ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2003, p. 2223–2230 Vol. 47, No. 7
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.7.2223–2230.2003

Detection of Inhibition of Bovine Viral Diarrhea Virus by Aromatic Cationic Molecules

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Received 8 October 2002/Returned for modification 12 December 2002/Accepted 24 March 2003

Bovine viral diarrhea virus (BVDV) is an economically significant pathogen of cattle and a problematic contaminant in the laboratory. BVDV is often used as an in vitro model for hepatitis C virus during drug discovery efforts. Aromatic dicationic molecules have exhibited inhibitory activity against several RNA viruses. Thus, the purpose of this research was to develop and apply a method for screening the aromatic cationic compounds for in vitro cytotoxicity and activity against a noncytopathic strain of BVDV. The screening method evaluated the concentration of BVDV in medium and cell lysates after 72 h of cell culture in the presence of either a 25 or 5 μM concentration of the test compound. Five of 93 screened compounds were selected for further determination of inhibitory (90 and 50%) and cytotoxic (50 and 10%) concentration endpoints. The screening method identified compounds that exhibited inhibition of BVDV at nanomolar concentrations while exhibiting no cytotoxicity at 25 μM concentrations. The leading compounds require further investigation to determine their mechanism of action, in vivo activity, and specific activity against hepatitis C virus.

Bovine viral diarrhea virus (BVDV), which is the prototype of the *Pestivirus* genus of the *Flaviviridae* family, causes early embryonic death, abortion, teratogenesis, respiratory problems, chronic wasting syndrome, and immune system dysfunction in cattle throughout the world. Acute infections of immunocompetent cattle with different strains of BVDV have caused mortality rates of 17 to 32% (8, 15, 31). Vaccines have provided inconsistent protection against infection with BVDV (36).

In addition to being a pathogen of cattle, BVDV can be a problematic contaminant in the laboratory. Two biotypes of BVDV (noncytopathic and cytopathic), which are based on their effects in cell cultures, are recognized (3). Noncytopathic biotypes of BVDV have been identified in commercially available lots of fetal bovine serum despite testing by the manufacturer (6, 45). Consequently, BVDV has been identified in commercially available bovine, canine, feline, and primate cell lines (16, 19), human viral vaccines for measles-mumps-rubella (18, 21), and interferons for human use (20). Viral contamination of biologicals for human use may be the reason BVDV-specific antibodies have been found in some samples of human serum (17). No antiviral pharmaceuticals are currently available for controlling BVDV in the laboratory or on the farm.

Hepatitis C virus (HCV), a member of the *Hepacivirus* genus of the family *Flaviviridae*, is a major cause of human liver disease throughout the world. The World Health Organization estimates that 170 million people are chronically infected with HCV (2). The organization of the HCV genome encoding the proteins for viral replication is very similar to that of BVDV (with the exception of the 5′-terminal protease) (4). The inability to propagate HCV efficiently by cell culture has caused researchers to adopt BVDV as a viral model for HCV (2). Because of the relatedness of BVDV and HCV, research with the inhibitors of BVDV replication is useful both for the control of animal diseases and for efforts to discover drugs effective against HCV. Aromatic cationic molecules have exhibited inhibitory activity against respiratory syncytial virus (14), rotavirus (39), and human immunodeficiency virus (24). Therefore, the purpose of this research was to develop and apply a method of screening aromatic cationic molecules for in vitro toxicity and activity against BVDV. These cationic molecules were selected for study in view of their recognition of specific RNA sequences in other RNA viruses (27).

MATERIALS AND METHODS

Compounds. All of the compounds screened in this study were synthesized in the laboratory of one of the authors (D.W.B.). Stock solutions of 5 or 10 mM were made in sterile distilled water or dimethyl sulfoxide (DMSO) and stored at −80°C until use. While screening results for 41 compounds are described, 52 additional compounds were screened. These additional compounds exhibited cytotoxicity and/or lack of antiviral effect. Information concerning these compounds is available upon request. The synthesis and physical properties of the following compounds have been described in the references indicated: compound DB18 (12); compounds DB60, DB75, and DB99 (11); compound DB289 (7); compounds DB293, DB294, and DB302 (22); compound DB501 (41); compounds DB606, DB771, and DB772 (26); and compounds DB701, DB711, DB748, DB762, and DB763 (35). Brief descriptions of the synthesis and physical properties of DB779, DB458, DB456, and DB459 are given here. The descriptions of the synthesis of the remaining compounds will be published separately.

Melting points (mp) were determined in open capillary tubes with a Mel-Temperature 3.0 capillary mp apparatus and are reported uncorrected. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity + 300 or a Varian VRX 400 instrument. Coupling constants are reported in hertz. Mass spectra were recorded on a VG Instruments 70-SE spectrometer at the Georgia Institute of Technology, Atlanta. Elemental analyses were performed by Atlantic Microlab, Norcross, Ga. All of the chemicals and solvents were purchased from Aldrich Chemical Co., Fisher Scientific, or Acros Organics.

