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CYCLOPENTENYL CYTIDINE ANALOGUE

AN INHIBITOR OF CYTIDINE TRIPHOSPHATE SYNTHESIS IN HUMAN COLON CARCINOMA CELLS

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Abstract—The mechanism of action of the cyclopentenyl analogue of cytidine, cCyd, was investigated in human colon carcinoma cell line HT-29. Upon exposure of cells to 10^{-6} M cCyd, cell viability was reduced to 20% of control, whereas cytocidal activity was not present after 2 hr of drug exposure. Cell lethality was partially reversible by Urd, Cyd or dCyd at 10^{-6} M cCyd, and fully reversible by these nucleosides at 2.5×10^{-7} M cCyd. The incorporation of [^{14}C]dThd and [^{3}H]Urd into DNA and RNA was inhibited by 50% by exposure for 2 hr to 2.5×10^{-7} and 1.5×10^{-6} M cCyd respectively. Upon 24 hr of drug exposure, the IC_{50} for RNA synthesis was reduced 2.5-fold, whereas DNA synthesis was almost totally inhibited. cCyd produced a rapid and preferential reduction of CTP synthesis with a half-life of 1 hr at 10^{-6} M drug. The IC_{50} of cCyd for reducing CTP concentrations after 2 hr of drug exposure was 4×10^{-7} M. Concomitant with the reduction of CTP levels was the inhibition of transcription of rRNA and, to a lesser extent, tRNA, without changes in the processing nucleolar RNA. No changes in the size of DNA were produced following treatment with cCyd. These results indicate that cCyd is a potent and rapid inhibitor of CTP synthesis and that this effect correlates with its cytocidal activity.

Cyclopentenyl nucleoside analogues such as neplanocin A possess anticancer [1, 2] and antiviral [3] activities. The unique biological activity of this antibiotic prompted its full synthesis [4–6], as well as the synthesis of pyrimidine analogues in this series [7]. The latter compounds, viz. the cyclopentenyl derivatives of uridine and cytidine (Fig. 1), were tested for their cytotoxicity in L1210 and KB cells. The uridine analogue, cUrd§, was noncytotoxic, whereas the cytidine analogue, cCyd, was a potent inhibitor of cell growth [4, 7]. Since cCyd appeared to have potential as a chemotherapeutic agent, we assessed its cytocidal effects and mechanism of action in human colon carcinoma cells, and these studies form the basis for this report.

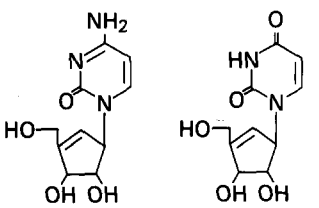

Fig. 1. Structures of cCyd and cUrd.

MATERIALS AND METHODS

Materials. [5-^{3}H]Urd (30 Ci/mmole), [5-^{3}H]Cyd (28 Ci/mmole), [2,8-^{3}H]Ado (32 Ci/mmole) and [*methyl*-^{12}C]dThd (53 mCi/mmole) were purchased from the New England Nuclear Corp., Boston, MA. cUrd and cCyd were prepared as described [7]. Cyd, dCyd and Urd were purchased from the Sigma Chemical Co., St. Louis, MO.

Tissue culture. HT-29 cells were grown under 5% CO_2:air in RPMI medium 1640 supplemented with 10% fetal calf serum, 40 mM Hepes (pH 7.4) and gentamicin, 50 μg/ml. Cell inoculums were 10^5 cells/10 ml of medium in 25 cm^2 plastic flasks.

Drug treatment. Log phase (3 day) cells were treated for 2 or 24 hr with cUrd or cCyd. After drug treatment, cells were harvested by trypsinization as described previously [8].

Cell viability determinations. Soft agar cloning was performed as described previously, except that colonies were counted directly without staining [9].

