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Quinoline tricyclic derivatives. Design, synthesis and evaluation of the antiviral activity of three new classes of RNA-dependent RNA polymerase inhibitors

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ABSTRACT

In this study three new classes of linear *N*-tricyclic compounds, derived by condensation of the quinoline nucleus with 1,2,3-triazole, imidazole or pyrazine, were synthesized, obtaining triazolo[4,5-*g*]quinolines, imidazo[4,5-*g*]quinolines and pyrido[2,3-*g*]quinoxalines, respectively. Title compounds were tested in cell-based assays for cytotoxicity and antiviral activity against RNA viruses representative of the three genera of the *Flaviviridae* family, that is BVDV (*Pestivirus*), YFV (*Flavivirus*) and HCV (*Hepacivirus*). Quinoline derivatives were also tested against representatives of other RNA virus families containing single-stranded, either positive-sense ($ssRNA^+$) or negative-sense (RNA^-), and double-stranded genomes (dsRNA), as well as against representatives of two DNA virus families. Some quinolines showed moderate, although selective activity against CVB-5, Reo-1 and RSV. However, derivatives belonging to all classes showed activity against BVDV. Among the most potent were the bis-triazoloquinoline **1m**, the imidazoquinolines **2e** and **2h**, and the pyridoquinoxalines **4h**, **4j** and **5n** (EC_{50} range 1–5 μM). When tested in a replicon assay, compound **2h** was the sole derivative to also display anti-HCV activity (EC_{50} = 3.1 μM). In enzyme assays, **1m**, **2h**, **5m** and **5n** proved to be potent inhibitors of the BVDV RNA-dependent RNA polymerase (RdRp), while only **2h** also inhibited the recombinant HCV enzyme.

1. Introduction

The *Flaviviridae* family consists of single-stranded, positive-sense RNA ($ssRNA^+$) viruses which cause significant diseases in humans and animals. They are distributed into three genera.

The *Flavivirus* genus comprises Dengue Fever, Yellow Fever, West Nile, Japanese Encephalitis and Tick-borne Encephalitis viruses, which are human pathogens prevalent throughout the world and cause acute febrile illness, encephalitis and haemorrhagic fevers. Although an effective vaccine against YFV has been available since the late 1930s, utilization is incomplete in many areas.[1]

The *Pestivirus* genus includes animal pathogens of major economic impact for the livestock industry, such as Bovine Viral Diarrhoea virus (BVDV), Border Disease and Classical Swine Fever viruses. They cause a range of clinical manifestations including abortion, teratogenesis, respiratory problems, chronic wasting disease, immune system dysfunction and predisposition to secondary viral and bacterial infections. BVDV can also establish a persistent infection in animals, that remain viremic throughout life, serve as continuous virus reservoirs and often succumb to fatal mucosal disease.[2] Furthermore, BVDV shows the ability to cross the placenta of susceptible animals causing a variety of fetal infections.[3]

The *Hepacivirus* genus includes, as sole representative, the Hepatitis C virus (HCV), while viruses such as GBV-A, GBV-A-like, GBV-D and GBV-C (also known as Hepatitis G virus, HGV), although closely related to HCV, represent unassigned members of *Flaviviridae*. HCV is a major cause of human hepatitis.[4] According to WHO estimates, over 170 million people worldwide are presently infected with this virus.[5,6] Most infections become persistent and about 60% of cases progress towards chronic liver disease. Chronic HCV infection can lead to development of cirrhosis, hepatocellular carcinoma and liver failure.[7,8] Pegylated interferon in combination with ribavirin is used in the clinic for hepatitis due to HCV. Unfortunately, this therapy has limited efficacy and is often associated with severe and adverse events.[9]

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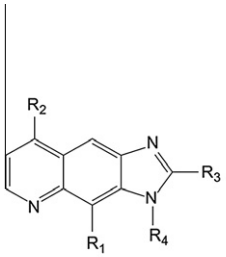
With the exception of YFV, no vaccines exist against *Flaviviridae* pathogens, as well as no selective antiviral drugs are yet available in the clinic to prevent and/or treat their infections. Hence the need to identify new lead compounds targeted at virus-specific steps of the *Flaviviridae* replication cycle.

Recently, we reported the synthesis of the [4,7]phenantroline nucleus, an angular *N*-tricyclic system derived by expansion of the quinoline nucleus with pyridine and several related derivatives. These compounds emerged as a new class of antiviral agents endowed with in vitro selective activity against ssRNA$^+$ viruses.[10] Molecular modeling studies detailed the interactions between [4,7]phenantrolines and the RNA-dependent RNA polymerase (RdRp) of BVDV and HCV,[11] thus giving clue to further design, synthesis and antiviral evaluation of these *N*-tricyclic systems.

Here, we will present studies on triazolo[4,5-*g*]quinolines, imidazo[4,5-*g*]quinolines and pyrido[2,3-*g*]quinoxalines. Since 2000, several classes derived from unsubstituted nuclei have been prepared and evaluated in vitro by us for antibacterial, antifungal and anticancer activity.[12–14] At the beginning of this study, we re-synthesized compounds **1a–j**, **2a–c** and **3a–d** (Figs. 1–3) with the aim to evaluate their cytotoxicity and antiviral activity. Encouraged by the positive results obtained, we designed, synthesized and evaluated the cytotoxicity and antiviral activity of a second generation of linear *N*-tricyclic systems, represented by compounds **1k–m**, **2d–q**, **3e–f**, **4g–j** and **5k–o** (Figs. 1–3). Selected leads were then evaluated for anti-HCV activity in a replicon system and tested in enzyme assays for activity against the RNA-dependent RNA polymerases (RdRp) of BVDV and HCV.

2. Chemistry

Triazolo[4,5-*g*]quinolines **1a–c**, imidazo[4,5-*g*]quinolines **2a–h** and **2k–q**, and pyrido[2,3-*g*]quinoxalines **3a–f**, **4g–j** and **5k–o** were prepared, starting from the proper 6,7-diaminoquinolines **6a–c**, as summarized in Scheme 1.[15] According to previous procedures, **1a–c** were obtained (in 42–44% yield) subjecting the diamines **6a–c** to ring closure by diazotization with HNO_2, followed by cyclization of the corresponding diazonium salt at room temperature, as previously reported.[12,14] Imidazo derivatives **2a–h**, **2k–q** were, in turn, synthesized by condensation of **6a–c** with formic acid obtaining the known **2a–c** in 80–90% yield,[12,14] or by reaction with Bertagnini's salts of the aldehydes obtaining the new derivatives **2d–h** and **2k–p** in 32–95% yield. Pyridoquinoxalines **3a–f** were obtained by condensation of diaminoquinolines **6a–c** with α,β-dicarbonyls in refluxing ethanol for 1–2 h, obtaining the known derivatives **3a–d** in 45–90% yield[12,14] and the new **3e** and **3f** derivatives in 87% and 69% yield, respectively. When diamines **6a–c** were reacted with α-ketocarboxylic derivatives in refluxing ethanol for 3–15 h, or in 10% aqueous solution of sulfuric acid at 45–50 °C for 2 h, mixtures of two 2/3-oxo-isomers were obtained in 10–80% yield (the known **4g–i** and **5k–l**,[13] or the new **4j** and **5m–o**), which were then separated and purified by chromatography.



2a-q

a) R_1=R_2=R_3=R_4=H;
b) R_1=Cl, R_2=R_3=R_4=H;
c) R_1= Cl, R_2=OH, R_3=R_4=H;
d) R_1=R_2=R_4=H, R_3=Ph;
e) R_1=Cl, R_2=R_4 =H, R_3=Ph;
f) R_1=R_2=R_4=H, R_3=4-Cl-Ph;
g) R_1=R_2=R_4=H, R_3=4-NO_2-Ph;
h) R_1=Cl, R_2=R_4 =H, R_3=4-NO_2-Ph;
i) R_1=Cl, R_2=R_4=H, R_3=4-NH_2-Ph;
j) R_1=Cl, R_2=R_4=H, R_3=4-NH($COCH_3$)-Ph;
K) R_1=R_2=R_4=H, R_3=2-OCH_3-Ph;
l) R_1=R_2=R_4=H, R_3=3,4-diCl-Ph;
m) R_1=R_2=R_4=H, R_3=4-Ph-Ph;
n) R_1=R_2=R_4=H,R_3=4-NO_2-(α)naphtyl;
o) R_1= Cl, R_2=R_4=H, R_3=cyclohexyl;
p) R_1= Cl, R_2=OH, R_3=Ph, R_4=H;
q) R_1=Cl, R_2=H, R_3=4-NO_2-Ph; R_4=cyclohexyl.

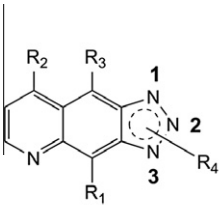
Figure 2. Imidazo[4,5-*g*]quinolines (**2a–q**).

Triazolo[4,5-*g*]quinolines **1d–m** were prepared following the procedures previously reported and described in Scheme 2. 7,8-Dichloro-6-nitroquinolines **7d** and **7j**, subjected to reaction with a large excess of hydrazine hydrate in ethanol in a sealed steal vessel at 70 °C for 90 h, afforded good yields (56–90%) of the corresponding triazolo[4,5-*g*]quinoline-1-oxides 1d and **1j**.[12,14]

Nitration of both **1b** and **1j** by treatment with potassium nitrate in concentrated sulfuric acid at 50 °C for 3 h, gave the corresponding 9-nitro-derivatives **1e** and **1f**, respectively, in 56–90% yield.[16] A mixture of **1g** and **1h** in about 1:1 ratio (70% yield) was obtained by reaction of **1b** with chloroacetonitrile in dimethylformamide (DMF), catalyzed by KOH, while **1i** was obtained with acetic anhydride in 70% yield.[16] Finally, reaction of **1b** with 1,3-dichloroacetone **8**, under the same conditions, afforded a mixture of **1k** and **1l** in 70% total yield when the molar ratio of reactive reagents was 1:10, while, when the latter was 2:1, the mixture **1m** was obtained in 35% yield. The mixture of 1,3-bis-(4-chloro-1*H*(3*H*)-triazolo[4,5-*g*]quinoline)propan-2-ones in 1:1 ratio (**1m**) was not resolved by preparative chromatography.

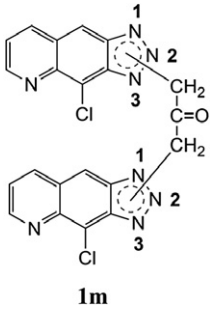
Hydrogenation in Parr apparatus of the nitroimidazoquinoline **2h**, catalyzed with 10% palladized charcoal, gave **2i** (88% yield) which, subjected to reaction with acetic anhydride, afforded **2j** in 83% yield, as shown in Scheme 3.

Finally, the imidazoquinoline **2q** was prepared following a three-step procedure (Scheme 4). The known dichloroquinoline **7j**[10] was subjected to nucleophilic substitution of the chlorine atom at position 7 by cyclohexylamine in dimethylformamide, to afford the aminoquinoline **9** (48% yield). The latter, after reduction of the nitro group by catalytic hydrogenation, gave the diamino derivative **10** in 88% yield. **10** was then cyclized to the imidazoquinoline **2q** (25% yield) as described above for derivatives **2d–h** and **2k–p**.