2,5-Bis(3-ethoxy-4-guanidinophenyl)furan dihydrochloride (DB779). 2-Nitro-5-bromophenetole (64% yield; mp, 78 to 79°C [ethanol-water]) was produced by

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the reaction of 3,4-dinitrobromobenzene with sodium ethoxide in ethanol (37). Coupling of the bromo compound with 2,5-bis(tributylstannyl)furan gave, after recrystallization from *N*,*N*-dimethylformamide–methanol, 2,5-bis(3-ethoxy-4-nitrophenyl)furan as a yellow-orange fluffy solid (75% yield; mp, 192 to 194°C). ^{1}H NMR (DMSO-d_6): 1.38 (t, 6H), 4.34 (q, 4H), 7.51 (s, 2H), 7.59 (dd, J = 8.4, 1.8, 2H), 7.69 (d, J = 1.8 Hz, 2H), 7.97 (d, J = 8.7, 2H). Analysis calculated for $C_{20}H_{18}N_2O_7$ (398.36): C, 60.30; H, 4.55; N, 7.03. Found: C, 60.34; H, 4.58; N, 6.93.

Hydrogenation with Pd on C gave, after crystallization from methanol-water, 2,5-bis(4-amino-3-ethoxyphenyl)furan as a light green and tan solid (85% yield). ^{1}H NMR (DMSO-d_6): 1.36 (t, 6H), 4.07 (q, 4H), 4.85 (br s, 4H), 6.63 to 6.68 (m, 4H), 7.10 (m, 4H). From the diamine, the title bis-guanidine was prepared as a light green hygroscopic solid (76% yield for a two-step procedure). ^{1}H NMR (DMSO-d_6): 1.38 (q, 6H), 4.21 (q, 4H), 7.21 (d, 2H), 7.27 (dd, J = 8.1, 2.1, 2H), 7.42 (br s, 8H), 7.44 to 7.49 (m, 4H), 9.40 (br s, 2NH). Mass spectrum (electrospray): *m/e* 423.3 (60% yield; $M^+ - 2HCl$). Analysis calculated for $C_{22}H_{26}N_6O_3 \cdot 2HCl \cdot 0.5H_2O$ (504.41): C, 52.38; H, 5.79; N, 16.67. Found: C, 52.25; H, 5.81; N, 16.52.

2-[5(6)-{*N*-Isopropylamidino}-2-benzimidazoyl]-5-(4-nitrophenyl)furan (DB458). A mixture of 5-(4-nitrophenyl)furfural (0.434 g, 0.002 mol), 4-*N*-isopropylamidino-1,2-phenylenediamine hydrochloride hydrate (0.493 g, 0.002 mol), and 1,4-benzoquinone (0.216 g, 0.002 mol) in 40 ml of ethanol (under nitrogen) was heated at reflux for 6 h. The volume of the reaction mixture was reduced to about 15 ml under reduced pressure, the mixture was cooled, and the resultant solid was collected by filtration to yield 0.66 g (80%) of the monohydrochloride salt. The monohydrochloride salt was dissolved in 100 ml of ethanol and acidified with HCl-saturated ethanol, and after cooling in an ice bath, the resultant solid was filtered, washed with ether, and dried for 24 h in a vacuum oven at 75°C to yield 0.7 g (91%) at an mp of >300°C. ^{1}H NMR (DMSO-d_6/D_2O): 8.26 (d, J = 8.8, 2H), 8.11 (d, J = 8.8 Hz, 2H), 8.01 (d, J = 1.2, 1H), 7.77 (d, J = 8.8, 1H), 7.59 (dd, J =1.2, 8.8, 1H), 7.50 (d, J = 7.6, 1H), 7.42 (d, J = 7.6, 1H), 4.04 (septet, J = 6.8, 1H), 1.3 (d, J = 6.8, 6H). ^{13}C NMR (DMSO-d_6): 162.7, 153.8, 147.2, 145.2, 144.8, 140.7, 138.2, 135.2, 125.4, 124.7, 124.0, 123.5, 116.3, 115.9, 115.3, 112.6, 45.6, 21.4. Fast atom bombardment mass spectrum (FABMS): *m/e* 376 (M^+ + 1). Analysis calculated for $C_{21}H_{19}N_5O_3 \cdot 2HCl \cdot 2.0H_2O$: C, 49.71; H, 5.16; N, 13.80. Found: C, 49.65; H, 5.11; N, 13.50.

