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BROAD-SPECTRUM ANTIVIRAL ACTIVITY OF CARBODINE, THE CARBOCYCLIC ANALOGUE OF CYTIDINE

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Abstract—Carbocyclic cytidine (C-Cyd) is a broad-spectrum antiviral agent active against DNA viruses [pox (vaccinia)], (+)RNA viruses [toga (Sindbis, Semliki forest), corona], (−)RNA viruses [orthomyxo (influenza), paramyxo (parainfluenza, measles), rhabdo (vesicular stomatitis)] and (±)RNA viruses (reo). The target enzyme of C-Cyd is supposed to be CTP synthetase that converts UTP to CTP. In keeping with this assumption are the observations that (i) C-Cyd effects a dose-dependent inhibition of RNA synthesis in both virus-infected and uninfected cells, and (ii) exogenous addition of either Urd or Cyd reverses both the antiviral and cytocidal activity of C-Cyd, whereas addition of dThd or dCyd fails to do so. The selectivity of C-Cyd against Sindbis, vesicular stomatitis and reo virus is markedly increased when C-Cyd is combined with Cyd (10 μg/mL). This combination may therefore be worth pursuing as a chemotherapeutic modality for the treatment of virus infections.

Carbocyclic nucleoside analogues, which contain a cyclopentyl or -pentenyl ring instead of the usual ribose or 2-deoxyribose moiety, have received considerable attention as potential chemotherapeutic (i.e. antitumor and antiviral) agents. These carbocyclic analogues are resistant to phosphorolysis by nucleoside phosphorylases which cleave the *N*-glycosidic linkage of regular nucleosides and thereby abrogate their antiviral or antitumor activity. Various carbocyclic derivatives of pyrimidine nucleosides have been synthesized [1–10]. When derived from 5-substituted 2′-deoxyuridines with anti-herpes virus activity, the carbocyclic compounds, akin to their parent compounds, appeared to specifically block herpes simplex virus (HSV) replication [4, 6, 7, 9]. The carbocyclic analogues of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (IVDU) were shown to react equally well with the viral 2′-deoxythymidine kinase (TK) as their parent compounds [11] and, following phosphorylation by the virus-infected cells to their triphosphate forms, both IVDU and its carbocyclic counterpart may be incorporated into DNA of these cells [12].

The cyclopentyl and cyclopentenyl derivatives of cytosine, termed C-Cyd and Ce-Cyd respectively, differ from the carbocyclic dUrd derivatives in that they are not only active against TK^+ HSV, but also TK^- HSV [10, 13] and other herpes viruses [varicella-zoster (VZV), cytomegalovirus (CMV)] [10] as well as influenza A virus [14]. Ce-Cyd has been pursued primarily as an antitumor agent [10, 15–17]. Its mode of cytostatic action has been attributed to a depletion of CTP pools, resulting from an inhibitory effect of the triphosphate metabolite of Ce-Cyd on CTP synthetase, the enzyme that converts UTP to CTP [10, 15, 17].

Also, C-Cyd is assumed to interact with CTP synthetase after it has been phosphorylated intracellularly to the 5′-triphosphate [14]. C-Cyd (also referred to as carbodine) has proved active against various influenza virus strains *in vitro* [14, 18]. In preliminary experiments it did not show efficacy against lethal influenza virus infections in mice when administered systemically or intranasally in doses up to apparent dose-limiting toxicity [14]. The present studies were undertaken to (i) delineate the antiviral activity spectrum of C-Cyd; (ii) explore its mechanism of antiviral action; and (iii) work out a therapeutic modality to increase its antiviral selectivity.

MATERIALS AND METHODS

Compounds. C-Cyd (carbodine) was synthesized as described by Shealy and O'Dell [1, 2]. The synthesis of C-c^{3}Ado, the carbocyclic analogue of 3-deazaadenosine has been described by Montgomery *et al.* [19]. Ribavirin (Virazole) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). The formulae of the test compounds are presented in Fig. 1. The nucleosides 2′-deoxythymidine (dThd), uridine (Urd), 2′-deoxycytidine (dCyd) and cytidine (Cyd) were obtained from the Sigma Chemical Co. (St Louis, MO).