1a-l

a) R_1=R_2=R_3=R_4=H;
b) R_1=Cl, R_2=R_3=R_4=H;
c) R_1=Cl, R_2=OH, R_3=R_4=H,
d) R_1=Cl, R_2=OH, R_3=H, R_4= N_1-OH;
e) R_1=Cl, R_2=R_4=H, R_3=NO_2;
f) R_1=Cl, R_2=H, R_3=NO_2, R_4=N_1-OH;
g) R_1=Cl, R_2=R_3=H, R_4=N_1-CH_2CN;
h) R_1=Cl, R_2=R_3=H, R_4=N_3-CH_2CN;
i) R_1=Cl, R_2=R_3=H, R_4=N_1-$COCH_3$;
j) R_1=Cl, R_2=R_3=H, R_4=N_1-OH;
k) R_1=Cl, R_2=R_3=H, R_4=N_1-CH_2COCH_2Cl;
l) R_1=Cl, R_2=R_3=H, R_4=N_3-CH_2COCH_2Cl.



1m

Figure 1. Triazolo[4,5-*g*]quinolines **1a–m**.

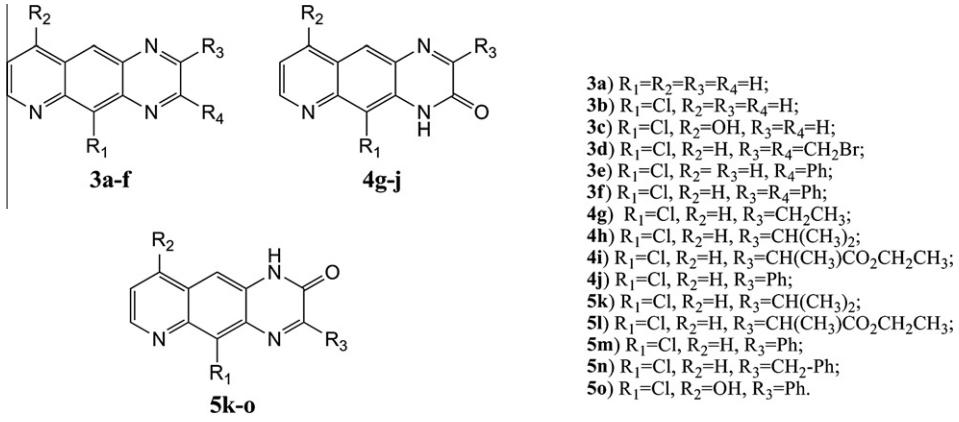
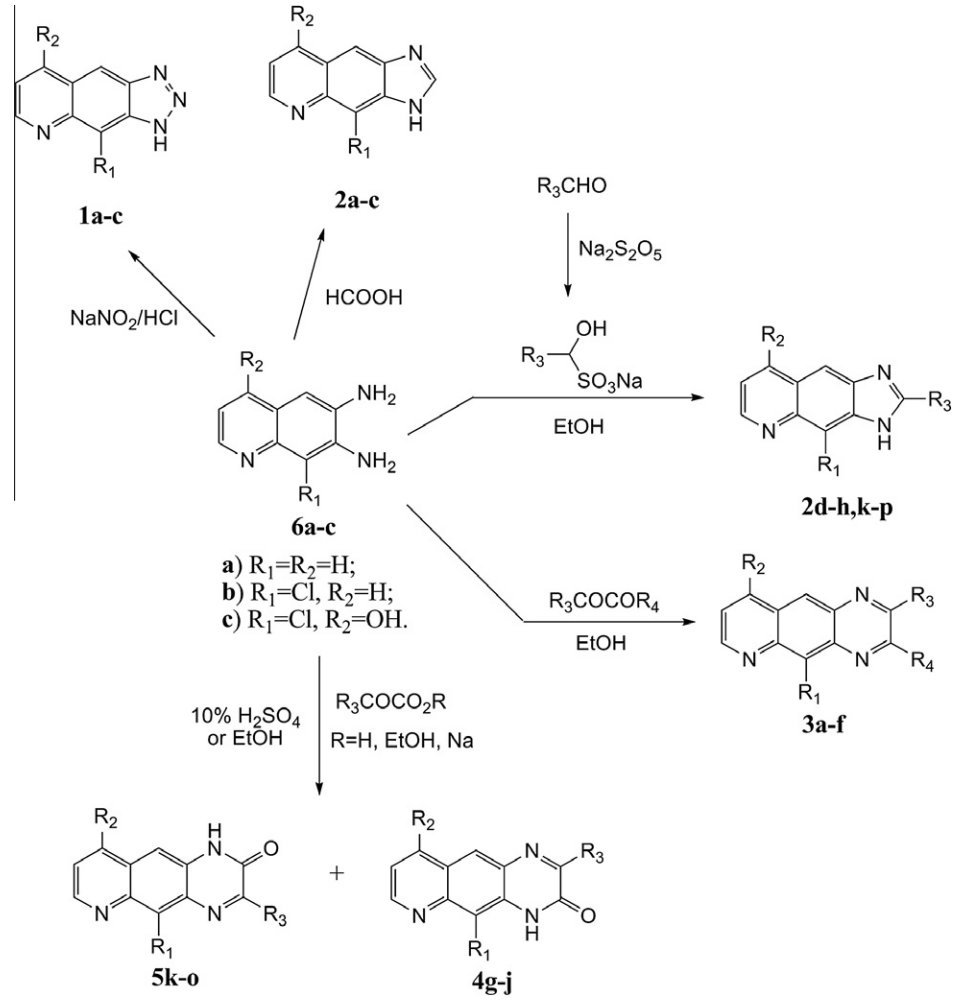


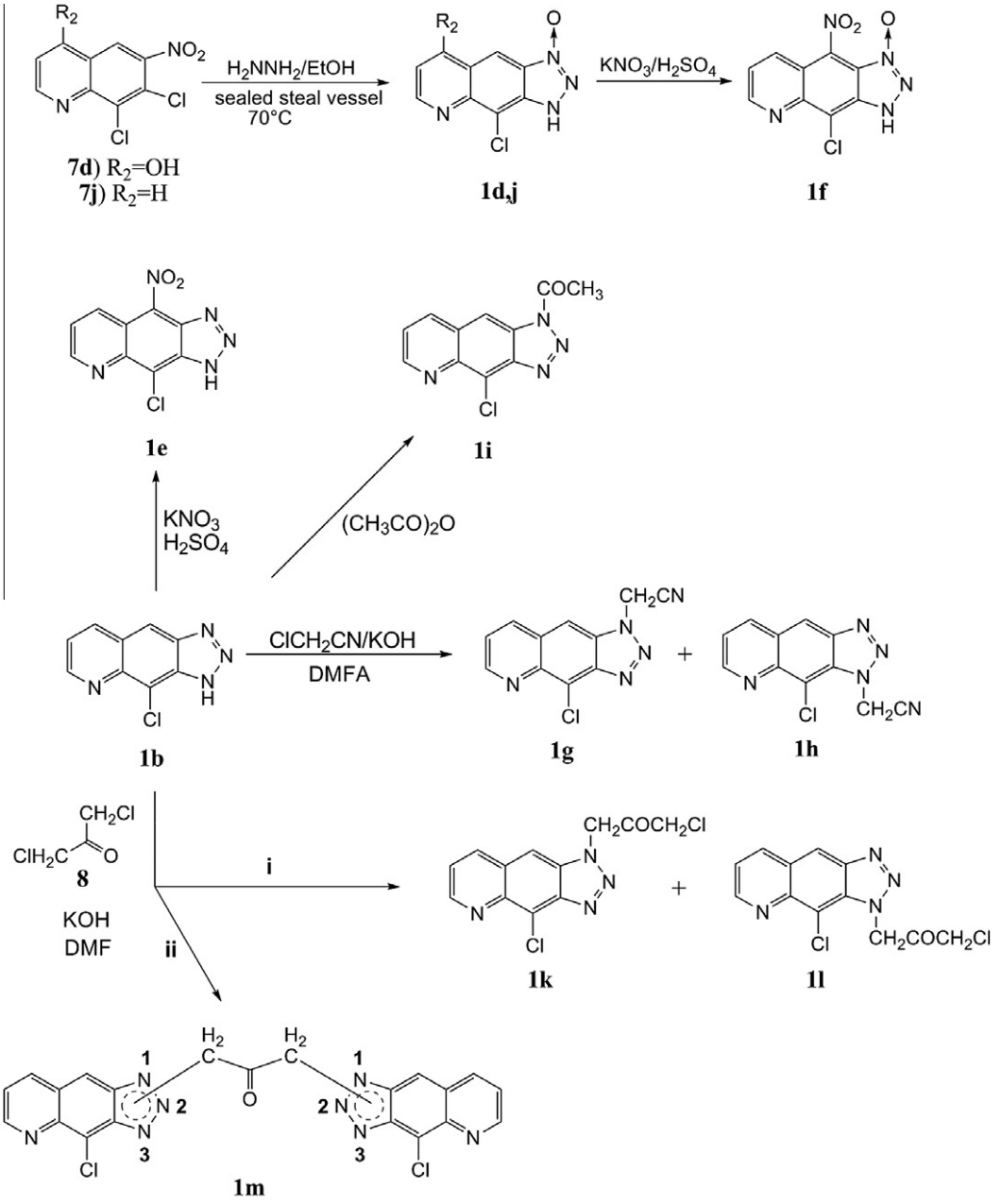
Figure 3. Pyrido[2,3-*g*]quinoxalines **3a–f**, **4g–j** and **5k–o**.



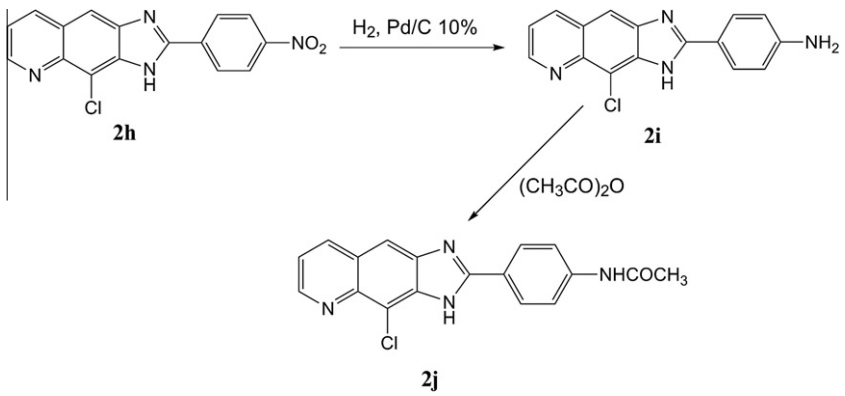
Scheme 1. Synthesis of derivatives **1a–c**, **2a–h,k–p**, **3a–f**, **4g–j** and **5k–o**.