2-[5(6)-{2-Imidazolinyl}-2-benzimidazoyl]-5-(4-aminophenyl)furan (DB456). The monohydrochloride salt of the nitro analog described above (0.5 g, 0.0013 mol) and 0.2 g of 10% Pd or C in 130 ml of methanol were subjected to hydrogenation at 50 lb/in^2 for 4 h. The catalyst was removed by filtration over diatomaceous earth and by washing with warm methanol. The solvent volume was reduced to approximately half under reduced pressure. The flask containing the solution was placed in an ice bath and saturated with HCl gas. The mixture was stirred at room temperature for 4 h and treated with dry ether, and the solid was collected by filtration. The solid was dried under vacuum at 75°C for 24 h to yield 0.55 g (86%) at an mp of >300°C. ^{1}H NMR (DMSO-d_6/D_2O): 8.24 (d, J = 1.2, 1H), 7.88 (d, J = 8.0, 2H), 7.80 (s, 2H), 7.51 (d, J = 3.6, 1H), 7.21 (d, J = 8.4, 2H), 7.10 (dd, J = 1.2, 3.6, 1H), 4.0 (s, 4H). ^{13}C NMR (DMSO-d_6/D_2O): 165.8, 156.4, 145.8, 142.0, 140.9, 137.9, 126.2, 123.7, 121.0, 117.0, 116.8, 115.3, 108.5, 44.6. FABMS: *m/e* 344 (M^+ + 1). Analysis calculated for $C_{20}H_{17}N_5O \cdot 3HCl \cdot 2.1H_2O$: C, 48.96; H, 4.97; N, 14.27. Found: C, 48.58; H, 4.32; N, 14.27.

2-[5(6)-*N*-Isopropylamidino-2-benzimidazoyl]-5-(4-aminophenyl)furan (DB 459). The monohydrochloride salt of the nitro analog described above (0.411 g, 0.001 mol) and 0.3 g of 10% Pd or C in 120 ml of methanol were subjected to hydrogenation at 50 lb/in^2 for 4 h. The catalyst was removed by filtration over Filteraid. The solvent volume was reduced to approximately half under reduced pressure. The flask containing the solution was placed in an ice bath and saturated with HCl gas. The mixture was stirred at room temperature for 4 h and treated with dry ether, and the solid was collected by filtration. The solid was dried under vacuum at 80°C for 24 h to yield 0.41 g (87%) at an mp of >300°C. ^{1}H NMR (DMSO-d_6/D_2O): 8.04 (d, J = 1.6, 1H), 7.91 (d, J = 8.4, 2H), 7.80 (d, J = 8.4, 1H), 7.64 (dd, J = 1.6, 8.4, 1H), 7.60 (d, J = 4.0, 1H), 7.24 (d, J = 8.4, 2H), 7.14 (d, J = 4.0, 1H), 4.05 (septet, J = 6.4, 1H), 1.3 (d, J = 6.4, 6H). ^{13}C NMR (DMSO-d_6): 162.4, 156.8, 144.4, 140.9, 138.8, 137.6, 135.0, 126.3, 125.4, 124.6, 124.1, 121.1, 118.0, 115.6, 114.9, 108.6, 45.6, 21.3. FABMS, *m/e* 360 (M^+ + 1). Analysis calculated for $C_{21}H_{21}N_5O_3 \cdot 3HCl$: C, 53.80; H, 5.15; N, 14.93. Found: C, 54.22; H, 4.75; N, 15.05.

Test organism and medium. A genotype I, noncytopathic strain of BVDV (SD-1) was used for the determination of viral inhibition (13). Stock virus, initially isolated from the serum of a persistently infected cow, was propagated in BVDV-free Madin Darby bovine kidney (MDBK) cells cultured in minimum essential medium with Earle's salts supplemented with 10% (vol/vol) equine serum, 0.75 mg of sodium bicarbonate/ml, 0.29 mg of *L*-glutamine/ml, and antibiotics (100 U of penicillin G/ml, 100 μg of streptomycin/ml, and 0.25 μg of amphotericin B/ml) (MEM-eq). Virus was harvested by freezing and thawing and was stored as aliquots in cryovials at −80°C until needed. The 50% cell culture infective doses ($CCID_{50}$) of virus per milliliter were determined by the statistical method of Reed and Muench (33). Antiviral testing was performed in MEM-eq.

Virus inhibition test. Initially, 93 compounds were screened at 25 and/or 5 μM for antiviral effects and cytotoxicity. Compounds were selected for further testing based on their inhibition of viral replication without visible cytotoxicity in cell culture. For five selected compounds (DB456, DB459, DB606, DB771, and DB772), antiviral activity and cytotoxicity were evaluated in twofold dilutions of the compound at concentrations of 25 to 0.007 μM to determine the inhibitory concentrations at 90% (IC_{90}) and at 50% (IC_{50}).

