}Cl_2N_2O$: C, 57.36; H, 3.44; N, 9.56. Found: C, 57.00; H, 3.44; N, 9.50.

5.5. 2-Phenylbenzimidazoles. Method 'c' of ring closure

To a solution of 4-trifluoromethyl-1,2-phenylenediamine (5 mmol) in 7 mL of anhyd dioxane, a solution of 3,5-dimethoxybenzoyl chloride (5 mmol) in 8 mL of anhyd dioxane was added dropwise and the mixture was refluxed for 7 h with stirring. The solvent was removed, and the residue was taken up in 4 N HCl (20 mL) and refluxed for 6 h. After cooling, the acidic solution was basified with concd NH_3 and extracted with $CHCl_3$. The organic layer was dried (Na_2SO_4) and evaporated to afford the benzimidazole that was purified by CC (Al_2O_3/Et_2O).

5.5.1. 2-(3,5-Dimethoxyphenyl)-5-trifluoromethyl-1*H*-benzimidazole (7)

Yield: 30%. Mp 182–184 °C (Et_2O). ^{1}H NMR (DMSO-d_6): 3.87 (s, 6H, OCH_3); 6.82 (t, J = 2.2, 1 arom. H); 7.36–7.60 (m, 3 arom. H); 7.82 (d, J = 8.6, 1 arom. H); 7.97 (s, 1 arom. H); 13.28 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{16}H_{13}F_3N_2O_2$: C, 59.63; H, 4.06; N, 8.69. Found: C, 59.50; H, 4.22; N, 8.80.

5.6. Reduction of 2-(nitrophenyl)benzimidazoles. Method 'a'

A solution of the suitable nitro derivative (3 mmol) in 25 mL of EtOH was hydrogenated at rt and atmospheric pressure in the presence of 10% Pd/C (0.1 g). After 1 h the calculated volume of H_2 was absorbed. The catalyst was removed and the solvent was evaporated in vacuo, leaving an almost quantitative yield of amino compound that was crystallized from EtOH or dry Et_2O.

5.6.1. 2-(2-Amino-4-methoxyphenyl)-5-trifluoromethyl-1*H*-benzimidazole (11)

Yield: 95%. Mp 184–185 °C (EtOH). ^{1}H NMR ($CDCl_3$): 3.85 (s, OCH_3); 6.20–6.57 (m, 4 arom. H); 7.44 (d, J = 8.5, 1 arom. H); 7.52 (s, 1 arom. H); 7.86 (br s, NH_2, collapse with D_2O); 9.42 (s, NH of benzim., collapses with D_2O). Anal. Calcd for $C_{15}H_{12}F_3N_3O$: C, 58.63; H, 3.94; N, 13.68. Found: C, 58.63; H, 3.83; N, 13.70.

5.6.2. 2-(4-Aminophenyl)-5-trifluoromethyl-1*H*-benzimidazole (36)

Yield: 53%. Mp 210–212 °C (Et_2O). ^{1}H NMR (DMSO-d_6): 5.77 (br s, NH_2, collapses with D_2O); 6.84 (d, J = 8.7, 2 arom. H); 7.58–8.10 (m, 3 arom. H and 7.65, d, J = 8.7, 2 arom. H superimposed); 12.57 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{14}H_{10}F_3N_3$: C, 60.65; H, 3.64; N, 15.16. Found: C, 60.54; H, 3.80; N, 15.47.

5.6.3. 2-(4-Aminophenyl)-1-methyl-5-trifluoromethyl-1*H*-benzimidazole (59)

Yield: 96%. Mp 184–186 °C (EtOH). ^{1}H NMR (DMSO-d_6): 4.05 (s, CH_3); 5.71 (br s, NH_2 collapses with D_2O); 6.88 (d, J = 10, 2 arom. H); 7.64–8.17 (m, 3 arom. H and 7.69, d, J = 10, 2 arom. H superimposed). Anal. Calcd for $C_{15}H_{12}F_3N_3$: C, 61.85; H, 4.15; N, 14.43. Found: C, 61.64; H, 4.21; N, 14.43.

5.6.4. 2-(4-Aminophenyl)-1-cyclohexyl-5-trifluoromethyl-1*H*-benzimidazole (69)

Yield: 99%. Mp 151–153 °C (EtOH). ^{1}H NMR (DMSO-d_6): 1.15–2.39 (m, 10H, cyclohexane nucleus); 4.20–4.45 (m, 1H, cyclohexane nucleus); 5.45 (br s, NH_2 collapses with D_2O); 6.72 (d, J = 8.9 Hz, 2 arom. H); 7.35–8.18 (m, 3 arom. H and 7.79, d, J = 8.9 Hz, 2 arom. H superimposed). Anal. Calcd for $C_{20}H_{20}F_3N_3$: C, 66.84; H, 5.61; N, 11.69; Found: C, 66.60; H, 5.51; N, 11.49.

5.6.5. 1-(1-Adamantyl)-2-(4-aminophenyl)-5-trifluoromethyl-1*H*-benzimidazole (72)

Yield: 98%. Mp 223–225 °C (EtOH). ^{1}H NMR (DMSO-d_6): 1.49–1.77 (m, 6H, adamantane nucleus); 2.06–2.37 (m, 9H, adamantane nucleus); 4.95 (br s, NH_2 collapses with D_2O); 7.41–7.56 (m, 1 arom. H and 7.46, d, J = 9.0, 2 arom. H superimposed); 7.66 (d, J = 8.9, 2 arom. H); 7.95 (s, 1 arom. H); 8.19 (d, J = 9.6, 1 arom. H). Anal. Calcd for $C_{24}H_{24}F_3N_3$: C, 70.06; H, 5.88; N, 10.21. Found: C, 70.23; H, 5.81; N, 10.06.

5.7. Method 'b'

The nitro compound **22** (4.0 mmol) was dissolved in 35 mL of EtOH, and treated with a solution of $SnCl_2{\cdot}2H_2O$ (14 mmol) in 20 mL of concd HCl and the mixture was refluxed for 1 h with stirring. On cooling to room temperature, the precipitate was filtered and taken up with lukewarm water. The solution was neutralized with 2 M NaOH and the precipitate was collected by filtration and crystallized from EtOH.

5.7.1. 2-[4-(2-Amino-4-methoxyphenyl]-5,6-dichloro-1*H*-benzimidazole (23)

Yield: 66%. Mp 222–223 °C (EtOH). ^{1}H NMR ($CDCl_3$): 3.76 (s, OCH_3); 6.32–6.52 (m, 2 arom. H); 7.31 (br s, NH_2, collapse with D_2O); 7.65–7.92 (m, 3 arom. H); 12.72 (br s, NH of benzim., collapses with D_2O) Anal. Calcd for $C_{14}H_{11}Cl_2N_3O$: C, 54.57; H, 3.60; N, 13.64. Found: C, 54.32; H, 3.50; N, 13.73.