3. Results and discussion

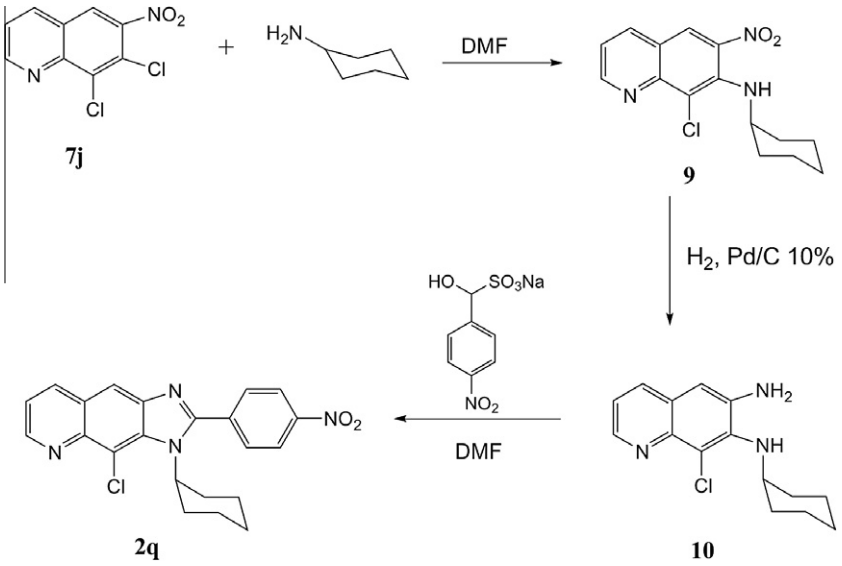
Triazolo[4,5-*g*]quinolines, imidazo[4,5-*g*]quinolines and pyrido[2,3-*g*]quinoxalines were tested in cell-based assays against representative members of several virus families (Tables 1–3). Among ssRNA$^+$ viruses, were: human immunodeficiency virus type-1 (HIV-1) (*Retroviridae*), BVDV and YFV (*Flaviviridae*), two *Picornaviridae*, human enterovirus B (coxsackie virus B5, CVB-5) and human enterovirus C (polio virus type-1, Sb-1). Among ssRNA$^-$ viruses, were: human respiratory syncytial virus (RSV) (*Paramyxoviridae*) and vesicular stomatitis virus (VSV) (*Rhabdoviridae*). Among dsRNA viruses, we tested reovirus type-1 (Reo-1) (*Reoviridae*). Finally,



Scheme 2. Synthesis of triazoloquinolines **1d**–**m**. Reaction conditions: (i) **1a** and **8** in 1:1 molar ratio; (ii) **1a** and **8** in 2:1 molar ratio.



Scheme 3. Synthesis of imidazoquinolines **2i**–**j**.



Scheme 4. Synthesis of the imidazoquinoline **2q**.

representatives of two DNA virus families were also included: vaccinia virus (VV) (*Poxviridae*) and human herpesvirus 1 (herpes simplex type-1, HSV-1) (*Herpesviridae*).

The cytotoxicity was evaluated in parallel with the antiviral activity. Efavirenz, 2′-C-methyl-guanosine, 2′-C-methyl-cytidine, 2′-C-ethynyl-cytidine, 6-azauridine, mycophenolic acid and acyclovir were used as reference inhibitors.

Among the compounds endowed with selective antiviral activity, **2d**, **2e**, **2i**, **2m**, **2q**, **5l**–**n** showed cytotoxicity for exponentially growing MT-4 cells at concentrations of 8–29 μM. On the other hand, only compounds **2i** and **2d**, **2e**, **2i**, **2o** proved moderately cytotoxic (26–42 μM) for cells (MDBK and BHK, respectively) in stationary growth.

As far as the antiviral activity is concerned, none of title compounds turned out active against HIV-1. Some showed activity against YFV, Sb-1, VSV, VV and HSV-1, but nothing more than a first indication for the further search for new, more potent leads. Moderate, although selective, activity was shown by the imidazoquinolines **2d**, **2m** and **2q** against CVB-5 (EC_{50} range = 3–19 μM), and **2d** and **2e** against Reo-1 (EC_{50} range = 10–13 μM), as well as by pyridoquinoxalines **4g** and **5m** against RSV (EC_{50} range = 12–18 μM). Viceversa, compounds belonging to all classes proved to be endowed with interesting anti-BVDV activity. In this regard, none of the three unsubstituted tricyclic systems (compounds **1a**, **2a** and **3a**) exhibited anti-BVDV activity, thus evidenciating the importance of a side chain. Triazolo[4,5-*g*]quinoline derivatives (Table 1) resulted less active than compounds belonging to the other two classes. Only compounds **1e** and **1g**, bearing a nitro group at position 9 and an acetonitrile at *N*-1, respectively, exhibited selective, although not very potent, anti-BVDV activity. Noteworthy, **1m** [the mixture 1,3-bis-(4-chloro-1*H*(3*H*)-triazolo[4,5-*g*]quinoline)propan-2-one in 1:1 ratio, obtained by dimerization of the inactive 4-chlorotriazolo[4,5-*g*]quinoline **1b** and 1,3-dichloroacetone] showed potent anti-BVDV activity (EC_{50} = 1 ± 0.09 μM) associated with lack of cytotoxicity (CC_{50} >100 μM; S.I. >100) for MDBK and the other cell lines.

The imidazo[4,5-*g*]quinoline class (Table 2) showed a large number of derivatives (**2d**–**j**, **o**) endowed with significant anti-BVDV activity. The most potent compound, **2h**, showed an EC_{50} of 1.2 ± 0.07 μM and total lack of cytotoxicity (CC_{50} >100 μM; S.I. >100) for MDBK and the other cell lines. Structure/activity relationship (SAR) studies indicate that potent activity is associated with the presence of a chlorine atom at position 4 (compare **2e** with **2d**), a phenyl at position 2 (compare **2e** with **2o**, and **2d** with **2m** and **2n**) and no substituents at both positions 8 (compare **2e** with **2p**) and 3 (compare **2h** with **2q**). Furthermore, electron withdrawing substituents on the phenyl moiety confer better activity than releasing substituents (compare **2h** with **2i**, **2j** and **2d** with **2f**, **2k**, **2l**).

As far as pyrido[2,3-*g*]quinoxalines are concerned (Table 3), compounds **3e**, **4g**–**j** and **5l**–**n** showed activity against BVDV in the range 2.6–19 μM. SAR studies of this linear *N*-tricyclic system suggest that for this nucleus, as well as for the imidazoquinoline nucleus, a phenyl or benzyl side chain is generally more favorable than an aliphatic substituent (compare **4j**, **5m** and **5n** with **4g**–**i**, **5k** and **5l**). Among the latter compounds, 2-oxo-substituted derivatives exhibited higher activity than unsubstituted counterparts (compare **4j** and **5m** with **3e**). Phenyl substituents at both positions 2 and 3, as well as an hydroxy substituent at position 9, had negative effects on the anti-BVDV activity (compare **3e** with **3f** and **5m** with **5o**, respectively).

Due to their potent activity against BVDV, and to the fact that this virus is widely used as a surrogate for hepatitis C virus (HCV),[18] the bis-triazoloquinoline **1m**, the imidazoquinoline **2h** and the pyridoquinoxalines **5m** (chosen because of its dual activity against BVDV and RSV) and **5n** were tested against HCV in a subgenomic replication assay that allows viral replication in a human hepatoma cell line (GS4.1). As shown in Table 4, **2h** also inhibited the HCV replication (EC_{50} = 3.1 ±0.3 μM); however, being significantly cytotoxic for GS4.1 cells (CC_{50} = 12 ± 0.9 μM), its selectivity index (S.I. = 4) was rather low. On the contrary, **1m**, **5m** and **5n** proved inactive in the replicon system.

To gain more insights into the target of quinoline tricyclic derivatives, the above leads were evaluated in enzyme assays performed with the recombinant BVDV and HCV RdRps. In these assays, the rate of RNA product formation was monitored by using a non-isotopic detection method based on the capability of the dye RiboGreen to detect RNA transcripts by fluorescence. As shown in Table 4 and in dose–response curves (Fig. 4), compound **2h**, in addition to inhibiting the BVDV RdRp (IC_{50} = 0.06 μM), also inhibited the HCV enzyme (IC_{50} = 8.0 μM), although with a 100-fold lower potency. Viceversa, **1m**, **5m** and **5n** only inhibited the BVDV recombinant enzyme (Table 4).

The higher cytotoxicity of **2h** for GS4.1 cells, compared to that shown for the other cell lines used in the antiviral assays, prompted us to evaluate its cytotoxicity also for a panel of human

Table 1
Cytotoxicity and antiviral activity of triazoloquinolines (**1a–m**) against ssRNA$^+$ (HIV-1, BVDV, YFV, CBV-5, Sb-1), ssRNA$^-$ (RSV, VSV), dsRNA (Reo-1) and DNA (VV, HSV-1) viruses

Compd	MT-4 CC_{50}[a]	HIV-1 EC_{50}[b]	MDBK CC_{50}[c]	BVDV EC_{50}[d]	BHK CC_{50}[e]	YFV EC_{50}[f]	Reo-1 EC_{50}[g]	Vero-76 CC_{50}[h]	CVB-5 EC_{50}[i]	Sb-1 EC_{50}[j]	RSV EC_{50}[k]	VSV EC_{50}[l]	VV EC_{50}[m]	HSV-1 EC_{50}[n]
1a	40	>40	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
1b	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
1c	>100	>100	>100	>100	>100	>100	$\geqslant$100	>100	>100	>100	>100	>100	>100	>100
1d	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	ND
1e	>100	>100	>100	**23**	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
1f	>100	>100	>100	>100	>100	>100	ND	>100	>100	>100	>100	>100	>100	ND
1g	62	>62	>100	**23**	>100	>100	>100	>100	>100	>100	>100	>100	>100	51
1h	100	>100	>100	100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
1i	46	>46	30	>30	72	>72	ND	>100	>100	>100	>100	>100	>100	>100
1j	>100	>100	>100	>100	>100	>100	ND	>100	86	>100	>100	>100	>100	>100
1k	21	>21	53	>53	85	>85	>85	>100	>100	>100	>100	>100	>100	>100
1l	23	>23	21	>21	65	>65	>65	>100	>100	>100	>100	>100	>100	>100
1m	>100	>100	>100	**1.0**	>100	>100	50	>100	>100	>100	>100	>100	ND	>100
EFV	40	**0.002**												
2′-MeGuo			>10	**1.1**	>10	**1.9**								
2′-MeCyt					>100		**16**							
2′-EtynCyt								>100	**27**	**23**				
6-AUdr								$\geqslant$12.5			**1.2**			
MA								$\geqslant$12.5					**1.5**	
ACG								>100						**3**

ND = Not determined.
EFV = Efavirenz; 2′-MeGuo = 2′-C-methyl-guanosine; 2′-MeCyt = 2′-C-methyl-cytidine; 2′-C-EtyCyt = 2′-C-ethynyl-cytidine; 6-AU = 6-aza-uridine; MA = mycophenolic acid; ACG = acycloguanosine.
Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%.
[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 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 cells 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 Polio-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.