MDBK cells (4×10^4 to 8×10^5/2.0-cm^2-surface-area well) that were free of BVDV were incubated in 200 μl of medium containing the test compound (or no compound for the negative control) for 15 min before inoculation with BVDV (strain SD-1) at a multiplicity of infection of 0.5. The cells were cultured with this inoculum for 1 h at 38.5°C with 5% CO_2 in humidified air. After this 1-h incubation, culture medium was removed and the cells were washed with 1 ml of Dulbecco's phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$. Immediately after the cells were washed, 1 ml of antiviral test medium that contained the test compound (or maintained as the negative control) was placed on the inoculated monolayer. After 72 h of incubation at 38.5°C with 5% CO_2 in humidified air, the cell monolayers were observed for cytotoxic effect with an inverted culture microscope at a magnification of ×400. Culture medium was then removed from the cell monolayer and stored at −80°C for later viral assay. One milliliter of fresh medium containing no test compound was added to the cell monolayers prior to freezing at −80°C and thawing to lyse MDBK cells for the purpose of releasing any intracellular BVDV.

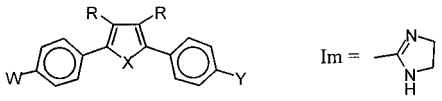
Virus isolation and detection. BVDV was detected and quantified from cell culture medium and cell lysate samples by endpoint dilution immunoperoxidase monolayer assays (1). Samples were assayed in triplicate in a 96-well culture plate by adding 50 μl of MEM-eq containing approximately 2.5×10^3 MDBK cells to 25-μl aliquots of sample diluted in 75 μl of MEM-eq. One-hundred-microliter aliquots of serial dilutions (10^{-1} to 10^{-7}) of all of the samples were also assayed for BVDV in triplicate. Plates were incubated for 72 h at 38.5°C in a humidified atmosphere of 5% CO_2 and air before the immunoperoxidase labeling technique was performed.

After incubation for 72 h and subsequent fixation with 20% acetone, potentially infected cells were incubated with monoclonal antibody D89 (38, 43), which is specific for E2 (gp53), a major envelope glycoprotein of BVDV (44), and monoclonal antibody 20.10.6, which is specific for NS3 (p80) or unprocessed NS2/3 (p125), a conserved nonstructural protein (10). After the cells were washed with PBS and Tween 20 to remove unbound antibodies, peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Jackson Immuno Research Lab, West Grove, Pa.) was added. After a short incubation period, unbound peroxidase-conjugated antibody was removed by washing with PBS and Tween 20. Finally, the enzyme substrate, aminoethyl carbazole (Zymed Laboratories, South San Francisco, Calif.), which produces a reddish-brown color when oxidized by horseradish peroxidase, was added. Color change was visualized under light microscopy and compared to those of the known positive and negative controls on each plate.

Toxicity evaluation. For five selected compounds (DB456, DB459, DB606, DB771, and DB772), the viability of drug-treated cell cultures was quantitated by using the tetrazolium-based compound XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (42) in twofold dilutions of the compound at concentrations from 100 to 1.56 μM to determine the 50 and 10% cytotoxic concentrations (CC_{50} and CC_{10}).

A cell suspension of MDBK cells was divided into aliquots in antiviral test medium in 96-well plates to give a final cell count per well of approximately 10^4. The plates were incubated at 37°C for 1 to 2 h to allow for cell attachment. Following visual confirmation of cell attachment, dilutions of selected compounds were added to the appropriate experimental wells to give a final well concentration of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, or 0 μM. The plates were incubated at 37°C for 24 h. Each plate contained multiple controls of untreated cells and medium without cells. Each compound was assayed for toxicity at each concentration in six replicates. Prior to addition of the XTT reagent, the antiviral test medium in the 96-well plate was removed and replaced with 100 μl of medium lacking phenol red/well. The XTT reagent was then added (25 μl/well), and the plate was incubated at 37°C for 20 min. The optical density (OD) of each well was then read with a SPECTRAFluor Plus microplate fluorometer (TECAN) at 620 and 450 nm by using the 405/450/492/620-nm absorbance filter. The amount of reduced XTT was calculated by subtracting the sample's OD at 620 nm (OD_{620}; turbidity) from its OD_{450} ($OD_{450} - OD_{620}$ = OD due to XTT

TABLE 1. Structures and in vitro activities of cationic diaryl 5-membered ring compounds



Compound no.	Molecule at[a]:				BVDV remaining (% of control[b]) at indicated concn of compound			
					25 μM		5 μM	
	X	R	W	Y	Medium	Cell lysates	Medium	Cell lysates
DB18	S	H	Am	Am	—[c]	—	—	—
DB60	O	H	Im	Im	—	—	—	—
DB75	O	H	Am	Am	<0.001	<0.001	180	8
DB99	O	CH_3	Am	Am	0.6	0.3	31	32
DB289	O	H	*O*-MeAm	*O*-MeAm	180	32	180	18
DB606	O	H	OCH_3	Im	—	—	<0.001	<0.001
DB777	O	H	OCH_3	NHAm	—	—	10	31
DB784	O	H	OCH_3	NH(C═NH)-2-pyridyl	3.2	1.8	32	18

[a] Am, C(═NH)NH_2; *O*-MeAm, C(═NOMe)NH_2.
[b] Percentage of control = ($CCID_{50}$ of BVDV in compound sample)/($CCID_{50}$ of BVDV in control sample lacking compound).
[c] —, cytotoxic effect of compound observed after 72 h.

reduction). This final OD value was calculated for the cell and medium controls, as well as for the compound assays.

