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Synergistic Antitumor Activity of Troxacitabine and Camptothecin in Selected Human Cancer Cell Lines

Tracy E. Kim, Shin-Young Park, Chih-Hung Hsu, Ginger E. Dutschman, and Yung-Chi Cheng

Yale Cancer Center, Medical Oncology (T.E.K.) and Department of Pharmacology (S.-Y.P., C.-H.H., G.E.D., Y.-C.C.), Yale University School of Medicine, New Haven, Connecticut

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ABSTRACT

Troxacitabine (L-OddC) is an L-configuration deoxycytidine analog currently in phase II trials for the treatment of cancer. The cytotoxicity of L-OddC in combination with other anticancer agents has not been studied systematically. In the present study, we assessed the cytotoxic effects produced by the combinations of L-OddC and several commonly used chemotherapy drugs in a panel of cultured human cancer cell lines. Growth inhibition resulting from simultaneous exposure to two-drug combinations was determined using the methylene blue staining method. Camptothecin (CPT) and analogs exhibited additives to synergistic interactions with L-OddC by isobologram analysis. These effects were cell type-specific, with the most pronounced synergism being observed in KB oropharyngeal carcinoma and CPT-resistant KB100 cell lines. In KB cells, the total cellular uptake and DNA incorporation of L-OddC were increased by the addition of CPT. One explanation that emerged from enzyme assays of deoxycytidine kinase (dCK) and deoxycytidine monophosphate kinase (dCMPK), key enzymes involved in L-OddC phosphorylation, was that CPT protected against L-OddC–induced reduction in dCK and dCMPK activity. The resulting increase in L-OddC metabolites and incorporation into DNA was associated with enhanced L-OddC cytotoxicity. These findings will be useful in designing future clinical trials of combination chemotherapy with L-OddC and CPT analogs with the potential for a broad use against both hematological and solid tumors.

Nucleoside analogs constitute an important class of drugs used widely in the treatment of cancer. L-OddC is a novel deoxycytidine analog (Fig. 1). Its L-configuration differs from the naturally occurring nucleosides and nucleoside analogs currently approved for anticancer therapy. In the early 1990s, L-2′,3′-dideoxythiocytidine (lamivudine) was shown to be more active than its D-stereoisomer against hepatitis B and human immunodeficiency viruses (Chang et al., 1992; Coates et al., 1992; Schinazi et al., 1992). Several L-nucleoside analogs possessing antiviral activity have since been identified, and among them, L-OddC was found to exhibit potent cytotoxicity (Kim et al., 1992). This recognition has led to its subsequent development as an antineoplastic agent.

The metabolism of L-OddC in human cancer cells differs from that of other cytidine analogs in several aspects. For one, cellular uptake of L-OddC occurs predominantly via passive diffusion, relatively independent of nucleoside transporters (Gourdeau et al., 2001b) and unaffected by the multidrug resistance transporter P-glycoprotein (Bowlin et al., 1997; Gourdeau et al., 2002). Mono- and diphosphorylation of L-OddC are catalyzed by dCK (Grove et al., 1995) and cytidine/uridine monophosphate kinase (Liou et al., 2002), respectively, as with cytarabine and gemcitabine. The di-to-triphosphate conversion, however, is mediated preferentially by 3-phosphoglycerate kinase (PGK) rather than by nucleoside diphosphate kinase and is the rate-limiting step in the activation of L-OddC (Krishnan et al., 2002). Its triphosphate form is incorporated readily into DNA of replicating cells (Kukhanova et al., 1995), and lacking a hydroxyl group at the 3′-position causes immediate chain termination. Inefficient removal by 3′→5′ exonucleases results in prolonged retention of L-OddC within DNA and may be a contributing factor to its cytotoxicity (Kukhanova et al., 1995; Chou et al., 2000). L-OddC also inhibits repair DNA polymerases (Kukhanova et al., 1995), whereas inhibitions of RNA and ribonucleotide reductase are negligible (Grove and Cheng, 1996).

In contrast to cytarabine and gemcitabine, which are rap-

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T.E.K. and S.-Y.P. contributed equally to this work.

ABBREVIATIONS: L-OddC, troxacitabine, β-L-dioxolane-cytidine; dCK, deoxycytidine kinase; dCMPK, deoxycytidine monophosphate kinase; PGK, 3-phosphoglycerate kinase; dCD, deoxycytidine deaminase; dCyd, deoxycytidine; topo, topoisomerase; CPT-11, irinotecan; TPT, topotecan; PLDB, protein-linked DNA break; TCA, trichloroacetic acid; DTT, dithiothreitol; CPT, camptothecin; PBS, phosphate-buffered saline; dsDNA, double-stranded DNA; SN-38, 7-ethyl-10-hydroxycamptothecin.