Table 2
Cytotoxicity and antiviral activity of imidazoquinolines (**2a**–**q**) against ssRNA$^+$ (HIV-1, BVDV, YFV, CBV-5, Polio-1), ssRNA$^-$ (RSV, VSV), dsRNA (Reo-1) and DNA (VV, HSV-1) viruses

Compd	MT-4 CC_{50}[a]	HIV-1 EC_{50}[b]	MDBK CC_{50}[c]	BVDV EC_{50}[d]	BHK CC_{50}[e]	YFV EC_{50}[f]	Reo-1 EC_{50}[g]	Vero-76 CC_{50}[h]	CVB-5 EC_{50}[i]	Sb-1 EC_{50}[j]	RSV EC_{50}[k]	VSV EC_{50}[l]	VV EC_{50}[m]	HSV-1 EC_{50}[n]
2a	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	ND	>100	>100	>100
2b	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	ND	>100	>100	>100
2d	28	>28	80	**10**	40	>40	**13**	>100	**19**	37	>100	>100	>100	>100
2e	29	>29	65	**4.0**	35	>35	**10**	>100	36	53	>100	>100	>100	>100
2f	100	>100	>100	**18**	>100	>100	>100	>100	43	>100	>100	>100	>100	>100
2g	>100	>100	90	**20**	>100	>100	67	95	35	>95	>95	>95	>95	ND
2h	>100	>100	>100	**1.2**	>100	>100	>100	>100	>100	>100	>100	>100	>100	44
2i	15	>15	45	**10**	42	>42	>42	>100	78	>100	>100	>100	>100	>100
2j	>100	>100	>100	**11**	>100	>100	51	>100	>100	>100	>100	>100	>100	>100
2k	14	>14	11	⩾11	32	>32	>32	75	47	>75	>75	>75	>75	>75
2l	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
2m	8	>8	⩾100	60	20	>20	>20	80	**3**	>80	>80	>80	>80	>80
2n	6	>6	18	>18	>100	>100	50	>100	>100	>100	>100	>100	>100	ND
2o	57	>57	73	**19**	26	>26	>26	>100	43	80	>100	⩾100	⩾100	>100
2p	>100	>100	>100	>100	75	>75	>75	>100	>100	>100	>100	>100	>100	>100
2q	23	>23	>100	39	>100	>100	>100	>100	**14**	>100	>100	>100	>100	ND
EFV	40	**0.002**												
2′-MeGuo			>10	**1.1**	>10	**1.9**								
2′-MeCyt					>100		**16**							
2′-EtyCyt								>100	**27**	**23**				
6-AUdr								⩾12.5			**1.2**			
MA								⩾12.5					**1.5**	
ACG								>100						**3**

*Cytotoxicity data were higher due to longer incubation times.
ND = Not determined.
EFV = Efavirenz; 2′-MeGuo = 2′-C-methyl-guanosine; 2′-MeCyt = 2′-C-methyl-cytidine; 2′-C-EtyCyt = 2′-C-ethynyl-cytidine; 6-AU = 6-aza-uridine; MA = mycophenolic acid; ACG = acycloguanosine.
Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%.
[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 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 cells 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 Polio-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.

Table 3
Cytotoxicity and antiviral activity of pyridoquinoxalines (**3a**–**5o**) against ssRNA$^+$ (HIV-1, BVDV, YFV, CBV-5, Polio-1), ssRNA$^-$ (RSV, VSV), dsRNA (Reo-1) and DNA (VV, HSV-1) viruses

Compd	MT-4 CC_{50}[a]	HIV-1 EC_{50}[b]	MDBK CC_{50}[c]	BVDV EC_{50}[d]	BHK CC_{50}[e]	YFV EC_{50}[f]	Reo-1 EC_{50}[g]	Vero-76 CC_{50}[h]	CVB-5 EC_{50}[i]	Sb-1 EC_{50}[j]	RSV EC_{50}[k]	VSV EC_{50}[l]	VV EC_{50}[m]	HSV-1 EC_{50}[n]
3a	>100	>100	>100	>100	>100	>100	ND	>100	>100	>100	ND	>100	>100	>100
3b	82	>82	6.5	>6.5	70	>70	>70	90	50	>90	>90	>90	57	90
3c	42	>42	63	>63	85	>85	>85	90	>90	>90	>90	>90	>90	90
3d	16	>16	20	>20	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
3e	>100	>100	>100	**18**	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
3f	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
4g	51	>51	75	**6.5**	95	>95	36	100	>100	>100	**18**	35	>100	>100
4h	48	>48	100	**5.0**	>100	>100	47	>100	>100	>100	38	62	>100	>100
4i	59	>59	>100	**19**	>100	>100	>100	>100	>100	⩾100	>100	⩾100	>100	⩾100
4j	61	>61	>100	**5.0**	>100	>100	100	>100	>100	>100	>100	100	39	>100
5k	100	>100	>100	100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
5l	11	>11	>100	**10**	75	50	>75	>100	>100	>100	62	>100	>100	>100
5m	22	>22	>100	**11**	>100	>100	>100	>100	>100	>100	**12**	>100	>100	>100
5n	15	>15	>100	**2.6**	>100	>100	>100	88	>88	>88	>88	>88	>88	>88
5o	>100	>100	>100	100	>100	>100	>100	⩾100	>100	>100	>100	>100	>100	⩾100
EFV	40	**0.002**												
2′-MeGuo			>10	**1.1**	>10	**1.9**								
2′-MeCyt					>100		**16**							
2′-EtyCyt								>100	**27**	**23**				
6-AUdr								⩾12.5			**1.2**			
MA								⩾12.5					**1.5**	
ACG								>100						**3**

() Selectivity index.
ND = Not determined.
EFV = Efavirenz; 2′-MeGuo = 2′-C-methyl-guanosine; 2′-MeCyt = 2′-C-methyl-cytidine; 2′-C-EtyCyt = 2′-C-ethynyl-cytidine; 6-AU = 6-aza-uridine; MA = mycophenolic acid; ACG = acycloguanosine.
Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%.

[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 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 cells 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 Polio-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.

Table 4
Comparative activity of **1m**, **2h**, **5m** and **5n** against BVDV and HCV in cell-based and recombinant RdRp assays

Compd	Cell-based[a]				RdRp[a]	
	BVDV		HCV-1b		BVDV	HCV-1b
	CC_{50}[b] (μM)	EC_{50}[c] (μM)	CC_{50}[d] (μM)	EC_{50}[e] (μM)	IC_{50}[f] (μM)	
1m	>100	1.0 ± 0.09	>100	>100	0.4 ± 0.05	>10
2h	>100	1.2 ± 0.07	12 ± 0.9	3.1 ± 0.3	0.06 ± 0.01	8.0 ± 0.8
5m	>100	11 ± 1.1	11 ± 1.3	>11	1.0 ± 0.3	>10
5n	>100	2.6 ± 0.3	65 ± 9.0	>65	1.2 ± 0.2	>10

[a] Data represent mean values for three independent determinations.
[b] Compound concentration (μM) required to reduce the viability of mock-infected MDBK cells by 50%, as determined by the MTT method.
[c] Compound concentration (μM) required to achieve 50% protection of MDBK cells from BVDV-induced cytopathogenicity, as determined by the MTT method.
[d] Compound concentration (μM) required to reduce the viability of GS4.1 cells by 50%, as described in the Section 5.
[e] Compound concentration (μM) required to achieve 50% protection of GS4.1 cells from cytopathogenicity, as described in the Section 5.
[f] Compound concentration (μM) providing 50% of inhibition, as described in the Section 5.

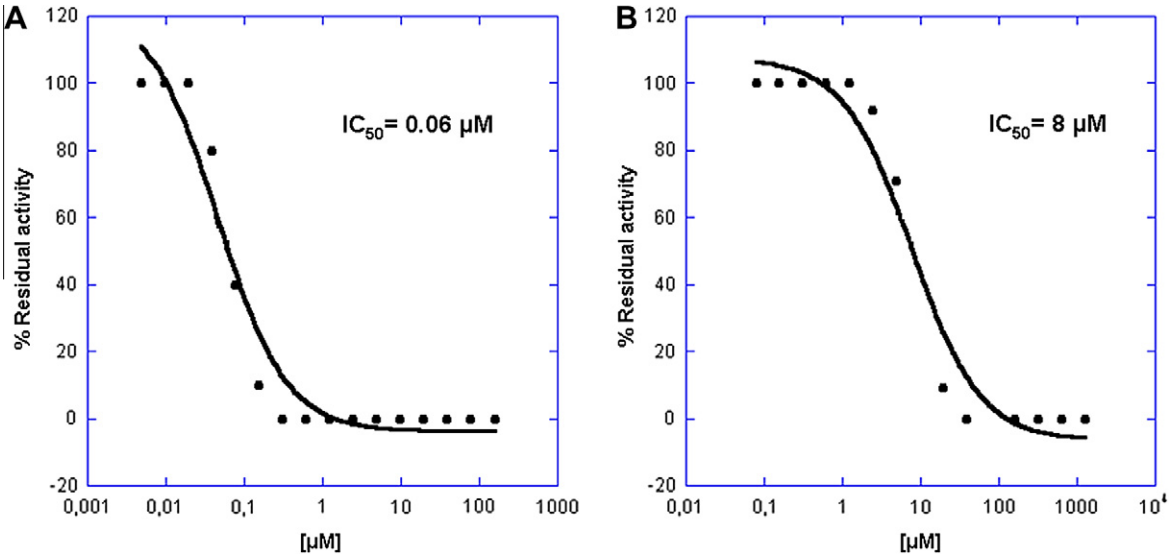


Figure 4. **2h** dose–response curves from enzyme assays with BVDV (A) and HCV (B) RNA-dependent RNA polymerases.

Table 5
Cytotoxicity of **1m**, **2h**, **5m** and **5n** against human leukaemia/lymphoma (CCRF-CEM, WIL-2NS, CCRF-SB), solid tumour (SK-MEL28, MCF7, SKMES-1, HepG2, DU145) and 'normal' (MRC5, CRL7065) cell lines

Compd	CCRF-CEM[b]	WIL-2NS[c]	CCRF-SB[d]	SK-MEL28[e]	MCF7[f]	SKMES-1[g]	HepG2[h]	DU145[i]	MRC-5[l]	CRL7065[m]
	CC_{50}[a]									
1m	40 ± 13	>100	53 ± 9	>100	ND	>100	>100	>100	ND	>100
2h	49 ± 1	>100	>100	>100	>100	>100	10.3 ± 0.9	>100	>100	>100
5m	16 ± 0.8	ND	19 ± 2	5.6 ± 1	7.6 ± 0.8	8.0 ± 3	8.0 ± 3	7.4 ± 2	ND	16 ± 2
5n	19 ± 3	33 ± 0.5	25 ± 5	>100	>100	>100	72 ± 8	⩾100	ND	>100
Camptotecin	0.003 ± 0.0005	0.005 ± 0.0007	0.004 ± 0.0005	0.07 ± 0.01	0.02 ± 0.01	0.03 ± 0.004	0.02 ± 0.01	0.015 ± 0.0004	0.2 ± 0.1	0.3 ± 0.1

ND = Not determined.
Data represent mean values for three independent determinations.
[a] Compound concentration (μM) required to reduce cell proliferation by 50%, as determined by the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication.
[b] $CD4^+$ human acute T-lymphoblastic leukaemia.
[c] Human splenic B-lymphoblastoid cells.
[d] Human acute B-lymphoblastic leukemia.
[e] Human skin melanoma.
[f] Human breast adenocarcinoma.
[g] Human lung squamous carcinoma.
[h] Human hepatocellular carcinoma.
[i] Human prostate carcinoma.
[l] Human lung fibroblasts.
[m] Human foreskin fibroblasts.

cells derived from both haematological and solid tumours or from normal tissues. The cytotoxicity of **1m**, **5m** and **5n** was also evaluated. As shown in Table 5, **2h** showed no cytotoxicity for the various cell lines tested, exception made for HepG-2, a human hepatocellular carcinoma cell line. In this case, **2h** showed a CC_{50} value of 10.3 ± 0.9 μM, comparable to that shown for GS4.1 cells,

suggesting that it targets some structure or metabolic step peculiar to hepatoma cells. While **5m** proved equally cytotoxic for all cell lines tested, **1m** and **5n** showed only moderate antiproliferative activity.

4. Conclusions

As part of an ongoing project to develop new therapeutic agents against *Flaviviridae*, we here report the cytotoxicity and the antiviral activity of three new classes of linear *N*-tricyclic systems derived by expansion of the quinoline nucleus with 1,2,3-triazole, imidazole or pyrazine, that originate triazolo[4,5-*g*]quinolines, imidazo[4,5-*g*]quinolines and pyrido[2,3-*g*]quinoxalines, respectively.

When tested against representatives of ssRNA$^+$, ssRNA$^-$, dsRNA and DNA virus families, quinoline derivatives proved to be mainly active against BVDV, though some of them showed selective, although not very potent, activity against an additional ssRNA$^+$ virus (CVB-5), a dsRNA virus (Reo-1) and a ssRNA$^-$ virus (RSV).