Radiochemicals. The radiolabeled precursors [*methyl*-^{3}H]-2′-deoxythymidine, [5-^{3}H]uridine and [4,5-^{3}H]leucine, used to monitor the synthesis of cellular DNA, RNA and protein, were obtained from Amersham (Bucks, U.K.). Their specific radioactivity was 40, 30 and 52 Ci/mmol, respectively.

Viruses. The origin of all viruses used in the present assay has been documented previously [20], except for rhinovirus type 1A (ATCC VR-242) and coronavirus (strain 229E) (ATCC VR-740) which were obtained from the American Type Culture Collection (Rockville, MD).

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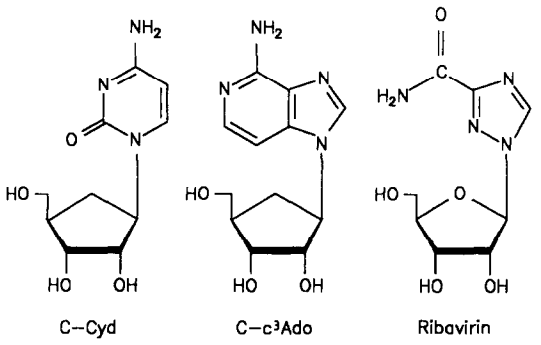

Fig. 1. Formulae of carbocyclic cytidine (C-Cyd, carbodine), carbocyclic 3-deazaadenosine (C-c^{3}Ado) and ribavirin (virazole).

Cells. The cell lines used for the antiviral activity assays were: PRK (primary rabbit kidney), HeLa (a human epithelial cell line derived from a cervix carcinoma), Vero (a simian fibroblast cell line derived from African green monkey kidney), WI-38 (human embryonic lung diploid fibroblasts, ATCC CCL75), E_6SM (human embryonic skin-muscle fibroblasts), HK (a human kidney fibroblast cell line), HEp-2 (a human epithelial cell line derived from a larynx carcinoma), RK13 (a rabbit kidney cell line), BSC-1A (a simian epithelial cell line derived from African green monkey kidney), CV-1 (a simian fibroblast cell line derived from African green monkey kidney), BHK-21 (a baby hamster kidney fibroblast cell line), and BALB/3T3 (murine fibroblasts derived from BALB/C mouse embryos). The cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum.

Antiviral activity. Inhibition of virus-induced cytopathogenicity was measured following well-established procedures [20, 21]. In all viral cytopathogenecity assays the virus inoculum as 100 $CCID_{50}$ per microtiter well (1 $CCID_{50}$ corresponding to the virus stock dilution that proved infective for 50% of the cell cultures).

Inhibition of virus multiplication was measured in HeLa cells infected with vesicular stomatitis virus, again following a well-established procedure [22]. In this particular assay the virus inoculum was 100 PFU (plaque forming units) per petri dish.

Antimetabolic activity. Inhibition of host cell macromolecule (DNA, RNA and protein) synthesis was monitored by incorporation of [*methyl*-^{3}H]dThd, [5-^{3}H]Urd and [4,5-^{3}H]Leu, respectively, over an incubation period of 16 hr of exponentially growing cells in the presence of the test compound.

Cytocidal activity. Inhibition of Vero and HeLa cell proliferation was assessed during their exponential growth phase and monitored by counting the number of viable cells (following staining with trypan blue). The procedure was similar to that described previously for murine leukemia L1210 cells [23].

