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BROAD-SPECTRUM ANTIVIRAL AND CYTOCIDAL ACTIVITY OF CYCLOPENTENYLCYTOSINE, A CARBOCYCLIC NUCLEOSIDE TARGETED AT CTP SYNTHETASE

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Abstract—Cyclopentenylcytosine (Ce-Cyd) is a broad-spectrum antiviral agent active against DNA viruses [herpes (cytomegalo), pox (vaccinia)], (+)RNA viruses [picorna (polio, Coxsackie, rhino), toga (Sindbis, Semliki forest), corona], (−)RNA viruses [orthomyxo (influenza), paramyxo (parainfluenza, measles), arena (Junin, Tacaribe), rhabdo (vesicular stomatitis)] and (±)RNA viruses (reo). Ce-Cyd is a more potent antiviral agent than its saturated counterpart, cyclopentylcytosine (carbodine, C-Cyd). Ce-Cyd also has potent cytocidal activity against a number of tumor cell lines. The putative target enzyme for both the antiviral and antitumor action of Ce-Cyd is assumed to be the CTP synthetase that converts UTP to CTP. In keeping with this hypothesis was the finding that the antiviral and cytocidal effects of Ce-Cyd are readily reversed by Cyd and, to a lesser extent, Urd, but not by other nucleosides such as dThd or dCyd. In contrast, pyrazofurin and 6-azauridine, two nucleoside analogues that are assumed to interfere with OMP decarboxylase, another enzyme involved in the biosynthesis of pyrimidine ribonucleotides, potentiate the cytocidal activity of Ce-Cyd. Ce-Cyd should be further pursued, as such and in combination with OMP decarboxylase inhibitors, for its therapeutic potential in the treatment of both viral and neoplastic diseases.

Various pyrimidine nucleoside analogues, i.e. 5-fluoro-2′-deoxyuridine [1], 5-iodo-2′-deoxyuridine [2], (*E*)-5-(2-bromovinyl)-2′-deoxyuridine [3], 5-ethyl-2′-deoxyuridine [4], 3′-amino-2′,3′-dideoxythymidine [5], 5-iodo-2′-deoxycytidine [6] and 5-iodo-1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)cytosine [7], are known as potent antiviral or antitumor agents. To make these nucleoside analogues resistant to degradation by nucleoside phosphorylases that cleave the N-glycosidic bond, carbocyclic derivatives of these nucleosides have been synthesized, whereby the sugar part is replaced by a cycloalkyl ring [1–7]. As a rule, these carbocyclic derivatives retained only part of the antitumor or antiviral activity of the parent compounds. Also, carbocyclic analogs, both cyclopentyl and cyclopentenyl derivatives, of the normal nucleoside cytidine have been prepared, and these derivatives, termed C-Cyd (carbodine) and Ce-Cyd respectively (Fig. 1), have proved effective as both antiviral and antitumor agents [8–11].

Ce-Cyd has shown antitumor activity in several human tumor xenografts in athymic mice; *in vitro* it has proved active against herpesviruses (herpes simplex, cytomegalo and varicella-zoster), vaccinia, influenza A, vesicular stomatitis virus, Japanese encephalitis virus and Punta Toro virus [11].

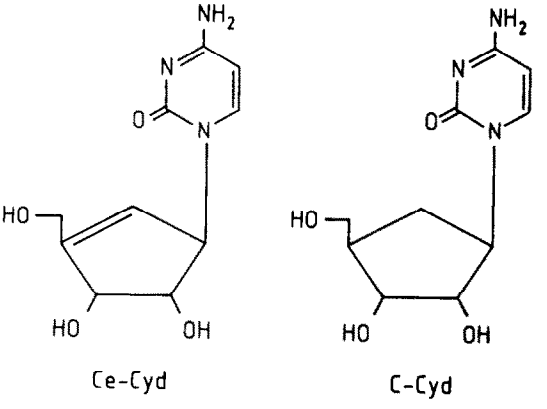

Fig. 1. Formulae of cyclopentenylcytosine (Ce-Cyd) and cyclopentylcytosine (C-Cyd, carbocyclic cytidine, carbodine).