The derivatives endowed with anti-BVDV activity were more numerous among imidazoquinolines and pyridoquinoxalines than among triazoloquinolines. Nevertheless, each class contained at least one potent and selective derivative. Noteworthy, **1m**, **2h** and **5n** proved active against BVDV with potencies comparable to those of 2′-methyl-nucleosides, such as 2′-C-methyl-guanosine (see Tables 1–3), which target the RdRp of *Flaviviridae* and other ssRNA$^+$ and dsRNA virus families.[21]

Of particular interest is the fact that the imidazoquinoline **2h**, endowed with the more potent and selective activity against BVDV, also proved active, although moderately selective, against HCV. In fact, **2h** proved less selective in the replicon assay because of a cytotoxicity specifically directed against the GS4.1 cells. Moreover, in enzyme assays **2h** proved 100 fold less potent against the HCV rather than the BVDV recombinant RdRp, a result suggesting that, although with different efficiency, **2h** targets the viral RdRps.

In vitro selection of BVDV resistant mutants and in silico docking studies are in progress to clarify the molecular basis of drug resistance to the above leads and their interaction modes with the RdRps.

Since test compounds represent attractive leads for the development of antiviral agents against *Pestiviruses*, and possibly HCV, one or more leads belonging to each quinoline class will be further modified to obtain more active derivatives. In particular, bis-triazoloquinolines (**1m**) could be developed through the synthesis of new, more potent dimers, while imidazoquinolines (**2e**,**h**) and pyridoquinoxalines (**4h**,**j** and **5m**,**n**) could be developed through the introduction of different electron withdrawing substituents on the phenyl moiety.

5. Experimental section

5.1. Synthetic methods

Melting points were uncorrected and were taken in open capillaries in a Digital Electrothermal IA9100 melting point apparatus. ^{1}H NMR spectra were recorded on a Varian XL-200 (200 MHz) instrument, using TMS as internal standard. The chemical shift values are reported in ppm (δ) and coupling constants (J) in Hertz (Hz). Signal multiplicities are represented by: s (singlet), d (doublet), dd (double doublet), t (triplet), q (quadruplet) and m (multiplet). MS spectra were performed on a combined Liquid Chromatograph-Agilent 1100 series Mass Selective Detector (MSD). Column chromatography was performed using 70–230 mesh (Merck Silica Gel 60). Light petroleum refers to the fraction with bp 40–60 °C. The progress of the reactions and the R_f were monitored by TLC using Merck F-254 commercial plates. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values.

5.1.1. Starting materials, intermediates and known compounds

Aldehydes, α,β-dicarbonyls, α-ketocarboxylic derivatives, chloroacetonitrile, acetic anhydride, 1,3-dichloroacetone, cyclohexylamine, and inorganic reagents were commercially available. Dichloroquinolines **7d**,**j** and 6,7-diaminoquinoline **6a**–**c** intermediates were prepared following the procedure previously described.[10,15] Bertagnini's salts were obtained reacting the aldehydes with $Na_2S_2O_5$ in hydro alcoholic solution.[19] Analytical and spectroscopical data of the known triazolo[4,5-*g*]quinolines **1a**–**j**, imidazoderivatives **2a**–**c** and pyridoquinoxalines **3a**–**d**, **4g**–**i** and **5k**–**l** have been previously reported.[12–14,16]

5.1.2. General procedure for preparation of triazolo[4,5-*g*] quinolines (1a–c)

A solution of sodium nitrite (3 mmol) in water (3 mL) was added dropwise to a stirred solution of 4 mmol of the opportune diamine **6a**–**c** in 2 M hydrochloric acid solution (10 mL). After the addition was complete, the stirring was continued for an additional 12 h, when the pH was adjusted to 6 with concentrated ammonia aqueous solution. The resulting precipitate was filtered off and washed with water obtaining the triazolo[4,5-*g*]quinolines **1a**–**c**[12,14] in 42–44% yield.

5.1.3. General procedure for preparation of triazolo[4,5-*g*] quinolines (1d,j)

A solution of 4 mmol of the opportune 7,8-dichloro-6-nitroquinolines **7d**,**j** in ethanol (120 mL) and hydrazine hydrate (2.5 mL) were heated in a sealed steal vessel at 70 °C for 90 h. On cooling, resulting precipitate was collected, washed with ethanol and dried in a oven to give **1d**[14] in 56% yield and **1j**[16] in 90% yield.

5.1.4. General procedure for preparation of triazolo[4,5-*g*] quinolines (1e,f)

A solution of 3 mmol of the opportune triazoloquinolines **1b**,**j** in 4 mL of concentrated sulphuric acid was added dropwise at room temperature of a solution of potassium nitrate (9 mmol) in concentrated sulfuric acid (5 mL). Then the temperature was raised up to 50 °C for 3 h under continuous stirring. The mixture was poured into crushed ice (50 g) when the pH was adjusted to 9 with concentrated ammonia aqueous solution. The formed solid collected was washed with water and dried in a oven to give **1e** in 90% yield and **1f** in 56% yield.[16]

5.1.5. General procedure for preparation of triazolo[4,5-*g*] quinolines 1g–i,k–m

A solution of 5 mmol of **1b** in dry dimethyl formamide (5 mL) was added of KOH pellets (5.5 mmol) and stirred to room temperature until complete dissolution. To this solution 10 mmol of acetonitrile in 5 mL of dimethyl formamide; 50 mmol of 1,3-dichloroacetone in 10 mL of dimethyl formamide; 2.5 mmol of 1,3-dichloroacetone in 10 mL of dimethyl formamide; or 10 mL of acetic anhydride were alternatively added and the mixture heated under stirring at 120 °C for 22 h, 120 °C for 24 h, 120 °C for 120 h or 100 °C for 4 h respectively. On cooling, the mixture reactions were poured into 150 mL of rushed ice. The solid obtained in each reaction was collected, washed with water and dried in a oven to give: a mixture of **1g** and **1h** (70% yield in about 1:1 ratio); a mixture of **1k** and **1l** (70% yield in about 1:1 ratio); the mixture **1m** (35% yield in about 1:1 ratio) or **1i** (70% yield), respectively. Mixture **1g**/**1h** was chromatographed on silica gel column eluting with a mixture of diethyl ether/acetone in the ratio of 9:1, obtaining in the order **1h** in 33% yield and **1g** in 37% yield.[16] Mixture of **1k** and **1l** was chromatographed on silica gel column eluting with a mixture of diethyl ether/ethanol in the ratio of 95:5, obtaining in the order **1l** in 30% yield and **1k** in 40% yield. The assignment of the correct structure to the isomers with **1l**

and **1k** was achieved from NOE experiments. Compound identified as 1-chloro-3-(4-chloro-1*H*-triazolo[4,5-*g*]quinolin-1-yl)propan-2-one (**1k**) showed a negative NOE effect on H-9 when the singlet at 6.01 ppm was irradiated. No NOE effect was instead observed for the other compound when the methylene at 6.31 ppm was irradiated, according to the structure of 1-chloro-3-(4-chloro-3*H*-triazolo[4,5-*g*]quinolin-3-yl)propan-2-one (**1l**). Mixture 1,3-bis-(4-chloro-1*H*(3*H*)-triazolo[4,5-*g*]quinoline)propan-2-one in 1:1 ratio (**1m**) was not resolved by chromatography. Compound (**1i**) taken up with diethyl ether was crystallized by ethanol.[16]

5.1.5.1. 1-Chloro-3-(4-chloro-3*H*-triazolo[4,5-*g*]quinolin-3-yl) propan-2-one (1l). Mp >300 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 9.13 (dd, 1H, J = 4.0 and 1.4 Hz, H-6), 8.64 (s, 1H, H-9), 8.52 (dd, 1H, J = 8.6 and 1.4 Hz, H-8), 7.53 (dd, 1H, J = 8.6 and 4.0 Hz, H-7), 6.31 (s, 2H, N–CH_2), 4.59 (s, 2H, Cl–CH_2). LC/MS: 295 (M+H). Anal. Calcd for ($C_{12}H_8Cl_2N_4O$): C, 48.84; H, 2.73; N, 18.98; Cl, 24.03. Found: C, 48.51; H, 2.98; N, 18.79; Cl, 24.31.

5.1.5.2. 1-Chloro-3-(4-chloro-1*H*-triazolo[4,5-*g*]quinolin-1-yl) propan-2-one (1k). Mp >300 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 9.11 (dd, 1H, J = 4.2 and 1.6 Hz, H-6), 8.35 (dd, 1H, J = 8.6 and 1.6 Hz, H-8), 7.93 (s, 1H, H-9), 7.54 (dd, 1H, J = 8.6 and 4.2 Hz, H-7), 6.01 (s, 2H, N–CH_2), 4.44 (s, 2H, Cl–CH_2). LC/MS: 295 (M+H). Anal. Calcd for ($C_{12}H_8Cl_2N_4O$): C, 48.84; H, 2.73; N, 18.98; Cl, 24.03. Found: C, 49.07; H, 3.01; N, 19.30; Cl, 23.80.

5.1.5.3. Mixture 1,3-bis-(4-chloro-1*H*(3*H*)-triazolo[4,5-*g*]quinoline)propan-2-one in 1:1 ratio (1m). Mp 245 °C (dec.). ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 9.10–8.90 (m, 4H), 8.60–8.20 (m, 8H), 7.40–7.60 (m, 4H), 6.51–6.34 (m, 8H). LC/MS: 463 (M+H). Anal. Calcd for ($C_{21}H_{12}Cl_2N_8O$): C, 54.44; H, 2.61; N, 24.19; Cl, 15.31. Found: C, 54.71; H, 2.89; N, 24.50; Cl, 15.02.

5.1.6. General procedure for preparation of imidazo[4,5-*g*] quinolines 2a–c

A mixture of the diamines **(6a–c)** (5 mmol) and formic acid (10 g) was stirred at 100 °C for 2 h. After cooling to rt, the solution was neutralized with 50% sodium hydroxide aqueous solution until pH 5. The precipitate obtained was filtered off, washed with ethanol and dried to give the imidazo[4,5-*g*]quinolines **2a–c** in 80–90% yield.[12,14]

5.1.7. General procedure for preparation of imidazo[4,5-*g*] quinolines 2d–h,k–p

Compounds in title were synthesized by condensation of the diaminoquinolines **6a–c** (1–3 mmol) with an equimolar amount of the suitable activated aldehyde (Bertagnini's salt), in refluxed ethanol (10–30 mL) for 8 h or in DMF (dimethylformamide) at 130 °C for 4 h. On cooling, a small amount of inorganic compound was filtered off and the ethanol mother liquors were evaporated to dryness in vacuo. The solid residues, colored from orange to dark red, were purified by recrystallization from ethanol in good yields.

5.1.7.1. 2-Phenyl-3*H*-imidazo[4,5-*g*]quinoline (2d). Solvent ethanol. Time of reaction 8 h. Yield 60%. Mp 162–164 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 9.01 (d, 1H, J = 4.0 Hz, H-6), 8.85 (d, 1H, J = 8.0 Hz, H-8), 8.47 (s, 1H, H-4), 8.40–8.30 (m, 3H), 7.60–7.50 (m, 4H). LC/MS: 246 (M+H). Anal. Calcd for ($C_{16}H_{11}N_3$): C, 78.35; H, 4.52; N, 17.13. Found: C, 78.61; H, 4.32; N, 17.40.

