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Original article

Synthesis and antiviral activity of new phenylimidazopyridines and *N*-benzylidenequinolinamines derived by molecular simplification of phenylimidazo[4,5-*g*]quinolines

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ABSTRACT

Continuing our program of research concerning the antiviral activity of a wide series of new angular and linear azolo bicyclic and tricyclic derivatives, now we have simplified and modified the 4-chloro-2-(4-nitrophenyl)-3*H*-imidazo[4,5-*g*]quinoline **1**, which previously resulted the most active derivative, through either the elimination of the central ring or the opening of the imidazole ring, obtaining various imidazopyridines and *N*-benzylidenequinolinamines respectively.

Title compounds were tested in cell-based assays for cytotoxicity and antiviral activity against representatives of two DNA virus families as wells as against representatives of RNA virus families containing single-stranded, either positive-sense (ssRNA$^+$) or negative-sense (ssRNA$^-$), and double-stranded genomes (dsRNA). Some imidazo[4,5-*b*]pyridines emerged as new derivatives endowed with antiviral activity against Vaccinia Virus (VV) at concentrations ranging from 2 to 16 μM. In particular, compound **2b** demonstrate to be about 10 times more potent than Cidofovir, used as reference drug. Similarly, the imidazo[4,5-*c*]pyridines and *N*-benzylidenequinolinamines derivatives resulted active against Bovine Viral Diarrhoea virus (BVDV), at concentrations ranging from 1.2 to 28 μM. Above all compounds **1**, **3a** and **3f** showed an EC_{50} of the same order of magnitude of the reference drug, the 2′-C-methyl-guanosine. Moreover, several *N*-benzylidenequinolinamines showed an interesting activity against Respiratory Syncytial Virus (RSV) at concentrations between 12 and 26 μM.

1. Introduction

Over 200 species of DNA and RNA viruses are known to be able to infect and cause significant diseases in humans [1,2]. Examples of DNA pathogen viruses are Herpesvirus and Poxvirus [3]. Herpes viruses belongs to the *Herpesviridae* family, subdivided into numerous genera, comprehending Herpes simplex virus type 1 (HSV-1), that causes facial cold-sores, Herpes simplex virus type 2 (HSV-2), also called genital herpes, and Varicella-Zoster virus (VZV), which causes chickenpox in children and shingles in adults.

Particularly we focused our attention on HSV-1, the most common etiologic agent of sporadic fatal encephalitis worldwide [4]. The clinical syndrome is often characterized by the rapid inception of fever, headache and nausea, followed by acute or subacute onset of an encephalopathy whose symptoms include lethargy, confusion, and delirium [5]. It is estimated to affect at least 1 in 500,000 per year [6]. Nucleoside analogues acyclovir (ACV) and its derivatives with better bioavailability such as famciclovir, valacyclovir and penciclovir have become the first line drugs for prophylaxis and treatment of HSV infections. However, their wide use to treat Herpes Simplex infections and their long-term administration for the treatment of chronic infections in the immunocompromised host (such as the organ transplant patient or patient with AIDS) can lead to the development of ACV-resistant mutant strain [7]. As result, the efficacy of these nucleoside analogues has been compromised.

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Poxviruses (members of the *Poxviridae* family) are viruses that can infect both vertebrate and invertebrate animals. They are subdivided in four genera, among which the Orthopoxvirus genus include Variola virus, Vaccinia virus (VV), Cowpox virus and Monkeypox virus [8]. Before the introduction of vaccination and eradication of Variola virus's infection (smallpox) in 1970s, it caused significant morbidity in human populations with anywhere from 1 to 25 percent of mortality [9]. In recent years, however, the potential of the use of Variola virus or another Orthopoxvirus, as a bioterrorism weapon, has heightened our awareness as to our vulnerability to this disease since vaccination for smallpox was discontinued in 1980 [10–14]. This has stimulated efforts to develop new drugs for treatment of Poxvirus infections. This potential threat has resulted in a resurgent effect to identify and develop more agents that can be used in an emergency situation to treat these viral diseases.

RNA viruses include more than 350 different human pathogens and most of the etiological agents of emerging diseases. According to their type of genetic material, RNA viruses are classified as single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA). Further classification accounts for the ssRNA viruses polarity into negative-sense and positive-sense, or ambisense. Unfortunately, most of these human pathogens have no available vaccine, and only a few selective antiviral drugs are utilizable in the clinic to prevent and/or treat their infections. Between the ssRNA, viruses belonging to the *Flaviviridae* family cause clinically significant diseases in humans and animals. This virus family includes three genera: Pestiviruses (i.e., Bovine Viral Diarrhoea virus [BVDV]), Flaviviruses (i.e., Yellow Fever [YFV], Dengue [DENV], and West Nile [WNV]), and Hepaciviruses (Hepatitis C virus [HCV]). Actually, with the exception of YFV, there is no vaccine and some new drugs are actually in study [15–23].

Other important ssRNA viruses pathogens are those belonging to the *Picornaviridae* (Coxsackievirus, Poliovirus) and *Paramixoviridae* (Respiratory syncytial virus) and Rhabdoviridae (Vesicular Stomatitis Virus, VSV) families. These viruses cause a variety of illnesses, and at present, no specific antiviral therapy is available for the treatment [24,25].

Hence the need to identify new lead compounds targeting a distinct stages of replication cycle in a virus-specific way. In this context, according to an antiviral research multiannual program, our group described the series of 2-[4-substituted naphtyl] [26], 2-[4-substituted biphenyl] [27], substituted-2-styryl [28] and 5-acetyl-2-[2,3,4-substituted aryl] benzimidazoles [29]. After biological evaluation, most of these compounds emerged for their selective activity against Respiratory Syncytial virus (RSV), Yellow Fever virus (YFV) and Coxsackie virus type B5 (CVB-5). Afterwards, with the aim to increase the antiviral behaviour, we operated a bioisosteric modification replacing the benzimidazole ring with a benzotriazole nucleus to obtain the series of *N*-[4-(1*H*(2*H*)-benzotriazol-1(2)-yl)phenyl]alkylcarboxamides [30] and *N*,*N*′-bis[4-(1*H*(2*H*)-benzotriazol-1(2)-yl)phenyl]alkyldicarboxamides [31]. Meantime, to provide molecular diversity of benzotriazole and benzimidazole nucleus for further structure activity relationship (SAR) analysis, we synthesized and tested for antiviral activity three new classes of angular [32] and linear N-tricyclic compounds [33–35].

Recently we have reported new linear azolo tricyclic derivatives as selective inhibitors of the RNA-dependent RNA polymerases (RdRps) of various RNA virus [36]. Between them, imidazo[4,5-*g*]quinoline derivatives showed an interesting activity against some ssRNA+ (BVDV, HCV and CVB), and dsRNA (Reo) viruses. Notably the 4-chloro-2-(4-nitrophenyl)-3*H*-imidazo[4,5-*g*]quinoline (**1**), resulted endowed with high and selective activity against BVDV and HCV. Now, with the purpose to extend the SAR analysis we have took in account the simplification of imidazo[4,5-*g*]quinoline nucleus through the elimination of the central ring or molecular modification by the opening of the imidazole ring, obtaining imidazopyridines (**2**) and *N*-benzylidenequinolinamines (**3**) derivatives respectively (Fig. 1).

Here we report the chemical synthesis and the biological assessment of their antiviral activity.

2. Chemistry

All chemical structures of the synthesized compounds are depicted in Figs. 2 and 3. The nitrogen atom positions in imidazopyridines and the substituents of all compounds were chosen with the aim to evaluate the influence of electron-withdrawing groups, lipophilicity and/or steric hindrance, on the antiviral activities.

The synthetic route to obtain the designed imidazopyridines **2a**–**l** (Fig. 2) and their intermediates is described in Scheme 1. The diamines **4a**–**c** underwent nucleophilic attack by the corresponding bisulphite compounds **5a**–**e**, to give the imidazo[4,5-*b*] (**2a**–**j**) and [4,5-*c*]pyridine (**2k**,**l**).

In turn *N*-benzylidenequinolinamines **3a**,**b**,**f**–**h** were obtained by condensation of diaminoquinolines **6a**,**b** with bisulphite compounds **5a**,**b**,**f**,**g**, operating by soft condition in refluxed ethanol (Scheme 2). Whereas more extreme conditions, DMF at 130 °C, in past principally afforded the corresponding imidazo[4,5-*g*]quinolines [36].

Bisulphites **5a**–**g** were obtained in high yields from the commercially available corresponding aldehydes (Aldrich) with $Na_2S_2O_5$ in ethanol, according to the procedure used by Shriner and Land [37].