RNA and DNA determinations. During the last hour of drug exposure, cells were pulse-labeled with 0.5 μCi each of [5-^{3}H]Urd and [*methyl*-^{14}C]dThd. Trichloroacetic acid-precipitable radioactivity was measured as described previously [8].

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§ Abbreviations: cCyd, cyclopentenyl cytidine analog, 1-[(1*R*,2*S*,3*R*)-4-hydroxymethyl-2,3-dihydroxy-4-cyclopenten-1-yl] cytosine; cUrd, cyclopentenyl derivative of uridine; HPLC, high pressure liquid chromatography; Cyd, cytidine, Urd, uridine; dCyd, 2′-deoxycytidine; Ado, adenosine; dThd, thymidine, IC_{50}, concentration producing 50% inhibition; and Hepes, 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid.

RNA extraction. RNA was prelabeled for 24 hr with 1.5 μCi of [^{14}C]Urd and labeled for 24 hr with 150 μCi of [^{3}H]Ado and 10^{-6} M 2′-deoxycoformycin concurrently with drug treatment. RNA was extracted and separated by polyacrylamide: agarose gel electrophoresis as described previously [10].

Agarose gel electrophoresis of DNA. Cells were prelabeled with 1 μCi of [^{3}H]dThd and labeled during the last hour of drug treatment with 10 μCi of [^{14}C]-dThd. Cells were washed with phosphate-buffered saline (6.6 mM Na_2HPO_4, 0.8 mM KH_2PO_4, 0.154 M KCl, pH 7.4) and lysed in 30 mM NaOH: 2 mM EDTA (pH 12.2) at 100° for 15 min. Lysates were applied directly to 0.7% agarose gels prepared in the same buffer and electrophoresed at 50 V [11, 12].

Ribonucleotide determinations. Following drug treatment, cells were extracted with 5% trichloroacetic acid and immediately neutralized with 2 vol. of 0.5 M trioctylamine in trifluoro-trichloroethane. Extracts were chromatographed by anion-exchange HPLC as described previously [8]. In some experiments, cells were pulse-labeled during the last hour of drug treatment with 10 μCi of [^{3}H]-Urd or [^{3}H]Cyd.

RESULTS

Cell viability. The cytocidal effects of cCyd and cUrd were determined by a soft agar clonogenic assay (Fig. 2). Neither drug was cytotoxic after 2 hr of drug exposure, whereas 80% reduction in colony formation was produced by 24-hr treatment with 10^{-6} M cCyd with a subsequent plateau of activity at higher drug concentrations. cUrd was nontoxic at concentrations up to 10^{-4} M. There was no further reduction in cell viability from coincubation of cCyd with 10^{-4} M tetrahydrouridine, and Cyd deaminase in HT-29 cell extracts did not deaminate cCyd (results not shown).

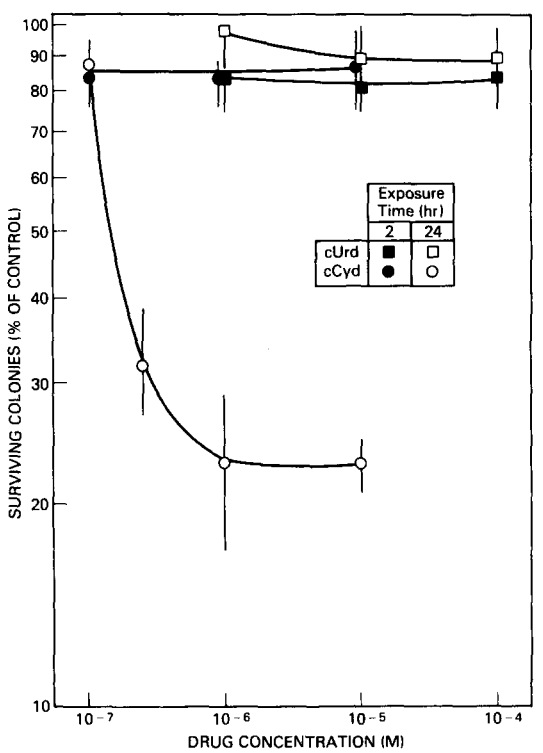

Fig. 2. Viability of HT-29 cells following exposure to cUrd or cCyd. Cell viability was determined by colony formation in a soft agar clonogenic assay [9]. Values are the means ± S.E. of three to five determinations.