5.1.7.2. 4-Chloro-2-phenyl-3*H*-imidazo[4,5-*g*]quinoline (2e). Solvent ethanol. Time of reaction 8 h. Yield 82%. Mp 185–187 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 8.96 (dd, 1H, J = 4.0 and 1.8 Hz, H-6), 8.47 (d, 1H, J = 8.0 Hz, H-8), 8.40–8.32 (m, 2H), 8.06 (s, 1H, H-9), 7.60–7.56 (m, 3H), 7.45 (1H, dd, J = 8.1 and 4.0 Hz, H-7). LC/MS: 282 (M+H). Anal. Calcd for ($C_{16}H_{10}ClN_3$): C, 68.70; H, 3.60; Cl, 12.67; N, 15.02. Found: C, 69.04; H, 3.38; Cl, 12.29; N, 15.31.

5.1.7.3. 2-(4-Chlorophenyl)-3*H*-imidazo[4,5-*g*]quinoline (2f). Solvent ethanol. Time of reaction 8 h. Yield 71%. Mp >300 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 8.83 (dd, 1H, J = 4.1 and 1.6 Hz, H-6), 8.39 (d, 1H, J = 8.0 Hz, H-8), 8.32–8.25 (m, 2H), 8.21 (s, 1H, H-9), 8.10 (s, 1H, H-4), 7.60–7.30 (m, 3H). LC/MS: 282 (M+H). Anal. Calcd for ($C_{16}H_{10}ClN_3$): C, 68.70; H, 3.60; Cl, 12.67; N, 15.02. Found: C, 68.41; H, 3.83; Cl, 12.31; N, 14.78.

5.1.7.4. 2-(4-Nitrophenyl)-3*H*-imidazo[4,5-*g*]quinoline (2g). Solvent ethanol. Time of reaction 8 h. Yield 32%. Mp >300 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 9.09 (dd, 1H, J = 4.0 and 1.6 Hz, H-6), 8.93 (d, 1H, J = 8.4 Hz, H-8), 8.61–8.45 (m, 6H), 8.30 (s, 1H, H-4), 8.10 (s, 1H, H-9), 7.74 (dd, 1H, J = 8.2 and 4.0 Hz, H-7). LC/MS: 293 (M+H). Anal. Calcd for ($C_{16}H_{10}N_4O_2$): C, 66.20; H, 3.47; N, 19.30. Found: C, 66.52; H, 3.18; N, 19.76.

5.1.7.5. 4-Chloro-2-(4-nitrophenyl)-3*H*-imidazo[4,5-*g*]quinoline (2h). Solvent DMF. Time of reaction 4 h. Yield 76%. Mp >300 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 8.97 (d, 1H, J = 4.0 Hz, H-6), 8.67 (d, 2H, J = 8.0 Hz, H-3′,5′), 8.42 (d, 2H, J = 8.0 Hz, H-2′,6′), 8.10 (d, 1H, J = 8.2 Hz, H-8), 8.02 (s, 1H, H-9), 7.49 (dd, 1H, J = 8.2 and 4.0 Hz, H-7). LC/MS: 327 (M+H). Anal. Calcd for ($C_{16}H_9ClN_4O_2$): C, 59.18; H, 2.79; Cl, 10.92; N, 17.25. Found: C, 59.51; H, 3.01; Cl, 11.25; N, 17.51.

5.1.7.6. 2-(2-Methoxyphenyl)-3*H*-imidazo[4,5-*g*]quinoline (2k). Solvent ethanol. Time of reaction 8 h. Yield 69%. Mp 231–233 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 9.10–9.03 (m, 2H, H-6,8), 8.68 (s, 1H, H-4), 8.54 (s, 1H, H-9), 8.51 (d, 2H, J = 8.2 Hz, H-6′), 7.85–7.60 (m, 2H, H-4′,5′), 7.28–7.16 (m, 2H, H-7,3′), 4.20 (s, 3H, CH_3). LC/MS: 276 (M+H). Anal. Calcd for ($C_{17}H_{13}N_3O$): C, 74.17; H, 4.76; N, 15.26. Found: C, 74.43; H, 5.01; N, 14.89.

5.1.7.7. 2-(3,4-Dichlorophenyl)-3*H*-imidazo[4,5-*g*]quinoline (2l). Solvent ethanol. Time of reaction 8 h. Yield 80%. Mp >300 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 8.94 (dd, 1H, J = 4.2 and 1.8 Hz, H-6), 8.66 (dd, 1H, J = 8.2 and 1.8 Hz, H-6′), 8.52 (d, 1H, J = 1.8 Hz, H-2′), 8.37 (s, 1H, H-4), 8.16 (dd, 1H, J = 8.2 and 1.8 Hz, H-8), 7.91 (s, 1H, H-9), 7.73 (d, 2H, J = 8.2 Hz, H-5′), 7.56 (dd, 1H, J = 8.2 and 4.2 Hz, H-7). LC/MS: 318 (M+H). Anal. Calcd for ($C_{16}H_9Cl_2N_3$ + 0.5H_2O): C, 59.46; H, 3.12; Cl, 21.94; N, 13.0. Found: C, 59.81; H, 3.12; Cl, 22.31; N, 13.09.

5.1.7.8. 2-(Biphenyl-4-yl)-3*H*-imidazo[4,5-*g*]quinoline (2m). Solvent ethanol. Time of reaction 8 h. Yield 75%. Mp 179–181 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 9.03 (d, 1H, J = 4.1 Hz, H-6), 8.94 (d, 1H, J = 8.0 Hz, H-8), 8.59 (s, 1H, H-4), 8.46 (d, 2H, J = 8.6 Hz, H-2′,6′), 7.41 (s, 1H, H-9), 7.84 (d, 2H, J = 8.6 Hz, H-3′,6′), 7.75–7.60 (m, 2H), 7.55–7.40 (m, 3H). LC/MS: 322 (M+H). Anal. Calcd for ($C_{22}H_{15}N_3$): C, 82.22; H, 4.70; N, 13.08. Found: C, 82.60; H, 4.45; N, 13.39.

5.1.7.9. 2-(4-Nitronaphthalen-1-yl)-3*H*-imidazo[4,5-*g*]quinoline (2n). Solvent ethanol. Time of reaction 8 h. Yield 70%. Mp >300 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 9.28 (m, 1H, H-5′), 9.01–8.84 (m, 2H, H-6,8′), 7.60–8.20 (m, 5H), 7.90–7.80 (m, 2H), 7.50 (dd, 1H, J = 8.0 and 4.0 Hz, H-7). LC/MS: 341 (M+H). Anal. Calcd for ($C_{20}H_{12}N_4O_2$): C, 70.58; H, 3.55; N, 16.46. Found: C, 70.23; H, 3.86; N, 16.28.

5.1.7.10. 4-Chloro-2-cyclohexyl-3*H*-imidazo[4,5-*g*]quinoline (2o). Solvent ethanol. Time of reaction 8 h. Yield 95%. Mp 146–148 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 8.96 (dd, 1H, J = 4.0

and 1.6 Hz, H-6), 8.45 (dd, 1H, *J* = 8.4 and 1.8 Hz, H-8), 8.00 (s, 1H, H-9), 7.47 (dd, 1H, *J* = 8.4 and 4.0 Hz, H-7), 3.10 (m, 1H, C1–H), 1.80–1.70 (m, 4H, C2–H_2 + C6–H_2), 2.15–1.43 (m, 6H, C3–H_2 + C4–H_2 + C5–H_2). LC/MS: 288 (M+H). Anal. Calcd for ($C_{16}H_{16}ClN_3$): C, 67.25; H, 5.64; Cl, 12.41; N, 14.70. Found: C, 66.92; H, 5.91; Cl, 12.52; N, 14.40.

5.1.7.11. 4-Chloro-2-phenyl-3*H*-imidazo[4,5-*g*]quinolin-8(5*H*)-one (2p). Solvent ethanol. Time of reaction 8 h. Yield 80%. Mp >300 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 8.36 (s, 1H, H-9), 8.32 (m, 2H, H-2′,6′), 8.42 (d, 2H, *J* = 8.0 Hz, H-2′,6′), 8.04 (d, 1H, *J* = 7.2 Hz, H-7), 7.60–7.50 (m, 3H, H-3′,4′,5′). 6.32 (d, 1H, *J* = 7.2 Hz, H-6). LC/MS: 298 (M+H). Anal. Calcd for ($C_{16}H_{10}ClN_3O$): C, 64.98; H, 3.41; Cl, 11.99; N, 14.21. Found: C, 64.63; H, 3.75; Cl, 11.81; N, 14.53.

5.1.8. General procedure for preparation of imidazo[4,5-*g*] quinolines (2i,j)

A suspension of 4-chloro-2-(4-nitrophenyl)-3*H*-imidazo[4,5-*g*]quinoline (**2h**) (4 mmol) and of 10% palladized charcoal (0.20 g) in ethanol (200 mL) was hydrogenated in Parr at 20–25 °C and 3 atm for 3 h. After filtration of the catalyst, the solvent was evaporated in vacuo. The solid residue, purified by crystallization from ethanol, afforded the imidazoquinoline **2i** in good yield. Compound **2i** (2 mmol) was suspended in acetic anhydride and the mixture heated at 90 °C for 1 h under stirring. Then the excess of solvent was removed in vacuo to give a residue that taken up with diethyl ether gave the acetyl derivative **2j** in good yield.

5.1.8.1. 4-(4-Chloro-3*H*-imidazo[4,5-*g*]quinolin-2-yl)aniline (2i). Yield 88%. Mp >300 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 8.89 (dd, 1H, *J* = 4.0 and 1.4 Hz, H-6), 8.38 (d, 1H, *J* = 8.2 Hz, H-8), 8.07 (d, 2H, *J* = 8.8 Hz, H-2′,6′), 8.07 (s, 1H, H-9), 7.41 (dd, 1H, *J* = 8.2 and 4.0 Hz, H-7), 6.75 (d, 2H, *J* = 8.8 Hz, H-3′,5′), 5.57 (s, 2H, NH_2). LC/MS: 297 (M+H). Anal. Calcd for ($C_{16}H_{11}ClN_4$): C, 65.20; H, 3.76; Cl, 12.03; N, 16.01. Found: C, 64.88; H, 4.02; Cl, 12.31; N, 18.87.

5.1.8.2. *N*-(4-(4-Chloro-3*H*-imidazo[4,5-*g*]quinolin-2-yl)phenyl) acetamide (2j). Yield 83%. Mp >300 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 10.32 (s, 1H, NHCO), 9.01 (d, 1H, *J* = 4.0 Hz, H-6), 8.72 (d, 1H, *J* = 8.2 Hz, H-8), 8.34 (d, 2H, *J* = 8.6 Hz, H-2′,6′), 8.09 (s, 1H, H-9), 7.86 (d, 2H, *J* = 8.6 Hz, H-3′,5′), 7.63 (dd, 1H, *J* = 8.2 and 4.0 Hz, H-7), 2.16 (s, 3H, CH_3). LC/MS: 339 (M+H). Anal. Calcd for ($C_{18}H_{13}ClN_4O$): C, 64.19; H, 3.89; Cl, 10.53; N, 16.64. Found: C, 63.91; H, 4.04; Cl, 10.88; N, 16.33.

