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CYCLOPENTENYL URACIL: AN EFFECTIVE INHIBITOR OF URIDINE SALVAGE *IN VIVO*

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Abstract—Cyclopentenyl uracil, a non-cytotoxic inhibitor of uridine kinase, was found to effectively block the salvage of circulating uridine by host and tumor tissues in the intact mouse. Dose–response characteristics of the inhibition were determined. Large doses (1 g/kg) of cyclopentenyl uracil were required, and the effect of a single dose fell rapidly over a 24-hr period. A sustained inhibition of uridine salvage of >64–79% could be maintained by multiple doses of 1 g/kg given on an every 8-hr schedule. Mice given cyclopentenyl uracil (1 g/kg) every 8 hr for 5 days continued to gain weight and showed no signs of toxicity; however, the combination of cyclopentenyl uracil with a non-toxic dose of *N*-(phosphonacetyl)-L-aspartic acid (PALA; 200 mg/kg daily for 5 days) was lethal to mice, indicating that circulating uridine modifies the toxicity of agents that act on enzymes of the *de novo* pyrimidine pathway. Although the duration of action and potency of cyclopentenyl uracil are not ideal, this is the first demonstration of an effective inhibition of uridine salvage in the intact mouse with a non-cytotoxic agent. This makes possible the evaluation of concurrent inhibition of *de novo* and salvage routes to pyrimidine nucleotides as an approach to chemotherapy.

Key words: cyclopentenyl uracil; uradine; inhibition of pyrimidine salvage *in vivo*

Inhibitors of pyrimidine biosynthesis *de novo*, such as PALA† and pyrazofurin, have had only marginal success in the clinic as single agents [1–5]. This failure may be explained by the use of the alternative salvage pathway by tumor cells for provision of cellular pyrimidine nucleotides. Previous work from this laboratory demonstrated that the concentration of uridine present in human plasma is sufficient to satisfy the cellular requirements for pyrimidines without synthesis *de novo* [6]. Although salvage of uridine by cells in culture has been studied intensely, much less is known concerning the process of salvage in normal physiology. Studies characterizing the salvage of the naturally occurring nucleosides by tissues of the mouse indicated that although uracil and the pyrimidine deoxynucleosides were only inefficiently salvaged, uridine and cytidine were well utilized for nucleotide synthesis *in vivo* [7]. In fact, the efficiency of the salvage of uridine and cytidine in mice exceeded 50%, in contrast to the situation in the rat wherein a much smaller percentage is anabolized [8]. Salvage occurs as a sequential process of transport to transverse the cell membrane followed by trapping intracellularly by phosphorylation by uridine kinase, thus providing two possible targets for pharmacologic intervention.

Inhibitors of nucleoside transport such as dipyridamole and *p*-nitrobenzylthioinosine, although potent inhibitors of nucleoside uptake by some lines of cultured cells, proved unable to block uridine salvage *in vivo* [9, 10]. Harrap and co-workers [11] demonstrated that the salvage of thymidine is also not inhibited effectively by dipyridamole *in vivo*. In the last few years, a number of new transport mechanisms for nucleosides have been reported, notably a concentrative transport of uridine by some tissues of the mouse, which is insensitive to the usual inhibitors of nucleoside transport [12–14]. It is possible that these alternative transport mechanisms allow tissues *in vivo* to circumvent the effects of dipyridamole and *p*-nitrobenzylthioinosine. The inability of the inhibitors of nucleoside transport to block uridine salvage *in vivo* led us to concentrate on the development of inhibitors of uridine kinase.