Statistical calculations. The $CCID_{50}$ of BVDV in cell culture medium and cell lysate samples were determined by the statistical method of Reed and Muench (33). BVDV in cell culture medium and cell lysate samples was evaluated by comparison to equivalent samples from temporal control cultures in which no compound was added before or after inoculation [percentage of control = ($CCID_{50}$ of BVDV in the compound sample)/($CCID_{50}$ of BVDV in the control sample lacking the compound)]. The IC_{90} and IC_{50} of selected compounds were calculated with JMP software by least-squares regression techniques using the logarithm of the percentage of the control for each sample (34).

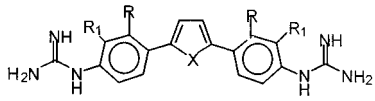
The toxicity of each compound at each concentration was calculated as a percentage of the untreated cell control by first subtracting the average final OD of the medium control wells from the final OD values of the cell controls and drug assays. The drug assay values were then compared to the untreated cell control average value for each plate and evaluated as percentage of control, with 100% being equivalent to the cell control value [(drug assay values/average cell control value) × 100 = percentage of untreated cell control]. The CC_{50} and CC_{10} of selected compounds were calculated with JMP software by least-squares regression techniques using the square of the percentage of control of each sample (34).

RESULTS

Screening assays. During screening assays at both 25 and 5 μM concentrations, 4 of 93 molecules were visibly toxic to MDBK cells in the exponential growth phase (Tables 1 through 5). At a concentration of only 25 μM, eight additional molecules were visibly toxic to the MDBK cells. Screening assays identified 5 of 93 aromatic cationic molecules that were selected for determination of CC_{50}, CC_{10}, IC_{90}, and IC_{50} for BVDV in cell culture (Fig. 1).

Analysis of structure-activity relationships: cationic diaryl five-membered ring compounds. As a group, the cationic diaryl five-membered ring compounds were cytotoxic in this assay (Table 1) compared to other groups of compounds. The inclusion of oxygen rather than sulfur within the central five-membered ring was noted to decrease cytotoxicity (compare results

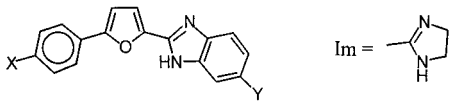
TABLE 2. Structures and in vitro activities of dicationic guanidino compounds



Compound no.	Molecule at:			BVDV remaining (% of control[b]) at indicated concn of compound			
				25 μM		5 μM	
	X	R	R_1	Medium	Cell lysates	Medium	Cell lysates
DB701	O	CF_3	H	0.2	0.6	NT	NT
DB711	O	OCH_3	H	7	320	NT	NT
DB748	O	CH_3	CH_3	31	320	NT	NT
DB752	S	CH_3	H	—	—	NT	NT
DB762	O	OEt	H	21	28	120	900
DB763	O	H	OCH_3	<0.001	<0.001	2	9
DB778	O	Oi-Pr	H	1.8	3	31	56
DB779	O	H	OEt	<0.001	<0.001	1.8	0.6

[a] NT, not tested.
[b] See Table 1, footnotes *b* and *c*, for details.

TABLE 3. Structures and in vitro activities of cationic benzimidazole-substituted aryl furans[a]



Compound no.	Molecule at[b]:		BVDV remaining (% of control[d]) at indicated concn of compound			
			25 μM		5 μM	
	X	Y	Medium	Cell lysates	Medium	Cell lysates
DB293	Am	Am	10	0.2	57	100
DB294	i-PrAm	i-PrAm	—	—	57	56
DB302	Im	Im	1,000	32	100	56
DB456	NH_2	Im	0.1	2	0.2	<0.001
DB457	NO_2	Im	0.003	0.006	NT	NT
DB458	NO_2	i-PrAm	—	—	NT	NT
DB459	NH_2	i-PrAm	<0.001	0.002	100	10
DB501	NH_2	Am	27	6	47	100
DB771	Am	H	<0.001	<0.001	<0.001	<0.001
DB772	Im	H	<0.001	<0.001	<0.001	<0.001
DB805	NO_2	c-PrAm	—	—	—	—

[a] For structure of Im, see Table 1.
[b] Am, C(═NH)NH_2, i-PrAm, C(═NH)NH-*i*-Pr; c-PrAm, C(═NH)NH-*c*-Pr.
[c] NT, not tested.
[d] See Table 1, footnotes *b* and *c*, for details.