3. Results, discussion and conclusion

Title compounds **2a**–**l** and **3a**,**b**,**f**–**h** were evaluated in cell based assays for their cytotoxicity and antiviral activity against a panel of 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-5, CVB-5, and Poliovirus type-1, Sabin strain, Sb-1), and viruses representative of two of the three genera of the Flaviviridae family, i.e., a Flavivirus (Yellow Fever Virus, YFV), and a Pestivirus (Bovine Viral Diarrhoea 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,

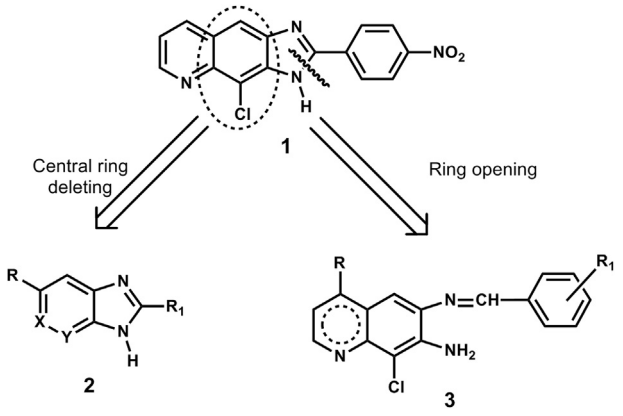


Fig. 1. Molecular modification of the lead compound **1**.

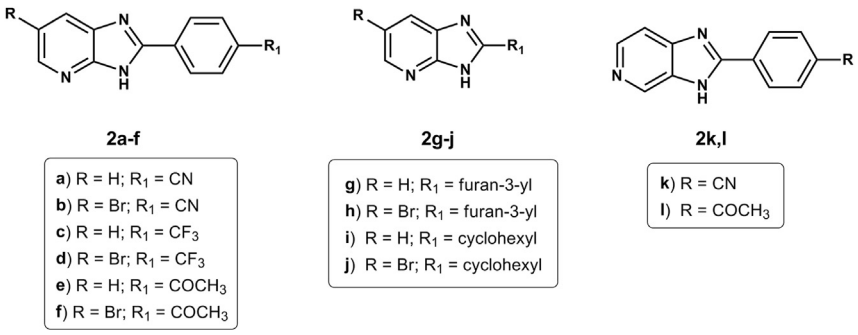


Fig. 2. Imidazo[4,5-*b*] and [4,5-*c*]pyridines **2a**–**l** synthesized.

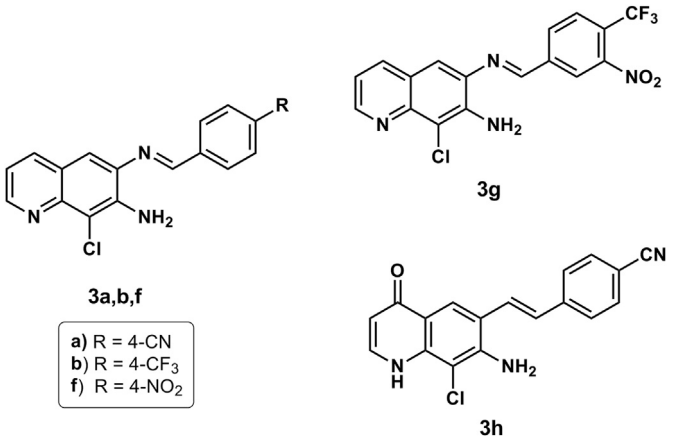


Fig. 3. *N*-benzylidenequinolinamines **3a,b,f**–**h** synthesized.

two representatives of DNA virus families were also included: Herpes Simplex Virus type-1, HSV-1 (*Herpesviridae*) and Vaccinia Virus, VV (*Poxviridae*).

Results are reported in Table 1. In general, they exhibited low cytotoxicity, with the exception of compounds **2f,h** and **3b,g** that resulted be slightly cytotoxic against some cell lines used in this study (CC_{50} between 20 and 40 μM against MT-4, MDBK and Vero-76).

As far as the antiviral activity is concerned, the imidazopyridines synthesized can be divided into derivatives connected by *b* (**2a**–**j**) or *c* (**2k,l**) sides on the pyridine ring and both the adjacent carbon atoms of the [1,2,3]triazole ring.

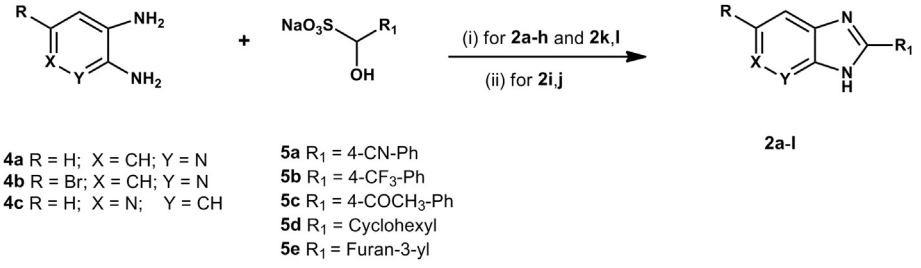
Three derivatives **2b,d,j** belonging to the first series are specifically active against Vaccinia Virus (VV) at concentrations ranging from 2 to 16 μM. In particular, compound **2b** showed an activity about 10 times more potent than the reference drug Cidofovir. Being Cidofovir molecular target a polymerase, and being known that various benzimidazole derivatives are endowed with the same mechanism of action [38,39], is acceptable to suppose that our compounds act similarly. In this direction actually are going more biological and *in silico* essays.

In all the active derivatives is present a bromine atom in 6 position, whereas their counterpart without Br **2a,c,i** are completely inactive. Surprisingly in the copies **2e/2f** and **2g/2h** also the 6-Br-derivatives resulted inactive. Probably the cause of this behaviour is the higher cytotoxicity of **2f** and **2h**, in comparison with **2b,d,j**, associated with the decrease of biological activity due to either 4-CH_3CO-phenyl or furan-3-yl substituents in position 2. Considering the virtual chemical reactivity paired with the bromine substitution, we investigated the chemical and enzymatic stability of this class of compounds. In view of the high activity of **2b** against VV, we chose this compound as an example of the entire class. Compound was then incubated in rat plasma and in a pH 7.4 buffered solution, and results showed a good stability in buffer ($t_{½} > 4$) h and plasma ($t_{½} = 2$) h.

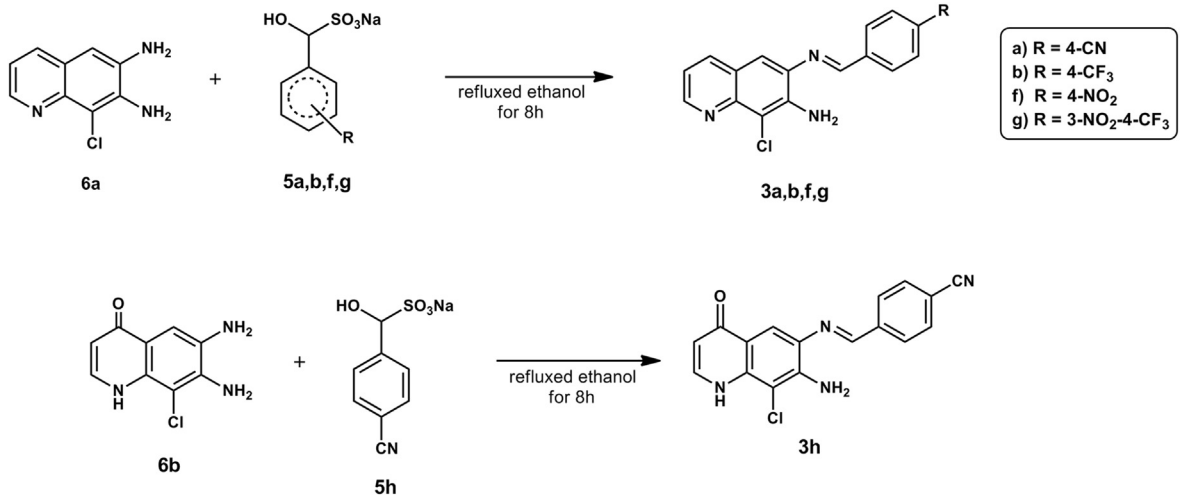
While, among the imidazo[4,5-*c*]pyridines, only **2k** resulted active against VV even if at high concentration ($EC_{50} = 56$ μM). In conclusion this preliminary SAR study suggests that the presence of both a 6-Br substituent and 4′–CN–phenyl (**2b**), 4′–CF_3–phenyl (**2d**) or cyclohexyl (**2j**) substituents in position 2 is associated to a selective antiviral activity against VV of the imidazo[4,5-*b*]pyridine series.

Furthermore, the imidazo[4,5-*b*]pyridine **2b** turned out to be active against the second representative DNA-virus tested, the Herpes Simplex type-1 (HSV-1), at the concentration of 15 μM. About the others virus tested only the derivatives **2c** and **2j** showed a moderate activity against CVB-5 in the range 18–20 μM but not against Sb-1.