In contrast to our failure to inhibit uridine salvage *in vivo* with the transport inhibitors, we found that 3-deazauridine, a competitive inhibitor of uridine kinase, blocks uridine salvage by tissues of the intact mouse, albeit at high toxic doses [10]. This finding prompted us to search for less toxic inhibitors of uridine kinase that could be used to inhibit uridine salvage *in vivo*. We evaluated 29 analogs of uridine and found that CPE-U is a modestly effective inhibitor of uridine kinase and an even more effective inhibitor of uridine salvage by intact cells [15]. CPE-U has a number of properties that make it attractive as a possible chemotherapeutic agent: it is not a substrate for uridine phosphorylase (a highly active degradative enzyme *in vivo*), it is non-cytotoxic (in contrast to 3-deazauridine), and it is a selective inhibitor of the salvage of uridine (and cytidine)

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† Abbreviations: CPE-U, cyclopentenyl uracil; PALA, *N*-(phosphonacetyl)-L-aspartic acid.

without effect on the salvage of deoxycytidine or thymidine [16]. The current report demonstrates that CPE-U is an effective inhibitor of uridine salvage *in vivo* and characterizes the pharmacologic parameters for this inhibition in the intact mouse.

MATERIALS AND METHODS

[5-^{3}H]Uridine, 20 Ci/mmol, was purchased from Moravek Biochemicals (Brea, CA), lyophilized before use, and reconstituted in sterile 0.9% sodium chloride for injections. Nucleotides, nucleosides, phosphodiesterase I (type VII), and alkaline phosphatase were purchased from the Sigma Chemical Co. (St. Louis, MO). CPE-U was synthesized as described previously [16]. L1210 cells used in *in vitro* studies were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Fischer's medium containing 10% horse serum at 2–6 × 10^5 cells/mL. P388 leukemia cells used in *in vivo* studies were obtained from the Developmental Therapeutics Program, National Cancer Institute, tumor repository and maintained by weekly passage in C57BL/6 × DBA/2 F1 male mice weighing 25–28 g. These mice had free access to Lab-Chow and water.

The salvage of radiolabeled uridine into total acid-soluble uracil nucleotides and RNA was measured as described previously [7]. Briefly, male BALB/c × DBA/2 mice (hereafter referred to as CDF_1) received radiolabeled uridine by injection into the tail vein in a total volume of 0.2 mL. After 20 min, the mouse was killed by cervical dislocation. The liver, both kidneys, and the spleen were removed and immediately immersed in liquid nitrogen. The entire intestine, from immediately below the stomach to the rectum, was removed and placed in ice-cold 0.9% sodium chloride. The intestine was flushed free of its contents, blotted lightly, and immersed in liquid nitrogen. The tissues were weighed and homogenized in 8 vol. of ice-cold 0.5 M perchloric acid with a mechanically driven glass–Teflon homogenizer. The acid-soluble fraction was neutralized as described by Khym [17], and then treated with phosphodiesterase and alkaline phosphatase to convert all uracil nucleotides to uridine. Uridine was separated by reverse-phase high performance liquid chromatography using a Gilson chromatograph equipped with a Waters automated sample injector. A C18 reverse-phase column (15 cm × 0.38 cm) of 5 μm particle size (Waters) was eluted with 1.5 mL/min of 0.2 M potassium phosphate, pH 5.1. Detection was by absorbance at 254 and 280 nm. Fractions of 0.5 or 1 mL were collected as required. Radioactivity was determined by liquid scintillation counting in ACS (Amersham). Efficiency was determined by the addition of an internal standard. Recovery of radiolabel from the chromatograph was >90%. The RNA fraction prepared by alkaline hydrolysis was adjusted to pH 9.0 with Tris base, and 50 U/mL of calf intestine alkaline phosphatase was added. This was incubated for 20 min at 37° and then boiled, the precipitated protein was removed by centrifugation, and the distribution of radiolabel was determined by HPLC. This procedure indicated that the radiolabel in the RNA fraction following administration of [5-^{3}H]uridine was >90% nucleoside after this treatment.

To determine the effect of drug treatments on L1210 cell replication, L1210 cells were grown in Fischer's medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% horse serum. The cultures were initiated at 10,000 cells/mL and incubated at 37° with constant exposure to inhibitor for 48 hr. Cell number was determined with a Coulter counter. Control cultures typically grew to 160,000 cells/mL. The value given is the mean of three or four independent determinations.