RESULTS

C-Cyd was evaluated in comparison with the two broad-spectrum antiviral agents, C-c^{3}Ado [22] and ribavirin [24], against a wide variety of RNA and DNA viruses (Table 1). C-Cyd showed marked activity against the following viruses: vaccinia, vesicular stomatitis, reo, parainfluenza, Sindbis, Semliki forest, measles, corona, TK$^-$ herpes simplex, influenza and SSPE. It was virtually inactive against TK$^+$ herpes simplex, polio, Coxsackie, rhino and respiratory syncytial virus (Table 1). As compared to C-Cyd, C-^{3}Ado was more active against vaccinia, vesicular stomatitis (in PRK) and parainfluenza but less active against the other viruses. Ribavirin was less active than C-Cyd against any of the viruses tested, except for polio, Coxsackie and respiratory syncytial virus. Against influenza, ribavirin proved equally effective as C-Cyd (Table 1). Neither C-Cyd nor C-c^{3}Ado or ribavirin were active against human immunodeficiency virus type 1 at concentrations that were below the concentration required to inhibit growth of the host (MT-4 lymphocyte) cells by 50% (0.06, 1.2 and 26 μg/mL, respectively) (data not shown).

As the antiviral potency of antiviral agents in general, and anti-herpes drugs in particular, may differ considerably depending on the choice of the cells [27, 28], C-Cyd was further evaluated for its activity against vesicular stomatitis virus in a wide variety of cell lines (Table 2). Whereas C-Cyd inhibited virus-induced cytopathogenecity in PRK, HeLa, E_6SM, Vero, CV-1, BHK-21 and BALB/3T3, it failed to do so in other cell lines (i.e. HEp-2, RK13, BSC-1A). The antiviral activity of C-c^{3}Ado and ribavirin also varied considerably from one cell line to another. Moreover, the cell-dependence pattern of the antiviral effects of C-Cyd, C-c^{3}Ado and ribavirin also differed from one to another (Table 2): i.e. C-c^{3}Ado was very active against vesicular stomatitis virus in HEp-2 cells where C-Cyd did not show much activity, whereas C-c^{3}Ado was inactive in CV-1 and BHK-21 cells where C-Cyd proved effective. Ribavirin was inactive against vesicular stomatitis virus in PRK cells where both C-Cyd and C-c^{3}Ado showed activity, whereas ribavirin was quite active in HK and RK13 cells where C-Cyd failed to show activity.

That the inhibitory effect of C-Cyd on virus-induced cytopathogenicity (Tables 1 and 2) reflected inhibition of virus multiplication was ascertained by following virus yield in HeLa cells which had been infected with vesicular stomatitis virus and treated with varying concentrations (0, 1, 10 and 100 μg/mL) of C-Cyd. The compound effected a dose-dependent reduction in virus yield, whether the virus content was determined at 24, 48 or 72 hr after infection (Fig. 2). The maximum reduction in virus yield (4.5 $\log_{10}$) was achieved with a concentration of 100 μg/mL at 24 hr post infection.

As shown in a variety of cells (Table 3), C-Cyd proved inhibitory to host cell DNA and RNA synthesis (as monitored by the incorporation of [*methyl*-^{3}H]dThd and [5-^{3}H]Urd, respectively) within the range of concentrations (0.5–5 μg/mL) exhibiting antiviral activity (Table 1). While inhibitory to DNA and RNA synthesis, C-Cyd did not affect protein synthesis in any of the examined cell lines (Table 3).

The dose–response curve for the inhibitory effect

Table 1. Antiviral activity spectrum of C-Cyd, as compared to the antiviral activity spectrum of two other broad-spectrum antiviral agents, C-c^{3}Ado and ribavirin

Virus	Cell	50% Inhibitory concentration* (μg/mL)		
		C-Cyd	C-c^{3}Ado	Ribavirin
Herpes simplex 1 (KOS)	PRK	100	>400	>400
Herpes simplex 2 (G)	PRK	400	200	>400
Vaccinia	PRK	15	0.5	15
Vesicular stomatitis	PRK	4	0.2	>400
Polio 1	HeLa	>400	>400	7
Coxsackie B4	HeLa	>400	>400	20
Reo 1	Vero	0.7	2	70
Parainfluenza 3	Vero	6	1	40
Sindbis	Vero	0·7	40	150
Semliki forest	Vero	3	8	25
Measles	Vero	0.7	2	50
Rhino 1A	WI-38	>100	>100	80
Corona (229E)	WI-38	7	150	150
TK$^-$ Herpes simplex 1 (B2006)†	PRK	4	150	>400
Respiratory syncytial‡	HeLa	>70	—	6
Influenza A, B, C§	MDCK	4.5	>16	4.5
SSPE‖	Vero	0.95	2	8