Among the second series, both the imidazo[4,5-*c*]pyridine derivatives **2k,l** turned out to be selective inhibitors of BVDV in the range 7–9 μM, this highlighting that the *c* side connection of the pyridine ring is determining for the anti-BVDV behaviour of this class of compounds.



Scheme 1. Synthesis of compounds **2a**–**l**. Reagents and conditions: (i) DMF, 130 °C for 2 h. (ii) EtOH, reflux for 8 h.



Scheme 2. Synthesis of compounds **3a**,**b**,**f**–**h**.

About the antiviral activity of the *N*-benzylidenequinolinamines **3a**,**b**,**f**–**h**, all the compounds resulted active against BVDV and recombinant BVDV RNA-dependent RNA polymerase (RdRp) [40]. In particular **3a** and **3f** resulted the most potent ($EC_{50} = 1.7$ and 2 μM respectively), showing an activity comparable with the reference drug 2′-C-methyl-guanosine ($EC_{50} = 1.1$ μM).

Moreover, compound **3f** can be identified as the opened analogue of 4-chloro-2-(4-nitrophenyl)-3H-imidazo[4,5g]quinoline, one of the lead compounds from the previously synthesized series of RpRd inhibitors [36]. This activity confirms the relevance of an electron withdrawing substituent on the 4 position of the phenyl moiety, as previously observed. At the same time the opening of the imidazole ring seem do not lead to an anti-BVDV activity loss, while causing a broader antiviral spectrum. In fact, with the sole exception of **3h**, all tested compounds exhibited an interesting activity against CVB-5, Sb-1 and RSV. In particular **3g** turned out as the most active against CVB-5 ($EC_{50} = 16$ μM), **3g** against Sb-1 ($EC_{50} = 22$ μM) and finally **3f** versus RSV ($EC_{50} = 12$ μM).

On the contrary of the imidazo[4,5-*c*]pyridines, the *N*-benzylidenequinolinamines resulted completely inactive towards VV. None of the tested compounds, however, resulted active against HIV-1, YFV, Reo-1 and VSV.

In the light of the above mentioned results, we can conclude that imidazo[4,5-*b*]pyridine derivatives showed, in general, to be endowed with good activity against VV, whereas *N*-benzylidenequinolinamines exhibited an interesting activity versus BVDV comparable to those of both reference drug and lead compound **1** ($EC_{50} = 1.1$ and 1.2 μM respectively). Owing to their good ratio of activity/cytotoxicity imidazo[4,5-*b*]pyridines might represent new interesting leads that can be further developed as new agents against poxvirus infections, through the introduction of alternative substituents on the positions 2 and 6, and submitted to *in vivo* experiments.

On the other hand, *N*-benzylidenequinolinamines turned out as attractive leads for the development of new derivatives endowed with an improved activity against BVDV and RSV, towards the introduction of either further electron withdrawing or electron donor substituents on the phenyl moiety and on the pyridine ring.

Finally we can affirm that the molecular simplification and opening ring of the lead compound **1** results favourable for differentiation and address the antiviral activity against either VV in the case of imidazo[4,5-*b*]pyridines or BVDV and RSV in the case of *N*-benzylidenequinolinamines.

4. Experimental

4.1. Chemistry

4.1.1. General remarks

Melting points were carried out with a Köfler hot stage or Digital Electrothermal melting point apparatus and are uncorrected. Infrared spectra were recorded as nujol mulls on NaCl plates with a PerkinElmer 781 IR spectrophotometer and are expressed in ν (cm^{-1}). Uv spectra are qualitative and were recorded in nm for solutions in EtOH with a PerkinElmer Lambda 5 spectrophotometer. Nuclear magnetic resonance (^{1}HNMR) spectra were determined in $CDCl_3$, DMSO-d_6, $CDCl_3$/DMSO-d_6 (1:3 ratio) and were recorded with a Varian XL-200 (200 MHz) spectrometer. Chemical shifts (δ scale) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) used as internal standard. Splitting patterns are designated, as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; br s, broad singlet; dd, double doublet. The assignment of exchangeable protons (O*H* and N*H*) was confirmed by the addition of D_2O. ^{13}C NMR were determined in DMSO-d_6 and were recorded at 100 MHz with Brucher 400 Avance Nanobay.

Chromatographic separations were performed using a 1090L Liquid Chromatograph (HewlettPackard, Palo Alto, USA) equipped with a diode array detector HP 1040A. All separations were accomplished on a Phenomenex Luna C18 (200 × 4.6 mm, particle size 5 μm).

Ms spectra were performed on combined HP 5790-HP 5970 GC/MS apparatus or with a combined Liquid Chromatograph-Agilent 1100 series Mass Selective Detector (MSD). All exact mass data (HRMS)were obtained with a "Thermo Scientific Quantitative Plus Orbitrap LC/MS/MS System" apparatus. Analytical thin-layer chromatography (TLC) was performed on Merck silica gel F-254 plates. Pure compounds showed a single spot in TLC. For flash chromatography, Merck silica gel 60 was used with a particle size 0.040–0.063 mm (230–400 mesh ASTM). Elemental analysis were performed on a Perkin-Elmer 2400 instrument at Laboratorio di Microanalisi, Dipartimento di Chimica e Farmacia, Università di Sassari, Italy, and the results were within ±0.4% of theoretical values.

4.1.2. Intermediates

The diamines (**4a**–**c**) were commercially available while bisulphite compounds (**5a**–**g**) were obtained in high yields from the

Table 1
Cytotoxicity and antiviral activity of imidazo[4,5-*b*] and [4,5-*c*]pyridine **2a**–**l** and *N*-benzylidenequinolinamine**s 3a**,**b**,**f**–**h** against ssRNA$^+$ (HIV-1, BVDV, YFV, CV-5, Sb-1), ssRNA$^-$ (RSV, VSV), dsRNA (Reo-1) and DNA (VV, HSV-1) viruses.

Compd	MT-4[a]	HIV-1[b]	MDBK[c]	BVDV[d]	BHK-21[e]	YFV[f]	Reo-1[g]	Vero-76[h]	CVB-5[i]	Sb-1[j]	RSV[k]	VSV[l]	VV[m]	HSV-1[n]
	CC_{50}	EC_{50}	CC_{50}	EC_{50}	CC_{50}	EC_{50}		CC_{50}	EC_{50}					
1	>100	>100	>100	1.2	>100	>100	>100	>100	>100	>100	>100	>100	>100	44
2a	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2b	53	>53	>100	>100	>100	>100	>100	73	>73	>73	>73	>73	2	15
2c	>100	>100	>100	>100	>100	>100	>100	>100	18	>100	>100	>100	>100	>100
2d	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	4	>100
2e	>100	>100	>100	65	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2f	28	>28	>100	>100	>100	>100	>100	50	>50	>50	>50	>50	>50	>50
2g	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2h	20	>20	40	>40	>100	≥100	≥100	40	>40	>40	>40	>40	>40	>40
2i	>100	>100	>100	>100	>100	>100	>100	>100	86	>100	>100	>100	>100	>100
2j	>100	>100	>100	>100	>100	>100	>100	>100	20	>100	>100	>100	16	>100
2k	>100	>100	>100	9	>100	>100	>100	>100	>100	>100	>100	>100	56	>100
2l	>100	>100	>100	7	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
3a	46	>100	>100	2	>100	>100	>100	>100	46	>100	26	>100	>100	>100
3b	49	>100	>100	14	25	>25	>25	90	29	57	12	>90	>90	>100
3f	53	>100	>100	1.7	>100	>100	>100	>100	>100	>100	14	>100	88	>100
3g	20	>100	>100	11	>100	>75	>100	90	16	22	>90	>90	>90	>100
3h	>100	>100	>100	28	>100	>87	>100	>100	>100	>100	>100	>100	>100	>100
EFV	40	0.002												
Met-Gua			>100	1.1	>100	1.9								
Met-Cyt						>100	16							
Eth-Cyt								>100	27	23				
Aza								≥12.5			1.2			
CDF								>100					19	
ACG								>100						3

Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%.
ND = Not determined.
EFV = *Efavirenz*.
MetGua = 2′-C-methyl-guanosine.
Met-Cyt = 2′-C-methyl-cytidine.
Eth-Cyt = 2′-C-ethynyl-cytidine.
Aza = 6-aza-uridine.
CDF = Cidofovir.
ACG = Acycloguanosine.
[a] Compound concentration (μM) required to reduce the proliferation of mock-infected MT-4 cells by 50%, as determined by the MTT method.
[b] Compound concentration (μM) required to achieve 50% protection of MT-4 cells from HIV-1 induced cytopathogenicity, 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-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 from YFV-induced cytopathogenicity, as determined by the MTT method.
[g] Compound concentration (μM) required to achieve 50% protection of BHK cells from Reo-1-induced cytopathogenicity, as determined by the MTT method.
[h] Compound concentration (μM) required to reduce the viability of mock-infected VERO-76 cells by 50% as determined by the MTT method.
[i] Compound concentration (μM) required to reduce the plaque number of CVB-5 by 50% in VERO-76 monolayers.
[j] Compound concentration (μM) required to reduce the plaque number of Sb-1 by 50% in VERO-76 monolayers.
[k] Compound concentration (μM) required to reduce the plaque number of RSV by 50% in VERO-76 monolayers.
[l] Compound concentration (μM) required to reduce the plaque number of VSV by 50% in VERO-76 monolayers.
[m] Compound concentration (μM) required to reduce the plaque number of VV by 50% in VERO-76 monolayers.
[n] Compound concentration (μM) required to reduce the plaque number of HSV-1 by 50% in VERO-76 monolayers.

commercially available corresponding aldehydes (Aldrich) with $Na_2S_2O_5$ in ethanol, according to the procedure used by Shriner and Land [37]. 6,7-Diamine-8-chloroquinolines (**6a**,**b**) was prepared according to procedure previously reported by us [41].