RESULTS AND DISCUSSION

Previous work from our laboratory using cultured L1210 cells demonstrated that the concentration of uridine present in human plasma, and thus available for salvage, is sufficient to prevent the growth inhibitory effects of PALA [6, 18–20], an inhibitor of pyrimidine synthesis *de novo*. Also, in the presence of plasma concentrations of uridine, L1210 cells turn off their *de novo* pathway and utilize their salvage pathway [6]. A recent study of murine

Table 1. Inhibition of L1210 cell growth by the combination of PALA and CPE-U

Drug	Increase in cells/well × 10^{-5}		
	Uridine (μM)		
	0	5	20
None	1.2	1.2	1.4
CPE-U (300 μM)	1.4	ND*	ND*
PALA (1 mM)	0.03	1.0	1.4
PALA (1 mM) and CPE-U (300 μM)	0.0	0.02	0.07

L1210 cells were grown in Fischer's medium with 5% dialyzed horse serum with continuous exposure to PALA, CPE-U, and PALA plus CPE-U. Values are the increase in cells per incubation well after a 48-hr incubation at 37°. Cells were seeded at 0.1 × 10^5 cells/mL. Cell number was determined with a Coulter counter, and the values given are the means of three independent determinations.

* ND = not determined.

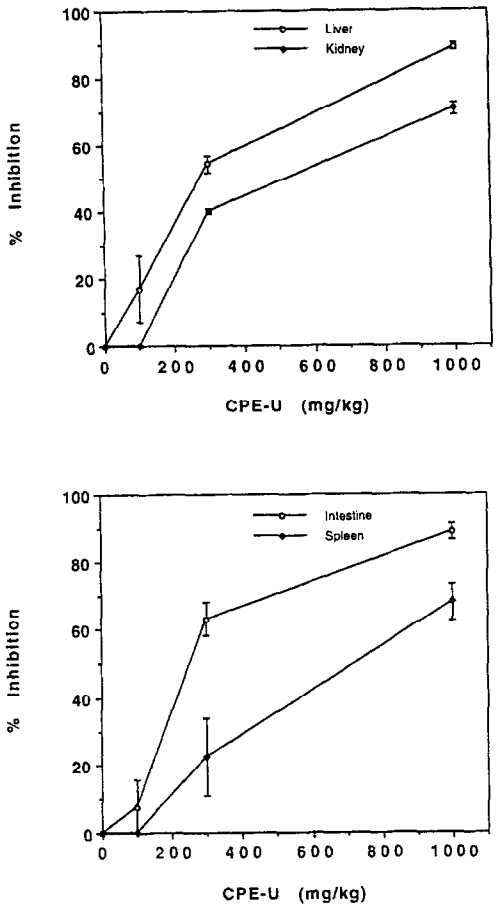

Fig. 1. Dose dependence of inhibition of uridine salvage by CPE-U. Male CDF_1 mice received the indicated dose of CPE-U (i.p.), and 2 hr later 20 μCi of [5-^{3}H]uridine was administered (i.v.). Mice were killed by cervical dislocation 20 min after administration of the uridine. Values are the percent inhibition relative to saline controls of incorporation of [^{3}H]uridine into RNA and uracil nucleotides [means ± SEM (bars), N = 5 control; N = 3 treated]. Values (μCi/g tissue wet weight) for tissues from non-treated control animals were: intestine, 0.110 ± 0.019 (RNA) and 0.294 ± 0.095 (uracil nucleotides); liver, 0.074 ± 0.021 (RNA) and 0.452 ± 0.096 (uracil nucleotides); kidney, 0.141 ± 0.025 (RNA) and 0.452 ± 0.096 (uracil nucleotides); and spleen, 0.116 ± 0.027 (RNA) and 0.216 ± 0.097 (uracil nucleotides). Each control value is the mean of determinations on 5 mice; variation is indicated as the SD.