Both C-Cyd and Ce-Cyd are assumed to interact with CTP synthetase, the enzyme that converts UTP to CTP, after they have been phosphorylated intracellularly to the 5′-triphosphate [9, 12, 13]. Direct measurements with CTP synthetase (from murine leukemia L1210 cells) have indicated that the 5′-triphosphate of Ce-Cyd is indeed inhibitory to the enzyme [14]. The depletion of CTP pools that results from such an inhibitory effect has been held responsible for the cytocidal action of Ce-Cyd *in*

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vitro [12] and its antitumor activity *in vivo* [13]. It has not been ascertained, however, whether the antiviral action of Ce-Cyd also results from an inhibitory effect on CTP synthetase.

The present studies were undertaken to (i) delineate the antiviral activity spectrum of Ce-Cyd (in comparison with the activity spectrum of C-Cyd [15]), and to (ii) assess the influence of exogenously added cytidine and other nucleosides on both the antiviral and cytocidal action of Ce-Cyd. In agreement with previous studies [11], Ce-Cyd was found to be a highly potent inhibitor of both virus replication and tumor cell proliferation. It proved active against a remarkably broad range of RNA and DNA viruses. Both the antiviral and cytocidal action of Ce-Cyd could be reversed by Cyd, but not dThd or dCyd. Combination of Ce-Cyd with pyrazofurin or 6-azauridine, two compounds which are known to inhibit OMP decarboxylase, resulted in an enhancement of its cytocidal activity [16–19].

MATERIALS AND METHODS

Compounds. Ce-Cyd was synthesized as described by Lim *et al.* [10] and Marquez *et al.* [11]. C-Cyd (carbodine) was synthesized as described by Shealy and O'Dell [8] and provided by J. A. Montgomery (Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, AL). Neplanocin A was obtained from Toyo Jozo Co. (Tagata-gun, Shizuoka-ken, Japan). Ribavirin (Virazole) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). The nucleosides 2′-deoxythymidine (dThd), uridine (Urd), 2′-deoxycytidine (dCyd) and cytidine (Cyd) were obtained from the Sigma Chemical Co. (St Louis, MO). The sources of the other compounds were as follows: pyrazofurin [3-(β-D-ribofuranosyl)-4-hydroxypyrazole-5-carboxamide], Calbiochem Behring Corporation (Lucerne, Switzerland); 6-azauridine, Sigma Chemical Co.; 5-fluorouracil, Sigma Chemical Co.; and cytarabine [1-(β-D-arabinofuranosyl)cytosine], Upjohn (Puurs, Belgium).

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 (Amersham, U.K.). Their specific radioactivity was 40, 30 and 52 Ci/mmol, respectively.

Viruses. The origin of the viruses and the preparation of the virus stocks have been documented in previous reports: herpes simplex virus type 1 (strain KOS) [20], herpes simplex virus type 2 (strain G) [20], thymidine kinase deficient (TK^-) herpes simplex virus type 1 (strain B2006) [21], cytomegalovirus (strains Davis and AD-169) [22], vaccinia virus [23], vesicular stomatitis virus [24], poliovirus type 1 [23], Coxsackie virus type B4 [23], reovirus type 1 [24], parainfluenza virus type 3 [24], Sindbis virus [23], Semliki forest virus [24], Junin virus [25], Tacaribe virus [25], coronavirus (strain 229E) [15], rhinovirus (types 1A and 9) [26], respiratory syncytial virus (strain Long) [27], measles [SSPE (subacute sclerosing panencephalitis), strain Niigata-1] [28], and influenza types A, B, C.

Cells. The cell lines used in the antiviral activity assays were: PRK (primary rabbit kidney), HEL (human embryonic lung fibroblast), 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 fibroblast), MDCK (Madin–Darby canine kidney), E_6SM (human embryonic skin-muscle fibroblast), RK13 (a rabbit kidney cell line), BSC-1A (a simian epithelial cell line derived from African green monkey kidney), GBK (Georgia bovine kidney), CV-1 (a simian fibroblast cell line derived from African green monkey kidney) and L-929 (a murine fibroblast cell line originally derived from an adult C3H mouse and mutagenized with methylcholanthrene). The cell lines used in the cytocidal activity assays were, besides Vero and HeLa cells, murine leukemia (L1210), murine mammary carcinoma (FM3A), human B-lymphoblast (Raji) and human T lymphoblast (Molt/4F) cells. 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, 24]. In all viral cytopathogenicity assays the virus inoculum was 100 $CCID_{50}$ per microtiter well (1 $CCID_{50}$ corresponding to the virus stock dilution that proved infective for 50% of the cell cultures). In the cytomegalovirus assays, plaque formation was measured instead of viral cytopathogenicity. In these assays the virus inoculum was 20 PFU (plaque forming units).