*4.1.3. General procedure for the preparation of the imidazo[4,5-b] pyridines (**2a**–**h**) and imidazo[4,5-c]pyridines (**2k,l**)*

A mixture of diamine (**4a**, **4b** or **4c**) (2) mmol and either sodium hydroxy(R-substituted-phenyl-1-yl)methanesulfonate (**5a**–**c**) or sodium furan-3-yl(hydroxy)methanesulfonate (**5d**) (2) mmol was stirred for 2 h in 4 mL of DMF at 130 °C (for **2a**–**h**, and **2k**,**l**). On cooling, the mixture was diluted with water and the formed precipitate was collected and dried on oven. The solid residues were purified by recrystallization from EtOH.

*4.1.3.1. 4-(3H-imidazo[4,5-b]pyridin-2-yl)benzonitrile (**2a**).* This compound was obtained in 90% yield by the protocol described in the general procedure starting from **4a** (218 mg, 2 mmol) and sodium (4-cyanophenyl)(hydroxy)methanesulfonate (**5a**) (470 mg, 2 mmol); m.p. 285–288 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.44; IR (nujol): ν 3554, 2224, 1597, cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.82 (1H, br s, NH), 8.40 (3H, J = 8.8 Hz, H-3′, 5′ and H-5 partially obscured), 8.06 (3H, d, J = 8.6 Hz, H-2′, 6′ and H-7 partially obscured), 7.31(1H, dd, J = 8.0 Hz and J = 4.6 Hz, H-6); ^{13}C-NMR ($CDCl_3$): δ 162.09 (s), 149.19 (s), 146.11 (d), 139.22 (s), 132.89 (d, 2 CH), 129.75 (s), 128.71 (d, 2 CH), 123.23 (d), 123.06 (d), 119.21 (s), 112.66 (s). LC/MS: m/z 221 [M + 1]. HRMS: 220.0741. Anal. Calcd. for $C_{13}H_8N_4$: C,70.90; H, 3.66; N, 25.44; found C, 70.70; H, 3.56; N, 25.42.

*4.1.3.2. 4-(6-Bromo-3H-imidazo[4,5-b]pyridin-2-yl)benzonitrile (**2b**).* This compound was obtained in 79% yield by the protocol described in the general procedure starting from **4b** (376 mg, 2 mmol) and sodium (4-cyanophenyl)(hydroxy)methanesulfonate

(**5a**) (470 mg, 2 mmol); m.p. 239–242 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.49; IR (nujol): ν 3554, 2231, 1597, cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.99 (1H, br s, NH), 8.40 (2H, $J = 8.4$ Hz, H-3′, 5′), 8.39 (1H, s, H-5), 8.25 (1H, s, H-7), 7.94 (2H, d, $J = 8.2$ Hz, H-2′, 6′); ^{13}C-NMR ($CDCl_3$): δ 161.91 (s), 151.06 (s), 148.79 (d), 140.46 (s), 139.51 (d), 133.19 (d, 2 CH), 132.05 (s), 127.85 (d, 2 CH), 119.44 (d), 118.81 (s), 113.41 (s). LC/MS: *m/z* 299 [M + 1]. HRMS: 297.9849. Anal. Calcd. for $C_{13}H_7BrN_4$: C,52.20; H, 2.36; N,18.73; found C, 52.16; H, 2.29; N, 18.69.

*4.1.3.3. 2-(4-(trifluoromethyl)phenyl)-3H-imidazo[4,5-b]pyridine (**2c**).* This compound was obtained in 67% yield by the protocol described in the general procedure starting from **4a** (218 mg, 2 mmol) and sodium hydroxy(4-(trifluoromethyl)phenyl)methanesulfonate (**5b**) (556 mg, 2 mmol); m.p. 272–274 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.44; IR (nujol): ν 3558, 1588, cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.99 (1H, br s, NH), 8.45 (2H, $J = 8.4$ Hz, H-3′, 5′), 8.38 (1H, d, $J = 4.2$ Hz, H-5), 8.01 (1H, d, $J = 8.2$ Hz, H-7), 7.83 (2H, d, $J = 8.8$ Hz, H-2′, 6′), 7.25 (1H, dd, $J = 8.2$ Hz and $J = 4.8$ Hz, H-6); ^{13}C-NMR ($CDCl_3$): δ 162.88 (s), 149.63 (s), 146.33 (d), 139.12 (s), 131.79 (s), 130.89 (s), 126.68 (d, 2 CH), 125.44 (d, 2 CH), 124.91 (q), 124.01 (s), 123.71 (d). LC/MS: *m/z* 264 [M + 1]. HRMS: 263.0645 Anal. Calcd. for $C_{13}H_8F_3N_3$: C, 59.32; H, 3.06; N, 15.96; found C, 59.29; H, 2.29; N, 15.90.

*4.1.3.4. 6-Bromo-2-(4-(trifluoromethyl)phenyl)-3H-imidazo[4,5-b] pyridine (**2d**).* This compound was obtained in 72% yield by the protocol described in the general procedure starting from **4b** (376 mg, 2 mmol) and sodium hydroxy(4-(trifluoromethyl)phenyl) methanesulfonate (**5b**) (556 mg, 2 mmol); m.p. 288–291 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.45; IR (nujol): ν 3556, 1615, cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.93 (1H, br s, NH), 8.44 (2H, $J = 7.8$ Hz, H-3′, 5′), 8.42 (1H, s, H-5), 8.23 (1H, s, H-7), 7.84 (2H, d, $J = 7.6$ Hz, H-2′, 6′); ^{13}C-NMR ($CDCl_3$): δ 161.48 (s), 150.93 (s), 148.57 (d), 140.07 (d), 138.21 (s), 131.65 (s), 130.77 (s), 126.94 (d, 2 CH), 124.36 (d, 2 CH), 123.71 (d), 120.34 (s). LC/MS: *m/z* 342 [M + 1]. HRMS: 340.9769. Anal. Calcd. for $C_{13}H_7BrF_3N_3$: C, 45.64; H, 2.06; N, 12.28; found C, 45.54; H, 2.00; N, 12.21.

*4.1.3.5. 1-(4-(3H-imidazo[4,5-b]pyridin-2-yl)phenyl)ethanone (**2e**).* This compound was obtained in 72% yield by the protocol described in the general procedure starting from **4a** (218 mg, 2 mmol) and sodium (4-acetylphenyl)(hydroxy)methanesulfonate (**5c**) (504 mg, 2 mmol); m.p. >300 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.35; IR (nujol): ν 3556, 1672, 1615, cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.96 (1H, br s, NH), 8.38 (2H, $J = 8.8$ Hz, H-3′, 5′ and H-7), 8.10 (3H, d, $J = 8.8$ Hz, H-2′, 6′), 7.24 (1H, dd, $J = 8.2$ and $J = 4.8$ Hz, H-6), 2.65 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$): δ 196.67 (CO), 162.08 (s), 151.68 (s), 144.12 (d), 140.83 (s), 137.01 (s), 130.86 (s), 130.56 (d, 2 CH), 127.88 (d, 2 CH), 124.51 (d), 121.44 (d), 27.09 (q). LC/MS: *m/z* 238 [M + 1]. HRMS: 237.0912. Anal. Calcd. for $C_{14}H_{11}N_3O$: C, 70.87; H, 4.67; N, 17.71; found C, 70.80; H,4.58; N, 17.68.