5.1.9. Procedure for preparation of imidazo[4,5-*g*]quinoline (2q)

A solution of dichloroquinoline **7j**[10] (8 mmol) in DMF (30 mL) was added of cyclohexylamine (40 mmol) and heated at 100 °C under stirring for 8 h. On cooling, solvent and the excess of amine were evaporated to dryness in vacuo. The solid crude obtained was purified by chromatography on silica gel column, eluting with diethyl ether, to afford the intermediate **9**. A suspension of **9** (5 mmol) and of 10% palladized charcoal (0.20 g) in ethanol (150 mL) was hydrogenated in Parr at 20–25 °C and 3 atm for 2 h. After filtration of the catalyst, the solvent was evaporated in vacuo. The solid crude obtained was taken up with a mixture of diethyl ether and light petroleum (7:3) giving the diamine derivative **10** in good yield. Finally, diamine **10** was reacted with an equimolar amount of sodium hydroxy(4-nitrophenyl)methanesulfonate (Bertagnini's salt of the 4-nitrobenzaldehyde), in DMF at 130 °C for 4 h. On cooling, a small amount of inorganic compound was filtered off and the mother liquors were evaporated to dryness in vacuo. The solid residue was purified by recrystallization from ethanol to afford the imidazo[4,5-*g*]quinoline **2q** in low yield.

5.1.9.1. 8-Chloro-*N*-cyclohexyl-6-nitroquinolin-7-amine (9). Yield 48%. Oil dark red. ^{1}H NMR ($CDCl_3$): δ 9.07 (dd, 1H, *J* = 4.4 and 1.6 Hz, H-2), 8.38 (s, 1H, H-5), 8.15 (dd, 1H, *J* = 8.2 and 1.6 Hz, H-4), 7.38 (dd, 1H, *J* = 8.2 and 4.4 Hz, H-3), 5.88 (d, 1H, *J* = 8.6 Hz, NH-cyclohexyl), 3.66 (m, 1H, C1–H), 1.75–1.60 (m, 4H, C2–H_2 + C6–H_2), 1.38–1.17 (m, 6H, C3–H_2 + C4–H_2 + C5–H_2). LC/MS: 308 (M+H). Anal. Calcd for ($C_{15}H_{16}ClN_3O_2$): C, 58.92; H, 5.27; Cl, 11.60; N, 13.74. Found: C, 59.30; H, 5.09; Cl, 11.91; N, 13.50.

5.1.9.2. 8-Chloro-N^7-cyclohexylquinoline-6,7-diamine (10). Yield 92%. Oil red. ^{1}H NMR ($CDCl_3$): δ 8.74 (dd, 1H, *J* = 4.4 and 1.888 Hz, H-2), 7.87 (dd, 1H, *J* = 8.2 and 1.6 Hz, H-4), 7.24 (dd, 1H, *J* = 8.2 and 4.4 Hz, H-3), 6.87 (s, 1H, H-5), 5.02–4.75 (m, 3H, NH_2 + NH-cyclohexyl), 3.26 (m, 1H, C1–H), 1.95–1.62 (m, 6H, C2–H_2 + C5–H_2 + C6–H_2), 1.32–1.20 (m, 4H, C3–H_2 + C4–H_2). LC/MS: 278 (M+H). Anal. Calcd for ($C_{15}H_{18}ClN_3$): C, 65.33; H, 6.58; Cl, 12.86; N, 15.24. Found: C, 65.01; H, 6.81; Cl, 12.49; N, 15.00.

5.1.9.3. 4-Chloro-3-(cyclohexylmethyl)-2-(4-nitrophenyl)-3*H*-imidazo[4,5-*g*]quinoline (2q). Yield 25%. Mp 159–161 °C. ^{1}H NMR ($CDCl_3$): δ 9.10 (dd, 1H, *J* = 4.2 and 1.8 Hz, H-6), 8.44 (d, 2H, *J* = 8.8 Hz, H-3′,5′), 8.37 (dd, 1H, *J* = 8.4 and 1.6 Hz, H-8), 8.20 (s, 1H, H-9), 7.92 (d, 2H, *J* = 8.8 Hz, H-2′,6′), 7.47 (dd, 1H, *J* = 8.6 and 4.2 Hz, H-7), 2.18 (m, 1H, C1–H), 1.93–1.60 (m, 4H, C2–H_2 + C6–H_2), 1.34–1.12 (m, 6H, C3–H_2 + C4–H_2 + C5–H_2). LC/MS: 409 (M+H). Anal. Calcd for ($C_{22}H_{19}ClN_4O_2$): C, 64.94; H, 4.71; Cl, 8.71; N, 13.77. Found: C, 65.33; H, 4.44; Cl, 8.99; N, 13.39.

5.1.10. General procedure for preparation of pyrido[2,3-*g*] quinoxalines 3a–d

The known pyridoquinoxalines **3a–c** were obtained by slowly dropwise addition of glyoxal (60 mmol, 40% water), under stirring, to a refluxing solution of diaminoquinolines **6a–c** (4.0 mmol) in ethanol (20 mL) and the stirring continued for additional 2 h. After cooling the solution was evaporated and the solid residue purified by chromatography on silica gel column, eluting with a mixture of diethyl ether–acetone (7:3) to afford **3a–c** in 75–90% yield.[12,14] Condensation of **6c** with 1,4-dibromobutane (6 mmol) in the same condition, for 1 h, give **3d** in 45% yield.[14]

5.1.11. General procedure for preparation of pyrido[2,3-*g*] quinoxalines 3e–f, 4g–j and 5k–o

A solution (15 mL) of the 6,7-diamino-8-chloroquinoline (**6b**) (3 mmol) in 1 M H_2SO_4 was added of 4.0 mmol 2-oxo-2-phenylacetaldehyde, benzyl, sodium 2-oxobutanoate or sodium 2-oxo-3-phenylpropanoate and heated at 60 °C under stirring for 1 h. After cooling the resulting precipitate was filtered, while the acid mothers were neutralized by 2 N NaOH, the solids obtained were gathered together, washed, dried and crystallized by ethanol to give the pyrido[2,3-*g*]quinoxalines **3e–f**, **4g** and **5n**, respectively. In the same condition condensation of 6,7-diamino-8-chloroquinolin-4(1*H*)-one (**6c**) with 2-oxo-2-phenylacetic acid afforded the pyrido[2,3-*g*]quinoxaline **5o**. On the contrary, the reaction of **6b** with ethyl 3-methyl-2-oxobutanoate, diethyl 2-methyl-3-oxosuccinate or 2-oxo-2-phenylacetic acid, gave the mixture **4h**/**5k**; **4i**/**5l**; **4j**/**5m**, respectively, that were resolved by chromatography on silica gel column eluting with a mixture of diethyl ether/acetone in the ratio of 8:2.

5.1.11.1. 5-Chloro-3-phenylpyrido[3,2-*g*]quinoxaline (3e). Yield 87%. Mp 280–282 °C. ^{1}H NMR (DMSO-d_6): δ 9.82 (s, 1H, H-2), 9.41 (d, 1H, *J* = 4.6 Hz, H-7), 9.30 (d, 1H, *J* = 8.6 H-9), 9.01 (s, 1H, H-10), 8.54 (m, 2H, H-2′,6′), 8.07 (dd, 1H, *J* = 8.6 and 4.6 Hz, H-8), 7.67 (m, 3H, H-3′,4′,5′). LC/MS: 294 (M+H). Anal. Calcd for ($C_{17}H_{10}ClN_3$): C, 69.99; H, 3.45; Cl, 12.15; N, 14.40. Found: C, 7032; H, 3.19; Cl, 11.88; N, 14.73.

5.1.11.2. 5-Chloro-2,3-diphenylpyrido[3,2-*g*]quinoxaline (3f). Yield 69%. Mp 95–97 °C. ^{1}H NMR (DMSO-d_6): δ 8.10–7.85 (m, 5H), 7.80–7.70 (m, 4H), 7.65–7.50 (m, 5H). LC/MS: 370 (M+H). Anal. Calcd for ($C_{23}H_{14}ClN_3$): C, 75.10; H, 3.84; Cl, 9.64; N, 11.42. Found: C, 75.41; H, 3.59; Cl, 9.94; N, 11.17.

5.1.11.3. 5-Chloro-2-ethylpyrido[3,2-*g*]quinoxalin-3(4*H*)-one (4g). Yield 65%. Mp 269–270 °C. ^{1}H NMR (DMSO-d_6): δ 9.21 (s, 1H, NH), 9.04 (d, 1H, *J* = 4.2 Hz, H-7), 8.27 (d, 1H, *J* = 8.4 Hz, H-9), 8.22 (s, 1H, H-10), 7.43 (dd, 1H, *J* = 8.4 and 4.2 Hz, H-8), 2.97 (q, 2H, *J* = 7.2 Hz, CH_2), 1.32 (t, 3H, *J* = 7.2 Hz, CH_3). LC/MS: 262 (M+H). Anal. Calcd for ($C_{13}H_{10}ClN_3O$): C, 60.12; H, 3.88; Cl, 13.65; N, 16.18. Found: C, 60.34; H, 3.73; Cl, 13.69; N, 16.22.

5.1.11.4. 5-Chloro-2-isopropylpyrido[3,2-*g*]quinoxalin-3(4*H*)-one (4h). Yield 61%. Mp 274–275 °C. **^{1}H NMR ($CDCl_3$ + DMSO-d_6)**: **δ 11.49 (s, 1H, NH), 9.04 (d, 1H, *J* = 4.2 Hz, H-7), 8.44 (d, 1H, *J* = 8.4 Hz, H-9), 8.35 (s, 1H, H-10), 7.52 (dd, 1H, *J* = 8.4 and 4.2 Hz, H-8), 3.60 (m, 1H, *J* = 6.8 Hz, CH), 1.33 (d, 6H, *J* = 6.8 Hz, $2CH_3$)**. LC/MS: 276 (M+H). Anal. Calcd for ($C_{14}H_{12}ClN_3O$): C, 61.43; H, 4.42; Cl, 12.95; N, 15.35. Found: C, 61.09; H, 4.65; Cl, 13.30; N, 15.04.

5.1.11.5. Ethyl 2-(5-chloro-3-oxo-3,4-dihydropyrido[3,2-*g*]quinoxalin-2-yl)propanoate (4i). Yield 48%. Mp 269–272 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 10.86 (s, 1H, NH), 9.14 (d, 1H, *J* = 4.4 Hz, H-7), 8.57 (d, 1H, *J* = 8.4 Hz, H-9), 8.50 (s, 1H, H-10), 7.68 (dd, 1H, *J* = 8.4 and 4.4 Hz, H-8), 4.30 (q, 1H, *J* = 7.0 Hz, C*H*CH_3), 4.22 (q, 2H, *J* = 7.2 Hz, C$H_2$$CH_3$), 1.66 (d, 3H, *J* = 7.0 Hz, CHCH_3), 1.25 (t, 3H, *J* = 7.2 Hz, C$H_3$$CH_2$). LC/MS: 334 (M+H). Anal. Calcd for ($C_{16}H_{14}ClN_3O_3$): C, 57.93; H, 4.25; Cl, 10.69; N, 12.67. Found: C, 58.30; H, 4.19; Cl, 10.35; N, 12.91.

5.1.11.6. 5-Chloro-2-phenylpyrido[3,2-*g*]quinoxalin-3(4*H*)-one (4j). Yield 10%. Mp >300 °C. ^{1}H NMR (DMSO-d_6): δ 12.23 (s, 1H, NH), 9.10 (d, 1H, *J* = 4.0 Hz, H-7), 8.71 (d, 1H, *J* = 8.4 Hz, H-9), 8.60 (s, 1H, H-10), 8.28 (m, 2H), 7.61 (m, 4H). LC/MS: 310 (M+H). Anal. Calcd for ($C_{17}H_{10}ClN_3O$): C, 66.35; H, 3.28; Cl, 11.52; N, 13.65. Found: C, 66.68; H, 3.09; Cl, 11.84; N, 13.37.

