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Acridine derivatives as anti-BVDV agents

Michele Tonelli [a,*], Gerolamo Vettoretti [a], Bruno Tasso [a], Federica Novelli [a], Vito Boido [a], Fabio Sparatore [a], Bernardetta Busonera [b], Aicha Ouhtit [b], Pamela Farci [b], Sylvain Blois [b], Gabriele Giliberti [b], Paolo La Colla [b,*]

[a] Dipartimento di Scienze Farmaceutiche, Università di Genova, Viale Benedetto XV 3, 16132 Genova, Italy
[b] Dipartimento di Scienze e Tecnologie Biomediche, Università di Cagliari, Cittadella Universitaria, S.S. 554, Km 4.500, 09042 Monserrato, Cagliari, Italy

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ABSTRACT

Twenty-six 9-aminoacridine derivatives were evaluated in cell-based assays for cytotoxicity and antiviral activity against a panel of 10 RNA and DNA viruses. While seven compounds (**9**, **10**, **14**, **19**, **21**, **22**, **24**) did not affect any virus and two (**6**, **11**) were moderately active against CVB-5 or Reo-1, 17 compounds exhibited a marked specific activity against BVDV, prototype of pestiviruses which are responsible for severe diseases of livestock. Most anti-BVDV agents showed EC_{50} values in the range 0.1–8 μM, thus comparing favorably with the reference drugs ribavirine and NM 108. Some compounds, particularly those bearing a quinolizidinylalkyl side chain, displayed pronounced cytotoxicity. Further studies are warranted in order to achieve still better anti-BVDV agents, and to explore the potential antiproliferative activity of this kind of compounds.

1. Introduction

Infections of livestock (cattle, pigs and sheep) by members of *Pestivirus* genus (Flaviviridae family) produce heavy economic losses worldwide and availability of effective and inexpensive anti-pestiviruses agents is strongly needed (Houe, 2003). Bovine viral diarrhea virus (BVDV) is the prototype of the genus and, in addition to its intrinsic importance as an economic burden to the farming industry, it is also used as a surrogate for hepatitis C virus (HCV) (Buckwold et al., 2003; Paeshuyse et al., 2006; Finkielsztein et al., 2010) for the identification and development of new anti-HCV agents.

In the last years, several potent anti-BVDV agents have been developed (Fig. 1) and shown to target the RNA-dependent RNA-polymerase (RdRp), even if in several cases these compounds exhibited rather weak effect or were inactive on the purified enzyme (Baginski et al., 2000; King et al., 2002; Sun et al., 2003; Seio et al., 2004; Paeshuyse et al., 2006; Tabarrini et al., 2006; Okamoto et al., 2009), suggesting that viral or cellular factors are involved in the inhibitory effects of these drugs along with polymerase.

Very recently we have identified different classes of compounds active against BVDV, such as arylazoenamines (Tonelli et al., 2008), 4-[(*tert*-aminoalkyl)amino]arylazo compounds (Tonelli et al., 2009), and particularly some 2-phenylbenzimidazole derivatives (Tonelli et al., 2010), that exhibited an EC_{50} in the range 0.8–1.5 μM (Fig. 2).

Among the last, the 2-(4-acetylaminophenyl)-5,6-dichlorobenzimidazole was additionally found to inhibit the highly purified BVDV and HCV RdRps with IC_{50} of 3 and 5 μM, respectively.

Pursuing our efforts to identify other molecular types of anti-BVDV agents, we turned our interest versus some acridine derivatives that we are studying as possible inhibitors of hPrP90-231 prion fragment toxicity, in analogy to quinacrine (Villa et al., 2010). Indeed, the anti-prion agent quinacrine has been recently shown to affect multiple steps of RNA recruitment and replication of a positive stranded RNA virus (tomato bushy stunt virus, TBSV) (Sasvari et al., 2009).

Abbreviations: ACG, acyclovir; Ar, aromatic ring; ATCC, American type culture collection; AZT, 3′-azidothymidine; BHK, baby hamster kidney; BVDV, bovine viral diarrhea virus; CC, column chromatography; $CCDI_{50}$, cell culture infectious dose 50%; CVB-5, coxsackie virus, type 5; DMEM, Dulbecco's modified Eagle medium; DMF, dimethylformamide; DMSO, dimethylsulfoxide; ds-RNA, double-stranded RNA virus; EMCV, encephalomyocarditis virus; FBS, fetal bovine serum; HIV-1, human immunodeficiency virus, type 1; HCV, hepatitis C virus; HSV-1, herpes simplex virus, type 1; HTLV-1, human T-cell leukemia virus type 1; MDBK, Madin Darby bovine kidney; MEM-E, Minimum Essential Medium Eagle; m.o.i., multiplicity of infection; MT-4, $CD4^+$ human T-cells containing an integrated HTLV-1 genome; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffer solution; PFU, Plaque Forming Unit; RdRp, RNA-dependent RNA-polymerase; Reo-1, Reovirus, type 1; RPMI, Roswell Park Memorial Institut (medium); RSV, respiratory syncytial virus; Sb-1, poliovirus type 1-Sabin strain; SI, selectivity index; $ssRNA^+$, single-stranded positive RNA virus; $ssRNA^-$, single-stranded negative RNA virus; Tris/HCl, tris(hydroxymethyl)aminomethane hydrochloride; Vero-76, monkey kidney; VSV, vesicular stomatitis virus; VV, vaccinia virus; YFV, yellow fever virus.

* Corresponding authors. Tel.: +39 010 3538378; fax: +39 010 3538358 (M. Tonelli), tel.: +39 070 6754147; fax: +39 070 6754210 (P. La Colla).
E-mail addresses: michele.tonelli@unige.it (M. Tonelli), placolla@unica.it (P. La Colla).

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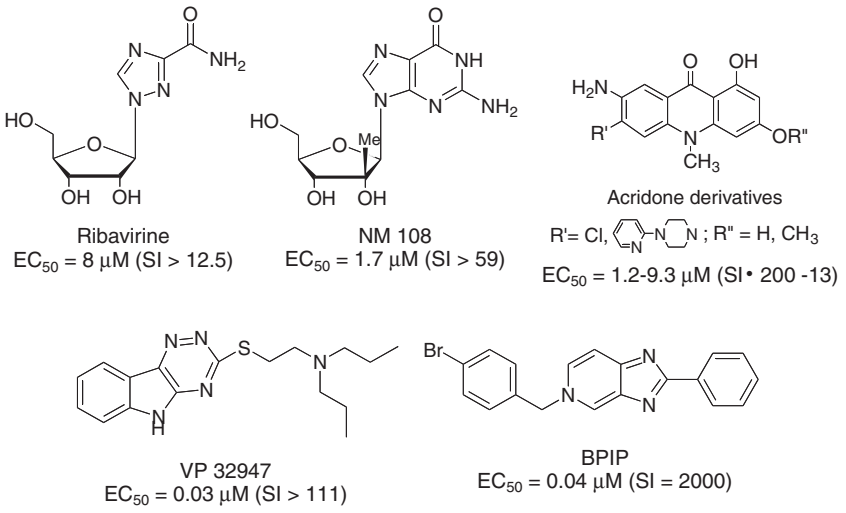


Fig. 1. Structures of some anti-BVDV agents.