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 (Vero) cells in the presence of varying concentrations of the test compounds.

Cytocidal activity. Inhibition of the proliferation of Vero, HeLa, L1210, FM3A, Raji and Molt/4F cells 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 initially described for L1210 cells [30].

RESULTS

Ce-Cyd was evaluated in comparison with C-Cyd [15] and two other broad-spectrum antiviral agents, neplanocin A [24] and ribavirin [31], against a wide variety of RNA and DNA viruses (Table 1). Of the four compounds, Ce-Cyd showed the most pronounced antiviral activity. Against some viruses (i.e. TK^- herpes simplex, vaccinia, polio, reo, Junin, Tacaribe, SSPE), Ce-Cyd proved effective at a 50% inhibitory concentration of ≤ 0.1 μg/mL, whereas normal cell morphology was not altered (at concentrations up to 400 μg/mL). Ce-Cyd was active against all viruses that are sensitive to C-Cyd, but, in addition, Ce-Cyd proved also active against picornaviruses (polio, Coxsackie, rhino) which are not sensitive to C-Cyd. Thus, the activity spectrum of Ce-Cyd includes all major virus groups [herpes (TK^- herpes simplex, cytomegalo), pox (vaccinia), picorna (polio,

Table 1. Antiviral activity spectrum of Ce-Cyd, as compared to activity spectrum of C-Cyd, neplanocin A and ribavirin

Virus	Cell	50% Inhibitory concentration* (μg/mL)			
		Ce-Cyd	C-Cyd	Neplanocin A	Ribavirin
Herpes simplex 1 (KOS)	PRK	>400	100†	20	>400
Herpes simplex 2 (G)	PRK	200	400†	20	>400
TK⁻ Herpes simplex 1 (B2006)	PRK	0.1‡	4‡	2‡	>400‡
Cytomegalo (Davis)	HEL	0.2	ND	0.15	250
Cytomegalo (AD-169)	HEL	0.6	ND	0.2	200
Vaccinia	PRK	0.02	15†	0.03	20
Vesicular stomatitis	PRK	0.7	4†	0.07	300
Polio 1	HeLa	0.1	>400†	>40	20
Coxsackie B4	HeLa	0.2	>400†	>40	40
Reo 1	Vero	0.1	0.7†	1	70
Parainfluenza 3	Vero	0.2	6†	1	70
Sindbis	Vero	0.2	0.7†	2	100
Semliki forest	Vero	0.4	3†	7	40
Junin	Vero	0.03	0.2	0.3	1
Tacaribe	Vero	0.03	0.3	0.3	1
Corona (229E)	WI-38	10	7†	15	300
Rhino 1A	WI-38	10	>100†	15	70
Rhino 9	WI-38	2	ND	10	70
Respiratory syncytial (Long)	HeLa	0.04	>70§	>20§	6§
SSPE (Niigata-1)	Vero	0.05‖	0.9‖	6‖	8‖
Influenza A, B, C	MDCK	4.5¶	4.5¶	>50¶	4.5¶
Cytotoxicity	PRK	>400	>400	40	>400
Cytotoxicity	HEL	>400	ND	40	>400
Cytotoxicity	HeLa	>400	>400	40	>400
Cytotoxicity	Vero	>400	>400	40	>400
Cytotoxicity	WI-38	>400	>400	40	>400

* Required to reduce virus-induced cytopathogenicity [or plaque formation (cytomegalovirus)] by 50%. For cytotoxicity measurements, a microscopically detectable alteration of normal cell morphology was taken as the end point.

† Data taken from De Clercq *et al.* [15].

‡ Data taken from De Clercq *et al.* [21].

§ Data taken from Kawana *et al.* [27].

‖ Data taken from Hosoya *et al.* [28].

¶ Data taken from Shigeta *et al.* [29].

ND, not determined.

Coxsackie, rhino), toga (Sindbis, Semliki forest), corona, orthomyxo (influenza), paramyxo (parainfluenza, measles), arena (Junin, Tacaribe), rhabdo (vesicular stomatitis) and reo] (Table 1).