5.8. Acylation of 2-(4-aminophenyl)benzimidazoles with anhydrides. Method 'a'

A suspension of the appropriate 2-(4-aminophenyl)-1-substituted-benzimidazole (1.2 mmol) in 2 mL of anhyd benzene and of Ac_2O or propionic anhydride (3.6 mmol) was refluxed for 2 h. After removing the solvent, the residue was taken up with 5 mL of EtOH and 1 mL of water and refluxed for 1–2 h. The solvent was evaporated under vacuum and the solid was taken up with water, filtered and washed with water. Acetyl derivatives and compound **71** were crystallized from the indicated solvents.

5.8.1. 2-(4-Acetylaminophenyl)-1-methyl-5-trifluoromethyl-1*H*-benzimidazole (60)

Yield: 50%. Mp 240–243 °C (EtOH). ^{1}H NMR (DMSO-d_6): 2.10 (s, CH_3CO); 3.94 (s, NCH_3); 7.62 (d, J = 8.8, 1 arom. H); 7.76 (m, 5 arom. H); 8.05 (s, 1 arom. H); 10.42 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{17}H_{14}F_3N_3O$: C, 61.26; H, 4.23; N, 12.61. Found: C, 61.41; H, 4.35; N, 12.57.

5.8.2. 2-(4-Acetylaminophenyl)-1-cyclohexyl-5-trifluoromethyl-1*H*-benzimidazole (70)

Yield: 85%. Mp 240–242 °C (Et_2O). 1.22–2.45 (m, 10H, cyclohexane nucleus and 2.08, s, CH_3CO, superimposed); 4.17–4.37 (m, 1H, cyclohexane nucleus); 6.85 (d, J = 8.2, 1 arom. H); 7.40 (d, J = 8.8, 2 arom. H); 7.68 (d, J = 8.8, 2 arom. H); 7.83–8.20 (m, 2 arom. H); 10.21 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{22}H_{22}F_3N_3O$: C, 65.82; H, 5.52; N, 10.47. Found: C, 65.78; H, 5.79; N, 10.27.

5.8.3. 2-(4-Acetylaminophenyl)-1-(1-adamantyl)-5-trifluoromethyl-1*H*-benzimidazole (73)

Yield: 15%. Mp 267–269 °C (EtOH). ^{1}H NMR (DMSO-d_6): 1.52–1.82 (m, 6H, adamantane nucleus); 2.00–2.20 (m, 9H, adamantane nucleus and 2.09, s, CH_3CO superimposed); 7.37–7.57 (m, 1 arom. H and 7.43, d, J = 8.0, 2 arom. H superimposed); 7.67 (d, J = 8.0, 2 arom. H); 7.97 (s, 1 arom. H); 8.20 (d, J = 8.6, 1 arom. H); 10.15 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{26}H_{26}F_3N_3O$: C, 68.86; H, 5.78; N, 9.27. Found: C, 68.79; H, 6.09; N, 9.10.

5.8.4. 2–4-(Propionylaminophenyl)-1*H*-benzimidazole (31)

Yield: 72%. Mp 285–287 °C. ^{1}H NMR (DMSO-d_6): 1.12 (t, J = 7.6, 3H, CH_3CH_2CO); 2.38 (q, 2H, CH_3CH_2CO); 7.10–7.24 (m, 2 arom. H); 7.48–7.64 (m, 2 arom. H); 7.78 (d, J = 8.6, 2 arom. H); 8.11 (d, J = 8.6, 2 arom. H); 10.12 (s, NHCO, collapses with D_2O); 12.80 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{16}H_{15}N_3O + 0.5H_2O$: C, 70.05; H, 5.88; N, 15.32. Found: C, 70.48; H, 5.46; N, 15.35.

5.8.5. 2–4-(Propionylaminophenyl)-5-trifluoromethyl-1*H*-benzimidazole (38)

Yield: 60%. Mp 280–281 °C. ^{1}H NMR (DMSO-d_6): 1.12 (t, J = 5.8, 3H, CH_3CH_2CO); 2.38 (q, 2H, CH_3CH_2CO); 7.48–7.60 (m, 1 arom. H); 7.71–7.97 (m, 4 arom. H); 8.14 (d, J = 8.8, 2 arom. H); 10.17 (s, NHCO, collapses with D_2O); 12.72 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{17}H_{14}F_3N_3O + H_2O$: C, 58.12; H, 4.59; N, 11.96. Found: C, 57.92; H, 4.56; N, 11.85.

5.8.6. 5,6-Dichloro-2-(4-propionylaminophenyl)-1*H*-benzimidazole (54)

Yield: 73%. Mp >300 °C. ^{1}H NMR (DMSO-d_6): 1.12 (t, J = 6.0, 3H, CH_3CH_2CO); 2.38 (q, 2H, CH_3CH_2CO); 7.75 (s, 2 arom. H); 7.78 (d, J = 10, 2 arom. H); 8.08 (d, J = 10, 2 arom. H); 10.08 (s, NHCO, collapses with D_2O); 12.48 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{16}H_{13}Cl_2N_3O + H_2O$: C, 54.56; H, 4.29; N, 11.93. Found: C, 54.32; H, 4.44; N, 11.66.

5.8.7. 1-Methyl-2-(4-propionylaminophenyl)-5-trifluoromethyl-1*H*-benzimidazole (61)

Yield: 92%. Mp 241–243 °C. ^{1}H NMR (DMSO-d_6): 1.13 (t, J = 7.4, 3H, CH_3CH_2CO); 2.39 (q, 2H, CH_3CH_2CO); 3.95 (s, NCH_3); 7.58–7.70 (m, 1 arom. H); 7.78–7.94 (m, 5 arom. H); 8.03 (s, 1 arom. H); 10.19 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{18}H_{16}F_3N_3O$: C, 62.24; H, 4.64; N, 12.10. Found: C, 62.31; H, 4.89; N, 12.02.

5.8.8. 1-Cyclohexyl-2-(4-propionylaminophenyl)-5-trifluoromethyl-1*H*-benzimida-zole (71)

Yield: 78%. Mp 121–122 °C (EtOH/H_2O, 3:1). ^{1}H NMR (DMSO-d_6): 1.10 (t, J = 7.0, 3H, CH_3CH_2CO); 1.20–2.45 (m, 10H, cyclohexane nucleus and 2H, CH_3CH_2CO); 4.21–4.40 (m, 1H, cyclohexane nucleus); 7.50–7.67 (m, 1 arom. H and d, J = 8.6, 2 arom. H); 7.83 (d, J = 8.6, 2 arom. H); 8.00–8.17 (m, 2 arom. H); 10.07 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{23}H_{24}F_3N_3O$: C, 66.49; H, 5.82; N, 10.11. Found: C, 66.10; H, 6.09; N, 9.98.