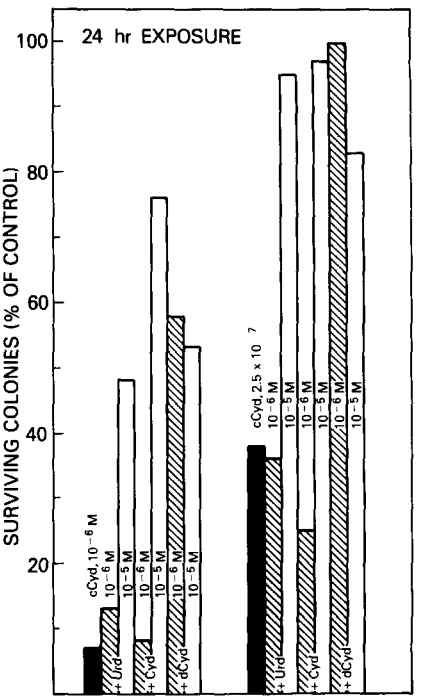

Fig. 3. Reversal of call lethality by Urd, Cyd or dCyd following treatment with cCyd. HT-29 cells were treated concurrently for 24 hr with 2.5×10^{-7} or 10^{-6} M cCyd and either 10^{-6} or 10^{-5} M Urd, Cyd or dCyd. Cell viability was determined by colony formation as described under Materials and Methods.

The cell lethality produced by 24 hr of exposure to cCyd was partially or fully reversible by simultaneous incubation with an excess of Urd, Cyd or dCyd depending on the concentration of cCyd (Fig. 3).

RNA and DNA synthesis. Cells were double-labeled with [^{3}H]Urd and [^{14}C]dThd to assess the synthesis of RNA and DNA respectively (Fig. 4). cCyd produced a rapid reduction in DNA and RNA synthesis within 2 hr, wherein the IC_{50} was 2.5×10^{-7} M and 1.5×10^{-6} M respectively (Fig. 4A). In contrast, cUrd only inhibited the incorporation of [^{3}H]Urd into RNA at an $IC_{50} = 4 \times 10^{-5}$ M. After 24 hr of treatment with cCyd, inhibition of DNA synthesis was virtually complete, whereas the IC_{50} for RNA synthesis was reduced to 6×10^{-7} M (Fig. 4B). Again, cUrd only inhibited [^{3}H]Urd incorporation into RNA, but the IC_{50} was reduced 10-fold.

To obtain a more detailed picture of RNA and DNA synthesis, these nucleic acids were resolved by electrophoresis. Continuous labeling of cells with [^{3}H]Ado concurrently with cCyd treatment revealed