Ce-Cyd and C-Cyd were also examined for their inhibitory effect on the replication of human immunodeficiency virus type 1 (HIV-1) in (exponentially growing) MT-4 cells (a human T-lymphocyte cell line). Ce-Cyd and C-Cyd were inhibitory to the growth of MT-4 cells at an IC_{50} of 1.2 and 50 ng/mL, respectively. They were not inhibitory to HIV-1 replication at concentrations below their IC_{50} for host cell growth (data not shown).

The antiviral potency of antiviral agents may differ considerably depending on the choice of the cells, as has been previously noted with C-Cyd and ribavirin when evaluated for their activity against vesicular stomatitis virus in different cell lines [15]. In contrast with C-Cyd and ribavirin, however, Ce-Cyd inhibited the cytopathogenicity of vesicular stomatitis virus in all cell lines in which it was examined (Table 2). Differences between C-Cyd and Ce-Cyd were particularly striking in RK13 and BSC-1A, two cell lines in which Ce-Cyd exhibited marked antiviral activity whereas C-Cyd did not. The concentrations at which Ce-Cyd inhibited vesicular stomatitis virus in the different cell lines ranged from 0.1 to 2 μg/mL, whereas, again, no alteration of normal cell morphology was observed at C-Cyd concentrations up to 400 μg/mL.

Ce-Cyd was not toxic to resting (confluent) cell monolayers, as used in the antiviral assays (Tables 1 and 2), at concentrations up to 400 μg/mL. However, Ce-Cyd proved inhibitory to (Vero) cell DNA and RNA synthesis (as monitored by the incorporation of [*methyl*-^{3}H]dThd and [5-^{3}H]Urd, respectively), at a concentration of 1.4 and 0.7 μg/mL, respectively (Table 3). These concentrations coincide with, or are only 10- to 100-fold higher than, the concentrations at which Ce-Cyd exhibit antiviral activity in Vero cells (Tables 1 and 2). Yet, it should be pointed out that during macromolecule synthesis the cells were in their exponential growth phase, whereas they were resting (confluent) in the antiviral activity assays.

Ce-Cyd proved inhibitory to the growth of HEL

Table 2. Activity of Ce-Cyd, C-Cyd, neplanocin A and ribavirin against vesicular stomatitis virus in different cell lines

Cell	50% Inhibitory concentration* (μg/mL)			
	Ce-Cyd	C-Cyd	Neplanocin A	Ribavirin
PRK	0.7	4†	0.07	300
HeLa	0.1	2†	2	20
E_6SM	0.1	10†	0.04	7
HEL	2	ND	15	200
Vero	1.5	7†	0.7	100
RK13	0.1	>400†	0.07	300
BSC-1A	0.2	300†	0.7	20
GBK	0.4	ND	0.7	70
CV-1	2	2†	70	150
L-929	0.4	ND	0.4	300

* Concentration required to reduce virus-induced cytopathogenicity by 50%. Microscopically visible alteration of normal cell morphology was not observed with Ce-Cyd or ribavirin at the highest concentration tested (400 μg/mL). With neplanocin, an alteration of normal cell morphology was observed in all lines at a concentration of ≥40 μg/mL. With C-Cyd, it was observed in E_6SM and CV-1 at a concentration of ≥200 μg/mL.

† Data taken from De Clercq *et al.* [15].

ND, not determined.

cells at an IC_{50} of 0.5 μg/mL (data not shown), and the growth of tumor cells (L1210, FM3A, Raji, Molt/4F and HeLa) was inhibited at even lower concentrations (Table 4). The IC_{50} at which Ce-Cyd inhibited the proliferation of L1210, FM3A, Raji, Molt/4F and HeLa cells was lower than the IC_{50} at which its purine counterpart, neplanocin A, inhibited the growth of these tumor cells (Table 4). (For a confirmatory study on the cytostatic activity of neplanocin, see Ref. 32.)