5.8.9. 1-(1-Adamantyl)-2-(4-propionylaminophenyl)-5-trifluoromethyl-1*H*-benzimidazole (74)

Yield: 25%. Mp 252–254 °C. ^{1}H NMR (DMSO-d_6): 1.12 (t, J = 7.4, 3H, CH_3CH_2CO); 1.50–1.81 (m, 6H, adamantane nucleus); 2.00–2.30 (m, 9H, adamantane nucleus); 2.50 (q, 2H, CH_3CH_2CO); 7.39–7.60 (m, 1 arom. H and 7.45, d, J = 8.6, 2 arom. H superimposed); 7.71 (d, J = 8.6, 2 arom. H); 7.97 (s, 1 arom. H); 8.21 (d, J = 8.9, 1 arom. H); 10.09 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{26}H_{28}F_3N_3O$: C, 69.36; H, 6.04; N, 8.99. Found: C, 69.35; H, 6.36; N, 8.65.

5.9. Method 'b'

To a suspension of 2-(4-aminophenyl)-5-trifluoromethylbenzimidazole (2 mmol) in 10 mL of anhyd benzene, succinic anhydride (2 mmol) was added and the mixture was refluxed for 18 h with stirring. After cooling the precipitate was collected by filtration and crystallized from anhyd benzene.

5.9.1. 2-(Succinylaminophenyl)-5-trifluoromethyl-1*H*-benzimidazole (48)

Yield: 88%. Mp >300 °C (benzene). ^{1}H NMR (DMSO-d_6): 2.44–2.56 (m, 4H, CH_2); 7.49 (d, J = 9.6, 1 arom. H); 7.72 (pseudo s, 1 arom. H); 7.78 (d, J = 10.6, 2 arom. H); 7.86 (pseudo s, 1 arom. H); 8.17 (d, J = 10.6, 2 arom. H); 10.33 (s, NHCO, collapses with D_2O); 13.01 (br s, 2H, NH of benzimidazole and COOH, collapse with D_2O). Anal. Calcd for $C_{18}H_{14}F_3N_3O_3$: C, 57.30; H, 3.74; N, 11.14. Found: C, 57.14; H, 3.87; N, 10.95.

5.10. Acylation of 2-(4-aminophenyl)benzimidazoles with acylchlorides. Method 'a'

To an ice-cooled solution of homolupinanoyl chloride hydrochloride[21,22] (3 mmol) in 8 mL of anhyd $CHCl_3$, a solution of 2-(4-aminophenyl)-5-substituted benzimidazole (3 mmol) in 22 mL of anhyd $CHCl_3$ and triethylamine (3 mmol) were added under N_2 and with stirring. The mixture was refluxed for 6 h. After cooling, the solvent was removed, water was added and the acidic solution was neutralized with 2 M NaOH and then shaken with CH_2Cl_2. The organic layer was dried (Na_2SO_4) and evaporated to afford an oily residue that was crystallized from dry Et_2O.

5.10.1. 2-{4-[(1*S*,9*aR*)-Octahydro-2*H*-quinolizin-1-ylacetylaminophenyl}-1*H*-benzimidazole (35)

Yield: 32%. Mp 265–270 °C (Et_2O). ^{1}H NMR (DMSO-d_6): 1.02–2.76 (m, 16H, octahydro-2*H*-quinolizine and 2H, CH_2CO); 7.20–7.36 (m, 2 arom. H); 7.45–7.76 (m, 2 arom. H); 7.82 (d, J = 8.8, 2 arom. H); 8.15 (d, J = 8.8, 2 arom. H); 10.18 (s, NHCO, collapses with D_2O); 13.16 (br s, NH of benzim., collapses with D_2O). Anal. Calcd for $C_{24}H_{28}N_4O + 0.75H_2O$: C, 71.70; H, 7.40; N, 13.94. Found: C, 71.76; H, 7.16; N, 14.33.

5.10.2. 2-{4-[(*1S*,*9a*R)-Octahydro-2*H*-quinolizin-1-ylacetylaminophenyl}-5-trifluoromethyl-1*H*-benzimidazole (47)

Yield: 19%. Mp 252–255 °C (Et_2O). ^{1}H NMR (DMSO-d_6): 1.04–2.80 (m, 16H, octahydro-2*H*-quinolizine and 2H, CH_2CO); 7.55 (d, J = 9.6, 1 arom. H); 7.73–8.0 (m, 2 arom. H and, 7.82, d, J = 9.0, 2 arom. H superimposed); 8.14 (d, J = 9.0, 2 arom. H); 10.24 (s, NHCO, collapses with D_2O); 13.24 (br s, NH of benzim., collapses with D_2O). Anal. Calcd for $C_{25}H_{27}F_3N_4O$: C, 65.78; H, 5.96; N, 12.27. Found: C, 65.79; H, 6.33; N, 12.23.

5.11. Method 'b'

To a suspension of 2-(4-aminophenyl)-5-trifluoromethylbenzimidazole (2 mmol) in 10 mL of anhyd benzene and of triethylamine (2 mmol), cooled in an ice bath, a solution of trichloromethyl chloroformiate (1 mmol) in 5 mL of anhyd benzene was added dropwise, under N_2 and with stirring. The mixture was refluxed for 5 h with stirring. After removing the solvent, the precipitate was taken up with water, filtered and washed with water. The residue was purified by CC (Al_2O_3/ethyl acetate+2% MeOH).

5.11.1. 1,3-Bis-{4-[(5-trifluoromethyl)-1*H*-benzimidazol-2-yl]phenyl}urea (49)

Yield: 43%. Mp >300 °C. ^{1}H NMR (DMSO-d_6): 7.51 (d, J = 10.6, 2 arom. H); 7.69 (d, J = 9.0, 4 arom. H); 7.78 (m, 2 arom. H); 7.91 (pseudo s, 2 arom. H); 8.18 (d, J = 9.0, 4 arom. H); 9.18 (s, 2H, NHCO, collapses with D_2O); 13.23 (br s, 2H, NH benzimidazole, collapses with D_2O). Anal. Calcd for $C_{29}H_{18}F_6N_6O$: C, 60.00; H, 3.13; N, 14.48. Found: C, 59.80; H, 3.69; N, 14.55.

5.12. Method 'c'

To an ice-cooled suspension of the suitable 2-(4-aminophenyl)benzimidazole derivative (2–6 mmol) in anhyd benzene (4–22 mL) and of triethylamine (2–6 mmol), a solution of chloroacetyl chloride (2–6 mmol) in 6 mL of anhyd benzene was added dropwise under N_2 and with stirring. The mixture was refluxed for 6 h and then evaporated to dryness; the residue was taken up with water, filtered and washed with water. Compound **75** was crystallized from EtOH/H_2O.