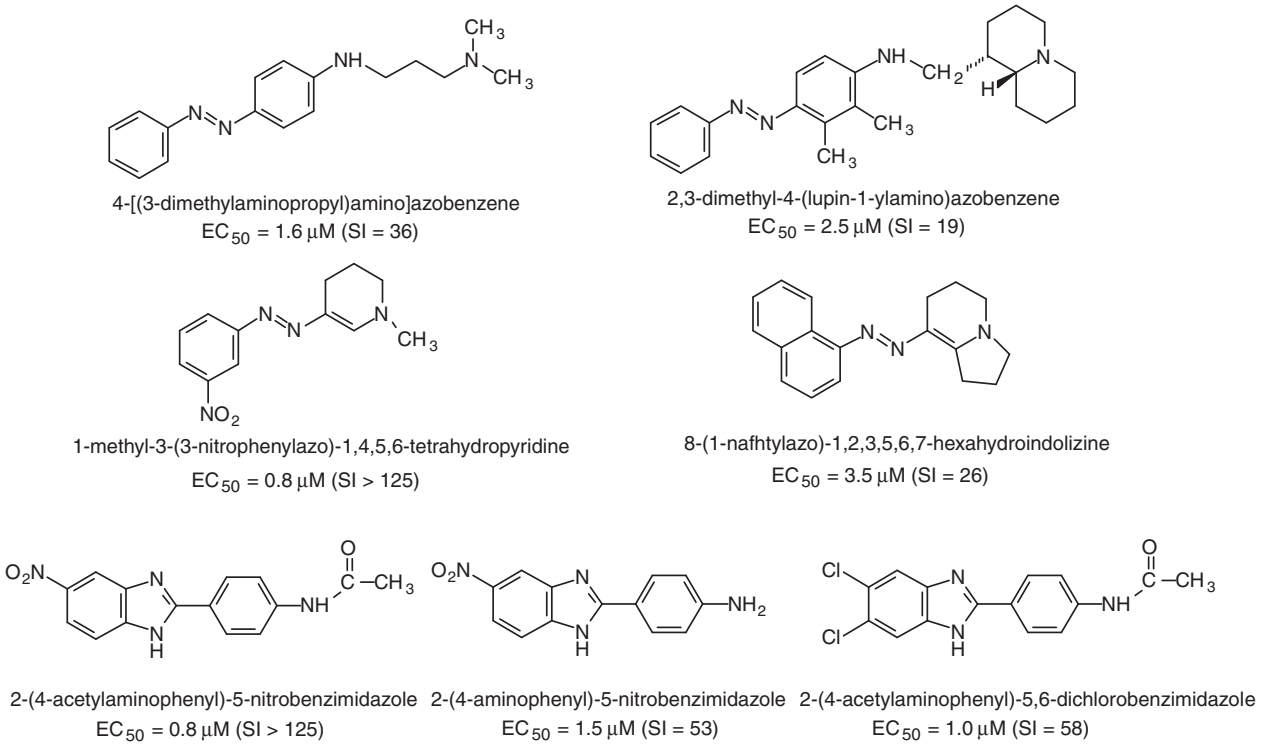


Fig. 2. Structures of some of our previously studied anti-BVDV agents.

Since the 50 several acridine derivatives were shown to inhibit the multiplication of a number of viruses (however not including BVDV), but only quinacrine possessed significant activity in the infected mouse (Hurst et al., 1952a,b; Greenhalgh et al., 1956; Glaz et al., 1973). Later it was demonstrated that quinacrine, and even more acranil, induced the formation of an interferon-like substance in mice, protecting the animals from the typical tail lesions induced by Vaccinia virus (Glaz et al., 1973; Glaz and Talas, 1975).

More recently aminacrine and quinacrine have been shown to interfer with several factors involved in the replication of different viruses (VSV, Herpes, HTLV-1, EMCV, polio virus) (Jamison et al., 1990; Goodell et al., 2006; Jung et al., 2008; Guendel et al., 2009; Gasparian et al., 2010), but more relevant for the present study is the specific anti-BVDV activity of some acridone derivatives (Fig. 1) (Tabarrini et al., 2006) that showed EC_{50} values in the range 1.2–9.3 μM. On such a base, novel acridone derivatives were identified (Manfroni et al., 2009) that resulted active against HCV (Hepacivirus genus, Flaviviridae family).

The compounds, object of the present study, are N-substituted derivatives of 9-amino-6-chloro-2-methoxyacridine scaffold, which characterizes the cited quinacrine and acranil. In order to explore the N-substituents best suited for the antiviral activity, diverse functionalized chains have been introduced on the amino group.

The simple 9-aminoacridine (aminacrine) has also been considered.

On the whole, 26 compounds (Fig. 3) have been evaluated in cell based assays for cytotoxicity and antiviral activity against BVDV and several other RNA and DNA viruses (see further).