Cyclopentenyluracil (Ce-Urd) [10], in sharp contrast with Ce-Cyd, did not show any inhibitory effect on the growth of L1210, FM3A, Raji or Molt/4F cells, even at a concentration of 1 mg/mL (data not shown). Likewise, Ce-Urd was inactive against

Table 4. Inhibitory effects of Ce-Cyd and neplanocin A on the growth of different tumor cell lines

Cell	50% Inhibitory concentration* (μg/mL)	
	Ce-Cyd	Neplanocin A
L1210	0.033 ± 0.001	0.104 ± 0.027
FM3A	0.048 ± 0.001	0.0583 ± 0.0008
Raji	0.033 ± 0.004	0.734 ± 0.196
Molt/4F	0.036 ± 0.008	1.84 ± 0.92
HeLa	0.0036 ± 0.0009	0.225 ± 0.110

Values are mean ± SD.

* Required to reduce tumor cell proliferation (during the exponential growth phase) by 50%.

any of the viruses (listed in Table 1) that were found susceptible to Ce-Cyd.

To obtain further insight into the mechanism of action of Ce-Cyd at the cellular level, the reversing effects of exogenously added nucleosides (dThd, Urd, dCyd and Cyd) on the antiviral and cytocidal activity of Ce-Cyd was examined (Tables 5 and 6). The antiviral assays were carried out with four viruses, representative of the DNA virus (vaccinia), (−)RNA virus (vesicular stomatitis), (±)RNA virus (reo) and (+)RNA virus (Semliki forest) groups. The deoxyribonucleosides dThd and dCyd did not affect the antiviral potency of Ce-Cyd, even if added at a concentration of 100 μg/mL (Table 5). In marked contrast, the ribonucleosides Cyd and, to a lesser extent, Urd reversed the activity of Ce-Cyd against all viruses tested: if added at 100 μg/mL, Cyd completely abolished the antiviral activity of Ce-Cyd, whereas Urd caused a reduction in antiviral potency (increase in IC_{50}) of about 25- to 50-fold. Even if added to a concentration of 10 μg/mL, Cyd annihilated the activity of Ce-Cyd against vaccinia virus and Semliki forest virus, whereas Urd reduced it by 3- to 5-fold.

Similar results were obtained with regard to the reversing effects of the nucleosides on the cytocidal activity of Ce-Cyd (Table 6). Thus, dThd and dCyd did not markedly influence the inhibitory effect of

Table 3. Antimetabolic activity of Ce-Cyd, C-Cyd, neplanocin A and ribavirin in Vero cells

Compound	50% Inhibitory concentration* (μg/mL)		
	DNA synthesis	RNA synthesis	Protein synthesis
	[*Methyl*-^{3}H]dThd incorporation	[5-^{3}H]Urd incorporation	[4,5-^{3}H]Leu incorporation
Ce-Cyd	1.4 ± 0.6	0.7 ± 0.3	>100
C-Cyd	0.65 ± 0.4	3.0 ± 2.5	>400
Neplanocin A	1.0 (0.5–1.7)†	1.4 (0.3–3.2)†	13 (7–19)†
Ribavirin	9 ± 6	20 ± 6.5	>400

Values are mean ± SD.

* Required to reduce incorporation of radiolabeled precursors by 50%.

† Data obtained for PRK cells [24].

Table 5. Reversing effect of different nucleosides on antiviral activity of Ce-Cyd

Nucleoside	Concentration (μg/mL)	50% Inhibitory concentration* (μg/mL) Ce-Cyd			
		Vaccinia virus (PRK)	Vesicular stomatitis virus (HeLa)	Reo 1 virus (Vero)	Semliki forest virus (Vero)
dThd	100	0.7	0.2	0.4	0.7
	10	0.7	0.1	0.1	0.7
	1	0.2	0.1	0.1	0.4
Urd	100	20	7	3	10
	10	2	0.4	0.3	2
	1	0.7	0.07	0.07	0.7
dCyd	100	0.7	0.1	0.07	0.4
	10	0.7	0.07	0.07	0.4
	1	0.7	0.07	0.07	0.4
Cyd	100	>100	>100	>100	>100
	10	>100	4	40	>100
	1	4	0.07	0.07	4
None	—	0.7	0.1	0.07	0.4

* Required to reduce virus-induced cytopathogenicity by 50%. At the concentrations used (1, 10 and 100 μg/mL), the nucleosides dThd, Urd, dCyd and Cyd did not interfere with virus-induced cytopathogenicity.