5.12.1. 2-(4-Chloroacetylaminophenyl)-5-trifluoromethyl-1*H*-benzimidazole (39)

Yield: 74%. Mp >300 °C. ^{1}H NMR (DMSO-d_6): 4.36 (s, CH_2Cl); 7.63 (d, J = 8.8, 1 arom. H); 7.8–8.31 (m, 6 arom. H); 10.80 (s, NHCO, collapses with D_2O); 12.68 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{16}H_{11}ClF_3N_3O + H_2O$: C, 51.70; H, 3.52; N, 11.30. Found: C, 52.09; H, 4.05; N, 11.52.

5.12.2. 2-(4-Chloroacetylaminophenyl)-5,6-dichloro-1*H*-benzimidazole (52**)

Yield: 96%. Mp >300 °C. ^{1}H NMR (DMSO-d_6): 4.31 (s, CH_2Cl); 7.85 (d, J = 8.7, 2 arom. H and 7.87, s, 2 arom. H superimposed); 8.14 (d, J = 8.7, 2 arom. H); 10.08 (s, NHCO, collapses with D_2O); 12.48 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{15}H_{10}Cl_3N_3O$: C, 50.80; H, 2.84; N, 11.85. Found: C, 51.12; H, 2.98; N, 11.62.

5.12.3. 2-(4-Chloroacetylaminophenyl)-1-methyl-5-trifluoromethyl-1*H*-benzimida- zole (59**)

Yield: 75%. Mp >300 °C. ^{1}H NMR (DMSO-d_6): 3.95 (s, CH_3); 4.33 (s, CH_2); 7.6 (d, J = 8.8, 1 arom. H); 7.78–8.08 (m, 6 arom. H); 10.63 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{17}H_{13}ClF_3N_3O$: C, 55.52; H, 3.56; N, 11.43. Found: C, 55.23; H, 3.76; N, 11.22.

5.12.4. 1-(1-Adamantyl)-2-(4-chloroacetylaminophenyl)-5-trifluoromethyl-1*H*-benzimidazole (75)

Yield: 75%. Mp >300 °C (EtOH/H_2O). ^{1}H NMR (DMSO-d_6): 1.50–1.81 (m, 6H, adamantane nucleus); 2.08- 2.41 (m, 9 H, adamantane nucleus); 4.31, (s, CH_2CO); 7.40–7.58 (m, 1 arom. H and 7.46, d, J = 8.2, 2 arom. H superimposed); 7.71 (d, J = 8.2, 2 arom. H); 7.98 (s, 1 arom. H); 8.10 (d, J = 8.6, 1 arom. H); 10.57 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{26}H_{25}ClF_3N_3O$: C, 64.00; H, 5.16; N, 8.61. Found: C, 64.18; H, 5.24; N, 8.32.

5.13. 2-[4-(Aminoacetyl)amino]phenylbenzimidazoles

A solution of 2-(4-chloroacetylaminophenyl)benzimidazole derivative (1.5 mmol) and the appropriate amine (3.0 mmol) in 15 mL of EtOH was refluxed under N_2 for 18 h with stirring. The solvent was removed and the residue was taken up with water, filtered, dried and purified by CC (Al_2O_3/CH_2Cl_2). Compounds were further purified by rinsing the eluate residue with Et_2O or CH_2Cl_2.

Compounds **32** and **43** were prepared using benzene as solvent, instead of EtOH, while in the case of compounds **55**, **56** and **57** the chloroacetyl derivative was treated with a large excess of secondary amine.

5.13.1. 2-[4-(1-Pyrrolidinyl)acetylaminophenyl]-1*H*-benzimidazole (32)

Yield: 38%. Mp 250–252 °C (toluene). ^{1}H NMR (DMSO-d_6): 170–1.182 (m, 4H, CH_2 of pyrrolidine nucleus); 2.61–2.78 (m, 4H, CH_2 of

pyrrolidine nucleus); 3.37 (s, CH_2CO); 7.10–7.32 (m, 2 arom. H); 7.40–7.76 (m, 2 arom. H); 7.83 (d, J = 8.8, 2 arom. H); 8.13 (d, J = 8.8, 2 arom. H); 10.04 (s, NHCO, collapses with D_2O); 12.68 (s, NH of benzim., collapses with D_2O). Anal. Calcd for $C_{19}H_{20}N_4O$: C, 71.22; H, 6.29; N, 17.49. Found: C, 70.94; H, 6.05; N, 17.84.

5.13.2. 2-[4-(1-Adamantylamino)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimidazole (40)

Yield: 41%. Mp 279–282 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 1.44–1.77 (m, 12H, adamantane nucleus); 1.92–2.15 (m, 3H, adamantane nucleus); 3.25 (s, CH_2CO); 4.20 (br s, NH-adamantane, collapses with D_2O); 7.50 (d, J = 8.9, 1 arom. H); 7.75 (d, J = 8.2, 1 arom. H); 7.80–7.96 (m, 1 arom. H and 7.84, d, J = 8.6, 2 arom. H); 8.17 (d, J = 8.6, 2 arom. H); 10.20 (s, NHCO, collapses with D_2O); 12.46 (s, NH of benzim., collapses with D_2O). Anal. Calcd for $C_{26}H_{27}F_3N_4O$: C, 66.65; H, 5.81; N, 11.96. Found: C, 66.42; H, 6.11; N, 11.79.

5.13.3. 2-[4-(Diethylamino)acetylaminophenyl]-5-trifluoromet hyl-1*H*-benzimida-zole (41)

Yield: 45%. Mp 209–211 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 1.07 (t, J = 7.2, 6H, CH_3 of $N(C_2H_5)_2$); 2.58 (q, 4H, CH_2 of $N(C_2H_5)_2$); 3.30 (s, CH_2CO); 7.48 (d, J = 8.6, 1 arom. H); 7.61–8.00 (m, 2 arom. H and 7.83, d, J = 8.9, 2 arom. H); 8.08 (d, J = 8.9, 2 arom. H); 10.18 (s, NHCO, collapses with D_2O); 13.11 (s, NH of benzim., collapses with D_2O). Anal. Calcd for $C_{20}H_{21}F_3N_4O$: C, 61.53; H, 5.42; N, 14.35. Found: C, 61.16; H, 5.60; N, 14.17.

5.13.4. 2-[4-(1-Pyrrolidinyl)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimida-zole (42)

Yield: 30%. Mp 242–243 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 1.81 (t, J = 7.2, 4H, CH_2 of pyrrolidine nucleus); 2.76 (t, J = 7.2, 4H, CH_2 of pyrrolidine nucleus); 3.40 (s, CH_2CO); 7.44 (d, J = 8.9, 1 arom. H); 7.70–8.00 (m, 2 arom. H and 7.86, d, J = 8.6, 2 arom. H superimposed); 8.16 (d, J = 8.6, 2 arom. H); 10.08 (s, NHCO, collapses with D_2O); 13.30 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{20}H_{19}F_3N_4O$: C, 61.85; H, 4.93; N, 14.43. Found: C, 61.66; H, 4.97; N, 14.06.