idly inactivated by dCD to their respective uridine derivatives, L-OddC is a poor substrate for dCD, resulting in sustained retention of active metabolites (Grove et al., 1995). Certain solid tumors and leukemias have high levels of dCD, representing one mechanism of resistance to cytarabine. L-OddC was superior to cytarabine in a resistant leukemia cell line and had in vivo activity against leukemic xenografts (Gourdeau et al., 2001a). L-OddC also showed potent activity against multiple solid tumor cell lines, including KB oropharyngeal, HepG2 hepatocellular, and DU145 and PC3 prostate carcinomas (Grove et al., 1995). In animal studies, tumor regressions were seen with refractory tumor types such as renal cell (Kadhim et al., 1997), prostate (Grove and Cheng, 1996; Rabbani et al., 1998), and pancreatic (Weitman et al., 2000) xenografts. Against Panc-01 pancreatic xenografts, L-OddC was more effective than gemcitabine (Weitman et al., 2000). In clinical testing to date, promising activity has been observed in the treatment of hematological malignancies (Giles et al., 2001, 2002). A dose-finding study in advanced solid tumors (de Bono et al., 2002) and an efficacy trial in metastatic renal cell carcinoma showing modest activity (Townsley et al., 2003) also were published recently.

Despite the putative advantages conferred by its unique L-configuration, L-OddC as a single agent is likely to have only limited clinical efficacy. Multiagent chemotherapy is a major strategy for overcoming drug resistance and improving response and cure rates. In general, agents with different targets of action are exploited in concurrent, sequential, or alternating combinations for possible biochemical synergism. We investigated the interaction of L-OddC with other chemotherapeutic agents. Previous studies in our laboratory tested the combinational effects of L-OddC and several anticancer drugs including cisplatin, doxorubicin, etoposide, vincristine, and fluorouracil in DU145 cells (Y.-C. Cheng, unpublished data). Cisplatin and etoposide were additive, doxorubicin and fluorouracil were subsynergistic, and CPT and vincristine were synergistic. Among all of the drugs tested, CPT exhibited the most pronounced synergism with L-OddC. CPT is a topo I poison isolated nearly 40 years ago from the Chinese tree *Camptotheca acuminata* (Wall et al., 1966). CPT-11 (Negoro et al., 1991) and TPT (Burris et al., 1994) are soluble semisynthetic analogs currently in clinical use.

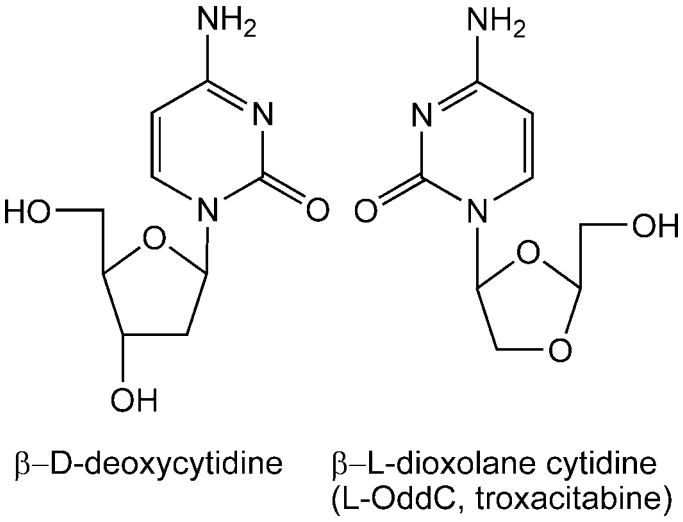

Fig. 1. Chemical structures of β-D-deoxycytidine and L-OddC.

The rationale for studying CPTs and L-OddC in combination is taken from their wide-ranging antitumor activity, different mechanisms of action, and nonoverlapping toxicities other than neutropenia. We looked for possible explanations for their synergy and tested the hypotheses that L-OddC enhances the topo I poisoning effects of CPT or, alternatively, that CPT augments incorporation of L-OddC into DNA.

Materials and Methods

Cell Lines. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The growth medium used was RPMI 1640 supplemented with 10% dialyzed fetal bovine serum and 100 μg/ml kanamycin. KB100 cells were maintained in the presence of 100 nM CPT to preserve their CPT-resistant phenotype (Beidler et al., 1996). Before each experiment, KB100 cells were grown in CPT-free media for 2 days.

Chemicals. L-OddC was provided by Shire Biochem, Inc. (Laval, Quebec, Canada) as a sterile lyophilized powder stored at −20°C. CPT was provided by Dr. Zong-Chao Liu of Cancer Institute at Sun Yat-Sen University of Medical Sciences (Guangzhou, China). SN-38 was provided by Pharmacia & Upjohn (Peapack, NJ). TPT was purchased from GlaxoSmithKline (Uxbridge, Middlesex, UK), and cisplatin and etoposide were purchased from Sigma Chemical (St. Louis, MO). Dilutions of all drugs were made with PBS from 10 mM stock solutions in 100% dimethyl sulfoxide stored at −70°C. L-OddC used in animal experiments was prepared as a suspension by mixing with Tween 80. CPT-11 was purchased from Pharmacia & Upjohn. [^{3}H]L-OddC and [^{14}C]dCyd were purchased from Moravek Biochemicals (Brea, CA). Bromovinyl-2′-deoxyuridine was purchased from Sigma.