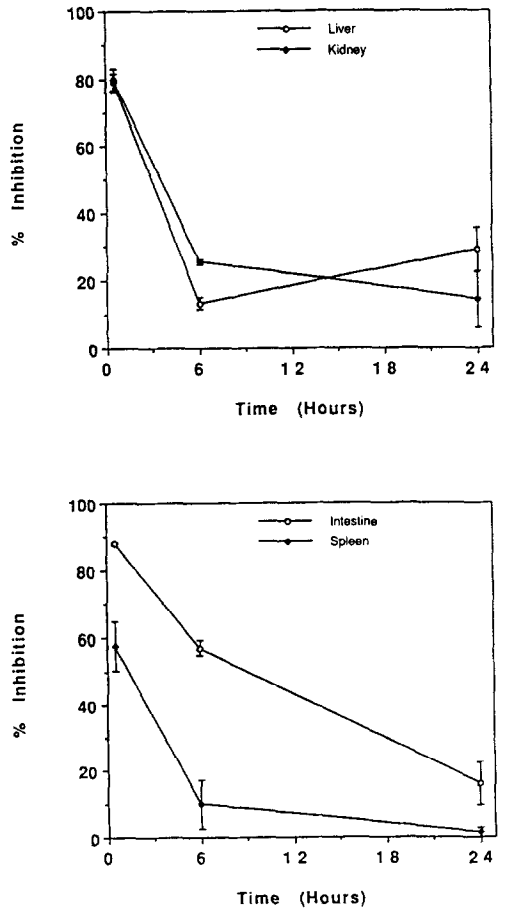

Fig. 2. Duration of inhibition of uridine salvage by CPE-U. Male CDF_1 mice received 1 g/kg of CPE-U (i.p.), and at the indicated time 20 μCi of [5-^{3}H]uridine was administered (i.v.). Mice were killed by cervical dislocation 20 min after administration of the uridine. Values are the percent inhibition relative to saline controls of incorporation of [^{3}H]uridine into RNA and uracil nucleotides [means ± SEM (bars), N = 4]. Values (μCi/g tissue wet weight) for tissues from non-treated control animals were: intestine, 0.086 ± 0.028 (RNA) and 0.223 ± 0.069 (uracil nucleotides); liver, 0.100 ± 0.070 (RNA) and 0.339 ± 0.123 (uracil nucleotides); spleen, 0.092 ± 0.022 (RNA) and 0.252 ± 0.082 (uracil nucleotides); and kidney, 0.130 ± 0.042 (RNA) and 0.718 ± 0.202 (uracil nucleotides). Each control value is the mean determination on 12 mice; variation is indicated as the SD.

tumors growing in intact animals demonstrated that at least one murine tumor switches to the salvage pathway when *de novo* pyrimidine synthesis is blocked [21]. These results indicate that uridine salvage is an important pathway for pyrimidine nucleotide synthesis *in vivo* and that circulating nucleosides might limit the therapeutic effectiveness of anticancer agents that act on enzymes of the *de novo* pyrimidine pathway. The lack of clinical success with inhibitors of pyrimidine synthesis *de novo* (such as PALA and pyrazofurin) has stimulated efforts to discover inhibitors of the salvage pathway so that concurrent inhibition of both pathways can be achieved.

The data in Table 1, obtained from *in vitro* experiments using cultured L1210 cells, support the concept that concurrent inhibition of both the *de novo* and salvage pathways for pyrimidine nucleotide synthesis is likely to produce a more effective growth inhibition than inhibition of only the *de novo* pathway. PALA is highly growth inhibitory when used in medium not containing uridine. However, the addition of uridine at concentrations found in human plasma renders cells resistant to the effects of PALA. The addition of CPE-U, at a concentration

Table 2. Inhibition of uridine salvage *in vivo* by CPE-U

Tissue	% Inhibition
Ascites	77 ± 6
Kidney	74 ± 19
Liver	64 ± 23
Spleen	79 ± 11
Intestine	79 ± 12