5.13.5. 2-[4-(1-Piperidinyl)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimidazole (43)

Yield: 37%. Mp 240–241 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 1.35–1.66 (m, 6H, CH_2 of piperidine); 2.37–2.60 (m, 4H, CH_2 of piperidine); 3.14 (s, CH_2CO); 7.48 (d, J = 8.9, 1 arom. H); 7.61–8.05 (m, 2 arom. H and 7.84 d, J = 8.7, 2 arom. H); 8.16 (d, J = 8.7, 2 arom. H); 9.97 (s, NHCO, collapses with D_2O); 13.30 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{21}H_{21}F_3N_4O$: C, 62.67; H, 5.26; N, 13.92. Found: C, 62.79; H, 5.41; N, 13.80.

5.13.6. 2-[4-(4-Morpholino)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimida-zole (44)

Yield: 46%. Mp 234–236 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 2.56 (t, J = 6.5, 4H, CH_2 of morpholine); 3.19 (s, CH_2CO); 3.68 (t, J = 6.5, 4H, CH_2 of morpholine); 7.50 (d, J = 8.6, 1 arom. H); 7.64–8.05 (m, 2 arom. H and 7.84 d, J = 8.8, 2 arom. H); 8.14 (d, J = 8.7, 2 arom. H); 10.03 (s, NHCO, collapses with D_2O); 13.22 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{20}H_{19}F_3N_4O_2$: C, 59.40; H, 4.74; N, 13.85. Found: C, 59.22; H, 4.87; N, 13.74.

5.13.7. 2-[4-(4-Thiomorpholino)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimidazole (45)

Yield: 35%. Mp 240–242 °C (Et_2O). ^{1}H NMR (DMSO-d_6): 2.63–2.85 (m, 8H, CH_2 of thiomorpholine); 3.22 (s, CH_2CO); 7.52 (d, J = 9.0, 1 arom. H); 7.75 (d, J = 8.5, 1 arom. H); 7.82–8.00 (m, 1 arom. H and 7.88, d, J = 8.7, 2 arom. H superimposed); 8.16 (d, J = 8.7, 2 arom. H); 9.97 (s, NHCO, collapses with D_2O); 13.26 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{20}H_{19}F_3N_4OS$: C, 57.13; H, 4.55; N, 13.33. Found: C, 56.86; H, 4.31; N, 12.99.

5.13.8. 2-[4-(4-Phenylpiperazin-1-yl)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimidazole (46)

Yield: 44%. Mp 232–236 °C (Et_2O). ^{1}H NMR (DMSO-d_6): 2.72 (pseudo s, 4H, CH_2 of piperazine); 3.23 (s, CH_2CO); 3.63 (pseudo s, 4H, CH_2 of piperazine); 6.68–7.32 (m, 5 arom. H); 7.53 (d, J = 9.0, 1 arom. H); 7.65–8.04 (m, 2 arom. H and 7.90, d, J = 8.7, 2 arom. H superimposed); 8.18 (d, J = 8.7, 2 arom. H); 10.11 (s, NHCO, collapses with D_2O); 13.31 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{26}H_{24}F_3N_5O + 0.25H_2O$: C, 64.52; H, 5.10; N, 14.47. Found: C, 64.54; H, 5.32; N, 14.54.

5.13.9. 5,6-Dichloro-2-[4-(1-pyrrolidinyl)acetylaminophenyl]-1*H*-benzimidazole (55)

Yield: 46%. Mp 253–256 °C (Et_2O). ^{1}H NMR (DMSO-d_6): 1.88 (t, J = 6.8, 4H, CH_2 of pyrrolidine nucleus); 2.70 (t, J = 6.8, 4H, CH_2 of pyrrolidine nucleus); 3.29 (s, CH_2CO); 7.56–7.85 (m, 4 arom. H); 8.00 (d, J = 8.6, 2 arom. H); 9.98 (s, NHCO, collapses with D_2O); 12.51 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{19}H_{18}Cl_2N_4O + H_2O$: C, 56.03; H, 4.64; N, 13.76. Found: C, 56.45; H, 4.77; N, 13.66.

5.13.10. 5,6-Dichloro-2-[4-(1-piperidinyl)acetylaminophenyl]-1*H*-benzimidazole (56)

Yield: 39%. Mp 259–261 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 1.38–1.71 (m, 6H, CH_2 of piperidine nucleus); 2.60 (t, J = 7.2, 4H, CH_2 of piperidine nucleus); 3.12 (s, CH_2CO); 7.64–7.96 (m, 4 arom. H); 8.05 (d, J = 8.8, 2 arom. H); 9.95 (s, NHCO, collapses with D_2O); 12.40 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{20}H_{20}Cl_2N_4O$: C, 59.56; H, 5.00; N, 13.89. Found: C, 59.88; H, 4.92; N, 13.50.

5.13.11. 5,6-Dichloro-2-[4-(4-morpholino)acetylaminophenyl]-1*H*-benzimidazole (57)

Yield: 38%. Mp 274–276 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 2.55 (t, J = 6.8, 4H, CH_2 of morpholine nucleus); 3.18 (s, CH_2CO); 3.66 (t, J = 6.8, 4H, CH_2 of morpholine nucleus); 7.77–8.03 (m, 4 arom. H); 8.15 (d, J = 9.4, 2 arom. H); 10.04 (s, NHCO, collapses with D_2O); 13.45 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{19}H_{18}Cl_2N_4O_2$: C, 56.30; H, 4.48; N, 13.83. Found: C, 55.95; H, 4.55; N, 13.58.

5.13.12. 5,6-Dichloro-2-[4-(4-methylpiperazin-1-yl)acetylaminophenyl]-1*H*-benzimidazole (58)

Yield: 32%. Mp 272–275 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 2.15 (s, NCH_3 of piperazine); 2.39 (pseudo s, 4H, CH_2 of piperazine); 2.50 (pseudo s, 4H, CH_2 of piperazine); 3.14 (s, CH_2CO); 7.80 (s, 2 arom. H); 7.83 (d, J = 9.6, 2 arom. H); 8.10 (d, J = 9.6, 2 arom. H); 9.92 (s, NHCO, collapses with D_2O); 13.17 (br s, NH benzim., collapses with D_2O). Anal. Calcd for $C_{20}H_{21}Cl_2N_5O + 0.5H_2O$: C, 56.21; H, 5.19; N, 16.39. Found: C, 56.72; H, 5.11; N, 16.30.

5.13.13. 2-[4-(Diethylamino)acetylaminophenyl]-1-methyl-5-trifluoromethyl-1*H*-benzimidazole (62)

Yield: 50%. Mp 116–118 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 1.04 (t, J = 7.0, 6H, CH_3 of $N(C_2H_5)_2$); 2.61 (q, 4H, CH_2 of $N(C_2H_5)_2$); 3.22 (s, CH_2CO); 3.96 (s, NCH_3); 7.50 (d, J = 8.9, 1 arom. H); 7.78–7.96 (m, 5 arom. H); 8.05 (s, 1 arom. H); 9.97 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{21}H_{23}F_3N_4O$: C, 62.37; H, 5.73; N, 13.85. Found: C, 62.07; H, 5.72; N, 13.72.