5.1.11.7. 5-Chloro-3-isopropylpyrido[3,2-*g*]quinoxalin-2(1*H*)-one (5k). Yield 15%. Mp 287–288 °C. **^{1}H NMR ($CDCl_3$ + DMSO-d_6)**: **δ 12.51 (s, 1H, NH), 8.96 (d, 1H, *J* = 4.2 Hz, H-7), 8.34 (d, 1H, *J* = 8.2 Hz, H-9), 7.64 (s, 1H, H-10), 7.56 (dd, 1H, *J* = 8.2 and 4.2 Hz, H-8), 3.57 (m, 1H, *J* = 6.4 CH), 1.35 (d, 6H, *J* = 6.4 Hz, $2CH_3$)**. LC/MS: 276 (M+H). Anal. Calcd for ($C_{14}H_{12}ClN_3O$): C, 61.43; H, 4.42; Cl, 12.95; N, 15.35. Found: C, 61.75; H, 4.31; Cl, 13.22; N, 15.68.

5.1.11.8. Ethyl 2-(5-chloro-2-oxo-1,2-dihydropyrido[3,2-*g*]quinoxalin-3-yl)propanoate (5l). Yield 24%. Mp 281–283 °C. ^{1}H NMR ($CDCl_3$ + DMSO-d_6): δ 11.75 (s, 1H, NH), 8.83 (d, 1H, *J* = 4.4 Hz, H-7), 8.31 (d, 1H, *J* = 8.4 Hz, H-9), 8.22 (s, 1H, H-10), 7.33 (dd, 1H, *J* = 8.4 and 4.4 Hz, H-8), 4.32 (q, 1H, *J* = 7.0 Hz, C*H*CH_3), 4.20 (q, 2H, *J* = 7.2 Hz, C$H_2$$CH_3$), 1.71 (d, 3H, *J* = 7.0 Hz, CHCH_3), 1.21 (t, 3H, *J* = 7.2 Hz, C$H_3$$CH_2$). LC/MS: 334 (M+H). Anal. Calcd for ($C_{16}H_{14}ClN_3O_3$): C, 57.93; H, 4.25; Cl, 10.69; N, 12.67. Found: C, 57.71; H, 4.42; Cl, 10.39; N, 12.34.

5.1.11.9. 5-Chloro-3-phenylpyrido[3,2-*g*]quinoxalin-2(1*H*)-one (5m). Yield 80%. Mp >300 °C. **^{1}H NMR (DMSO-d_6)**: **δ 12.76 (s, 1H, NH), 8.97 (d, 1H, *J* = 4.0 Hz, H-7), 8.46 (m, 3H), 7.62 (m, 5H)**. LC/MS: 310 (M+H). Anal. Calcd for ($C_{17}H_{10}ClN_3O$): C, 66.35; H, 3.28; Cl, 11.52; N, 13.65. Found: C, 66.02; H, 3.10; Cl, 11.19; N, 13.41.

5.1.11.10. 3-Benzyl-5-chloropyrido[3,2-*g*]quinoxalin-2(1*H*)-one (5n). Yield 66%. Mp 239–242 °C. ^{1}H NMR (DMSO-d_6): δ 11.60 (s, 1H, NH), 8.75 (d, 1H, *J* = 4.2 Hz, H-7), 8.41 (d, 1H, *J* = 8.2 Hz, H-9), 7.54 (m, 3H), 7.32 (m, 4H). LC/MS: 324 (M+H). Anal. Calcd for ($C_{18}H_{12}ClN_3O$): C, 67.19; H, 3.76; Cl, 11.02; N, 13.06. Found: C, 66.82; H, 3.155; Cl, 11.35; N, 13.42.

5.1.11.11. 5-Chloro-3-phenylpyrido[3,2-*g*]quinoxaline-2,9(1*H*,6*H*)-dione (5o). Yield 45%. Mp >300 °C. ^{1}H NMR (DMSO-d_6): δ 11.61 (s, 1H, N_6H), 12.41 (s, 1H, N_1H), 8.76 (s, 1H, H-10), 8.03 (d, 1H, *J* = 7.8 Hz, H-7), 7.77 (m, 2H), 7.39 (m, 3H), 6.25 (d, 1H, *J* = 7.8 Hz, H-8). LC/MS: 326 (M+H). Anal. Calcd for ($C_{17}H_{10}ClN_3O_2$): C, 63.07; H, 3.11; Cl, 10.95; N, 12.98. Found: C, 63.40; H, 3.29; Cl, 111.33; N, 12.69.

5.2. Biological assays

5.2.1. Test compounds

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

5.2.2. 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 CCL 22 (NBL-1) *Bos Taurus*]; Baby Hamster Kidney (BHK-21) [ATCC CCL 10 (C-13) *Mesocricetus auratus*] and Monkey kidney (Vero-76) [ATCC CRL 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 (CVB-5), strain Ohio-1 (ATCC VR-29)], 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-1540)]. 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) [vaccine strain Elstree-Lister (ATCC VR-1549)]; (vii) *Herpesviridae*: human herpes 1 (HSV-1) [strain KOS (ATCC VR-1493)].

5.2.3. 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% fetal bovine serum (FBS), 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.[20]

MDBK and BHK cells were seeded in 24-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), L-glutamine, 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 48–96 h at 37 °C by the MTT method.[20]

Vero-76 cells were seeded in 24-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 crystal violet staining method.

Cell lines derived from human haematological tumours [CD4+ human T-cells containing an integrated HTLV-1 genome (MT-4); CD4+ human acute T-lymphoblastic leukaemia (CCRF-CEM), human splenic B-lymphoblastoid cells (WIL-2NS), human acute B-lymphoblastic leukaemia (CCRF-SB)] were seeded at an initial density of 1×10^5 cells/ml in 96-well plates in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin G and 100 μg/ml streptomycin.

Cell lines derived from human solid tumours [skin melanoma (SK-28), breast adenocarcinoma (MCF-7), lung squamous carcinoma (SK-MES-1), hepatocellular carcinoma (HepG-2), prostate carcinoma (DU-145)] or normal tissues [foreskin fibroblasts (CRL-7065), lung fibroblasts (CCL.75) and (MRC-5)] were also seeded at 1×10^5 cells/ml in 96-well plates in specific media supplemented with 10% FCS and antibiotics, as above. 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 MTT method.[20]

5.2.4. Antiviral assays

Compound's activity against HIV-1 was based on inhibition of virus-induced cytopathogenicity in MT-4 cell 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.[20] Compound's 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. Compound's 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 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.[20]

Compound's activity against CVB-5, Sb-1, VV, HSV-1 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 50–100 PFU/well. Following removal of unadsorbed virus, 500 μL of maintenance medium [D-MEM with L-glutamine and 4500 mg/L D-glucose, supplemented with 1% inactivated FBS] containing 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-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.

5.2.5. 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.

5.2.6. HCV replicon assay

A human hepatoma cell line (Huh-7) bearing the HCV genotype 1b replicon (GS4.1 cells), kindly provided by C. Seeger (Fox Chase University, Philadelphia, PA, USA) through Idenix Pharmaceuticals, was grown in D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 110 mg/L sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.5 mg/mL G418 (Invitrogen). For dose-response testing, GS4.1 cells were seeded in 96-well plates at a density of 7.5×10^3 cells/well in 50 μL medium containing two-fold serial dilutions of test compounds (highest concentration, 75 μM). Huh-7 cells lacking the HCV replicon served as negative controls. Plates were then incubated for 72 h at 37 °C in a humidified, 5% CO_2 incubator. Inhibition of HCV replication was measured by quantification of the viral NS4A protein using an enzyme-linked immunosorbent assay (ELISA) as follows: plates were fixed for 1 min with 1:1 acetone–methanol, washed twice with PBS containing 0.1% Tween 20, left 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 three washes with PBS containing 0.1% Tween 20, plates were incubated for 1 h at 37 °C with anti-mouse immunoglobulin G-peroxidase conjugate in TNE buffer containing 10% FBS. After further washing as 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 absorbance was determined at 492 nm using a Sunrise Tecan (Durham, NC, USA) spectrophotometer. EC_{50} values were determined from % inhibition vs compound concentration data, using a sigmoidal non-linear regression analysis based on four parameters, with a Tecan Magellan software. For cytotoxicity evaluation, Huh-7 and GS4.1 cells were treated with compounds as described above, and cellular viability was monitored using the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega). CC_{50} values were determined from the % cytotoxicity versus compound concentration data with Tecan Magellan software, as described above.

5.2.7. Expression of the BVDV-NS5BΔ24 polymerase

Expression and purification of BVDV-NS5BΔ24 polymerase were done as previously described.[17] Briefly, the expression plasmid encoding the N-terminal His-tagged C-terminal 24-amino-acid-deleted BVDV-NS5B was introduced into the *Escherichia coli* strain Rosetta™ 2(DE3)pLysS (Novagene), by chemical transformation. Transformants were then cultured at 30 °C overnight in 5 mL of LB medium containing 25 μg/mL kanamycin and 30 μg/mL chloramphenicol. The cultures was diluted into 1 L of LB medium containing 25 μg/mL kanamycin and 30 μg/mL chloramphenicol and incubated at 30 °C until the A_{600} reached 0.6–0.7. The culture was then induced overnight with 1 mM isopropyl-b-D-thiogalactopyranoside, the cells were harvested by centrifugation and stored at −80 °C until the purification.

5.2.8. Expression of the 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 BamHI and XhoI 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 introduced into the *E. coli* strain Rosetta™ 2(DE3)pLysS (Novagene) by chemical transformation. A single colony expressing the 6xHis-tagged HCV1b-NS5BΔ21 protein was selected and cultured at 30 °C overnight in 5 mL of LB medium containing 100 μg/mL ampicillin and 30 μg/mL chloramphenicol. The culture was diluted into 1 L of LB medium and incubated at 30 °C until the A_{600} reached 0.6–0.7. The culture was then induced overnight at 25 °C with 1 mM isopropyl-b-D-thiogalactopyranoside, the cells were harvested by centrifugation and stored at −80 °C until the purification.

5.2.9. Purification of NS5B proteins

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 4 °C, 11,000 rpm 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, pH 8.0). 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 BSA as the standard. Following dialysis, the purified 6xHis-tagged HCV1b-NS5BΔ21 and 6xHis-tagged BVDV-NS5BΔ24 proteins were divided into aliquots and stored at −80 °C.

5.2.10. RNA-dependent RNA polymerase assays

Enzyme 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 (RNase inhibitor, 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 three-fold serial dilutions of test compounds in DMSO 0.5%, or DMSO 0.5% alone (as negative control), or of the nucleotide analog 3′-deoxyguanosine-5′-triphosphate (3′-dGTP) (tebu-bio) as positive control, were added, and the samples were incubated for 120 min at 37 °C (BVDV-NS5BΔ24) or 25 °C (HCV1b-NS5BΔ21). Reactions were stopped by the addition of 2 μL of 200 mM EDTA. 138 microliters of the PicoGreen Quantitation Reagent Molecular Probes diluted 1:345 in TE, were added to each sample, followed by incubation for 5 min at room temperature in the dark. After excitation at 480 nm, fluorescence was measured at 520 nm in a fluorescence microplate reader (VICTOR3 Multilabel Plate Reader, Perkin-Elmer). 'Relative fluorescence' was calculated by subtracting the mean fluorescence of the blanks and by converting it into % of activity. Percent of residual activity was then plotted vs compound concentrations. Dose–response curves were fit with Kaleidagraph (Synergy Software) to obtain the drug concentration providing 50% inhibition (IC_{50}).

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