Table 6. Reversing effect of different nucleosides on cytocidal activity of Ce-Cyd

Nucleoside	Concentration (μg/mL)	50% Inhibitory concentration* (μg/mL) Ce-Cyd		
		L1210	Vero	HeLa
dThd	100	0.0073	0.015	ND
	10	0.0058	0.034	0.0032
	1	0.0095	0.041	0.0032
Urd	100	3.9	3.4	4.1
	10	0.052	0.29	0.19
	1	0.016	0.061	0.0024
dCyd	100	0.059	0.055	0.026
	10	0.063	0.051	0.0048
	1	0.021	0.056	0.0030
Cyd	100	>100	>100	>100
	10	3.3	>10	0.80
	1	0.016	0.20	0.0022
None	—	0.015	0.036	0.0036

* Required to reduce cell proliferation (during the exponential growth phase) by 50%. At the concentrations used (1, 10 and 100 μg/mL), Urd, dCyd and Cyd did not cause any inhibition of L1210, Vero or HeLa cell growth; dThd, however, effected a 50% reduction in the growth of L1210, Vero and HeLa cells at a concentration of 20, 91 and 27 μg/mL, respectively.

ND, not determined.

Ce-Cyd on the proliferation of L1210, Vero or HeLa cells, whereas Cyd and, to a lesser extent, Urd counteracted the cytocidal activity of Ce-Cyd. At 100 μg/mL, Cyd completely reversed the inhibitory effect of Ce-Cyd on tumor cell growth; at 10 μg/mL, it effected a 200- to 300-fold increase in IC_{50}. When added at a concentration of 100 μg/mL, Urd also caused a significant (100- to 1000-fold) increase in the IC_{50} of Ce-Cyd.

Finally, different antimetabolites that are targeted at different enzymes in nucleic acid biosynthesis [OMP decarboxylase (pyrazofurin, 6-azauridine), RNA polymerase (5-fluorouracil), *S*-adenosylhomocysteine hydrolase (neplanocin A), DNA polymerase (cytarabine)] were examined in conjunction with Ce-Cyd. Whereas 5-fluorouracil, neplanocin A and cytarabine did not influence the inhibitory effect of Ce-Cyd on L1210 cell growth, pyrazofurin and 6-azauridine potentiated the cytocidal activity of Ce-Cyd. All antimetabolites were

Table 7. Potentiating effect of some nucleoside analogues on the cytocidal activity of Ce-Cyd

Nucleoside analogue added	Concentration (μg/mL)	50% Inhibitory concentration* (ng/mL) Ce-Cyd
Pyrazofurin	0.1	7.5 ± 2.5
	0.01	16.8 ± 1.8
	0.001	31.3 ± 4.4
6-Azauridine	0.1	4.0 ± 1.0
	0.01	19.4 ± 3.8
5-Fluorouracil	1	33.1 ± 6.1
	0.1	38.8 ± 8.1
	0.01	39.6 ± 5.1
Neplanocin A	1	17.3 ± 1.4
	0.1	23.0 ± 11.3
	0.01	34.3 ± 1.6
Cytarabine	0.1	34.7 ± 8.5
	0.01	47.0 ± 7.0
	0.001	40.6 ± 1.4
None	—	42.9 ± 4.2

Values are mean ± SD.

* Required to reduce L1210 cell proliferation (during the exponential growth phase) by 50%. The nucleoside analogues effected a 50% reduction in the growth of L1210 cells at the following concentrations: pyrazofurin (0.025 μg/mL), 6-azauridine (0.034 μg/mL), 5-fluorouracil (0.32 μg/mL), neplanocin A (0.40 μg/mL) and cytarabine (0.016 μg/mL).

used at concentrations below and above their IC_{50} for L1210 cell growth (Table 7). Under these conditions, pyrazofurin and 6-azauridine, when added at a concentration of 0.1 μg/mL (which is above their IC_{50} for L1210 cell growth), brought about a 6- to 10-fold decrease in the IC_{50} of Ce-Cyd.