5.13.14. 1-Methyl-2-[4-(1-pyrrolidinyl)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimidazole (63)

Yield: 38%. Mp 156–158 °C (CH_2Cl_2). ^{1}H NMR ($CDCl_3$): 1.84–1.95 (m, 4H, CH_2 of pyrrolidine nucleus); 2.68–2.80 (m, 4H, CH_2 of pyr-

rolidine nucleus); 3.34 (s, CH_2CO); 3.93 (s, NCH_3); 7.46 (d, J = 9.0, 1 arom. H); 7.54–7.63 (m, 1 arom. H); 7.72–7.91 (m, 4 arom. H); 8.10 (s, 1 arom. H); 9.38 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{21}H_{21}F_3N_4O$: C, 62.68; H, 5.26; N, 13.92. Found: C, 62.77; H, 5.26; N, 13.54.

5.13.15. 1-Methyl-2-[4-(1-piperidinyl)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimidazole (64)

Yield: 48%. Mp 161–163 °C (CH_2Cl_2). ^{1}H NMR ($CDCl_3$): 1.44–1.77 (m, 6H, CH_2 of piperidine nucleus); 2.63 (t, J = 7.0, 4H, CH_2 of piperidine nucleus); 3.14 (s, CH_2CO); 3.93 (s, CH_3); 7.44 (d, J = 9.2, 1 arom. H); 7.53–7.63 (m, 1 arom. H); 7.80 (pseudo s, 4 arom. H); 8.10 (s, 1 arom. H); 9.54 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{22}H_{23}F_3N_4O$: C, 63.45; H, 5.57; N, 13.45. Found: C, 63.51; H, 5.46; N, 13.51.

5.13.16. 1-Methyl-2-[4-(4-morpholino)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimidazole (65)

Yield: 49%. Mp 224–228 °C (Et_2O). ^{1}H NMR ($CDCl_3$): 2.70 (t, J = 5.8, 4H, CH_2 of morpholine); 3.22 (s, CH_2CO); 3.84 (t, J = 5.8, 4H, CH_2 of morpholine); 3.93 (s, NCH_3); 7.44 (d, J = 8.9, 1 arom. H); 7.59 (d, J = 8.8, 1 arom. H); 7.77–7.83 (m, 4 arom. H); 8.12 (dd, J = 9.0, 1.2, 1 arom. H); 9.38 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{21}H_{21}F_3N_4O_2 + 0.5H_2O$: C, 59.01; H, 5.19; N, 13.33. Found: C, 58.99; H, 5.49; N, 13.02.

5.13.17. 1-Methyl-2-[4-(4-thiomorpholino)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimidazole (66)

Yield: 73%. Mp 178–180 °C (Et_2O). ^{1}H NMR (DMSO-d_6): 2.64–2.87 (m, 8H, thiomorpholine nucleus); 3.21 (s, CH_2CO); 3.98 (s, NCH_3); 7.60 (d, J = 8.7, 1 arom. H); 7.80–7.97 (m, 5 arom. H); 8.03 (s, 1 arom. H); 10.00 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{21}H_{21}F_3N_4OS$: C, 58.05; H, 4.87; N, 12.90. Found: C, 58.12; H, 5.03; N, 12.84.

5.13.18. 1-Methyl-2-[4-(4-methyl-1-piperazinyl)acetylaminophenyl]-5-trifluoromethyl-1*H*-benzimidazole (67)

Yield: 52%. Mp 159–161 °C (Et_2O). ^{1}H NMR (DMSO-d_6): 2.17 (s, NCH_3 of piperazine); 2.26–2.40 (m, 8H, piperazine nucleus); 3.18 (s, CH_2CO); 3.97 (s, CH_3); 7.60 (d, J = 8.6, 1 arom. H); 7.80–7.96 (m, 5 arom. H); 8.02 (s, 1 arom. H); 10.02 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{22}H_{24}F_3N_5O$: C, 61.24; H, 5.61; N, 16.23. Found: C, 61.30; H, 5.49; N, 16.51.

5.13.19. 1-Methyl-2-[4-(4-phenyl-1-piperazinyl)acetylaminophenyl]-5-trifluoro-methyl-1*H*-benzimidazole (68)

Yield: 80%. Mp 165–167 °C (Et_2O). ^{1}H NMR (DMSO-d_6): 2.63–2.80 (m, 4H, piperazine nucleus); 3.18–3.35 (m, 4H, piperazine nucleus and 3.27, s, CH_2CO superimposed); 3.98 (s, NCH_3); 6.70–7.30 (m, 5 arom. H); 7.42 (d, J = 8.8, 1 arom. H); 7.81–7.98 (m, 5 arom. H); 8.03 (s, 1 arom. H); 10.10 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{27}H_{26}F_3N_5O$: C, 65.71; H, 5.31; N, 14.19. Found: C, 65.94; H, 5.63; N, 14.24.

5.13.20. 1-Adamantyl-2-[4-(4-phenyl-1-piperazinyl)acetylaminophenyl]-5-trifluoro-methyl-1*H*-benzimidazole (76)

Yield: 46%. Mp 238–241 °C (Et_2O). ^{1}H NMR ($CDCl_3$): 1.70–1.85 (m, 6H, adamantane nucleus); 2.16–2.41 (m, 9H, adamantane nucleus); 2.78–2.94 (m, 4H, piperazine nucleus); 3.20–3.45 (m, 4H, piperazine nucleus and 3.28, s, CH_2CO superimposed); 6.85–7.38 (m, 5 arom. H); 7.42–7.58 (m, 1 arom. H and 7.45, d, J = 8.8, 2 arom. H); 7.70 (d, J = 8.8, 2 arom. H); 7.92 (d, J = 9.2, 1 arom. H); 8.04 (s, 1 arom. H); 9.35 (s, NHCO, collapses with D_2O). Anal. Calcd for $C_{36}H_{38}F_3N_5O$: C, 70.45; H, 6.24; N, 11.41. Found: C, 70.66; H, 6.38; N, 11.17.

5.14. Cell-based assays

5.14.1. Compounds

Compounds were dissolved in DMSO at 100 mM and then diluted in culture medium.