*4.1.3.6. 1-(4-(6-bromo-3H-imidazo[4,5-b]pyridin-2-yl)phenyl)ethanone (**2f**).* This compound was obtained in 74% yield by the protocol described in the general procedure starting from **4b** (376 mg, 2 mmol) and sodium (4-acetylphenyl)(hydroxy)methanesulfonate (**5c**) (504 mg, 2 mmol); m.p. >300 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.45; IR (nujol): ν 3558, 1680, 1612, cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.90 (1H, br s, NH), 8.38 (3H, $J = 8.6$ Hz, H-3′, 5′and H-5), 8.19 (1H, s, H-7), 8.11 (2H, d, $J = 8.6$ Hz, H-2′, 6′), 2.65 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$): δ 198.29 (CO), 161.43 (s), 150.89 (s), 148.54 (d), 141.07 (s), 140.01 (d), 137.51 (s), 132.17 (s), 131.04 (d, 2 CH), 130.11 (s), 128.43 (d, 2 CH), 120.07 (s), 24.91 (q). LC/MS: *m/z* 317 [M + 1]. HRMS: 315.0017. Anal. Calcd. for $C_{14}H_{10}BrN_3O$: C, 53.19; H, 3.19; N, 13.29; found C, 53.00; H, 3.16; N, 3.26.

*4.1.3.7. 2-(Furan-3-yl)-3H-imidazo[4,5-b]pyridine (**2g**).* This compound was obtained in 18% yield by the protocol described in the general procedure starting from **4a** (218 mg, 2 mmol) and sodium furan-3-yl(hydroxy)methanesulfonate (**5e**) (400 mg, 2 mmol); m.p. 203–206 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.29; IR (nujol): ν 3558, 1631 cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.90 (1H, br s, NH), 8.35 (1H, s, H-2′), 8.30 (1H, dd, $J = 7.6$ Hz and $J = 1.8$ Hz, H-5), 7.96 (1H, dd, $J = 8.0$ Hz and $J = 1.8$ Hz, H-7), 7.55 (1H, d, $J = 1.6$ Hz, H-4′), 7.18 (1H, dd, $J = 8.0$ Hz and $J = 4.8$ Hz, H-6), 7.14 (1H, d, $J = 1.6$ Hz, H-5′); ^{13}C-NMR ($CDCl_3$): δ 150.33 (s), 147.41 (s), 145.41 (d), 144.57 (d), 138.65 (d), 130.09 (s), 129.81 (s), 108.77 (d), 123.36 (d), 121.96 (d). LC/MS: *m/z* 186 [M + 1]. HRMS: 185.0601. Anal. Calcd. for $C_{14}H_7N_3O$: C, 64.86; H, 3.81; N, 22.69; found C, 64.76; H, 3.78; N, 22.64.

*4.1.3.8. 6-Bromo-2-(furan-3-yl)-3H-imidazo[4,5-b]pyridine (**2h**).* This compound was obtained in 14% yield by the protocol described in the general procedure starting from **4b** (376 mg, 2 mmol) and sodium furan-3-yl(hydroxy)methanesulfonate (**5e**) (400 mg, 2 mmol); m.p. 274–277 °C (from EtOH); TLC (chloroform/methanol 95:0.5): R_f 0.31; IR (nujol): ν 3558, 1623 cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.38 (1H, s, H-5), 8.31 (1H, s, H-2′), 8.03 (1H, br s, NH), 7.96 (1H, s, H-7), 7.66 (1H, s, H-4′), 7.10 (1H, s, H-5′); ^{13}C-NMR ($CDCl_3$): δ 150.69 (s), 149.03 (d), 147.71 (s), 144.26 (d), 140.09 (d), 139.41 (d), 132.39 (s), 129.32 (s), 119.63 (s), 108.91 (d). LC/MS: *m/z* 265 [M + 1]. HRMS: 262.9711. Anal. Calcd. for $C_{10}H_6BrN_3O$: C, 45.48; H, 2.29; N, 15.91; found C, 45.38; H, 2.20; N, 15.88.

*4.1.3.9. 4-(3H-imidazo[4,5-c]pyridin-2-yl)benzonitrile (**2k**).* This compound, previously synthesized by D. W. Robertson et al. [42], was obtained in 51% yield by the protocol described in the general procedure starting from **4c** (218 mg, 2 mmol) and sodium (4-cyanophenyl)(hydroxy)methanesulfonate (**5a**) (470 mg, 2 mmol); m.p. >300 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.32. LC/MS: *m/z* 145 [M + 1]. HRMS: 220.0753. Anal. Calcd. for $C_7H_4N_4$: C, 58.33; H, 2.80; N, 38.87; found C, 58.29; H, 2.60; N, 38.70.

*4.1.3.10. 1-(4-(3H-imidazo[4,5-c]pyridin-2-yl)phenyl)ethanone (**2l**).* This compound was obtained in 55% yield by the protocol described in the general procedure starting from **4c** (218 mg, 2 mmol) and sodium (4-acetylphenyl)(hydroxy)methanesulfonate (**5c**) (470 mg, 2 mmol); m.p. 214–217 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.30; IR (nujol): ν 1672, 1600 cm^{-1}; ^1H NMR ($CDCl_3$): δ 9.08 (1H, s, H-4), 8.38 (3H, d, $J = 7.2$ Hz, H-3′, 5′ and 1H, partially obscured, H-6), 8.12 (2H, d, $J = 7.2$ Hz, H-2′, 6′) 7.72 (1H, d, $J = 5.0$ Hz, H-4), 2.62 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$): δ 196.77 (CO), 176.81 (s), 147.66 (s), 143.37 (d), 141.89 (d), 140.91 (s), 139.34 (s), 138.02 (s), 129.34 (d, 2 CH), 128.01 (d, 2 CH), 113.21 (d), 27.19 (q).LC/MS: *m/z* 238 [M + 1]. HRMS: 237.1005. Anal. Calcd. for $C_{14}H_{11}N_3O$: C, 70.87; H, 4.67; N, 17.71; found C, 70.77; H,4.58; N, 17.62.

*4.1.4. General procedure for the preparation of the imidazo[4,5-b] pyridines (**2i,j**)*

A mixture of diamine **4a** or **4b** (2) mmol and sodium cyclohexyl(hydroxy)methanesulfonate (**5d**) (2) mmol was refluxed in ethanol (10 mL) for 8 h. After cooling, an excess of the sodium salt was filtered off through filter paper and the mother liquors were evaporated to dryness under reduced pressure. The solid residues were purified by silica gel flash column chromatography, eluting with a mixture of chloroform-methanol in the ratio indicated under the R_f of each compound as described below.

*4.1.4.1. 2-Cyclohexyl-3H-imidazo[4,5-b]pyridine (**2i**).* This compound was obtained in 54% yield by the protocol described in the general procedure starting from **4a** (218 mg, 2 mmol) and sodium cyclohexyl(hydroxy)methanesulfonate (**5d**) (585 mg, 2 mmol); m.p. 197–198 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.60; IR (nujol): ν 1539 cm^{-1}; ^1H NMR ($CDCl_3$): δ 13.47 (1H, s a, NH), 8.33 (1H, d, J = 5.0 Hz, H-5), 8.06 (1H, d, J = 7.8 Hz, H-7) 7.26 (1H, dd, J = 7.8 Hz and J = 5.2 Hz, H-6), 3.12–3.00 (1H, m, CH), 2.30–1.35 (10H, m, H-cyclohexyl); ^{13}C-NMR ($CDCl_3$): δ 151.89 (s), 147.56 (s), 145.49 (d), 132.19 (s), 127.21 (d), 123.01 (d), 40.41 (d), 33.27 (t, 2 CH_2), 26.89 (t), 25.78 (t, 2 CH_2). LC/MS: m/z 201 [M + 1]. HRMS: 201,1204. Anal. Calcd. for $C_{12}H_{15}N_3$: C, 71.61; H, 7.51; N, 20.88; found C, 71.55; H, 7.42; N, 20.77.

*4.1.4.2. 6-Bromo-2-cyclohexyl-3H-imidazo[4,5-b]pyridine (**2j**).* This compound was obtained in 78% yield by the protocol described in the general procedure starting from **4b** (376 mg, 2 mmol) and sodium cyclohexyl(hydroxy)methanesulfonate (**5d**) (585 mg, 2 mmol); m.p. 226–227 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.50; IR (nujol): ν 1535 cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.29 (1H, d, J = 1.8 Hz, H-5), 7.97 (1H, d, J = 1.8 Hz, H-7), 2.60–2.83 (1H, m, CH), 2.10–1.36 (10H, m, H-cyclohexyl); ^{13}C-NMR ($CDCl_3$): δ 151.42 (s), 147.33 (s), 148.29 (d), 137.87 (d), 130.94 (s), 118.61 (s), 38.78 (d), 33.45 (t, 2 CH_2), 27.07 (t), 25.43 (t, 2 CH_2). LC/MS: m/z 280 [M + 1]. HRMS: 279.0348. Anal. Calcd. for $C_{12}H_{14}BrN_3$: C, 51.44; H, 5.04; N, 15.00; found C, 51.34; H, 5.00; N, 14.99.