DISCUSSION

Various enzymes of both viral or cellular origin have been shown to interact as either activators or targets for antiviral agents. The activity spectrum of these antiviral agents is determined by the enzymes with which they interact. For example, acyclovir [9-(2-hydroxyethoxymethyl)guanine] needs to be phosphorylated by the virus-encoded thymidine kinase (TK) before it will interact, as a chain terminator, with the viral DNA polymerase [33]. Hence, acyclovir is only active against those viruses, in particular herpes simplex virus type 1 (HSV-1) and 2 (HSV-2), that encode a viral TK recognizing acyclovir as substrate. The phosphonylmethoxyalkyl derivatives, i.e. HPMPA [(*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine] and HPMPC [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine], are not dependent on the viral TK for their phosphorylation, and, hence, these compounds are effective against a much broader spectrum of viruses, including TK^- herpesviruses and various other DNA viruses (i.e. adeno-, pox-, irido- and hepadnaviruses) [34].

Most compounds that have been recognized as selective antiviral agents interact with a specific viral protein: i.e. influenza A virus matrix (M_2) protein (amantadine) [35], HSV-1 DNA polymerase (acyclovir triphosphate) [36], HIV-1 reverse transcriptase (azidothymidine triphosphate) [37]. Yet, the target protein should not necessarily be of viral origin. Under certain conditions, host cell enzymes may serve as targets for antiviral agents. Thus, while not being a target for anti-HSV compounds in TK^+ HSV-1-infected cells, thymidylate synthase may be an important target in TK^- HSV-1-infected cells [21]. Also, *S*-adenosylhomocysteine (SAH) hydrolase, another host cell enzyme, has been identified as a target for the broad-spectrum antiviral action of several acyclic and carbocyclic adenosine analogues [38], including neplanocin A [24], 3-deazaneplanocin A and their 5′-nor derivatives [39]. Through their inhibitory effect on SAH hydrolase these compounds enhance the intracellular levels of SAH and thus inhibit transmethylation reactions starting from *S*-adenosylmethionine (SAM) as the methyl donor. This includes a number of transmethylation reactions catalysed by virus-associated methyltransferases that are required for the maturation of viral mRNAs.

Like SAH hydrolase, CTP synthetase is an example of a host cell enzyme that may be envisaged as a target enzyme for antiviral agents. CTP synthetase catalyses the last step in the biosynthesis of pyrimidine ribonucleoside 5′-triphosphates, that is the conversion of UTP to CTP (Fig. 2). There is circumstantial evidence for the role of CTP synthetase as target for the cytocidal activity of Ce-Cyd. As has been demonstrated in several cell lines, i.e. murine leukemia L1210 cells [13, 14], human colon carcinoma HT-29 cells [12], human promyelocytic leukemia HL-60 cells [40] and human colon carcinoma HCT 116 cells [41], Ce-Cyd treatment leads to a marked depletion of CTP pools, and this CTP pool reduction

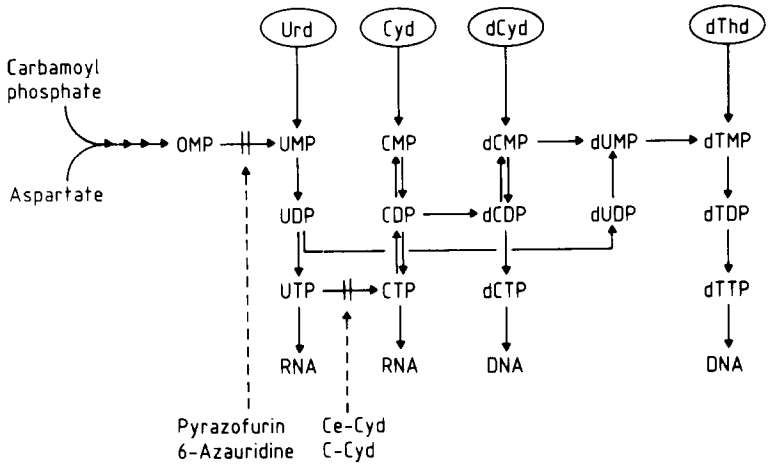

Fig. 2. Biosynthetic (*de novo* and salvage) pathways of pyrimidine nucleoside 5'-triphosphates. Targets for the action of Ce-Cyd, C-Cyd, pyrazofurin and 6-azauridine.

is closely correlated with the cytocidal activity of the compound. The depletion of CTP pools is most likely due to the inhibition of CTP synthetase, and, in fact, the triphosphates of both C-Cyd [9] and Ce-Cyd [14] have been shown to inhibit CTP synthetase.