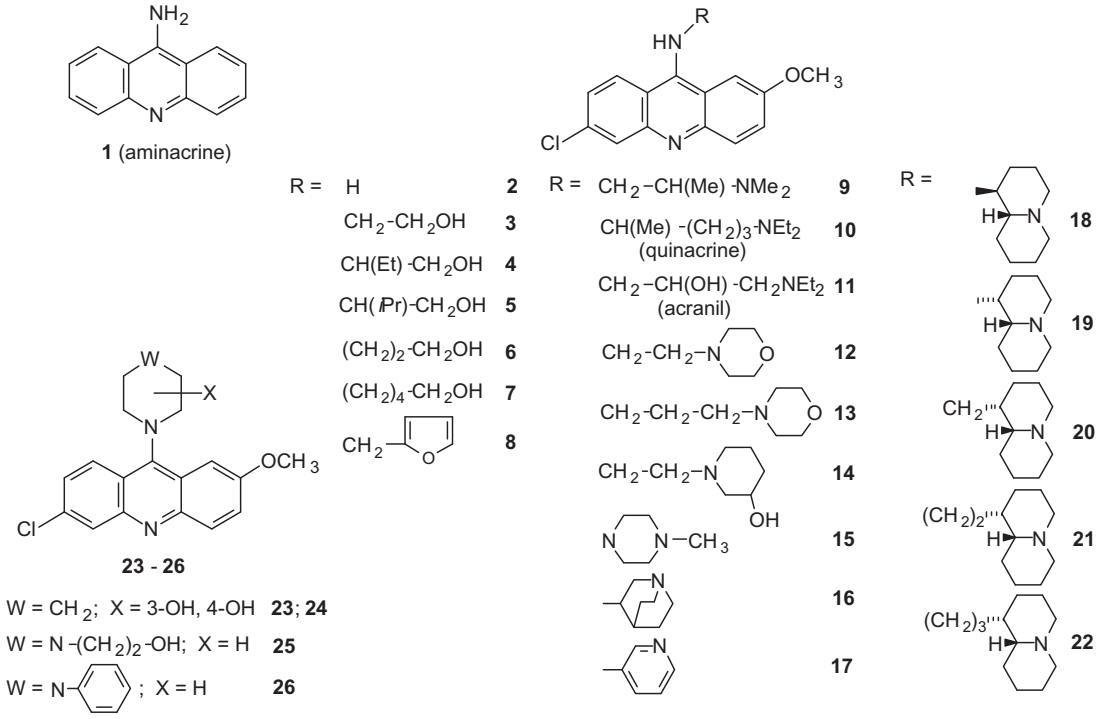


Fig. 3. Structures of the investigated compounds.

Fifteen of these compounds were already known, and some (aminacrine **1**, quinacrine **10** and acranil **11**) have been largely studied in the past as antibacterial, antiprotozoal and antitumoral agents (Goodman and Gilman, 1965; Wainwright, 2001; Denny, 2002).

2. Materials and methods

2.1. Compounds

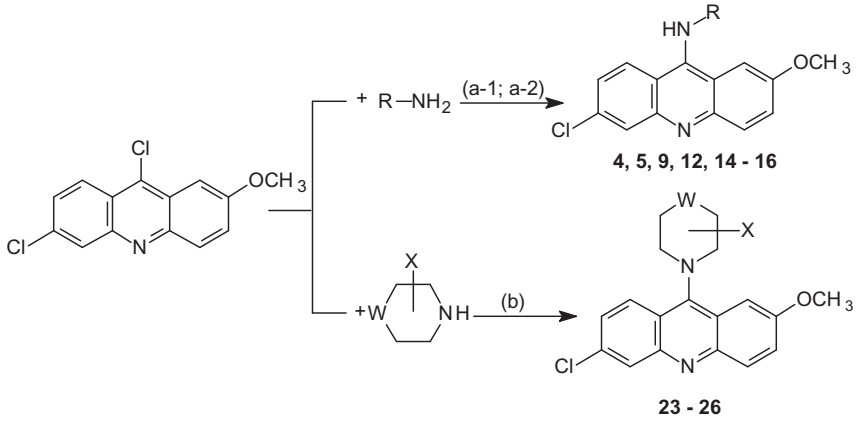
Fifteen out of 26 tested compounds (Fig. 3) were already known: aminacrine (**1**) and quinacrine (**10**) were purchased from Sigma–Aldrich, Milan, Italy, while the other 13 compounds were synthesized according to the literature procedures. Of these, the compounds **18**–**22** were already described by some of us (Boido Canu et al., 1989; Villa et al., 2010); for the remaining the references are as follows: **2** (Gerchuk et al., 1941), **3** and **17** (Burckhalter et al., 1943), **6** (Bolte et al., 1982), **7** (Constant et al., 1985), **8** (Korth et al., 2001), **11** (Magidson and Grigorovskii, 1936) and **13** (Magidson et al., 1938).

The novel compounds **4**, **5**, **9**, **12**, **14**–**16** and **23**–**26** were prepared as indicated in the Scheme 1, by reacting the 6,9-dichloro-2-methoxyacridine, previously dissolved in phenol or DMF, with the proper amine in a molar ratio 1:1 or 1:2 for primary or secondary amines, respectively.

During the final alkalinization of the reaction mixture, the separation of the formed monohydrochloride may occur (compounds **5** and **16**); in the case of **16** the amination was better effected in DMF solution and in the presence of diisopropylethylamine (Hünig base) in the ratio 1:1:1.

The 2-(3-hydroxypiperidin-1-yl)ethanamide and the 2-(3-hydroxypiperidin-1-yl)ethanamine, required for the synthesis of **14**, were prepared as previously described (Abood, 1960; Patterson et al., 2005).

All new compounds were characterized by elemental analyses and ^{1}H NMR spectra that are fully consistent with the described



Scheme 1. Reagents and conditions: (a-1) molar ratio 1:1 in phenol, 120 °C, 3 h; (a-2) for compound **16**, + Hünig base, molar ratio 1:1:1 in DMF, 140 °C, 3 h; (b) ratio 1:2 in DMF, 140 °C, 3 h.

structure. The purity of compounds (elemental analyses and ^{1}H NMR spectra) resulted in all cases $\geqslant$95%.

2.1.1. Experimental

Chemicals, solvents and reagents used for the syntheses were purchased from Sigma–Aldrich, Fluka or Alfa Aesar, and were used without any further purification. Column chromatography (CC): neutral alumina (Al_2O_3), activity 1 (Merck), or silica gel 60 (SiO_2) (Merck). Mps: Büchi apparatus, uncorrected. ^{1}H NMR spectra: Varian Gemini-200 spectrometer; $CDCl_3$ or DMSO-d_6; δ in ppm rel. to Me_4Si as internal standard. *J* in Hz. Elemental analyses were performed on a Carlo Erba EA-1110 CHNS-O instrument in the Microanalysis Laboratory of the Department of Pharmaceutical Sciences of Genoa University.

2.1.2. 9-Aminoacridine derivatives: general methods "a-1" and "a-2"

(a-1): A mixture of 6,9-dichloro-2-methoxyacridine (3.5 mmol), the appropriate amine (3.5 mmol) and phenol (2.20 g) was heated at 120 °C for 3 h. After cooling, the mixture was treated with 2 M NaOH till strong alkalinity and extracted with Et_2O. After removing the solvent, the residue was purified by CC.

In the cases of **5** and **16** the extraction with ether did not afford the expected free base, while with CH_2Cl_2 the corresponding monohydrochloride were obtained.

(a-2): Compound **16** was obtained as free base by reacting the 6,9-dichloro-2-methoxyacridine, previously dissolved in DMF with the quinuclidin-3-ylamine in the presence of the diisopropylethylamine (Hünig base) (ratio 1:1:1), at 140 °C for 3 h.