Growth-Inhibition Assays. Cells were plated at 1×10^4 cells/ml/well in 24-well plates. Serially diluted concentrations of drugs in triplicate were added to cells in logarithmic growth. After three-generation time, cells were stained with 0.5% methylene blue in 50% ethanol, and the cell layer was dissolved in 1% sarkosyl. The optical density at 595 nm was determined using a microplate reader. Fractional growth relative to control was calculated by dividing the optical density values in drug-treated wells by those in control wells. Data were plotted as fractional growth versus drug concentration.

Analysis of Combined Drug Effects. The traditional isobologram method (Loewe, 1953) was used to analyze the effects of binary drug combinations in growth-inhibition assays. The IC_{50} point was chosen as the effect of interest. Single-agent IC_{50} values were interpolated from the plots of fractional growth versus drug concentration. An isobole, defined as the line joining all dose pairs inducing the same specified effect, namely IC_{50}, was generated by plotting combination doses of each dose pair expressed as fractions of the single-agent IC_{50} values. An isobole is represented algebraically by the sum of the ratios of combination-to-single-agent doses of each drug producing an isoeffect, or $(A_{combination}/A_{single}) + (B_{combination}/B_{single})$, where A and B connote the two drugs being combined. When this sum is equal to 1, the interaction is said to be additive. A synergistic interaction appears below the line of additivity, whereas an antagonistic interaction appears above this line. In applying this method, we assumed that the action of one drug was completely independent of the action of the second drug and that their dose-response curves were linear.

Quantification of PLDB. A modified in vivo K-SDS coprecipitation assay was used to quantify steady-state PLDB levels as described previously (Beidler and Cheng, 1995). KB cells were pretreated with 1 μM L-OddC or PBS for 3 h before the addition of CPT to allow sufficient time for cellular uptake of L-OddC, generation of active metabolites, and incorporation into DNA. Cells were then exposed to increasing concentrations of CPT ranging from 100 to 3000 nM for 1 h.

CPT-Induced dsDNA Breaks. Double-stranded DNA breaks were measured as described previously (Wu et al., 2002). In brief, 2×10^6 cells in six-well plates were treated with drugs for 24 h. Cells were washed and spun down with PBS and resuspended in 1.0% low-melt agarose. Agarose plugs were incubated overnight in lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0, 1% sarkosyl, and 100 μg/ml proteinase K) at 50°C. The DNA/agarose plugs were washed three times with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1 μg/ml RNase A and were inserted into wells of 1.5% agarose gel prepared in 1× Tris, boric acid, and EDTA buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). After running at 4°C overnight, the gel was stained with ethidium bromide and photographed.

L-OddC Incorporation. Cells were incubated in 75-cm^2 flasks with the indicated concentrations of [^{3}H]L-OddC, unlabeled L-OddC, and CPT for 8 and 16 h. After the removal of radioactive media, the cell layer was washed with ice-cold 20 μM dipyridamole/PBS, harvested, and extracted with 15% TCA on ice. The acid-soluble material was neutralized by extraction with a mixture of 55% trichlorotriflouroethane and 45% trioctylamine and analyzed by high-performance liquid chromatography (Shimadzu, Braintree, MA) in a binary gradient of water and potassium phosphate buffer using an anion exchange column (Partisil-SAX; Whatman, Clifton, NJ). The acid-insoluble precipitates were resuspended in 15% TCA, spun down, washed with TCA, then dissolved in 100% dimethyl sulfoxide. The amount of L-OddCMP present in the precipitate was determined by scintillation counting using a Beckman radiospectrometer (Beckman Coulter, Fullerton, CA). The percentage change in the amount of L-OddC metabolites was expressed as [(L+C treatment) − (L treatment)]/L treatment × 100, where L indicates L-OddC and C indicates CPT.

Enzyme Activity Assays. Cells in logarithmic growth were treated with 100 nM CPT, 1 μM L-OddC, or a combination of two drugs for 16 h, washed with PBS, and lysed on ice for 1 h using lysis buffer (25 mM Tri-HCl, pH 7.4, 25 mM NaCl, 5 mM NaF, 2 mM DTT, 0.5% Triton X-100, 0.5% EDTA, and protease inhibitors). After centrifuging at 13,500 rpm for 20 min, the extracts were isolated for protein analysis and enzyme activity assays.

The enzyme assays were based on the DE-81 disc assay with minor modifications. For dCK, we used dCyd and L-OddC as substrates (Cheng et al., 1977). The assay was performed in a reaction buffer consisting of 100 μM Tri-HCl, pH 7.5, 10 mM NaF, 2 mM DTT, 0.04 mM tetrahydrouridine, and 6 mM ATP/Mg^{2+} supplemented with creatine phosphate and creatine kinase as ATP-regenerating systems. The substrates were either 100 μM of [^{14}C]dCyd (radiospecificity, 5 mCi/mmol) or 100 μM [^{3}H]L-OddC (radiospecificity, 100 mCi/mmol), respectively. To eliminate the effect coming from mitochondrial thymidine kinase, which is capable of phosphorylating dCyd, we also included 300 μM bromovinyl-2′-deoxyuridine, a known thymidine kinase inhibitor in the reaction (Cheng et al., 1981). The reaction was performed at 37°C for 2 h, with 75 μl total mixture for each reaction containing ~10 to 20 μl of cell extract. A 50-μl aliquot from each reaction mixture was spotted on DE-81 discs (Whatman). These were washed three times with 1 mM ammonium formate for 3 min, once with distilled water for 2 min, and then once with 95% ethanol for 2 min. To improve the detection of ^{3}H-labeled radioactive nucleotides, compounds were eluted from the discs by incubation with 0.1 N HCl plus 2 M NaCl for 20 min before scintillation reading.