*4.1.5. General procedure for the preparation of the N-benzylidenequinolinamines (**3a**,**b**,**f**–**h**)*

A mixture of diaminoquinolines **6a**, or **6b** (2) mmol and sodium hydroxy(R-substituted-phenyl-1-yl)methanesulfonate (**5a**,**b**,**f**,**g**) (2) mmol was stirred for 8 h in 15 mL of refluxed EtOH. On cooling, the mixture was diluted with water and the formed precipitate was collected and dried on oven. The solid residue was flash-chromatographed on silica gel column eluting with a mixture of chloroform/methanol in the ratio of 9:1, obtaining the *N*-benzylidenequinolinamines (**3a**,**b**,**f**–**h**) in 30-40% yield.

*4.1.5.1. 4-((7-Amino-8-chloroquinolin-6-ylimino)methyl)benzonitrile (**3a**).* This compound was obtained in 36% yield by the protocol described in the general procedure starting from **6a** (387 mg, 2 mmol) and sodium (4-cyanophenyl)(hydroxy)methanesulfonate (**5a**) (470 mg, 2 mmol); m.p. > 300 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.80; IR (nujol): ν 3358, 2228, 1598, cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.88 (1H, dd, J = 4.2 and 1.6 Hz, H-2), 8.70 (1H, s, N=CH), 8.09 (2H, d, J = 8.4 Hz, H-3′, 5′), 8.04 (1H, dd, J = 8.2 and 1.6 Hz, H-4), 7.82 (2H, d, J = 8.4 Hz, H-2′, 6′), 7.31 (1H, s, H-5), 7.25 (1H, dd, J = 8.2 and 4.2 Hz, H-3), 5.10 (2H, s, NH_2); ^{13}C-NMR ($CDCl_3$): δ 159.81 (d), 149.72 (d), 142.36 (s), 141.97 (s), 139.82 (s), 138.41 (s), 134.79 (d), 128.11 (d, 2 CH), 124.22 (d, 2 CH), 124.06 (s), 120.83 (d), 120.13 (d), 118.93 (s) 114.42 (s), 114.31 (s). LC/MS: m/z 309 [M + 1]. HRMS: 306.1064. Anal. Calcd. for $C_{17}H_{11}ClN_4$: C, 66.56; H, 3.61; N, 18.26; found C, 66.91; H, 3.44; N, 18.03.

*4.1.5.2. 8-Chloro-N^6-(4-(trifluoromethyl)benzylidene)quinoline-6,7-diamine (**3b**).* This compound was obtained in 40% yield by the protocol described in the general procedure starting from **6a** (387 mg, 2 mmol) and hydroxy(4-(trifluoromethyl)phenyl)methanesulfonate (**5b**) (556 mg, 2 mmol); m.p. 150-152 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.85; IR (nujol): ν 3383, 1627, cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.89 (1H, dd, J = 4.2 and 1.8 Hz, H-2), 8.71 (1H, s, N=CH), 8.10 (2H, d, J = 8.4 Hz, H-3′, 5′), 8.04 (1H, dd, J = 8.4 and 1.8 Hz, H-4), 7.62 (2H, d, J = 8.4 Hz, H-2′, 6′), 7.30 (1H, s, H-5), 7.24 (1H, dd, J = 8.4 and 4.2 Hz, H-3), 5.11 (2H, s, NH_2); ^{13}C-NMR ($CDCl_3$): δ 159.31 (d), 150.42 (d), 144.76 (s), 141.24 (s), 139.67 (s), 138.54 (s), 136.14 (d), 133. 11 (s), 129.34 (d, 2 CH), 125.90 (d, 2 CH), 122.30 (s), 121.05 (s), 118.58 (d), 112.13 (d), 111.91 (s). LC/MS: m/z 352 [M + 1]. HRMS: 349.1002. Anal. Calcd. for $C_{17}H_{11}ClF_3N_3$: C, 58.38; H, 3.17; N, 12.01; found C, 58.07; H, 3.32; N, 11.75.

*4.1.5.3. 8-Chloro-N^6-(4-nitrobenzylidene)quinoline-6,7-diamine (**3f**).* This compound was obtained in 30% yield by the protocol described in the general procedure starting from **6a** (387 mg, 2 mmol) and sodium hydroxy(4-nitrophenyl)methanesulfonate (**5f**) (510 mg, 2 mmol); m.p. 161–163 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.78; IR (nujol): ν 3375, 1603, cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.78 (1H, dd, J = 4.4 and 1.6 Hz, H-2), 8.68 (1H, s, N=CH), 8.27 (2H, d, J = 8.2 Hz, H-3′, 5′), 8.12 (1H, dd, J = 8.4 and 1.6 Hz, H-4), 7.70 (2H, d, J = 8.2 Hz, H-2′, 6′), 7.35 (1H, s, H-5), 7.29 (1H, dd, J = 8.4 and 4.4 Hz, H-3), 5.01 (2H, s, NH_2); ^{13}C-NMR ($CDCl_3$): δ 160.13 (d), 150.22 (s), 149.72 (d), 142.5 (s), 141.78 (s), 139.59 (s), 138.60 (s), 135.07 (d), 127.92 (d, 2 CH), 123.87 (d, 2 CH), 124.20 (s), 120.69 (d), 119.93 (d), 114.14 (s). LC/MS: m/z 329 [M + 1]. HRMS: 326.0511. Anal. Calcd. for $C_{16}H_{11}ClN_4O_2$: C, 58.82; H, 3.39; N, 17.15; found C, 59.21; H, 3.22; N, 17.35.

*4.1.5.4. 8-Chloro-N^6-(3-nitro-4-(trifluoromethyl)benzylidene)quinoline-6,7-diamine (**3g**).* This compound was obtained in 34% yield by the protocol described in the general procedure starting from **6a** (387 mg, 2 mmol) and sodium hydroxy(3-nitro-4-(trifluoromethyl) phenyl)methanesulfonate (**5g**) (626 mg, 2 mmol); m.p. 170–172 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.60; IR (nujol): ν 3389, 1624, cm^{-1}; ^1H NMR ($CDCl_3$): δ 9.17 (1H, s, N=CH), 8.91 (1H, dd, J = 4.4 and 1.8 Hz, H-2), 8.50–8.37 (3H, m, H-2′, 5′, 6′), 8.08 (1H, dd, J = 8.4 and 1.8 Hz, H-4), 7.39 (1H, s, H-5), 7.27 (1H, dd, J = 8.4 and 4.4 Hz, H-3), 5.11 (2H, s, NH_2); ^{13}C-NMR ($CDCl_3$): δ 160.19 (d), 149.50 (d), 144.94 (s), 142.23 (s), 140.55 (s), 138.89 (s), 138.41 (s), 135.88 (d), 135.41 (d), 126. 07 (s), 126.62 (d), 124.22 (s), 122.98 (d), 122.89 (s), 114.12 (s), 120.33 (d), 120.22 (d). LC/MS: m/z 397 [M + 1]. HRMS: 394.0409. Anal. Calcd. for $C_{17}H_{10}ClF_3N_4O_2$: C, 51.73; H, 2.55; N, 14.19; found C, 51.49; H, 2.68; N, 14.01.

*4.1.5.5. 4-((7-Amino-8-chloro-4-oxo-1,4-dihydroquinolin-6-ylimino) methyl)benzonitrile (**3h**).* This compound was obtained in 31% yield by the protocol described in the general procedure starting from **6b** (419 mg, 2 mmol) and sodium (4-cyanophenyl)(hydroxy)methanesulfonate (**5a**) (470 mg, 2 mmol); m.p. > 300 °C (from EtOH); TLC (chloroform/methanol 9:1): R_f 0.48; IR (nujol): ν 3359, 2225, 1605, cm^{-1}; ^1H NMR ($CDCl_3$): δ 10.95 (1H, d, J = 3.8 Hz, NH), 8.90 (1H, s, N=CH), 8.23 (2H, d, J = 8.4 Hz, H-3′, 5′), 7.94 (1H, s, H-5), 7.85 (2H, d, J = 8.4 Hz, H-2′, 6′). 8.04 (1H, d, J = 7.2 Hz, H-3), 6.03 (1H, dd, J = 7.2 and 3.8 Hz, H-2), 5.92 (2H, s, NH_2); ^{13}C_NMR ($CDCl_3$): δ 177. 31 (s), 159.94 (d), 140.88 (s), 147.98 (s), 146.95 (s), 132.98 (s), 129.89 (s), 139.21 (d), 132.45 (d, 2 CH), 126.88 (d, 2 CH), 123.08 (s), 122.03 (d), 118.71 (s) 114.96 (s), 108.55 (d). LC/MS: m/z 325 [M + 1]. HRMS: 323.1021. Anal. Calcd. for $C_{17}H_{11}ClN_4O$: C, 63.26; H, 3.44; N, 17.36; found C, 63.03; H, 3.58; N, 17.19.