*2.1.3. 6-Chloro-9-(1′-hydroxybut-2′-yl)]amino-2-methoxyacridine (**4**)*

Yield: 40%. M.p. 250 °C dec. (CH_2Cl_2). CC: SiO_2/CH_2Cl_2 + 2%MeOH. ^{1}H NMR (DMSO-d_6): 0.82 (t, *J* = 6.0, 3H, C(4)); 1.49–1.77 (m, 2H, C(3)); 3.50–3.64 (m, 2H, C(1)); 3.80–3.94 (m, 1H, C(2) and s, OCH_3 (3.90), superimposed); 4.97 (br. s, OH, collapses with D_2O); 6.40 (br. s, NH, collapses with D_2O); 7.37–7.90 (m, 5 arom. H); 8.47 (d, *J* = 10.0, 1 arom. H). Anal. calcd for $C_{18}H_{19}ClN_2O_2$ + 0.25 H_2O: C 64.41, H 5.80, N 8.35; found: C 64.28, H 6.03, N 7.92.

*2.1.4. 6-Chloro-9-(1′-hydroxyisopent-2′-yl)amino-2-methoxyacridine hydrochloride (**5**)*

Yield: 41%. M.p. >300 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 0.70 (d, *J* = 6.8, 3H, C(4)); 0.85 (d, *J* = 6.6, 3H, C(5)); 2.03–2.21 (m, ^{1}H, C(3)); 3.18 (br. s, OH, collapses with D_2O); 3.83–4.00 (m, 2H, C(1) and s, OCH_3 (3.93) superimposed); 4.20–4.37 (m, ^{1}H, C(2)); 5.44 (br. s, NH, collapses with D_2O); 7.41–8.25 (m, 5 arom. H); 8.80 (d, *J* = 9.6, 1 arom. H). Anal. calcd for $C_{18}H_{19}ClN_2O_2$ + HCl + 0.25 H_2O: C 59.10, H 5.83, N 7.26; found: C 58.93, H 5.28, N 7.09.

*2.1.5. 6-Chloro-9-[2′-(N,N-dimethylamino-1′-propan]amino-2-methoxyacridine (**9**)*

Yield: 42%. M.p. 125–126 °C (Et_2O). CC: SiO_2/Et_2O + 2%Et_2N. ^{1}H NMR (DMSO-d_6): 0.85 (d, *J* = 6.2, 3H, C(3)); 2.17 (s, $N(CH_3)_2$); 2.78–2.94 (m, ^{1}H, C(2)); 3.56–3.67 (m, 2H, C(1)); 3.88 (s, OCH_3); 6.78 (br. s, NH, collapses with D_2O); 7.24–7.87 (m, 5 arom. H); 8.25 (d, *J* = 10.0, ^{1}H, 1 arom. H). Anal. calcd for $C_{19}H_{22}ClN_3O$: C 66.37, H 6.45, N 12.22; found C 66.44, H 6.77, N 11.91.

*2.1.6. 6-Chloro-2-methoxy-9-[2′-(N-morpholino)ethyl]aminoacridine (**12**)*

Yield: 45%. M.p. 148–150 °C (Et_2O). CC: Al_2O_3/CH_2Cl_2. ^{1}H NMR ($CDCl_3$): 2.54 (t, *J* = 5.6, 4H, morpholine); 2.64 (t, *J* = 5.7, 4H, morpholine); 3.76–3.90 (m, 4H, *CH*$_2$-*CH*$_2$)); 3.95 (s, OCH_3); 6.05 (br. s, NH, collapses with D_2O); 7.20–8.14 (m, 6 arom. H). Anal. calcd for $C_{20}H_{22}ClN_3O_2$: C 64.60, H 5.96, N 11.30; found: C 64.87, H 6.11, N 11.39.

*2.1.7. 6-Chloro-9-[2-(3-hydroxypiperidin-1-yl)ethyl]amino-2-methoxyacridines (**14**)*

Yield: 42%. M.p. 186–188 °C (THF). ^{1}H NMR ($CDCl_3$): 1.43–2.00 (m, 4H, piperidine ring); 2.23–2.90 (m, 4H, piperidine ring, 2H, $HNCH_2$*CH*$_2$-piperidine, OH, exchanges with D_2O); 3.68 (t, 2H, HN*CH*$_2CH_2$-piperidine); 3.88–3.93 (m, 1H, piperidine ring and 3.91, s, OCH_3); 6.10 (NH, exchanges with D_2O); 7.00–7.38 (m, 3 arom. H); 7.86–8.01 (m, 3 arom. H). Anal. calcd for $C_{21}H_{24}ClN_3O_2$: C 65.36, H 6.27, N 10.89; found: C 65.63, H 6.23, N 10.61.

*2.1.8. 6-Chloro-2-methoxy-9-(4′-methylpiperazin-1′-yl)aminoacridine (**15**)*

Yield: 22%. M.p. 90–92 °C (CH_2Cl_2). CC: Al_2O_3/CH_2Cl_2. ^{1}H NMR (DMSO-d_6): 2.25 (s, CH_3); 2.50 (t, *J* = 5.2, 4H, piperazine); 2.72 (br. s, NH, collapses with D_2O); 3.31 (t, *J* = 5.2, 4H, piperazine); 3.78 (s, OCH_3); 7.04–7.18 (m, 3 arom. H); 7.65–8.28 (m, 3 arom. H). Anal. calcd for $C_{19}H_{21}ClN_4O$ + 0.2 H_2O: C 63.31, H 5.98, N 15.54; found: C 63.71, H 6.28, N 15.08.

*2.1.9. 6-Chloro-2-methoxy-9-(N-quinuclidin-3′-ylamino)acridine hydrochloride (**16**)*

Yield: 40%. M.p. 177–180 °C (CH_2Cl_2). ^{1}H NMR (DMSO-d_6): 1.23–2.27 (m, 4H, quinuclidine); 2.48–3.57 (m, 8H, quinuclidine); 3.94 (s, OCH_3); 4.16 (br. s, NH, collapses with D_2O); 7.21–8.47 (m, 6 arom. H); Anal. calcd for $C_{21}H_{22}ClN_3O$ + HCl + 0.25 H_2O: C 61.64, H 5.74, N 10.27; found: C 61.64, H 5.49, N 9.73.

Free base: Yield: 37%. M.p. 143–146 °C (Et_2O). CC: Al_2O_3/CH_2Cl_2 + 2%MeOH. Anal. Calcd for $C_{21}H_{22}ClN_3O$: C 68.56, H 6.03, N 11.42; found: C 68.59, H 6.27, N 11.09.

2.1.10. 9-Aminoacridine derivatives: general method "b"

A solution of 6,9-dichloro-2-methoxyacridine (3.5 mmol) and cyclic secondary amine (7.0 mmol) in DMF (5 mL) was heated at 140 °C for 3 h. The mixture was taken up with water, alkalinized with 2 M NaOH and extracted with CH_2Cl_2. The solvent was evaporated and the oily residue was purified by CC (silica gel/CH_2Cl_2 + 2%MeOH). The isolated compounds were crystallized from the indicated solvents.