5.14.2. Cells and viruses

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. Cell lines supporting the multiplication of RNA and DNA viruses were the following: $CD4^+$ human T-cells containing an integrated HTLV-1 genome (MT-4); Madin Darby Bovine Kidney (MDBK); Baby Hamster Kidney (BHK-21) and Monkey kidney (Vero 76) cells. Viruses were purchased from American Type Culture Collection (ATCC) except Yellow Fever Virus (YFV), and Human Immunodeficiency Virus type-1 (HIV-1). Viruses representative of positive-sense single-strand RNA ($ssRNA^+$) group used were: (i) *Retroviridae* family: the laboratory strain HIV-1_{IIIB} wild-type, obtained from the supernatant of the persistently infected H9/III_B cells (NIH 1983); (ii) *Flaviviridae* family: YFV strain 17-D vaccine (Stamaril Pasteur J07B01) and Bovine Viral Diarrhea Virus (BVDV) strain NADL (ATCC VR-534); (iii) *Picornaviridae* family: Human Coxsackievirus type B2 (CVB-2) strain Ohio-1 (ATCC VR-29) and Human Poliovirus type-1 Sabin (Sb-1) strain Chat (ATCC VR-1562). Viruses representative of a negative-sense single-strand RNA ($ssRNA^-$) group used were: *Paramyxoviridae* family: Vesicular Stomatitis Virus (VSV) strain Indiana Lab (ATCC VR-158) and Human Respiratory Syncytial Virus (RSV) strain A2 (ATCC VR-1540). A virus representative of a double-strand RNA (dsRNA) group used was: Reovirus type-1 (Reo-1) strain 3651 (ATCC VR-214). Viruses representatives of DNA group used were: (i) *Poxviridae* family: Vaccinia Virus (VV) strain Elstree-Lister Vaccine (ATCC VR-1549); (ii) *Herpesviridae* family: Human Herpesvirus 1 (HSV-1) strain KOS (ATCC VR-1493).

5.14.3. Cytotoxicity assays

Cytotoxicity assays were run in parallel with antiviral assays. Exponentially growing MT-4 cells were seeded at an initial density of 1×10^5 cells/mL in 96-well plates in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin G and 100 μg/mL streptomycin. Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 96 h at 37 °C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method.[38]

MDBK and BHK cells were seeded at an initial density of 6×10^5 and 1×10^6 cells/mL in 96-well plates, respectively, in culture medium (Minimum Essential Medium with Earle's salts (MEM-E) with L-glutamine, supplemented with 10% horse serum and 1 mM sodium pyruvate (for MDBK cells) or with 10% fetal bovine serum (FBS) (for BHK cells), 1% kanamycin). Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 48–96 h at 37 °C by the MTT method.

5.14.4. Antiviral assay

Compounds activity against HIV-1 was based on inhibition of virus-induced cytopathogenicity in MT-4 cells acutely infected with a multiplicity of infection (m.o.i.) of 0.01. Briefly, 50 μL of RPMI containing 1×10^4 MT-4 cells were added to each well of flat-bottom microtitre trays containing 50 μL of RPMI, without or with serial dilutions of test compounds. Then, 20 μL of a HIV-1 suspension containing 100 $CCID_{50}$ were added. After a 4-day incubation at 37 °C, cell viability was determined by the MTT method.

Compounds activity against YFV and Reo-1 was based on inhibition of virus-induced cytopathogenicity in BHK-21 cells acutely infected with a m.o.i. of 0.01. Compounds activity against BVDV was based on inhibition of virus-induced cytopathogenicity in MDBK cells acutely infected with a m.o.i. of 0.01. Briefly, BHK and MDBK cells were seeded in 96-well plates at a density of 5×10^4 and 3×10^4 cells/well, respectively, and were allowed to form confluent monolayers by incubating overnight in growth medium at 37 °C in a humidified CO_2 (5%) atmosphere. Cell monolayers were then infected with 50 μL of a proper virus dilution in maintenance medium (MEM-E with L-glutamine, supplemented with 0.5% inactivated FBS and 1 mM sodium pyruvate, 1% kanamycin) to give an m.o.i of 0.01. After 1 h, 50 μL of maintenance medium, without or with serial dilutions of test compounds, were added. After a 3–4-day incubation at 37 °C, cell viability was determined by the MTT method.

Compounds activity against CVB-2, Sb-1, VSV, VV, HSV-1 and RSV was determined by plaque reduction assays in infected Vero 76 cell monolayers. To this end, Vero 76 cells were seeded in 24-well plates at a density of 2×10^5 cells/well and were allowed to form confluent monolayers by incubating overnight in growth medium (Dulbecco's Modified Eagle Medium (D-MEM) with L-glutamine and 4500 mg/L D-glucose, supplemented with 10% fetal bovine serum and 1% Kanamycin) at 37 °C in a humidified CO_2 (5%) atmosphere. Then, monolayers were infected for 2 h with 250 μL of proper virus dilutions to give 50–100 PFU/well. Following removal of unadsorbed virus, 500 μL of maintenance medium (D-MEM medium with L-glutamine and 4500 mg/L D-glucose supplemented with 1% inactivated FBS and 0.75% methyl-cellulose), without or with serial dilutions of test compounds, were added. Cultures were incubated at 37 °C for 2 (Sb-1 and VSV), 3 (CVB-2, VV and HSV-1) or 5 days (RSV) and then fixed with PBS containing 50% ethanol and 0.8% crystal violet, washed and air-dried. Plaques were then counted and EC_{50} (50% effective concentration) was calculated by linear regression technique. The cytotoxicity of test compounds was determined in parallel on the same 24-well plate used for the EC_{50} determination.

5.14.5. Time-of-addition experiment

A time-of-addition experiment was carried out with MDBK cells. The confluent monolayers of MDBK cells, seed in 96-well tissue culture plates were inoculated at room temperature with 300 PFU of BVDV, corresponding to a multiplicity of infection of 0.01 PFU/cell. After adsorption for 60 min, the monolayers were washed two times with MEM-E + 1 mM NaPyr + 0.5% FBSi (Maintenance Medium in the presence of FBS inactivated) and incubated with the same medium at 5% CO_2 and 37 °C. The test medium containing 10× compound concentration was added at −1 to 0 (adsorption), 0 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 10, 10 to 12, or 12 to 14 h. After each incubation period, the monolayers were washed two times with maintenance medium and incubated with fresh medium until 14 h post-infection. Then, after 24–36 h post-infection the CPE was evaluated and the monolayers were collected, centrifuged and frozen at −80 °C. The viral titre was determined by a plaque reduction assay.

5.14.6. Expression of BVDV-NS5BΔ24 polymerase

Expression and purification of BVDV-NS5BΔ24 polymerase have been done as previously described.[39] Briefly, the expression plasmid encoding the N-terminal His-tagged C-terminal 24-amino-acid-deleted BVDV-NS5B was transformed into the *Escherichia coli* strain Rosetta™2 (DE3) pLysS (Novagene), and the transformants were then cultured in 5 mL of LB medium with 25 μg/mL kanamycin and 30 μg/mL chloramphenicol at 30 °C overnight. Cultures were diluted into 1 L of LB medium with 25 μg/mL kanamycin and 30 μg/mL chloramphenicol and incubated at 30 °C until the A_{600} reached 0.6–0.7. These cultures were then induced overnight with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested by centrifugation and stored at −80 °C until the purification.