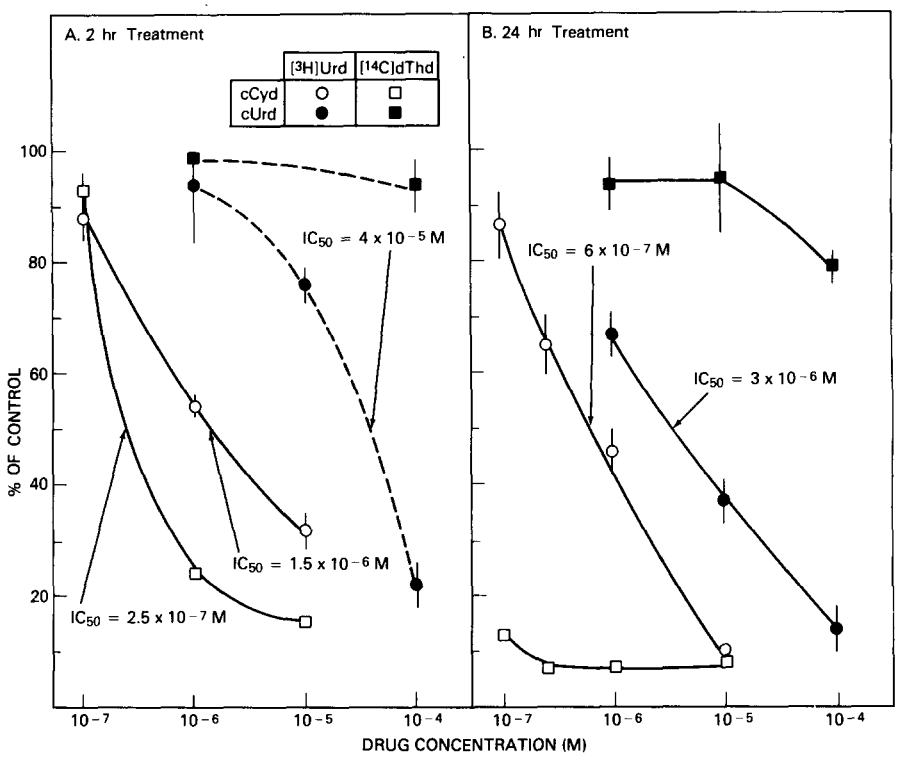

Fig. 4. RNA and DNA synthesis following treatment with cCyd. RNA and DNA synthesis was measured by the incorporation of [^{3}H]Urd or [^{14}C]dThd, respectively, into acid-precipitable material as described under Materials and Methods. Values are the means ± S.E. of three to five determinations.

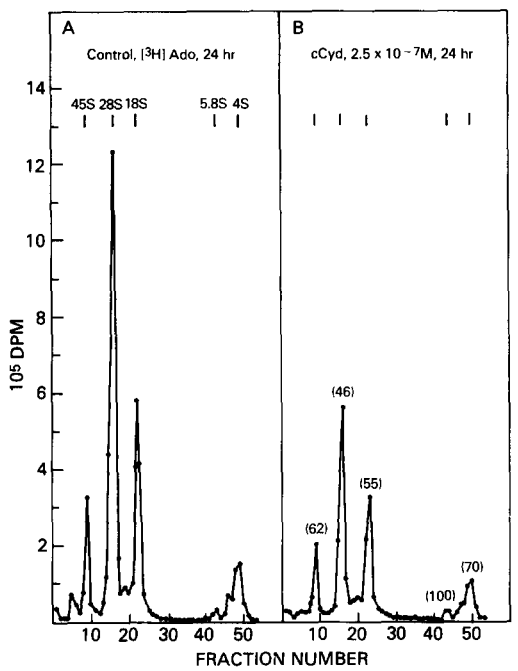

Fig. 5. Polyacrylamide-agarose gel electrophoresis of RNA following treatment with cCyd. HT-29 cells were prelabeled with [^{14}C]Urd and then treated concurrently for 24 hr with 2.5×10^{-7} M cCyd and [^{3}H]Ado. RNA was extracted and separated by electrophoresis as described under Materials and Methods.