*2.1.11. 6-Chloro-9-(3′-hydroxypiperidin-1′-yl)-2-methoxyacridine (**23**)*

Yield: 25%. M.p. >300 °C (CH_2Cl_2). ^{1}H NMR ($CDCl_3$): 1.66–1.92 (m, 2H, piperidine); 2.00–2.24 (t, 2H, piperidine); 2.90 (br. s, OH, collapses with D_2O); 3.29–3.50 (m, 3H, piperidine); 3.65–3.76 (m, 1H, piperidine); 3.94 (s, OCH_3); 4.05–4.16 (m, 1H, piperidine); 7.22–8.16 (m, 6 arom. H). Anal. calcd for $C_{19}H_{19}ClN_2O_2$: C 66.57, H 5.59, N 8.17; found: C 66.21, H 6.00, N 8.30.

*2.1.12. 6-Chloro-9-(4′-hydroxypiperidin-1′-yl)-2-methoxyacridine (**24**)*

Yield: 48%. M.p. 217–218 °C (Et_2O). ^{1}H NMR ($CDCl_3$): 1.81–2.00 (m, 2H, piperidine); 2.09–2.27 (m, 2H, piperidine and s, OH, collapses with D_2O, superimposed); 3.46–3.75 (m, 4H, piperidine); 3.97 (s, OCH_3); 4.05–4.28 (m, 1H, piperidine); 7.32–8.23 (m, 6 arom. H). Anal. calcd for $C_{19}H_{19}ClN_2O_2$: C 66.57, H 5.59, N 8.17; found: C 66.92, H 5.91, N 7.80.

*2.1.13. 6-Chloro-9-[4′-(hydroxyethyl)piperazin-1′-yl)-2-methoxyacridine (**25**)*

Yield: 51%. M.p. 161–163 °C (CH_2Cl_2). ^{1}H NMR ($CDCl_3$): 2.66–2.90 (m, 8H, piperazine); 3.47–3.65 (m, 2H, C(1) and s, OH, collapses with D_2O, superimposed); 3.75 (t, *J* = 5.6, 2H, C(2)); 3.97 (s, OCH_3); 7.24–8.31 (m, 6 arom. H). Anal. calcd for $C_{20}H_{22}ClN_3O_2$: C 64.60, H 5.96, N 11.30; found: C 64.46, H 6.27, N 11.09.

*2.1.14. 6-Chloro-2-methoxy-9-(4′-phenylpiperazin-1′-yl)acridine (**26**)*

Yield: 31%. M.p. 210–214 °C ($CH_3COOC_2H_5$). CC: SiO_2/CH_3CO-OC_2H_5. ^1H NMR ($CDCl_3$): 3.69 (t, J = 5.0, 4H, piperazine); 3.92 (t, J = 5.0, 4H, piperazine); 4.13 (s, OCH_3); 7.06–7.36 (m, 3 arom. H); 7.41–7.78 (m, 5 arom. H); 8.19–8.57 (m, 3 arom. H). Anal. calcd for $C_{24}H_{22}ClN_3O$: C 71.37, H 5.49, N 10.40; found: C 71.09, H 5.26, N 10.24.

2.2. Cell-based assays

2.2.1. Compounds

Compounds were dissolved in DMSO at 100 mM and then diluted in culture medium. The final DMSO concentration did not exceed 1% (commonly 0.1%) which did not affect the biological assay results.

2.2.2. 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) except Yellow Fever Virus (YFV), and Human Immunodeficiency Virus type-1 (HIV-1). Viruses representative of positive-sense single-strand RNA ($ssRNA^+$) group used were: (i) *Retroviridae* family: the laboratory strain HIV-1 IIIB wild-type, obtained from the supernatant of the persistently infected H9/IIIB cells (NIH 1983); (ii) *Flaviviridae* family: YFV [strain 17-D vaccine (Stamaril Pasteur J07B01)] and Bovine Viral Diarrhea Virus (BVDV) [strain NADL (ATCC VR-534)] (title compounds were evaluated *in vitro* for antiviral activity against viruses representative of only two of the three genera of the Flaviviridae family, i.e. Flaviviruses (Yellow Fever Virus, YFV) and Pestiviruses (Bovine Viral Diarrhea Virus, BVDV), as Hepaciviruses can hardly be used in routine cell-based assays); (iii) *Picornaviridae* family: Human Coxsackievirus type B5 (CVB-5) strain Ohio-1 (ATCC VR-29) and Human Poliovirus type-1 Sabin (Sb-1) [strain Chat (ATCC VR-1562)]. Virus representative of a negative-sense single-strand RNA ($ssRNA^-$) group used were: Vesicular Stomatitis Virus (VSV) [strain Indiana Lab (ATCC VR-158)] and Human Respiratory Syncytial Virus (RSV) [strain A2 (ATCC VR-1540)]. A virus representative of a double strand RNA (dsRNA) group used was: Reovirus type-1 [strain 3651 (SV-12, simian virus 12) (ATCC VR-214)]. Viruses representatives of DNA group used were: (i) *Poxviridae* family: Vaccinia Virus (VV) [strain Elstree-Lister Vaccine (ATCC VR-1549)]; (ii) *Herpesviridae* family: Human Herpesvirus 1 (HSV-1) [strain KOS (ATCC VR-1493)].

As reference inhibitors were used: EFV (efavirenz) for HIV-1 only, NM 108 (2′-C-methyl-guanosine), NM 176 (2′-C-ethynylcytidine) and Ribavirin for the other $ssRNA^+$ and dsRNA viruses; NM 299 (6-azauridine), ribavirine and M 5255 (mycophenolic acid) for the $ssRNA^-$viruses; M 5255 and ACG (acyclovir) for DNA viruses.

2.2.3. 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 U/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 (Pauwels et al., 1988). MDBK and BHK cells were seeded at an initial density of 6×10^5 and 1×10^6 cells/mL in 96-well plates, respectively, in culture medium (Minimum Essential Medium with Earle's salts (MEM-E) with L-glutamine, supplemented with 10% horse serum and 1 mM sodium pyruvate (for MDBK cells) or with 10% fetal bovine serum (FBS) (for BHK cells), 0.025 g/L kanamycin). Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 48–96 h at 37 °C by the MTT method. Vero-76 cells were seeded at an initial density of 4×10^5 cells/mL in 24-well plates, in culture medium (Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine, supplemented with fetal bovine serum (FBS), 0.025 g/L kanamycin). Cell cultures were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 48–96 h at 37 °C by the crystal violet staining method.