* Concentration required to reduce virus-induced cytopathogenicity by 50%. Cytotoxicity as could be judged by a microscopically visible alteration of normal cell morphology was not observed with either C-Cyd, C-c^{3}Ado or ribavirin at concentrations up to 400 μg/mL.

†,‡,§,‖ Data for these viruses taken from De Clercq *et al.* [13], Kawana *et al.* [25], Shigeta *et al.* [18] and Hosoya *et al.* [26], respectively.

MDCK, Madin-Darby canine kidney; SSPE, subacute sclerosing panencephalitis.

Table 2. Activity of C-Cyd, C-c^{3}Ado and ribavirin against vesicular stomatitis virus in different cell lines

Cell	50% Inhibitory concentration* (μg/mL)		
	C-Cyd	C-c^{3}Ado	Ribavirin
PRK	4	0.2	>400
HeLa	2	0.7	7
E_6SM	10 (200)	2	20
HK	85	0.4	0.7
HEp-2	200	0.07 (200)	20
Vero	7	7	100
RK13	>400	2	18
BSC-1A	300	>400	150
CV-1	2 (200)	>400	70
BHK-21	2 (100)	>400	20
BALB/3T3	5	300	20

‡ Concentration required to reduce virus-induced cytopathogenicity by 50%. Where a microscopically visible alteration of normal cell morphology was observed, the lowest concentrations at which such cytotoxicity was detected are listed in parentheses.

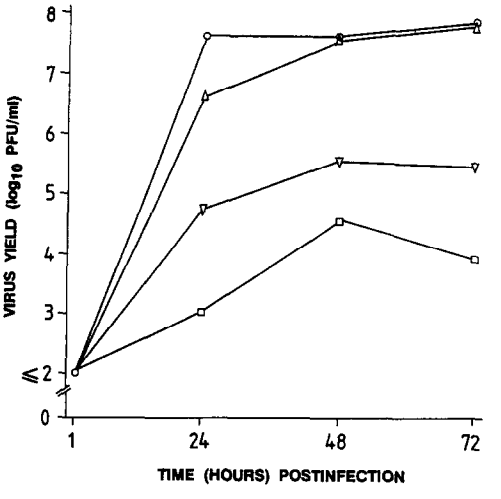

Fig. 2. Effect of Cyd on the multiplication of vesicular stomatitis in HeLa cell cultures. Virus input: 2 $\log_{10}$ PFU per petri dish. C-Cyd was added immediately after virus adsorption at either 0 μg/mL (○——○), 1 μg/mL (△——△), 10 μg/mL (▽——▽) or 100 μg/mL (□——□). Virus yield was determined by plaque formation in L-929 cells.

of C-Cyd on host cell RNA synthesis in Vero cells is presented in Fig. 3. To establish whether C-Cyd effected a comparable inhibition of viral RNA synthesis, Vero cells which had been infected with either Sindbis virus or reovirus were treated with actinomycin D (30 μg/mL) so as to completely block host cell DNA-directed RNA synthesis. The remaining viral RNA-directed RNA synthesis was inhibited by C-Cyd at concentrations which were only slightly (at the most 3- to 10-fold) higher than those required for inhibition of host cell RNA synthesis (in uninfected cells that were not treated with actinomycin D) (Fig. 3).

To obtain further insight into the mechanism of

Table 3. Antimetabolic activity of C-Cyd

Cell	50% Inhibitory concentration* (μg/mL)		
	DNA synthesis [*methyl*-^{3}H]dThd incorporation	RNA synthesis [5-^{3}H]Urd incorporation	Protein synthesis [4,5-^{3}H]Leu incorporation
PRK	0.5	1.1	>200
HeLa	2.6	2.6	>200
Vero	0.4	1.6	>200
WI-38	14	19.5	>200
CV-1	2.5	3.7	>200

* Concentration required to reduce incorporation of the radiolabeled precursors by 50%.