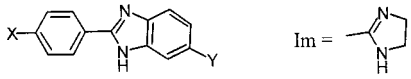
for DB18 and DB75), while the addition of methyl substituents to the central five-membered ring decreased antiviral efficacy. From this chemical group, DB606 was selected for further antiviral characterization.

Dicationic guanidino compounds. As a group, the dicationic guanidino compounds also exhibited a lack of toxicity when oxygen was substituted for sulfur within the central five-membered ring (Table 2). While two compounds within this group, DB763 and DB779, exhibited significant antiviral effects, they were not selected for further testing due to the identification of compounds in other chemical groups that were more efficacious at the 5 μM concentration.

Cationic benzimidazole-substituted aryl furans. Within this chemical group, several compounds exhibited effective inhibition of viral growth at 5 μM concentrations without evidence of cytotoxic effect at 25 μM concentrations (Table 3). Compounds DB456, DB459, DB771, and DB772 were selected for further antiviral characterization.

Cationic aryl benzimidazole compounds. This group of compounds exhibited limited antiviral effects at 5 μM concentra-

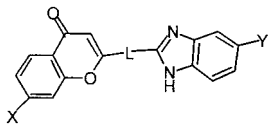
TABLE 4. Structures and in vitro activities of cationic aryl benzimidazole compounds



Compound no.	Molecule at[a]:		BVDV remaining (% of control[c]) at indicated concn of compound			
			25 μM		5 μM	
	X	Y	Medium	Cell lysates	Medium	Cell lysates
DB682	5-(p-$NH_2C_6H_4$)furan-2yl	Am	—	—	—	—
DB683	5-(p-$NH_2C_6H_4$)furan-2yl	Im	—	—	8	100
DB684	5-(p-$NH_2C_6H_4$)furan-2yl	i-PrAm	0.007	0.03	0.8	31
DB685	5-(p-$NO_2C_6H_4$)furan-2yl	THP	—	—	0.8	180
DB759	p-$MeOC_6H_4CH_2CH_2$	Im	—	—	21	280
DB787	p-$MeOC_6H_4CH_2CH_2$	Am	NT	NT	320	10
DB788	p-$MeOC_6H_4CH_2CH_2$	i-PrAm	NT	NT	180	100
DB789	p-$MeOC_6H_4CH_2CH_2$	c-pentyl	NT	NT	100	18
DB790	p-$EtC_6H_4CH_2CH_2$	Am	NT	NT	56	100
DB791	p-$EtC_6H_4CH_2CH_2$	Im	NT	NT	83	10
DB792	p-$EtC_6H_4CH_2CH_2$	i-PrAm	NT	NT	56	18
DB793	p-$EtC_6H_4CH_2CH_2$	c-pentyl	NT	NT	56	57
DB794	p-$FC_6H_4CH_2CH_2$	Am	NT	NT	180	10
DB795	p-$FC_6H_4CH_2CH_2$	Im	NT	NT	32	6
DB796	p-$FC_6H_4CH_2CH_2$	i-PrAm	NT	NT	100	6
DB797	p-$FC_6H_4CH_2CH_2$	c-pentyl	NT	NT	56	18

[a] Am = C(═NH)NH_2, i-PrAm = C(═NH)NH-*i*-Pr. For structure of Im, see Table 1.
[b] NT, not tested.
[c] See Table 1, footnotes *b* and *c*, for details.

TABLE 5. Structures and in vitro activities of cationic 4H-1-benzopyran-4-one substituted benzimidazoles



Compound no.	Molecule at[a]:			BVDV remaining (% of control[b]) at indicated concn of compound			
	X	Y	L	25 μM		5 μM	
				Medium	Cell lysates	Medium	Cell lysates
DB636	Ph	Am	Nil	36	280	20	90
DB637	Ph	i-PrAm	Nil	360	910	31	91
DB638	H	Am	1,4-phenylene	100	560	650	160
DB640	H	i-PrAm	1,4-phenylene	2,400	160	120	280
DB737	OCH_3	i-PrAm	1,4-phenylene	2,400	910	2,400	160
DB738	H	i-PrAm	2,5-dithienyl	36	28	65	28

[a] Am = C(=NH)NH_2, i-PrAm = C(=NH)NH-*i*-Pr.
[b] See Table 1, footnotes *b* and *c*, for details.

tions (Table 4). Only agents with significant antiviral effects at 5 μM were screened for effects at the 25 μM concentration. Of those agents screened at the higher concentration, four of five compounds exhibited cytotoxicity.