4.1.6. Chemical and enzymatic stability

Aliquots of **2b** were dissolved in pH 7.4 phosphate buffer (chemical stability) or in plasma (enzymatic stability). The solutions were maintained at 37 °C and aliquots were withdrawn every 1 h. Plasma samples were extracted with acetonitrile (1:2) and centrifuged at 3000 RPM (1000 g) for 10 min. The supernatant and buffer solutions were analysed by HPLC.

Chromatographic separations were performed using a Liquid Chromatograph, accomplished on a reversed phase HPLC column. The selected wavelength was 250 nm. The mobile phase used in the separation consisted of methanol/water [80:20]. The flow-rate was 1 mL/min with an injection volume of 20 μl.

Pseudo-first-order half-times (t½) determining the chemical and enzymatic stability were calculated from the linear slopes of plots of the logarithm of remaining compound against time. In results compound **2b** appeared quite stable in a pH 7.4 buffered solution ($t_{½} > 4$ h), slightly reduced in plasma ($t_{½} = 2$ h).

4.2. Biology

4.2.1. Test compounds

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

4.2.2. 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) [ATCC CCL22 (NBL-1) *Bos Taurus*], Baby Hamster Kidney (BHK-21) [ATCC CCL10 (C-13) *Mesocricetus auratus*] and Monkey kidney (Vero 76) [ATCCCRL 1587 *Cercopithecus Aethiops*]. Viruses were purchased from American Type Culture Collection (ATCC), with the exception of Yellow Fever Virus (YFV) and Human Immunodeficiency Virus type-1 (HIV-1). Viruses representative of positive-sense single stranded RNAs ($ssRNA^+$) were: (i) *Retroviridae*: the III_B laboratory strain of HIV-1, obtained from the supernatant of the persistently infected $H9/III_B$ cells (NIH 1983); (ii) *Flaviviridae*: yellow fever virus (YFV) [strain 17-D vaccine (Stamaril Pasteur J07B01)] and bovine viral diarrhoea virus (BVDV) [strain NADL (ATCC VR-534)]; (iii) *Picornaviridae*: human enterovirus B [coxsackie type B5 (CV-B5), strain Faulkner, (ATCC VR-185)], and human enterovirus C [poliovirus type-1 (Sb-1), Sabin strain Chat (ATCC VR-1562)]. Viruses representative of negative-sense, single-stranded RNAs (ssRNA-) were: (iv) *Paramyxoviridae*: human respiratory syncytial virus (RSV) [strain A2 (ATCC VR-1540)]; (v) *Rhabdoviridae*: vesicular stomatitis virus (VSV) [lab strain Indiana (ATCC VR 158)]. The virus representative of double-stranded RNAs (dsRNA) *Reoviridae* was reovirus type-1 (Reo-1) [simian virus 12, strain 3651 (ATCC VR- 214)]. DNA virus representatives were: (vi) *Poxviridae*: vaccinia virus (VV) [strain Elstree (Lister Vaccine) (ATCC VR-1549)]; and (vii) *Herpesviridae*: human herpesvirus 1 (HSV-1) [strain KOS (ATCC VR- 1493)].

4.2.3. 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% foetal bovine serum (FBS), 100 units/mL penicillin G and 100 mg/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 [43]. MDBK and BHK cells were seeded in 96-well plates at an initial density of 6×10^5 and 1×10^6 cells/mL, respectively, in Minimum Essential Medium with Earle's salts (MEM-E), Lglutamine, 1 mM sodium pyruvate and 25 mg/L kanamycin, supplemented with 10% horse serum (MDBK) or 10% foetal bovine serum (FBS) (BHK). 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 72 h at 37 °C by the MTT method. Vero-76 cells were seeded in 96-well plates at an initial density of 4×10^5 cells/mL, in Dulbecco's Modified Eagle Medium (D-MEM) with L-glutamine and 25 mg/L kanamycin, supplemented with 10% FBS. 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.

4.2.4. Antiviral assays

Antiviral 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. Antiviral 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. Activity of 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-Earl with L-glutamine, 1 mM sodium pyruvate and 0.025 g/L kanamycin, supplemented with 0.5% inactivated FBS] to give an m.o.i of 0.01. After 2 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.

Antiviral activity against CV-B5, Sb-1, VV, HSV-1, VSV and RSV was determined by plaque reduction assays in infected 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 and 0.025 g/L kanamycin, supplemented with 10% FBS] 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 to 100 PFU/well. Following removal of unadsorbed virus, 500 μL of maintenance medium [D-MEM with L-glutamine and 4500 mg/L Dglucose, supplemented with 1% inactivated FBS] containing 0.75% methylecellulose, without or with serial dilutions of test compounds, were added. Cultures were incubated at 37 °C for 2 (Sb-1 and VSV), 3 (CVB-5, 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.

4.2.5. Linear regression analysis

The extent of cell growth/viability and viral multiplication, at each drug concentration tested, were expressed as percentage of untreated controls. Concentrations resulting in 50% inhibition (CC_{50} or EC_{50}) were determined by linear regression analysis.

References

[1] S.J. Flint, L.W. Enquist, R.M. Krug, V.R. Racaniello, A.M. Skalka, Principles of Virology: Molecular Biology, Pathogenesis, and Control, 2000. Washington, D.C.
[2] M. Woolhouse, F. Scott, Z. Hudson, R. Howey, M. Chase-Topping, Human viruses: discovery and emergence, Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 367 (2012) 2864–2871.
[3] D.M. Knipe, P.M. Howley, D.E. Griffin, Virus-host cell interactions, in: W.W. Lippincott (Ed.), Fields Virology, 2001, pp. 133–170. Philadelphia.
[4] P.G. Kennedy, Viral encephalitis: causes, differential diagnosis, and management, J. Neurol. Neurosurg. Psychiatry 75 (Suppl. 1) (2004) i10–15.
[5] R.B. Domingues, F.D. Lakeman, C.S. Pannuti, M.C. Fink, A.M. Tsanaclis, Advantage of polymerase chain reaction in the diagnosis of herpes simplex