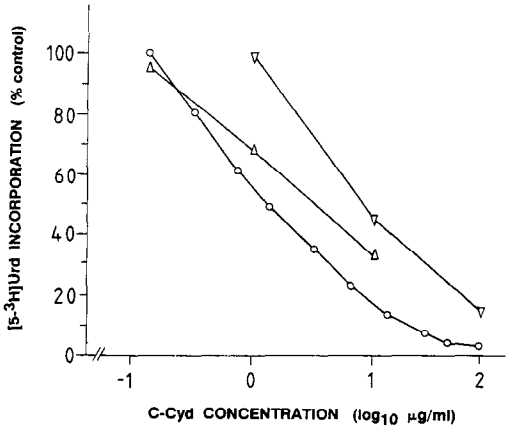

Fig. 3. Effect of C-Cyd on cellular and viral RNA synthesis: (○——○) cellular RNA synthesis in mock-infected Vero cells (control: 18400 counts/min); (△——△) viral RNA synthesis in Sindbis virus-infected Vero cells treated with actinomycin D at 30 μg/mL (control: 2856 counts/min); ▽——▽) viral RNA synthesis in Reovirus-1-infected Vero cells treated with actinomycin D at 30 μg/mL (control: 724 counts/min). All data represent average values for three separate experiments.

action of C-Cyd, attempts were undertaken to reverse its antiviral activity by the exogenous addition of nucleosides. The deoxynucleoside dThd and dCyd did not counteract the antiviral activity of C-Cyd in Vero or HeLa cells infected with either Sindbis, reo or vesicular stomatitis virus (Table 4). However, the ribonucleosides Urd and Cyd completely abrogated the antiviral effects of C-Cyd when added at a concentration of 100 μg/mL; this is evident from a more than 100-fold raise in the 50% virus-inhibitory concentration of C-Cyd following addition of Urd or Cyd at 100 μg/mL (Table 4). When added at 10 μg/mL, Urd brought about a 5- to 80-fold, and Cyd a 3- to 10-fold, raise in the 50% virus-inhibitory concentration of C-Cyd.

The ribonucleosides Urd and Cyd not only reversed the antiviral action of C-Cyd, but also abrogated its cytocidal effects (Table 5). In this respect, Cyd was more efficient than Urd: at a concentration of 10 μg/mL, Cyd reduced the cytocidal action of C-Cyd by about 250-fold, whereas a similar effect was accomplished by Urd only at a concentration of 100 μg/mL. Neither dThd or dCyd counteracted the cytocidal effects of C-Cyd on HeLa or Vero cells even if added at a concentration of 100 μg/mL (Table 5).

DISCUSSION

C-Cyd, the carbocyclic analogue of cytidine, has a unique spectrum of antiviral activity that encompasses DNA (pox) viruses and RNA [(−)RNA (orthomyxo, paramyxo, rhabdo), (+)RNA (toga, corona) and (±)RNA (reo)]viruses. The antiviral activity spectrum of C-Cyd is clearly different from that of ribavirin, which is active against picornaviruses (polio, Coxsackie) whereas C-Cyd is not (Table 1). Conversely, C-Cyd is quite active against various viruses, i.e. Sindbis, reo, corona, measles, TK$^-$ herpes simplex, which are not sensitive, or only slightly sensitive to ribavirin. Ribavirin is assumed to intereact with a number of target proteins: i.e. IMP dehydrogenase [29], mRNA 5′-capping enzymes [30] and viral mRNA polymerase complex proteins [31].