Cationic 4H-1-benzopyran-4-one-substituted benzimidazoles. The cationic 4H-1-benzopyran-4-one-substituted benzimidazoles exhibited no cytotoxicity and no significant antiviral effects (Table 5).

Compounds selected for IC_{90}, IC_{50}, CC_{50}, and CC_{10} endpoint determinations. Five agents were evaluated at multiple dilutions for determination of CC_{50} and CC_{10} by the XTT assay and for determination of IC_{90} and IC_{50} for BVDV in cell culture (Fig. 1). Of these agents, DB606 exhibited the greatest cytotoxicity at the 25 μM concentration. The IC_{90} results ranged from 15.9 (DB456) to 0.018 (DB772) μM concentrations. The IC_{50} results ranged from 13.6 (DB456) to 0.014 (DB772) μM concentrations. Agent DB772 exhibited IC_{90}s at 18 and 20 nM concentrations, but did not exhibit toxicity to transformed cells in exponential growth phase at a concentration of 25 μM (Fig. 1).

DISCUSSION

As BVDV is an economically significant pathogen of cattle (23), a problematic contaminant in the laboratory (18, 21), and a viral model for HCV to facilitate in vitro drug discovery efforts (46), the goal of this research was to screen aromatic cationic molecules for in vitro toxicity and activity against a noncytopathic strain of BVDV. The use of a noncytopathic rather than a cytopathic strain of BVDV permitted evaluation of the cytotoxic effects of chemical compounds without the confounding cytopathic effects of the virus. This choice of viral biotypes did increase the labor necessary to assess antiviral efficacy, as immunoperoxidase labeling with BVDV-specific antibodies was required for viral detection and quantitation. As cleavage of the nonstructural NS2/3 viral protein is the source of cytopathogenicity (25), compounds that prevent this cleavage could prevent cytopathic effects without altering viral attachment, infection, replication, packaging, or release. Thus, the use of a noncytopathic strain of BVDV prevented the inhibition of NS2/3 cleavage from being misinterpreted as inhibition of the viral life cycle. This screening method allowed the timely detection of compounds that exhibited a lack of in vitro cytotoxicity but that effectively inhibited the viral life cycle.

The screening method described in this report is an appropriate precursor to in vivo testing of the leading compounds for prevention or treatment of BVDV infections. Regardless of their therapeutic effects in vivo, compounds that exhibit nontoxic inhibition of the viral life cycle might be used to reduce the risks of contamination of vaccines and biologics produced in cell culture (45). If the leading compounds are effective against BVDV in vivo, their use during critical time periods might reduce the need to use antibiotics for treatment of the bacterial infections that are sequelae of the pulmonary tissue damage produced by BVDV.

The compounds selected by this screening method, particularly DB771 and DB772, are strong candidates for further testing to determine their efficacy against HCV. It is noteworthy that the most effective compounds identified by this screening method are monocations, which are significantly more potent than related dicationic molecules. Due to the inability to grow HCV effectively in cell culture, current investigations of potential therapeutic agents involve in vitro subgenomic HCV replicon assays with selected compounds advanced into a chimpanzee animal model or a transgenic mouse model for HCV infection (28). The limited availability and significant expense of the chimpanzee animal model and the transgenic mouse model require that in vitro screening be performed to identify the leading compounds. Subgenomic HCV replicon assays consist of transfecting permissive cells with a subset of the viral RNA which can be translated into nonstructural proteins that replicate the positive-sense viral RNA (5, 29). Thus, the replicon assay is limited to detection of antiviral agents that inhibit binding of the 5′nontranslated region of the HCV genome to cellular ribosomes, translation of the viral RNA, or replication of the viral RNA within the cytoplasm. The screening method described in this research has the potential to identify compounds that exhibit a mechanism of action beyond the scope of the subgenomic replicon assay. As the RNA-dependent RNA polymerase of HCV lacks proofreading ability and exhibits a