CTP pools were not measured in the host cells (i.e. PRK, HEL, Vero) used for the antiviral assays. However, we surmise that, since in all cell systems examined thus far [12–14, 40, 41] CTP pools are depleted as a consequence of Ce-Cyd treatment, this may also be the case for the cells used in the present study. At the concentrations at which Ce-Cyd was found effective in inhibiting virus-induced cytopathogenicity [for vaccinia virus, a concentration as low as 0.08 μM (Table 1)], it causes a significant reduction in CTP pools [12–14, 40, 41]. Also, the fact that exogenous addition of Cyd or Urd, but not dThd or dCyd, reverses the antiviral activity of C-Cyd [15] and Ce-Cyd (Table 5) supports the notion that the antiviral action of these cytidine analogues may be mediated by the inhibition of CTP synthetase (Fig. 2).

There are only a few compounds known to exert an antiviral effect through interference with the biosynthesis of pyrimidine ribonucleotides. Two antimetabolites that are supposed to do so are pyrazofurin [16, 17] and 6-azauridine [18]. These compounds are targeted at OMP decarboxylase (Fig. 2). Whether targeted at CTP synthetase (C-Cyd, Ce-Cyd) or OMP decarboxylase (pyrazofurin, 6-azauridine), all four compounds have antitumor, in addition to, antiviral properties. In fact, the antitumor potential of pyrazofurin [17] and Ce-Cyd [11] has been considered the more attractive for initial development of these drugs.

Why may some compounds, such as Ce-Cyd, have potential as both antitumor and antiviral agents? If viruses or tumor cells impose similar requirements on some essential metabolites for their growth, one may expect that a shortage in the supply of these metabolites leads to suppression of both virus growth and tumor cell growth. Through its inhibitory effect on CTP synthetase, Ce-Cyd abrogates the CTP supply that is needed for both virus replication and tumor cell growth. Consequently, both the antiviral and cytocidal effects of the compound are reversed if the CTP pools are restored by the exogenous addition of cytidine.

Whether Ce-Cyd has any practical utility for the treatment of virus infections in humans remains to be established. It could be argued that the antitumor and antiviral properties of Ce-Cyd may be mutually exclusive. This is certainly not the case if the antiviral and antitumor (or cytocidal) assays are conducted under different conditions, that is with resting confluent cells for the antiviral assays and exponentially growing cells for the cytocidal assays. Under these conditions, Ce-Cyd exhibits antiviral activity at concentrations which are by several orders of magnitude lower than the concentrations that are toxic to the host cells. If, however, rapidly growing cells have to be used to monitor antiviral activity, as is the case for HIV, the toxicity of the compound for the host (MT-4) cells precludes any specific action against the virus. If these considerations are extrapolated to the *in vivo* situation, it follows that, if Ce-Cyd were to be used as an antiviral drug, proper conditions have to be conceived so as to prevent the toxic effect of the compound on rapidly growing cells (i.e. bone marrow, small intestine). This goal may well be achieved by site-specific delivery whereby the drug is directly delivered to the site of virus infection, i.e. if the compound is administered as an aerosol in the treatment of virus infections of the respiratory tract.

Similar recommendations may be made for pyrazofurin, which also exhibits broad-spectrum antiviral activity *in vitro* [42], and, in addition, has proved inhibitory to the replication of respiratory syncytial virus (RSV) *in vivo*, in cotton rats given daily doses of 10 mg/kg [43]. Pyrazofurin may be too toxic for systemic administration, but, as an aerosol, it may prove particularly useful, not only in the treatment of RSV infection, but also in the

treatment of other respiratory tract virus infections. The high potency and wide-spectrum activity of pyrazofurin [26–28] and Ce-Cyd (Table 1) against ortho- and paramyxoviruses *in vitro* make these compounds attractive candidates in the pursuit of an effective chemotherapy of respiratory virus infections.

As Ce-Cyd and pyrazofurin interact with different steps of the biosynthesis of pyrimidine ribonucleotides (Fig. 2), they may act synergistically if combined. Such combinations should be pursued from both an antiviral and antitumor viewpoint. The combination of pyrazofurin with Ce-Cyd has already been examined in one tumor cell system (i.e. L1210) (Table 7), and in this system pyrazofurin potentiated the cytocidal activity of Ce-Cyd.

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