The antiviral activity spectrum of C-Cyd is also different from that of C-c^{3}Ado, in that the latter is much less active, or inactive, against Sindbis, corona, influenza and TK$^-$ herpes simplex (Table 1). Also, C-Cyd and C-c^{3}Ado show marked differences in their activity against vesicular stomatitis virus, depending on the nature of the cell line used (Table 2). C-c^{3}Ado is assumed to interact with *S*-adenosylhomocysteine hydrolase, a key enzyme in transmethylation reactions [32]. For a series of acyclic and carbocyclic adenosine analogues, including C-c^{3}Ado, a close correlation has been found betweeen their inhibitory effect on *S*-adenosylhomocysteine hydrolase and their activity against vaccinia and vesicular stomatitis virus [33].

From inspection of the activity spectrum of C-Cyd, relative to the spectra of ribavirin and C-c^{3}Ado (Tables 1 and 2), it can be inferred that C-Cyd must achieve its antiviral activity by a mechanism that is different from the mode of action of either ribavirin or C-c^{3}Ado. Shannon *et al.* [14] have demonstrated that C-Cyd is phosphorylated intracellularly to its 5′-triphosphate (C-CTP) and causes a specific decrease in the CTP pools. This points to an inhibitory effect

Table 4. Reversing effect of different nucleosides on antiviral activity of C-Cyd

Nucleoside added	Concentration (μg/mL)	50% Inhibitory concentration of C-Cyd* (μg/mL)		
		Sindbis virus (Vero)	Reo1 virus (Vero)	Vesicular stomatitis virus (HeLa)
dThd	100	0·7	0·5	0.8
	10	0.6	0.5	0.8
	1	0.6	0.6	0.8
Urd	100	165	60	141
	10	32	3	5
	1	4	0.8	1
dCyd	100	0.5	0.7	0.5
	10	0.4	0.7	0.6
	1	0.6	0.8	1.5
Cyd	100	80	100	400
	10	4	2.5	1.7
	1	0.7	1.6	0.3
None	—	0.4	0.6	0.5

* Concentration required to reduce virus-induced cytopathogenicity by 50%. The nucleosides dThd, Urd, dCyd and Cyd did not interfere with virus-induced cytopathogenicity at concentrations up to 200 μg/mL.

Table 5. Reversing effect of different nucleosides on cytocidal activity of C-Cyd

Nucleoside added	Concentration (μg/mL)	50% Inhibitory concentration of C-Cyd* (μg/mL)	
		Vero	Hela
dThd	100	0.13	0.08
	10	0.17	0.06
	1	0.13	0.17
Urd	100	50	26
	10	5.7	5.8
	1	0.13	0.2
dCyd	100	0.25	0.29
	10	0.21	0.22
	1	0.25	0.13
Cyd	100	>100	>100
	10	65	45
	1	3	0.26
None	—	0.17	0.25

* Concentration to reduce the viable cell number by 50%. The 50% inhibitory concentrations of the nucleosides dThd, Urd, dCyd and Cyd were: for Vero cells, 61, >100, >100 and >100 μg/mL, respectively; and for HeLa cells, 23, >100, >100 and >100 μg/mL, respectively.

of C-CTP at the CTP synthetase level, the last step in the *de novo* biosynthesis of CTP, which starts from aspartate and carbamoyl phosphate (Fig. 4).

If C-Cyd has to be converted to exert its inhibitory effect on CTP synthetase, it should not be the subject of premature degradation by pyrimidine nucleoside phosphorylases. Unpublished data of C. Desgranges and E. De Clercq indicate that C-Cyd is not a substrate for either Urd phosphorylase or dThd phosphorylase. It remains to be established how efficiently and by which enzymes C-Cyd is converted to its 5′-triphosphate.