The dCMPK assay (Liou et al., 2002) was performed using a buffer consisting of 1 mM of [^{3}H]dCMP (radiospecificity, 0.83 mCi/mmol), 100 μM Tri-HCl, pH 7.5, 10 mM NaF, 2 mM DTT, 100 μM TTP, 1 mM ATP, and 2 mM Mg^{2+} supplemented with creatine phosphate and creatine kinase. The reaction was performed at 37°C for 20 min with ~3 to 5 μl of cell extract. Subsequent procedures were performed as described above except for the washing conditions, which consisted of three washes of 50 mM formic acid plus 1 mM ammonium formate and a single wash of 95% ethanol for 3 min. Radioactivity was quantified by a scintillation counter (Beckman Coulter). The enzyme activities were defined as picomoles per minute per milligram of protein for dCK and nanomoles per minute per milligram of protein for dCMPK. The percentage of control of dCK or dCMPK activity was expressed as (treatment − control)/control × 100. Because dCK and dCMPK are enzymes working in sequential steps, the combined effect on dCK and dCMPK activities was calculated by multiplying the result from each assay.

Immunoblotting. The cell extracts obtained as described above were resolved by 14% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) by standard methods. The membranes were blocked with 0.2% Tween 20 in PBS containing 5% nonfat milk and then probed with primary antibodies, including a highly specific peptide-generated rabbit antiserum against dCK at 1:5000 dilution (Hatzis et al., 1998) and a recombinant protein-generated rabbit antiserum against dCMPK at 1:250 dilution (Liou et al., 2002), antiactin (Sigma), followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) and anti-mouse IgG (Sigma). Immunocomplexes were visualized by using enhanced chemiluminescence reagent (PekinElmer Life and Analytical Sciences, Boston, MA). Relative intensities of protein bands were quantified using Densitomer SI and ImageQuant 5.2 (Amersham Biosciences Inc., Piscataway, NJ).

Results

Synergistic Interactions of L-OddC and CPT in Select Human Cancer Cell Lines. The following mean IC_{50} values were determined for L-OddC during the course of our experiments: KB, 250 nM; KB100, 260 nM; RKO colorectal, 280 nM; H1299 non-small cell lung, 300 nM; and HepG2, 60 nM. HepG2 cells demonstrated the greatest sensitivity to L-OddC. The single-agent activity of CPT was similar across cell lines in the range of 6 to 26 nM, with the exception of CPT-resistant KB100 cells (350 nM). Simultaneous exposure to L-OddC and CPT produced varying degrees of additivity in a cell type-specific manner (KB, KB100 > RKO, H1299 > HepG2), as shown in Fig. 2A. No antagonism was observed. The addition of L-OddC to CPT resulted in a nearly 20-fold decrease in the IC_{50} value of CPT in KB100 cells (350→18 nM), bringing CPT sensitivity close but not equal to that of KB cells (6–10 nM).

CPT analogs, despite their shared mechanism of topo I inhibition, demonstrated different interactions with L-OddC (Fig. 2B). The parent compound CPT was synergistic with L-OddC. SN-38, the major active metabolite of CPT-11 generated by carboxylesterase in vivo, also exerted supra-additive effects but did so over a narrower dose range than CPT. The lack of synergism with TPT may be explained by differences among CPT analogs in terms of DNA binding sequence selectivity, stability of induced PLDB, and dependence on drug exposure time. For example, the optimal activity of TPT may require a more prolonged exposure than CPT or SN-38. Similar to our findings, gemcitabine has been reported to be additive with TPT (Tolis et al., 1999) and synergistic with CPT-11 (Bahadori et al., 2001). On the other hand, others have found that TPT and gemcitabine were antagonistic in an ovarian cell line (Distefano et al., 2000). Cisplatin and topo II inhibitor etoposide showed additivity with L-OddC, in concordance with our previous results.

CPT-Induced PLDB Formation Is Not Increased by L-OddC. Induction of PLDB is a prerequisite of CPT action. CPT treatment of KB cells produced a dose-dependent increase in the steady-state levels of PLDB. L-OddC had no effect on PLDB formation either alone or in combination with