encephalitis: presentation of 5 atypical cases, Scand. J. Infect. Dis. 29 (1997) 229–231.
[6] R.J. Whitley, Herpes simplex encephalitis: adolescents and adults, Antivir. Res. 71 (2006) 141–148.
[7] W.C. Tan, I.B. Jaganath, R. Manikam, S.D. Sekaran, Evaluation of antiviral activities of four local Malaysian Phyllanthus species against herpes simplex viruses and possible antiviral target, Int. J. Med. Sci. 10 (2013) 1817–1829.
[8] P. Chayavichitsilp, J.V. Buckwalter, A.C. Krakowski, S.F. Friedlander, Herpes simplex, Pediatr. Rev./Am. Acad. Pediatr. 30 (2009) 119–129 quiz 130.
[9] D.A. Henderson, Bioterrorism as a public health threat, Emerg. Infect. Dis. 4 (1998) 488–492.
[10] D.L. Heymann, M. Szczeniowski, K. Esteves, Re-emergence of monkeypox in Africa: a review of the past six years, Br. Med. Bull. 54 (1998) 693–702.
[11] Y.J. Hutin, R.J. Williams, P. Malfait, R. Pebody, V.N. Loparev, S.L. Ropp, M. Rodriguez, J.C. Knight, F.K. Tshioko, A.S. Khan, M.V. Szczeniowski, J.J. Esposito, Outbreak of human monkeypox, Democratic Republic of Congo, 1996 to 1997, Emerg. Infect. Dis. 7 (2001) 434–438.
[12] T. O'Toole, Smallpox: an attack scenario, Emerg. Infect. Dis. 5 (1999) 540–546.
[13] J.G. Breman, D.A. Henderson, Poxvirus dilemmas–monkeypox, smallpox, and biologic terrorism, N. Engl. J. Med. 339 (1998) 556–559.
[14] Russia, Iraq, and other potential sources of anthrax, smallpox, and other bioterrorist weapons: hearing before the Committee on International Relations, in: House of Representatives, One Hundred Seventh Congress, First Session, United States Congress, Washington, DC, 2001, p. 40.
[15] A.M. Di Bisceglie, J.H. Hoofnagle, Optimal therapy of hepatitis C, Hepatology 36 (2002) S121–S127.
[16] M.W. Fried, M.L. Shiffman, K.R. Reddy, C. Smith, G. Marinos, F.L. Goncales Jr., D. Haussinger, M. Diago, G. Carosi, D. Dhumeaux, A. Craxi, A. Lin, J. Hoffman, J. Yu, Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection, N. Engl. J. Med. 347 (2002) 975–982.
[17] F. Poordad, J. McCone Jr., B.R. Bacon, S. Bruno, M.P. Manns, M.S. Sulkowski, I.M. Jacobson, K.R. Reddy, Z.D. Goodman, N. Boparai, M.J. DiNubile, V. Sniukiene, C.A. Brass, J.K. Albrecht, J.P. Bronowicki, S.-Investigators, Boceprevir for untreated chronic HCV genotype 1 infection, N. Engl. J. Med. 364 (2011) 1195–1206.
[18] B.R. Bacon, S.C. Gordon, E. Lawitz, P. Marcellin, J.M. Vierling, S. Zeuzem, F. Poordad, Z.D. Goodman, H.L. Sings, N. Boparai, M. Burroughs, C.A. Brass, J.K. Albrecht, R. Esteban, H.R.-Investigators, Boceprevir for previously treated chronic HCV genotype 1 infection, N. Engl. J. Med. 364 (2011) 1207–1217.
[19] I.M. Jacobson, J.G. McHutchison, G. Dusheiko, A.M. Di Bisceglie, K.R. Reddy, N.H. Bzowej, P. Marcellin, A.J. Muir, P. Ferenci, R. Flisiak, J. George, M. Rizzetto, D. Shouval, R. Sola, R.A. Terg, E.M. Yoshida, N. Adda, L. Bengtsson, A.J. Sankoh, T.L. Kieffer, S. George, R.S. Kauffman, S. Zeuzem, A.S. Team, Telaprevir for previously untreated chronic hepatitis C virus infection, N. Engl. J. Med. 364 (2011) 2405–2416.
[20] S. Zeuzem, P. Andreone, S. Pol, E. Lawitz, M. Diago, S. Roberts, R. Focaccia, Z. Younossi, G.R. Foster, A. Horban, P. Ferenci, F. Nevens, B. Mullhaupt, P. Pockros, R. Terg, D. Shouval, B. van Hoek, O. Weiland, R. Van Heeswijk, S. De Meyer, D. Luo, G. Boogaerts, R. Polo, G. Picchio, M. Beumont, R.S. Team, Telaprevir for retreatment of HCV infection, N. Engl. J. Med. 364 (2011) 2417–2428.
[21] E.A. Gould, T. Solomon, Pathogenic flaviviruses, Lancet 371 (2008) 500–509.
[22] A.M. O'Connor, S.D. Sorden, M.D. Apley, Association between the existence of calves persistently infected with bovine viral diarrhea virus and commingling on pen morbidity in feedlot cattle, Am. J. Vet. Res. 66 (2005) 2130–2134.
[23] C.C. Chase, G. Elmowalid, A.A. Yousif, The immune response to bovine viral diarrhea virus: a constantly changing picture, the Veterinary clinics of North America, Food Anim. Pract. 20 (2004) 95–114.
[24] C. Centers for Disease, Prevention, Enterovirus surveillance–United States, 2002-2004, Morb. Mortal. Wkly. Rep. 55 (2006) 153–156.
[25] A.T. Borchers, C. Chang, M.E. Gershwin, L.J. Gershwin, Respiratory syncytial virus–a comprehensive review, Clin. Rev. Allergy Immunol. 45 (2013) 331–379.
[26] G. Vitale, A. Carta, M. Loriga, G. Paglietti, R. Loddo, B. Busonera, D. Collu, P. La Colla, 2-Arylbenzimidazoles as antiviral and antiproliferative agents. Part 1, Med. Chem. 4 (2008) 605–615.
[27] G. Vitale, P. Corona, M. Loriga, A. Carta, G. Paglietti, P. La Colla, B. Busonera, E. Marongiu, D. Collu, R. Loddo, 2-Arylbenzimidazoles as antiviral and antiproliferative agents. Part 2, Med. Chem. (2009) 507–516.
[28] G. Vitale, P. Corona, M. Loriga, A. Carta, G. Paglietti, C. Ibba, G. Giliberti, R. Loddo, E. Marongiu, P. La Colla, Styrylbenzimidazoles. Synthesis and biological activity – part 3, Med. Chem. 6 (2010) 70–78.
[29] G. Vitale, P. Corona, M. Loriga, A. Carta, G. Paglietti, G. Giliberti, G. Sanna, P. Farci, M.E. Marongiu, P. La Colla, 5-acetyl-2-arylbenzimidazoles as antiviral agents. Part 4, Eur. J. Med. Chem. 53 (2012) 83–97.
[30] A. Carta, G. Loriga, S. Piras, G. Paglietti, M. Ferrone, M. Fermeglia, S. Pricl, P. La Colla, B. Secci, G. Collu, R. Loddo, Synthesis and in vitro evaluation of the antiviral activity of N-[4-(1H(2H)-benzotriazol-1(2)-yl)phenyl]alkylcarboxamides, Med. Chem. 2 (2006) 577–589.
[31] A. Carta, M. Loriga, S. Piras, G. Paglietti, M. Ferrone, M. Fermeglia, S. Pricl, P. La Colla, G. Collu, T. Sanna, R. Loddo, Synthesis and anti-picornaviridae in vitro activity of a new class of helicase inhibitors the N,N'-bis[4-(1H(2H)-benzotriazol-1(2)-yl)phenyl] alkyldicarboxamides, Med. Chem. 3 (2007) 520–532.
[32] A. Carta, M. Loriga, G. Paglietti, M. Ferrone, M. Fermeglia, S. Pricl, T. Sanna, C. Ibba, P. La Colla, R. Loddo, Design, synthesis, and preliminary in vitro and in silico antiviral activity of [4,7]phenantrolines and 1-oxo-1,4-dihydro-[4,7] phenantrolines against single-stranded positive-sense RNA genome viruses, Bioorg. Med. Chem. 15 (2007) 1914–1927.
[33] P. Sanna, A. Carta, G. Paglietti, Synthesis of two novel tricyclic rings: triazolo [4,5-g]quinolines and pyrido[2,3-g]quinoxalines derived from 6,7-diaminoquinolines, Heterocycles 53 (2000) 423–432.
[34] A. Carta, P. Sanna, L. Gherardini, D. Usai, S. Zanetti, Novel functionalized pyrido [2,3-g]quinoxalinones as antibacterial, antifungal and anticancer agents, Farmaco 56 (2001) 933–938.
[35] A. Carta, G. Boatto, G. Paglietti, G. Poni, M.G. Setzu, P. Caredda, Synthesis and biological evaluation of triazolo[4,5-g]quinolines, imidazo[4,5-g]quinolines and pyrido[2,3-g]quinoxaline. Part II, Heterocycles 60 (2003) 833–842.
[36] A. Carta, I. Briguglio, S. Piras, P. Corona, G. Boatto, M. Nieddu, P. Giunchedi, M.E. Marongiu, G. Giliberti, F. Iuliano, S. Blois, C. Ibba, B. Busonera, P. La Colla, Quinoline tricyclic derivatives. Design, synthesis and evaluation of the antiviral activity of three new classes of RNA-dependent RNA polymerase inhibitors, Bioorg. Med. Chem. 19 (2011) 7070–7084.
[37] R.L. Shriner, A. Land, The structure of the bisulfite compound of acetaldehyde, J. Org. Chem. 6 (6) (1941) 888–894.
[38] F. Sparatore, M.I. La Rotonda, G. Caliendo, E. Novellino, C. Silipo, A. Vittoria, Structure-activity relationships for the auxin-like activity of benzotriazole derivatives, Farm. Ed. Sci. 43 (1988) 141–151.
[39] A. Boido, I. Vazzana, F. Sparatore, B. Cappello, V. Diurno, C. Matera, R. Marrazzo, S. Russo, M.L. Cenicola, E. Marmo, Synthesis and biological evaluation of N-oxides of 4′-(benzotriazol-2-yl)-phenyl and phenoxy alkanoic acids, Farm. Ed. Sci. 44 (1989) 257–277.
[40] Unpublished data, currently under study to evaluate, in enzyme assays and putative binding mode, the mechanism of action of all the title compounds in comparison with the tricyclic derivatives previously reported.
[41] A. Carta, M. Palomba, P. Corona, Synthesis of substituted aminoquinolines as useful intermediates for preparation of aromatic N-tricyclic systems, Heterocycles 68 (2006) 1715–1722.
[42] D.W. Robertson, E.E. Beedle, J.H. Krushinski, G.D. Pollock, H. Wilson, V.L. Wyss, J.S. Hayes, Structure-activity relationships of arylimidazopyridine cardiotonics: discovery and inotropic activity of 2-[2-methoxy-4-(methylsulfinyl) phenyl]-1H-imidazo[4,5-c]pyridine, J. Med. Chem. 28 (1985) 717–727.
[43] R. Pauwels, J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyter, E. De Clercq, Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds, J. Virol. Methods 20 (1988) 309–321.