2.2.4. Antiviral assays

Compounds activity against HIV-1 was based on inhibition of virus-induced cytopathogenicity in MT-4 cells acutely infected with a multiplicity of infection (m.o.i.) of 0.01. Briefly, 50 μL of RPMI containing 1×10^4 MT-4 cells were added to each well, of flat-bottom microtitre trays, containing 50 μL of RPMI, without or with serial dilutions of test compounds. Then, 20 μL of a HIV-1 suspension containing 100 $CCID_{50}$ were added. After a 4-days incubation at 37 °C, cell viability was determined by the MTT method. Compounds activity against YFV and Reo-1 was based on inhibition of virus-induced cytopathogenicity in BHK-21 cells acutely infected with a m.o.i. of 0.01. Compounds activity against BVDV was based on inhibition of virus-induced cytopathogenicity in MDBK cells acutely infected with a m.o.i. of 0.01. Briefly, BHK and MDBK cells were seeded in 96-well plates at a density of 5×10^4 and 3×10^4 cells/well, respectively, and were allowed to form confluent monolayers by incubating overnight in growth medium at 37 °C in a humidified CO_2 (5%) atmosphere. Cell monolayers were then infected with 50 μL of a proper virus dilution in maintenance medium (MEM-E with L-glutamine, supplemented with 0.5% inactivated FBS, 1 mM sodium pyruvate and 0.025 g/L kanamycin) to give an m.o.i of 0.01. After 1 h, 50 μL of maintenance medium, without or with serial dilutions of test compounds, were added. After a 3–4 days incubation at 37 °C, cell viability was determined by the MTT method.

Compounds activity against CVB-5, Sb-1, VSV, VV, HSV-1 and RSV was determined by plaque reduction assays in infected Vero 76 cell monolayers. To this end, Vero 76 cells were seeded in 24-well plates at a density of 2×10^5 cells/well and were allowed to form confluent monolayers by incubating overnight in growth medium (Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and 4500 mg/L D-glucose, supplemented with 10% FBS and 0.025 g/L Kanamycin) at 37 °C in a humidified CO_2 (5%) atmosphere. Then, monolayers were infected for 2 h with 250 μL of proper virus dilutions to give 50–100 PFU/well. Following removal of unadsorbed virus, 500 μL of maintenance medium (DMEM medium with L-glutamine and 4500 mg/L D-glucose supplemented with 1% inactivated FBS and 0.75% methyl-cellulose), without or with serial dilutions of test compounds, were added. Cultures were incubated at 37 °C for 2 (Sb-1 and VSV), 3 (CVB-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 and EC_{50} (50% effective concentration) was calculated by linear regression technique. The cytotoxicity of test compounds was determined in parallel on the same 24-well plate used for the EC_{50} determination.

Linear regression analysis: viral and cell growth at each drug concentration was expressed as percentage of untreated controls and concentrations resulting in 50% (EC_{50} and CC_{50}) growth inhibition were determined by linear regression analysis.

3. Results

Acridine derivatives were tested in cell-based assays against representative members of a number of virus families. In addition to the flaviviridae BVDV and YFV, among $ssRNA^+$ viruses were a retrovirus (human immunodeficiency virus type-1, HIV-1), two picornaviruses (Coxsackie virus type B5, CVB-5, and polio virus type-1, Sabin strain, Sb-1). Among $ssRNA^-$ virus was a paramyxovirus (respiratory syncytial virus, RSV) and a Rhabdovirus (Vesicular Stomatitis virus, VSV). Among dsRNA viruses, a Reovidae family member (Reo-1) was included. Two representatives of the DNA virus families were also included: herpes symplex type-1, HSV-1 (Herpesviridae) and vaccinia virus, VV (Poxviridae). Cytotoxicity was evaluated in parallel with the antiviral activity. Efavirenz, 2′-C-methyl-guanosine, 2′-C-ethynyl-cytidine, 6-azauridine, mycophenolic acid and acyclovir were used as reference inhibitors of $ssRNA^+$, $ssRNA^-$ and DNA viruses. The cytotoxicity and antiviral activity of test and reference compound are reported in Tables 1 and 2.

4. Discussion

As far as the activity against Flaviviridae was concerned, very interestingly 17 of the 26 tested compounds (Table 1) exhibited a very specific activity against BVDV, in a low micromolar range, leaving unaffected the remaining viruses at concentrations up to the CC_{50} value for the corresponding host cells or up to the highest concentration used (100 μM) (Table 2).

When tested against representative members of other virus families (Table 2), tested acridine derivatives resulted mainly inactive, with the exceptions of compound **6** which resulted moderately active against CVB-5 (EC_{50} = 20 μM) and compound **11** which resulted moderately active against Reo-1 (EC_{50} = 20 μM).

Quinacrine (**10**) and acranil (**11**), that in previous studies (see Introduction) were found endowed with some activity against several viruses, failed to affect any of the presently studied viruses, or, in the case of **11**, affected moderately only one virus (Reo-1).

While the activity against CVB-5 and Reo-1 was only moderate and will not be discussed further, the sought-after activity against BVDV was rather high; indeed fourteen compounds exhibited $EC_{50} \leqslant 8$ μM, thus resulting comparable or more potent than the reference drug ribavirine (EC_{50} = 8 μM). Five of these compounds had $EC_{50} \leqslant 1.5$ μM and were, therefore, comparable or even more effective than the experimental compound NM 108 (EC_{50} = 1.7 μM) and of the best acridone derivatives recently described (Tabarrini et al., 2006), (7-amino-1,3-dihydroxy-10-methyl-5-[4-(2-pyridinyl)-1-piperazinyl]-9(10*H*)acridone; EC_{50} = 1.2 μM).

The best compound was the simple 9-amino-6-chloro-2-methoxyacridine (**2**) ($EC_{50} \leqslant 0.1$ μM), whose potency was about one third or one fourth of that of VP32947 (3-{[(2-dipropylamino)-ethyl]thio}-5*H*-1,2,4-triazino[5,6-b]indole; $EC_{50} \leqslant 0.03$ μM) and BPIP (5-[(4-bromophenyl)methyl]-2-phenyl-5*H*-imidazo[4,5-c]-pyridine; $EC_{50} \leqslant 0.04$ μM), which still remain unsurpassed as the most potent inhibitors of BVDV replication.

Taking into account the cytotoxicity for MDBK cell line, used to grow the BVDV, it is observed that four of the active compounds had a $CC_{50} > 100$ μM, while the remaining 13 compounds exhibited CC_{50} values in the range from 5.3 to 75 μM. Correspondingly, the selectivity indices (SI: CC_{50}/EC_{50}) for the active compounds ranged from 1.5 to 90, and many of them were comparable or better than the SI of ribavirine (SI > 12.5) and NM 108 (SI > 59). The most active compound **2** exhibited a good SI (90), even if it remains lower than the SI of VP32947 (>111) and of BPIP (∼2000).