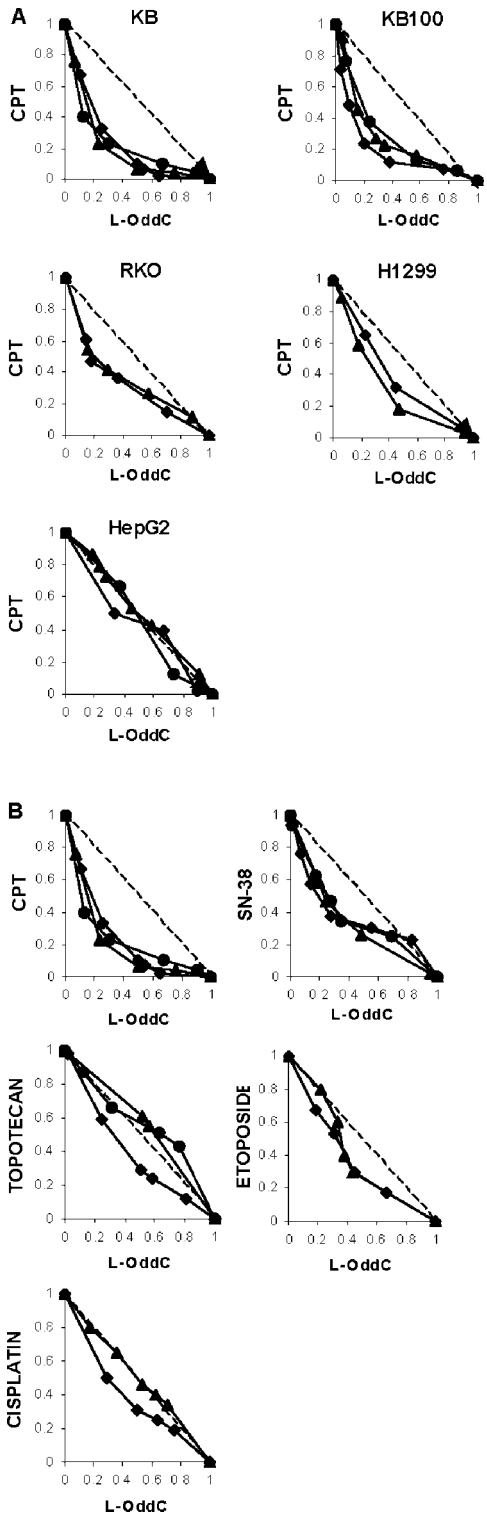

Fig. 2. Isobolograms of in vitro binary drug combinations. Numbers along each axis denote fractions of the single-agent IC_{50} for the drug indicated. Each datum point represents a dose pair of two drugs producing an effect equivalent to the IC_{50} of either drug used alone. Broken lines represent the line of additivity. Data from two to three independent experiments (each done in triplicate) are presented; ▲, first experiment; ♦, second experiment; ●, third experiment. A, the results of the simultaneous treatment of L-OddC and CPT in various cultured human cancer cell lines. In KB and KB100 cells, the data points were within the region of supra-additivity. In HepG2 cells, most data points fell along the line of additivity. In RKO and H1299 cells, data points fell within an intermediate zone between additivity and synergism. B, the results of the simultaneous treatment of L-OddC and various chemotherapy drugs in KB cell line.

CPT. Representative results (in triplicate) are presented in Fig. 3A. The lack of effect of L-OddC on PLDB formation was in contrast to the recent reports of cytarabine (Pourquier et al., 2000) and gemcitabine (Pourquier et al., 2002) and their ability to trap topo I-DNA complexes in the absence of CPT. One possible explanation is that L-and D-configurations of incorporated nucleoside analog exert different effects to trap topo I. In addition, unlike cytarabine and gemcitabine, L-OddC does not support continued DNA elongation after its incorporation because it is a dideoxy compound. Furthermore, drug treatment conditions were different. In our experiments, L-OddC treatment was for 4 h at 1 μM, whereas in their studies, cytarabine and gemcitabine treatments were either with a low dose (1 μM) and longer time (up to 24 h) or a high dose (10 μM) and shorter time (3 h). Whether the different methods used to detect the topo I-DNA complexes (modified in vivo K-SDS coprecipitation assay versus in vivo complex of enzyme bioassay) contributed to the different results cannot be determined unless we test all three drugs using one method.