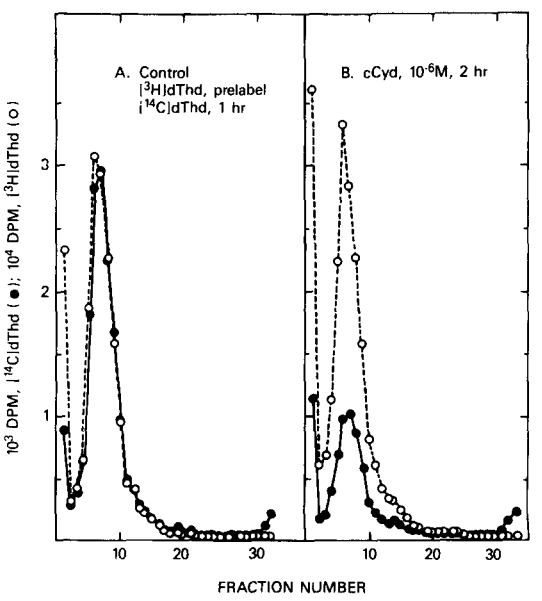

Fig. 6. Alkaline agarose gel electrophoresis of DNA. HT-29 cells were prelabeled with [^{3}H]dThd and then treated for 2 hr with 10^{-6} M cCyd. Cells were labeled during the last hour with [^{14}C]dThd, and DNA was separated by electrophoresis as described under Materials and Methods.

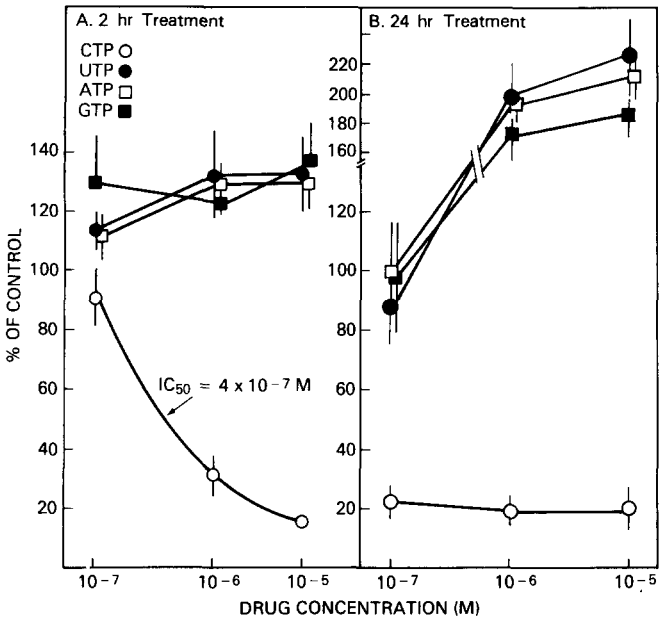

Fig. 7. Ribonucleoside triphosphate levels following treatment with cCyd. Ribonucleoside triphosphate concentrations were measured in neutralized trichloroacetic acid extracts from control and treated cells by anion-exchange HPLC as described previously [8]. Values are the means ± S.E. of three to four determinations.

that no accumulation of 45S precursor RNA occurred but that 28S and 18S rRNA were equally inhibited (Fig. 5). Less inhibition of tRNA occurred following 24-hr treatment with cCyd, and no inhibition of 5.8S RNA was evident.

The size of DNA following 2 hr of treatment with cCyd was analyzed by alkaline agarose gel electrophoresis (Fig. 6). Both prelabeled parental DNA and pulse-labeled newly synthesized DNA were of equivalent molecular weights. Exposure for 2 hr to cCyd did not affect the size of single-stranded DNA, but did produce an inhibition of DNA replication.

CTP synthesis. The possibility that cCyd may be functioning as an inhibitor of pyrimidine nucleotide synthesis was investigated by measurements of nucleoside triphosphate levels following drug treatment (Fig. 7). cCyd produced a rapid dose-dependent reduction of only CTP levels within 2 hr, wherein the $IC_{50} = 4 \times 10^{-7}$ M, a value similar to the IC_{50} for DNA synthesis (Fig. 4). Prolongation of treatment for 24 hr resulted in a 70–80% reduction in CTP levels at all concentrations of cCyd examined.