CDF_1 male mice bearing P388 ascites were injected twice at 12-hr intervals with 1 g/kg CPE-U. At 8 hr after the last CPE-U injection, the mice received 20 μCi of [^{3}H]uridine by intravenous (tail vein) injection. The tissue content of the sum of labeled uridine nucleotides and RNA was determined as described in Materials and Methods. The values given are the percent inhibition of incorporation for treated animals versus the control animals of the same experiment. Values (μCi/g tissue wet weight) for tissues from non-treated control animals were: ascites, 0.13 ± 0.045; kidney, 0.93 ± 0.13; liver, 1.38 ± 0.29; spleen, 1.14 ± 0.19; and intestine, 0.99 ± 0.16. Each value is the mean of determinations on 4 or 5 mice. Variation is indicated as SD.

that blocks uridine salvage *in vitro* [7] and that is not cytotoxic, restored the sensitivity of the cells to PALA. These data show that concentrations of uridine found in human plasma protect cells from the growth inhibitory effects of PALA; this protective effect can be prevented by blocking uridine salvage with CPE-U.

The next series of experiments show that CPE-U can inhibit uridine salvage in the intact mouse and establish pharmacologic parameters for this inhibition. Salvage of uridine is a two-step process: transport followed by phosphorylation. The phosphorylation of uridine that has been transported into the cell is limited by the competing catabolic reaction carried out by uridine phosphorylase [7]. Thus, the extent to which uridine is utilized by various tissues depends on the kinase to phosphorylase ratio. Inhibition of uridine kinase by CPE-U should allow more of the transported uridine to be catabolized by uridine phosphorylase, thus preventing an accumulation of intracellular uridine and the development of metabolic resistance to kinase inhibition. To obtain a true value for the amount of uridine salvaged (not just the amount transported into cells and therefore subjected to both anabolic and catabolic reactions), we determined the amount of radiolabeled uridine that is incorporated into pyrimidine nucleotides and nucleic acids of the tissues studied. Figure 1 shows the dose–response characteristics of the inhibition of uridine salvage by tissues of the mouse 2 hr after the indicated dose of CPE-U. Although CPE-U effectively inhibited uridine salvage by all the tissues studied, high doses (1 g/kg) were required. Figure 2 shows the time course of inhibition of uridine salvage following a single injection of CPE-U at a dose of 1 g/kg. The inhibitory effect of CPE-U fell rapidly over the 24-hr period, indicating that frequent dosing is required for a sustained inhibition of uridine salvage.

Table 2 shows the effect of two injections of CPE-U (1 g/kg), 12 hr apart, on uridine salvage measured 8 hr after the second injection. This schedule produced a minimum of 64% inhibition of uridine salvage in every tissue examined at 8 hr after the last dose. Mice injected with CPE-U at a dose of 1 g/kg every 8 hr for 5 days showed no decrease in weight gain compared with control animals for the 14-day duration of the experiment. When this dose schedule was used in combination with a dose schedule of PALA that also did not affect weight gain (200 mg/kg, daily for 5 days), all mice receiving both PALA and CPE-U died during the 5-day treatment period. These limited results show that uridine salvage is an important factor modulating the toxic effects of PALA. Additional experimentation is needed to determine if CPE-U can improve the anticancer activity of PALA.

In summary, CPE-U is a non-cytotoxic inhibitor of uridine kinase that is an effective inhibitor of uridine salvage in the intact mouse. By comparison, inhibitors of uridine transport (dipyridamole and *p*-nitrobenzylthioinosine) were ineffective in inhibiting uridine salvage in the intact mouse [9, 10]. Although the duration of action and potency of CPE-U are less than might be desired, the important point of these data is that an effective inhibition of uridine salvage can be produced in the mouse for the first time. This makes possible the evaluation of concurrent inhibition of *de novo* and salvage routes to pyrimidine nucleotides as an approach to chemotherapy. Furthermore, the non-cytotoxic properties of CPE-U make it a useful biological tool to study the physiology of uridine salvage *in vivo*.

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