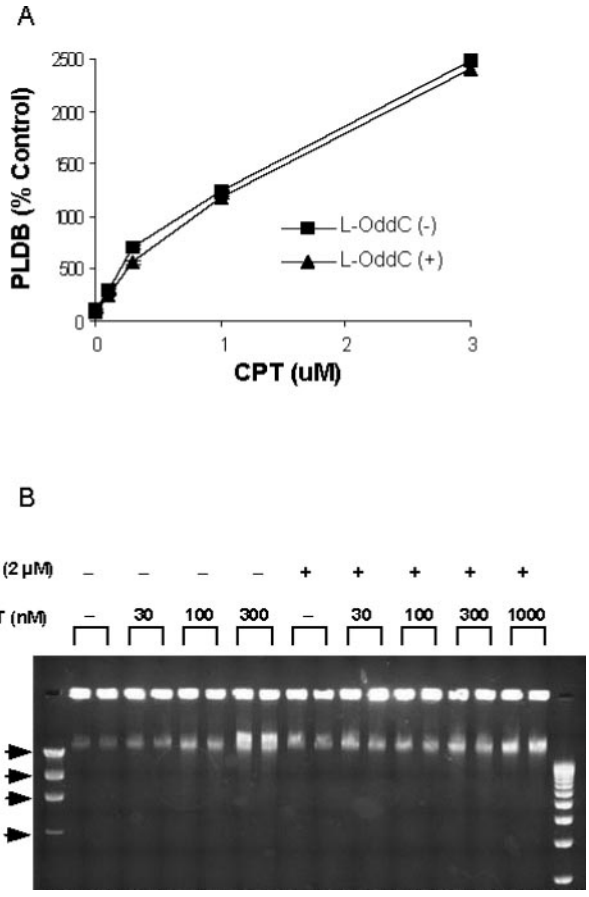

Fig. 3. A, CPT-induced PLDB formation. DNA of midlog phase cultures was labeled with [^{14}C]thymidine for 24 h. KB cells were harvested, counted, and seeded onto six-well plates in [^{14}C]thymidine-free media for 16 h. Cells were pretreated with L-OddC 1 μM or PBS for 3 h before the addition of CPT. After 1-h exposure to CPT, PLDB levels were determined by modified in vivo K-SDS coprecipitation assay. The line graphs represent steady-state levels of PLDB after drug treatments from one representative experiment done in triplicate; vertical bars, S.D. is not apparent. B, photograph of an ethidium bromide gel showing dsDNA breaks. KB cells were treated with the indicated concentrations of CPT in the presence (+) or absence (−) of 2 μM of L-OddC for 24 h. Cells were harvested and normalized by the cell number. DNA was subjected to agarose gel electrophoresis. The experiment was done in duplicate.

CPT-Induced dsDNA Breaks Are Not Increased by L-OddC. Creation of irreversible dsDNA breaks from single-stranded DNA cleavable complexes is believed to be the main mechanism of CPT cytotoxicity. In KB cells, dsDNA breaks increased in a dose-proportional manner upon exposure to CPT. L-OddC did not increase the amount of CPT-induced dsDNA breaks. In fact, at a high, clinically irrelevant concentration of CPT, cotreatment with L-OddC resulted in a slight decrease in dsDNA breaks compared with CPT alone. The decreased production of dsDNA breaks in the presence of L-OddC may be explained by the fact that L-OddC is an immediate DNA chain terminator, halting DNA synthesis and further progression of dsDNA breaks caused by CPT. Figure 3B represents a photograph of the gel.

DNA Incorporation of L-OddC Is Increased in KB Cells (Synergistic) but Not in HepG2 Cells (Additive). According to our previous work in DU145 prostate cancer cells, DNA incorporation of L-OddC correlates with its cytotoxicity (Grove and Cheng, 1996). Because L-OddC did not have the effect of increasing dsDNA breaks triggered by CPT, the synergistic interaction between L-OddC and CPT could be initiated by CPT causing more L-OddC to be incorporated into DNA, resulting in enhanced cell death. In a representative experiment using KB cells, the amount of L-OddC incorporated into DNA was increased by 62% after 16 h of coincubation with CPT compared with L-OddC alone (Fig. 4). With the addition of CPT, the intracellular L-OddC triphosphate amount determined by high-performance liquid chromatography was increased by 55 and 69% at 8 and 16 h, respectively, as was the total cellular uptake of L-OddC (31 and 24%). This experiment was repeated several times with the consistent finding of the same pattern of change, albeit with variations in the absolute quantity of change. We also observed a consistent trend of a reduction in the intracellular L-OddC monophosphate pool with corresponding increases in di- and triphosphates, although only in the range of 5 to 7%, raising the possibility of enhanced enzymatic action of activating kinases. In HepG2 cells, no significant difference was detected for the cellular uptake and DNA incorporation of L-OddC between two treatments (Fig. 5).

Activities of dCK and dCMPK Were Changed by CPT and L-OddC. To find an explanation for the enhanced L-OddC uptake in KB cells, we assayed the activities of enzymes responsible for the serial phosphorylation of L-OddC, namely dCK, dCMPK, and PGK. Drug treatment conditions were identical with those of L-OddC incorporation experiments. As shown in Table 1, the dCK activity measured by dCyd as the substrate was increased in KB and HepG2 cells by treatment with CPT, L-OddC, and two-drug combinations, but the degree of activation was higher in KB than in HepG2 cells. In KB cells, phosphorylated dCyd was increased to 153 to 154% of control by the single drugs and to 174% by L-OddC/CPT. The dCK activity in HepG2 cells was also increased by all drug treatments without statistically significant differences between samples.

Next, we studied the effect of dCK activity on phosphorylation of L-OddC. In KB cells, phosphorylated L-OddC was decreased to 80% of control in L-OddC–treated cells and unchanged in CPT-treated cells. The suppression of L-OddC phosphorylation by L-OddC was not only reversed but increased up to 113% by combining with CPT. In contrast, L-OddC phosphorylation in HepG2 cells was decreased with