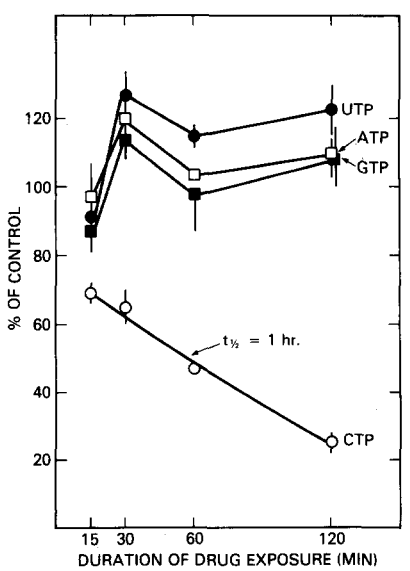

Fig. 8. Time course of inhibition of CTP synthesis by cCyd. CTP levels were measured as described in Fig. 7. Values are the means ± S.E. of three to four determinations.

Table 1. Urd and Cyd phosphorylation following treatment with cCyd*

Treatment	[^{3}H]UTP	[^{3}H]CTP
2 Hr	(dpm/10^6 cells)	
Control	17,200	5,500
	(% of control)	
cCyd, 10^{-7} M	142	91
10^{-6} M	150	40
10^{-5} M	103	26
24 Hr	(dpm/10^6 cells)	
Control	30,050	2,400
	(% of control)	
cCyd, 10^{-7} M	68	31
10^{-6} M	99	25
10^{-5} M	24	12

* Cells were treated for 2 or 24 hr with cCyd and labeled during the last hour of drug exposure with either 10 μCi of [^{3}H]Cyd or [^{3}H]Urd. UTP and CTP were separated by HPLC, and their radioactivity was determined as described under Materials and Methods.

The half-life for the reduction of CTP concentrations by 10^{-6} M cCyd was approximately 1 hr (Fig. 8).

Assessment of the pyrimidine salvage pathway indicated that the incorporation of [^{3}H]Urd into UTP was not affected except at 10^{-5} M cCyd, but that the conversion of [^{3}H]Cyd to CTP was preferentially inhibited (Table 1).

At the present, we have not been able to quantitate the formation of nucleotide metabolites of cCyd by HPLC using u.v. absorbance. The retention time of the nucleotide analogue, if it exists, is probably masked by the absorbance of ATP in the cell extracts. In contrast, cUrd does form a nucleotide metabolite with a retention time intermediate between UTP and ATP (data not shown).

DISCUSSION

The cyclopentenyl analogue of Cyd, cCyd, appears to be a potent inhibitor of CTP synthesis in HT-29 cells. The inhibition of CTP synthesis was rapid and preceded inhibition of DNA and RNA synthesis. Prolonged inhibition of CTP synthesis leads to a reduction in cell viability with an exponential plateau survival response [13]. This response is indicative of antimetabolites which are cell cycle stage specific, and it has been noted previously in HT-29 cells treated with 5-fluorouracil [14], sangivamycin [9] and the cyclopentenyl analogue of adenosine, neplanocin A [2]. It is noteworthy that the cyclopentyl derivative of Cyd, carbodine, also inhibits CTP synthesis and possesses antitumor activity against L1210 leukemia [15, 16].

The differential effect by cCyd on DNA and RNA synthesis, particularly after only a 2-hr exposure interval, can probably be attributed to depletion of dCTP. The normally lower cellular concentrations of deoxyribonucleoside triphosphates vs ribonucleoside triphosphates would render the former nucleotide pool more susceptible to depletion and, thus, become rate-limiting for DNA synthesis.

It is interesting that, whereas both cUrd and cCyd are inhibitors of the pyrimidine salvage pathway, only cCyd produced cytotoxicity. This phenomenon was noted previously in L1210 cells *in vitro* [7]. These results implicate the importance of inhibition of CTP synthesis *de novo* by cCyd, but not by cUrd, as the cause of cell lethality. However, cUrd may still find utility in chemotherapy in conjunction with cCyd, wherein blockage of both salvage and *de novo* pyrimidine biosynthetic pathways should lead to a synergistic antitumor response.

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