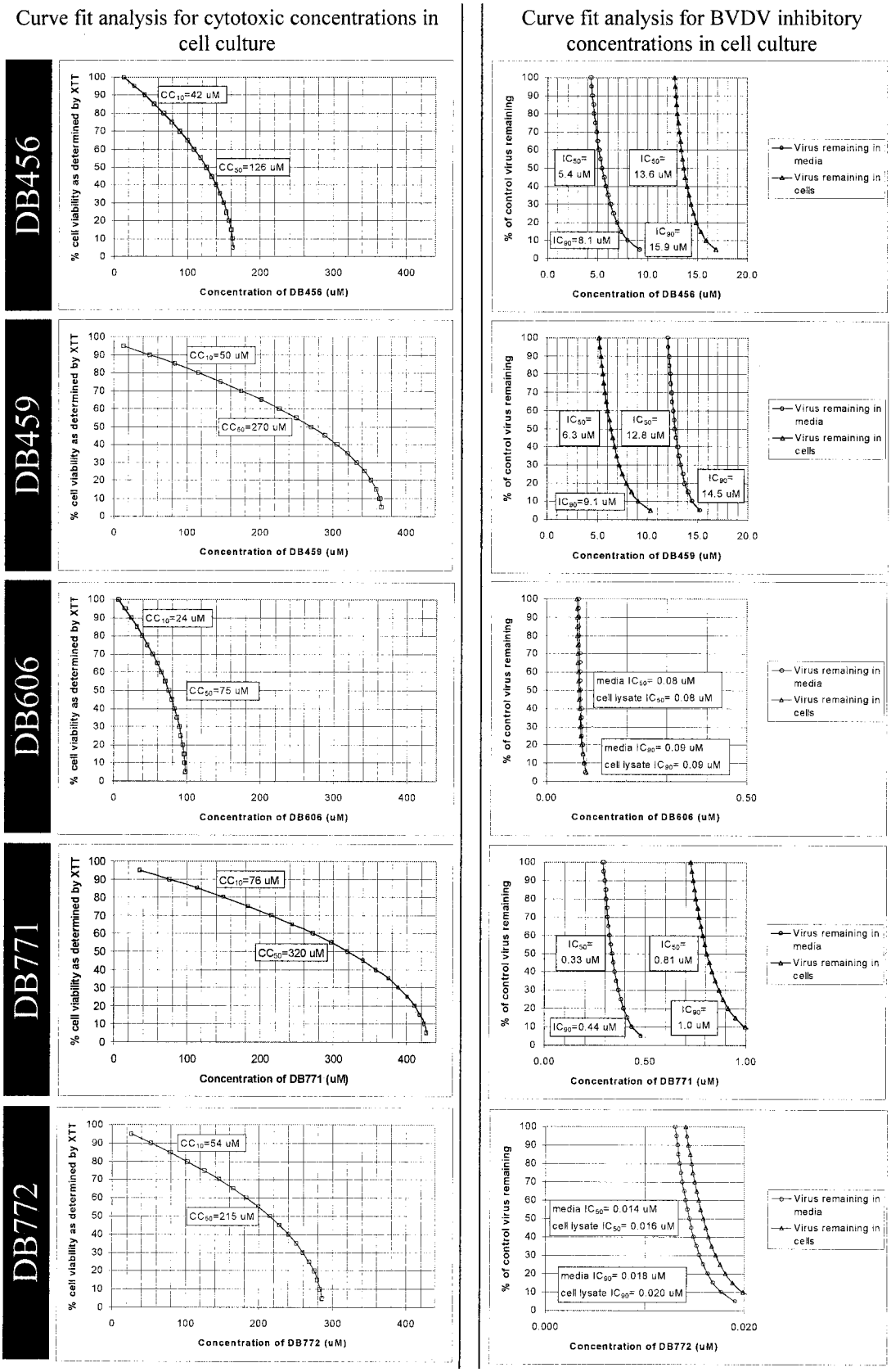


FIG. 1. Curve fit analysis for cytotoxic concentrations and BVDV inhibitory concentrations in cell culture of selected aromatic cationic molecules.

mutation rate of approximately 5×10^{-3}/site per year (9), a number of antiviral agents directed at multiple targets will be required to reduce or eliminate HCV quasispecies that have developed resistance to other drugs (5). If the replicon assay is used as the sole determinant in selecting HCV antiviral candidates, then compounds with antiviral targets involving viral attachment and entry into cells, packaging of viral RNA within the virion, or viral release will be regrettably overlooked. We believe that the screening methods described in this research complement the subgenomic HCV replicon assay and provide utility for the identification of compounds or mechanisms of action that might effectively inhibit HCV infection and replication.

The mechanisms of action of the antiviral agents identified in this research are yet to be determined. Since related compounds have been identified as protease inhibitors (14, 39) and helicase inhibitors (32) and have been shown to be effective in binding to nucleic acids (24, 40), multiple mechanisms of action might be employed by the nontoxic antiviral compounds reported here. Mechanisms of action of other anti-BVDV agents include inhibition of viral RNA-dependent RNA polymerase (2) and of α-glucosidase, which results in the misfolding of viral proteins (30). Further research will likely identify additional targets for compounds that inhibit replication of pestiviruses and HCV.

In conclusion, a method of in vitro screening with a noncytopathic strain of BVDV enabled the identification of aromatic cationic molecules that inhibit the BVDV life cycle. These leading compounds, which inhibit BVDV at nanomolar concentrations and exhibit limited cytotoxicity at 25 μM, merit further investigation to determine their mechanisms of action, in vivo efficacies, and specific activities against HCV.

ACKNOWLEDGMENTS

This work was supported by an NIH development grant (NIAID AI-01728-01) and Immtech International, Inc., Vernon Hills, Ill.

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