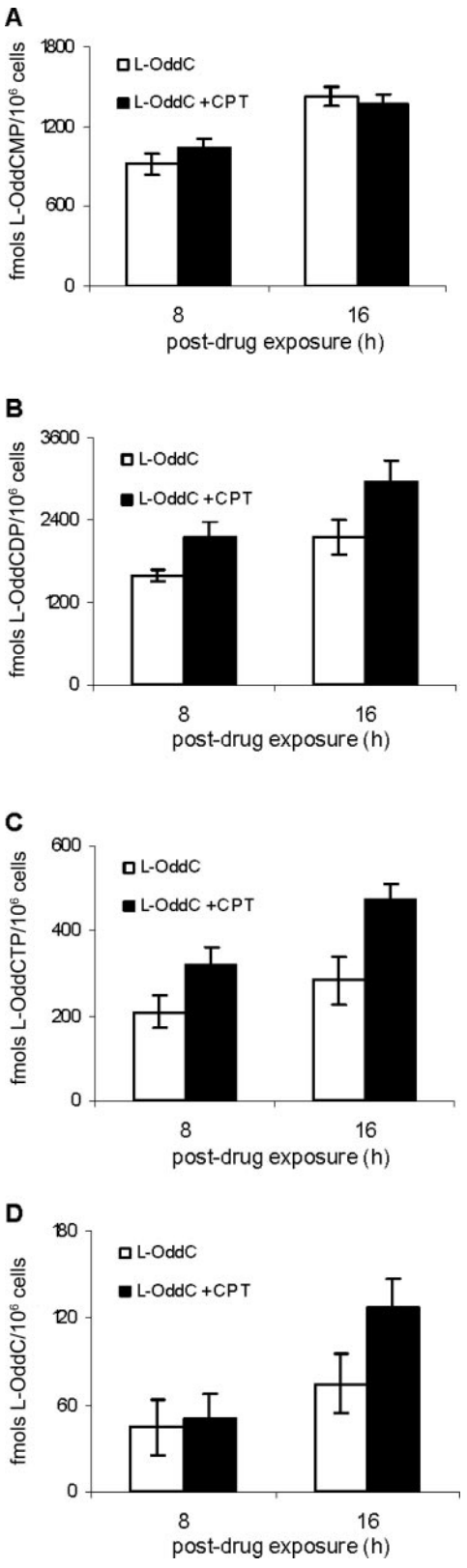

Fig. 4. L-OddC metabolism in KB cells. KB cells were incubated with 1.22 μM [^{3}H]L-OddC (2.65 Ci/mmol) in the presence or absence of 100 nM CPT for the indicated lengths of time. The acid-soluble metabolites of L-OddC were extracted with 15% TCA and analyzed by high-performance liquid chromatography (A, L-OddCMP; B, L-OddCDP; C, L-OddCTP). D, the amount of L-OddCMP present in DNA was determined by scintillation counting. Each bar graph represents three data points in one representative experiment; vertical bars, S.D.

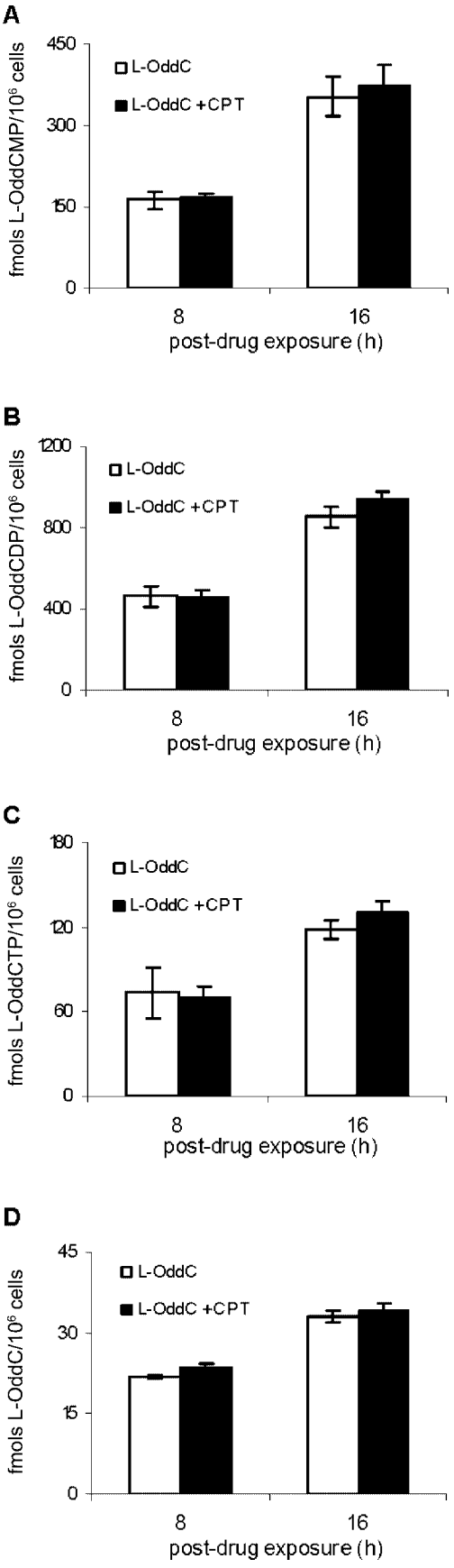

Fig. 5. L-OddC metabolism in HepG2 cells. HepG2 cells were treated under the same conditions as KB cells. A, L-OddCMP. B, L-OddCDP. C, L-OddCTP. D, the amount incorporated into DNA.

all treatments. The decrease in L-OddC phosphorylation by L-OddC was similar in KB and HepG2 cells, but the response of L-OddC phosphorylation induced by CPT was significantly different. CPT-treated KB cells showed 105% phosphorylation compared with control, whereas HepG2 cells showed 70% phosphorylation. L-OddC alone and L-OddC/CPT combination-treated HepG2 cells showed no difference in L-OddC phosphorylation.