5.14.7. Expression of HCV1b-NS5BΔ21 polymerase

The gene coding for the C-terminal 21-amino-acid-deleted NS5B polymerase (NS5BΔ21) of HCV BK strain (genotype 1b) C-terminally fused with a 6xHis-tag was cloned between the *Bam*HI and *Xho*I cloning sites of the pET-21a(+) expression plasmid (Novagen). The construct encoding the 6xHis-tagged HCV1b-NS5BΔ21 protein under the control of the T7 RNA polymerase promoter was confirmed by dideoxynucleotide sequencing and transformed into the *E. coli* strain Rosetta™ 2(DE3)pLysS (Novagene). A single colony expressing the 6xHis-tagged HCV1b-NS5BΔ21 protein was selected and cultured in 5 mL of LB medium supplemented with 100 μg/mL ampicillin and 30 μg/mL chloramphenicol at 30 °C overnight. The culture was diluted into 1 L of the same culture medium and incubated at 30 °C until the absorbance reached 0.6–0.7 at 600 nm. The culture was then induced overnight at 25 °C with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested by centrifugation and stored at −80 °C until the purification.

5.14.8. Purification of NS5B proteins

The cell pellets were thawed and immediately lysed by the addition of 10 mL of CelLytic B (Sigma). Any insoluble material was removed by centrifugation at 11,000 rpm 4 °C for 60 min. The soluble extract was applied to a 5-mL column of nickel–nitrilotriacetic acid–agarose (Qiagen) previously equilibrated with the lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH8.0). The column was washed extensively with the wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH 8.0) and then, the proteins were eluted stepwise with the elution buffer containing increasing concentration of imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 50–250 mM imidazole pH 8.0). The polypeptide composition of the column fractions was monitored by Coomassie stained SDS–PAGE analysis. Fractions enriched in pure 6xHis-tagged NS5B proteins recovered in the 130–250 mM imidazole eluates were pooled and dialyzed against the buffer containing 25 mM Tris–HCl, pH 7.5, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 50% glycerol. The protein concentration was determined by the micro-Bradford method (Bio-Rad) with bovine serum albumin as the standard. Following dialysis, the purified 6xHis-tagged HCV1b-NS5BΔ21 and 6xHis-tagged BVDV-NS5BΔ24 proteins were aliquoted and stored at −80 °C.

5.14.9. In vitro RNA-dependent RNA polymerase activity assays

In vitro synthesis assays were performed in 96-well plates using 10 μg/mL poly(rC) (GE Healthcare, formerly Amersham Biosciences) as template and 0.1 μg/mL oligo $(rG)_{12}$ (Invitrogen) as primer and 80 μM GTP (Invitrogen) as substrate in a 20 μL reaction mixture containing 20 mM Tris/HCl pH 7.0, 1 mM dithiothreitol, 25 mM NaCl, 20 U/mL RNasin (Promega), 0.5 mM $MnCl_2$ or 5 mM $MgCl_2$, 5% DMSO, 5% glycerol and 500–600 ng of each purified protein. After an enzyme/drug pre-incubation for 30 min at room temperature, reactions were started by the addition of GTP. One microliter of threefold serial dilutions of test compounds, or DMSO alone (as a negative control of inhibition), or the nucleotide analog 3′-deoxyguanosine-5′-triphosphate (3′-dGTP) (tebu-bio) (as a positive control of inhibition), was added to the reactions and incubated for 120 min at 37 °C (for BVDV-NS5BΔ24) or 25 °C (for HCV1b-NS5BΔ21), then stopped by the addition of 2 μL of 200 mM EDTA. One hundred thirty-eight microliters of PicoGreen Quantitation Reagent (Molecular Probes), diluted 1/345 in TE, were added into each sample and incubated for 5 min at room temper-

ature protected from light. After an excitation at ~480 nm, the fluorescence was measured at ~520 nm in a fluorescence microplate reader (VICTOR^3 Multilabel Plate Reader, PerkinElmer). 'Relative fluorescence' was calculated by subtracting the mean fluorescence of the blank from all samples and converted into percent of activity. Percent of residual activity was then plotted versus the compound concentrations. Dose–response curves were fit with Kaleidagraph (Synergy Software) to obtain the drug concentration that provides 50% of inhibition (IC_{50}).

5.14.10. HCV replicon assays

Huh-7 cells containing HCV Con1 subgenomic replicon (GS4.1 cells), kindly provided by C Seeger (Fox Chase University, Philadelphia, PA, USA), were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 110 mg/L sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/mL penicillin–streptomycin and 0.5 mg/mL G418 (Invitrogen Corp, Carlsbad, CA, USA). For dose–response testing, the cells were seeded in 96-well plates at 7.5×10^3 cells/well in a volume of 50 μL of 10 twofold serial dilutions of compounds (highest concentration, 75 μM) were added and cell cultures were incubated at 37 °C/5% CO_2 in the presence of 0.5% DMSO. Alternatively, compounds were tested at a single concentration of 15 μM. In all cases, Huh-7 cells lacking the HCV replicon served as a negative control. The cells were incubated in the presence of compounds for 72 h after which they were monitored for expression of the NS4A protein by ELISA. For this, the plates were then fixed for 1 min with 1:1 acetone–methanol, washed twice with PBS containing 0.1% Tween 20, blocked for 1 h at room temperature with TNE buffer containing 10% FBS and then incubated for 2 h at 37 °C with the anti-NS4A mouse monoclonal antibody A-236 (ViroGen, Watertown, MA, USA) diluted in the same buffer. After washing three times with PBS containing 0.1% Tween 20, the cells were incubated for 1 h at 37 °C with anti-mouse immunoglobulin G–peroxidase conjugate in TNE buffer with 10% FBS. After washing as described above, the reaction was developed with *o*-phenylenediamine (Zymed, San Francisco, CA, USA). The reaction was stopped after 30 min with 2 N H_2SO_4 and the absorbance was read at 492 nm using Sunrise Tecan (Durham, NC, USA) Spectrophotometer. EC_{50} values were determined from the% inhibition versus concentration data using a sigmoidal non-linear regression analysis based on four parameters with Tecan Magellan software. When screening at a single concentration, the results were expressed as% inhibition at 15 μM. For cytotoxicity evaluation, GS4.1 were treated with compounds as described above and cellular viability was monitored using the Cell Titer 96 Aqueous one solution cell proliferation assay (Promega Corp, Madison, WI, USA). CC_{50} values were determined from the% cytotoxicity versus concentration data with Tecan Magellan software as described above.

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Supplementary data

Supplementary data (References for the preparation of already known compounds (**1**–**4**, **9**, **20**, **24**, **25**, **29**, **30**, **33**, **34**, **50**–**52**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.02.037.

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