Nevertheless, compound **2** (9-amino-6-chloro-2-methoxyacridine) and some of its N-substituted derivatives (**3**, **7**, **8** and **15**) are appreciable for combining good activity and structural simplicity (and corresponding low cost), thus deserving further investigation to define the *in vivo* efficacy and toxicity. Should these characteristics result favorable for some of them, such compounds could be of practical therapeutic interest even if less potent than the more expensive compounds VP32947 and BPIP.

On the other hand, the synthesis of other acridine derivatives should be pursued in order to, eventually, find out even more potent and still inexpensive anti-pestivirus agents. To this regard, we attempted to define some correlations between structure and activity for the tested compounds.

First of all comparing aminacrine **1** with compound **2**, it is observed that the introduction of substituents on the acridine nucleus strongly enhanced the activity and reduced the toxicity. Thus the effect of introducing other kinds of substituents on the heterocyclic ring should be explored.

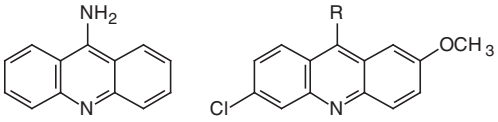
On the other hand, it is observed that, so far, none of the 15 N-substituted active compounds exhibited better antiviral activity than the N-unsubstituted compound **2**. Anyhow, the highest levels of activity were observed when the side chain terminates with an oxygenated group (**3**, **5**, **7**, **8**, **12**: EC_{50} = 0.6–2 μM; **4**, **13**, **23**, **25**: EC_{50} = 5–8 μM). However, this structural feature is not per se determinant of good activity, since some hydroxylated compounds (**6**, **14** and **24**) are inactive, and also compounds with a basic head are endowed with good activity (**15**–**17**: EC_{50} = 3–6 μM). The introduction of substituents on the amino group of **2** strongly modified the cytotoxicity of this compound. Indeed, substituents of very different dimensional and chemical characteristics may be able to even suppress the toxicity on MDBK cells ($CC_{50} > 100$ μM), while maintaining different degrees of antiviral activity (compare compounds **4**, **15**, **17** and **26**).

Moreover, among a more homogeneous group of compounds, as the ω-hydroxyalkyl derivatives **3**–**7**, somewhat bewildering results are observed when the chain is elongated or branched. While the 2-hydroxyethyl derivative (**3**) is very active, but rather toxic (SI = 16.7), the 3-hydroxypropyl homologue (**6**) is devoid of activity and toxicity; however the further elongation of the chain (**7**) restored activity and toxicity even with a better SI (26.7). When the hydroxyethyl chain of **3** was branched with the introduction of an ethyl (**4**) or isopropyl (**5**) group, striking different effects were observed. In compound **4** the cytotoxicity and the activity of **3** were, respectively, completely abolished and strongly reduced, while in compound **5** both activity and toxicity of **3** remained practically unaffected.

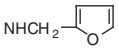
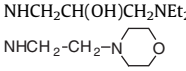
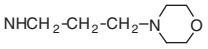
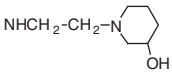
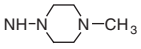
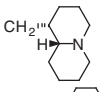
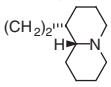
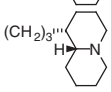
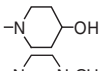
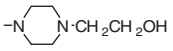
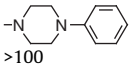
Somewhat unaccountable variations of activity and/or toxicity as consequence of minor structural modifications are also observed for compounds **12**–**13**, **19**–**22** and **23**–**24**. Anyhow the foregoing observations allow to conclude that other interesting anti-BVDV compounds might be expected also by further modifications of substituent on the 9-amino group of **2**.

Finally, some of the tested compounds elicit additional interest for their general high cytotoxicity displayed against the four host cell lines used to support the replication of the ten viruses (see: mean CC_{50} in Table 2), and, particularly, against the exponentially growing lymphoblastoid human cells MT-4 (used to grow HIV-1). Indeed against this cell line, 14 compounds (**1**–**3**, **5**, **7**, **10**–**13**, **16** and **20**–**22**) exhibited $CC_{50} \leqslant 2$ μM and 3 of them (**5**, **20** and **21**) had CC_{50} = 0.5–0.7 μM. For comparison, etoposide exhibited

Table 1
Cytotoxicity and anti-BVDV activity of 9-aminocridine (aminacrine) and of its derivatives **2**–**26**. CC_{50} and EC_{50} (μM).



1 **2 - 26**

Comp.	R	MDBK CC_{50}[a]	BVDV EC_{50}[b]	SI CC_{50}/EC_{50}
1 (aminacrine)		5.3	3.5	1.5
2	NH_2	9.0	0.1	90
3	$NHCH_2CH_2OH$	10.0	0.6	16.7
4	$NHCH(Et)CH_2OH$	>100	8.0	>12.5
5	$NHCH(iPr)CH_2OH$	8.0	0.6	13.3
6	$NHCH_2CH_2CH_2OH$	>100	>100	/
7	$NHCH_2(CH_2)_3CH_2OH$	40	1.5	26.7
8		41	1.3	31.5
9	$NHCH_2CH(Me)NMe_2$	12	>12	/
10 (quinacrine)	$NHCH(Me)(CH_2)_3NEt_2$	7	>7	/
11 (acranil)	$NHCH_2CH(OH)CH_2NEt_2$	24	>24	/
12		8	2	4
13		50	5	10
14		22	>22	/
15		>100	3	>33.3
16		55	3	18.3
17		>100	6	>16.7
18		75	19	3.9
19		43	>43	/
20		34	11	3.1
21		8	>8	/
22		9	>9	/
23		43	8	5.37
24		26	>26	/
25		55	6	9.17
26		>100	31	>3.2
NM 108 (2′-C-metilguanosine)	>100	1.7	>58.8	
Ribavirine	>100	8	>12.5	
NM 176 (2′-C-ethynylcytidine)	>100	38	>2.6	
NM 299 (6-azauridine)	>100	>100	/	
M 5255 (mycophenolic acid)	42	>42	/	
ACG (acyclovir)	>100	>100	/	

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

CC_{50} = 0.1 μM against the MT-4 cell line. Thus the above compounds deserve to be tested for antiproliferative activity against other tumoral cell lines derived from both leukemia/lymphoma and solid tumors.

Table 2
Cytotoxicity and antiviral activity of 9-aminocridine (**1**) and of its derivatives **2**–**26**. CC_{50} and EC50 (μM).