The inhibitory effects of C-Cyd on both cellular and viral RNA synthesis (Fig. 3) are in agreement with the postulated inhibition of CTP synthetase by C-CTP. Also consistent with the inhibition of CTP synthesis is the inhibitory effect of C-Cyd on DNA synthesis (Table 3), because inhibition of CTP synthesis also leads to a reduction in the supply of the pyrimidine deoxynucleoside 5′-triphosphates (dCTP, dTTP) as outlined in Fig. 4. From Fig. 4 it is also clear that if the mode of action of C-Cyd is based upon inhibition of the UTP → CTP step, additional supply of UTP and CTP through the Urd and Cyd salvage pathways may be expected to overcome the inhibitory effects of C-Cyd. This premise was borne out, as both the antiviral activity (Table 4) and cytocidal activity (Table 5) of C-Cyd could be

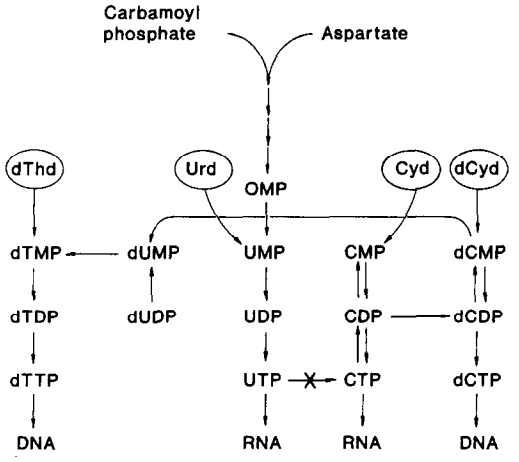

Fig. 4. *De novo* and salvage pathways for the biosynthesis of pyrimidine nucleoside 5'-triphosphates. (×) Target enzyme (CTP synthetase) for C-Cyd.

selective as an antiviral agent. However, the antiviral selectivity of C-Cyd can be markedly increased when combined with Cyd: addition of Cyd (at 10 μg/mL) reverses the cytocidal activity of C-Cyd to a significantly greater extent (Table 4) than its antiviral activity (Table 5), thus resulting in a marked increase in the antiviral selectivity index of C-Cyd (Table 6). This marked increase in selectivity has been observed on both Vero and HeLa cells infected with either a (+)RNA virus (Sindbis), (±)RNA virus (reo) or (−)RNA virus (vesicular stomatitis). The combination of C-Cyd with Cyd (10 μg/mL) represents a new therapeutic modality that deserves to be further explored in the treatment of various RNA virus infections.

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Table 6. Antiviral selectivity of C-Cyd in the presence of various nucleosides

Nucleoside added	Concentration (μg/mL)	Selectivity index of C-Cyd*		
		Sindbis virus (Vero)	Reo1 virus (Vero)	Vesicular stomatitis virus (HeLa)
dThd	100	0.18	0.26	0.10
	10	0.28	0.34	0.07
	1	0.22	0.22	0.21
Urd	100	0.30	0.83	0.18
	10	0.18	1.9	1.16
	1	0.03	0.16	0.2
dCyd	100	0.50	0.36	0.58
	10	0.62	0.30	0.37
	1	0.42	0.31	0.09
Cyd	100	>1.25	>1	>0.25
	10	16	26	26
	1	4.3	1.9	0.9
None	—	0.42	0.28	0.5

* Ratio of 50% inhibitory concentration for cell growth [cytocidal activity assays (Table 5)] to 50% inhibitory concentration for virus-induced cytopathogenicity [antiviral activity assays (Table 4)].

reversed by exogenous addition of Urd and Cyd, but not dThd or dCyd.

Thus, CTP synthetase can be considered as an important target in the design of broad-spectrum antiviral compounds. As CTP synthetase is also needed for host cell RNA and DNA synthesis, it may also serve as a target enzyme for antitumor agents. In fact, Ce-Cyd is assumed to exert its antitumor (i.e. cytocidal) properties through inhibition of CTP synthetase and depletion of intracellular CTP pools [15–17].

If CTP synthetase acts as a target enzyme for both the antiviral and cytocidal activities of C-Cyd, how could C-Cyd be envisaged to exert any selectivity in its antiviral action? From the ratios of the 50% inhibitory concentration for cell growth (exponentially growing cells) to the 50% inhibitory concentration for viral cytopathogenicity (in stationary cells) (Table 6) C-Cyd does not appear particularly

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