Comp.[a]	MT-4 CC_{50}[b]	HIV-1 EC_{50}[c]	BHK-21 CC_{50}[d]	YFV EC_{50}[e]	Reo-1 EC_{50}[f]	Vero-76 CC_{50}[g]	HSV-1 EC_{50}[h]	VV EC_{50}[i]	VSV EC_{50}[j]	CVB-5 EC_{50}[k]	Sb-1 EC_{50}[l]	RSV EC_{50}[m]	Mean CC_{50} of the Four host cell lines[n]
1	1.2	>[o]	7	>	>	8	>	>	>	>	>	>	5.37
2	1.8	>	9	>	>	10	>	>	>	>	>	>	7.45
3	1.8	>	20	>	>	4	>	>	>	>	>	>	8.95
4	9.6	>	56	>	>	>100	>	>	>	>	>	>	>66.4
5	0.7	>	6	>	>	5	>	>	>	>	>	>	4.92
6	59.0	>	>100	>	>	>100	>	>	>	20	>	>	>89.75
7	2.0	>	17	>	>	35	>	>	>	>	>	>	23.5
8	7.0	>	35	>	>	7	>	>	>	>	>	>	22.5
9	5.7	>	47	>	>	6	>	>	>	>	>	>	17.67
10	1.8	>	9	>	>	20	>	>	>	>	>	>	9.45
11	1.7	>	39	>	20	7	>	>	>	>	>	>	17.92
12	1.8	>	18	>	>	12	>	>	>	>	>	>	9.95
13	1.9	>	22	>	>	52	>	>	>	>	>	>	31.47
14	1.5	>	38	>	>	14	>	>	>	>	>	>	18.87
15	8.0	>	32	>	>	10	>	>	>	>	>	>	>37.5
16	1.9	>	12	>	>	35	>	>	>	>	>	>	25.97
17	9.0	>	>100	>	>	>100	>	>	>	>	>	>	>77.25
18	6.4	>	16	>	>	60	>	>	>	>	>	>	39.35
19	9.3	>	47	>	>	70	>	>	>	>	>	>	42.32
20	0.5	>	37	>	>	11	>	>	>	>	>	>	20.62
21	0.7	>	11	>	>	3	>	>	>	>	>	>	5.67
22	1.3	>	7	>	>	3	>	>	>	>	>	>	5.07
23	6.2	>	>100	>	>	45	>	>	>	>	>	>	>48.55
24	6.2	>	41	>	>	60	>	>	>	>	>	>	33.3
25	9.0	>	58	>	>	55	>	>	>	>	>	>	44.25
26	>100	>	>100	>	>	>100	>	>	>	>	>	>	>100
EFV[p]	40	0.003	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	/
NM 108	>100	>	>100	1.5	2.4	>100	>	>	>	20	>	>	>100
NM 176	>100	>	>100	>	>	>100	>	>	>	30	25	>	>100
Ribavirine	31	>	>100	>	>	>100	>	>	>	>	>	7	>82.75
NM 299	2.0	>	>100	26	>	20	>	11	>	>	>	1.2	>55.5
M 5255	0.2	>	>100	>	>	20	>	2	nt	>	>	0.6	>40.5
ACG	>100	>	>100	>	>	>100	1.2	>	>	>	>	>	>100

[a] For structures see Table 1.
[b] Compound concentration (μM) required to reduce the viability of mock-infected MT-4 (CD4+ human T-cells containing an integrated HTLV-1 genome) cells by 50%, as determined by the MTT method.
[c] Compound concentration (μM) required to achieve 50% protection of MT-4 cells from the HIV-1 (Human Immunodeficiency Virus, type-1) induced cytopathogenicity, as determined by the MTT method.
[d] 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.
[e] Compound concentration (μM) required to achieve 50% protection of BHK cells from the YFV (Yellow Fever Virus) induced cytopathogenicity, as determined by the MTT method.
[f] Compound concentration (μM) required to achieve 50% protection of BHK cells from the Reo-1 (Reovirus, type-1) induced cytopathogenicity, as determined by the MTT method.
[g] Compound concentration (μM) required to reduce the viability of mock-infected Vero-76 (Monkey normal kidney) monolayers by 50%.
[h] Compound concentration (μM) required to reduce the plaque number of HSV-1 (Herpes simplex virus, type-1) by 50% in Vero-76 monolayers.
[i] Compound concentration (μM) required to reduce the plaque number of VV (Vaccinia virus) by 50% in Vero-76 monolayers.
[j] Compound concentration (μM) required to reduce the plaque number of VSV (Vesicular Stomatitis Virus) by 50% in Vero-76 monolayers.
[k] Compound concentration (μM) required to reduce the plaque number of CVB-5 (Coxsackievirus B5) by 50% in Vero-76 monolayers.
[l] Compound concentration (μM) required to reduce the plaque number of Sb-1 (Poliovirus type-1, Sabin strain) by 50% in Vero-76 monolayers.
[m] Compound concentration (μM) required to reduce the plaque number of RSV (Respiratory Syncytial Virus) by 50% in Vero-76 monolayers.
[n] Mean of CC_{50} values of MDBK, MT-4, BHK-21 and Vero-76 cells (for MDBK: see Table 1).
[o] The sign '>' indicates that the EC_{50} is higher than the CC_{50} for the corresponding host cell line.
[p] Efavirenz. nt = not tested.

5. Conclusions

Twenty-six 9-aminoacridine derivatives were evaluated for *in vitro* antiviral activity against a panel of 10 RNA and DNA viruses. Seventeen compounds exhibited a specific activity against BVDV, and most of them had EC_{50} in the range 0.1–8 μM, with the corresponding selectivity index up to 90, thus comparing favorably with the reference drugs ribavirine and NM 108. The observed specificity for BVDV appears rather peculiar and deserves further investigations to define the molecular target and viral or cellular factors involved in the antiviral activity.

Both the activity and cytotoxicity are related to the presence and nature of substituents on the tricyclic system and on the 9-amino group. Thus, further efforts are warranted to explore the effect on activity of other kinds of substituents on the 9-aminoacridine scaffold in order to achieve more effective and low cost anti-BVDV agents, that are of importance to relieve the heavy economic burden produced by BVDV infections of livestock (reduced dairy production, cattle mortality, etc.).

Moreover some of the tested compounds, particularly those bearing a quinolizidinylalkyl side chain, exhibited pronounced cytotoxicity, and may, therefore, be of interest as hits for antiproliferative activity. This activity could be related to the DNA minor/major groove intercalation of the acridine nucleus, while the terminal basic head or the oxygenated function of the side chain might exert a stabilizing function through the electrostatic interaction with phosphate group or additional hydrogen bonds formation. Quinacrine and other acridine derivatives are enjoying

a renewed interest as antitumoral agents, being able to target DNA without induction of DNA damage (Gurova, 2009).

Despite a large number of acridine derivatives have already been studied as anticancer agents (Denny, 2002; Sebestik et al., 2007; Gálvez-Peralta et al., 2009; Guo et al., 2009; Gurova, 2009; Neznanov et al., 2009), the present cytotoxic compounds contain novel, unexplored structural features whose relevance in the field deserve investigation.

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