We also evaluated the activity of dCMPK responsible for phosphorylating L-OddC monophosphate to diphosphate. In KB cells, the phosphorylation of dCMP was decreased in L-OddC–treated cells (87% of control) but not significantly changed in the CPT-treated case. The combination of L-OddC with CPT returned dCMPK activity to baseline levels. As for HepG2 cells, the dCMPK activity was decreased in all treated groups. Its activity was the lowest in L-OddC–treated cells (77% of control). PGK, which phosphorylates L-OddC diphosphate to its triphosphate form, showed unaltered activity in both cell lines (data not shown).

The Protein Levels of dCK and dCMPK Are Generally Unchanged. Because the enzyme activity for dCK and dCMPK showed various degrees of modulation by drug treatment, we asked whether the change in enzyme activity was directly related to protein quantity. In terms of dCK protein levels, there was no significant change for both KB and HepG2 cells (Fig. 6). The greatest change was observed in combination-treated KB cells, which exhibited approximately 40% increase in dCK protein, but the protein levels did not correlate with dCK activity in both cell lines. This indicated that the altered dCK activity or L-OddC phosphorylation seen in KB or HepG2 cells after different drug treatments could not be entirely caused by a change in dCK protein amounts. On the other hand, the pattern of dCMPK protein levels was markedly different for the two cell lines. In KB cells, the dCMPK protein was decreased in cells treated with L-OddC but was unchanged with other treatment conditions. This pattern correlated with the changes seen in enzyme activity. However, in HepG2 cells, the protein levels remained unchanged despite a 10 to 25% reduction in dCMPK activity.

Discussion

In this study, the effects of combining L-OddC with other anticancer drugs were examined. Among the cell types used in our experiments, we observed that the simultaneous exposure to L-OddC and CPT was synergistic in oropharyngeal KB cells and even in CPT-resistant KB100 cells but only additive in HepG2 cells. Thus, the synergistic effect observed was not a general phenomenon but rather cell line-specific.

To elucidate the possible mechanism behind this synergism, we set out to test the known determinants of CPT and L-OddC cytotoxicity. From our PLDB and dsDNA break experiments, we observed that CPT toxicity determinants were not affected by the addition of L-OddC. We then asked whether CPT could augment L-OddC cytotoxicity, namely its incorporation into DNA. In the KB cell line, L-OddC incorporation into DNA was increased by the addition of CPT, and L-OddC di- and triphosphates were also increased. In the HepG2 cell line, however, these increases were not observed. It is intriguing to note that despite the amount of L-OddC metabolite accumulation and DNA incorporation being sig-

nificantly less in HepG2 versus KB cells (Figs. 4 and 5), sensitivity to L-OddC in growth-inhibition assays was approximately 4-fold greater in HepG2 than in KB cells. Although it is probably true that the rate of DNA incorporation is proportional to cytotoxicity within the same cell line, the same claim cannot be made across different cell lines, and we must invoke the existence of additional, perhaps cell type-specific, mechanisms of cytotoxicity.

The changes in L-OddC metabolites led us to perform a series of experiments measuring activity and protein levels of key enzymes involved in the phosphorylation of L-OddC. These studies were carried out in KB and HepG2 cell lines to represent synergism and additivity, respectively. In the cytoplasmic extract of KB cells treated with L-OddC, CPT, or the combinations, we found no difference in the activity of PGK, but differences in dCK and dCMPK activity were observed.

dCK is a key enzyme in the metabolism of deoxynucleosides and their analogs, phosphorylating nucleosides to their monophosphate form. dCK activity has been shown to be stimulated not only by deoxynucleoside analogs but also by aphidicolin (Csapo et al., 2001), etoposide (Ooi et al., 1996), and γ-irradiation (Csapo et al., 2003), all of which disturb DNA synthesis and/or cause DNA damage resulting in increased demand for deoxynucleotide metabolism to supply DNA precursors. We assayed dCK activity using two substrates, dCyd and L-OddC. In terms of dCyd phosphorylation, dCK activity was increased by all treatments in both cell lines, possibly in response to DNA damage induced by the drugs, but the degree of activation was higher in KB than in HepG2 cells. Although the L-OddC/CPT combination in KB cells did induce a greater phosphorylation of dCyd than either L-OddC or CPT alone, it was not enough to explain the synergism, because the combination of L-OddC/CPT in HepG2 cells also increased dCK.

With L-OddC as the substrate, we observed no change to a slight increase in dCK activity in KB cells and a decreased activity in HepG2 cells. L-OddC treatment reduced the amount of phosphorylated L-OddC to similar levels in KB and HepG2 cells, but L-OddC phosphorylation was increased by the addition of CPT in KB cells only. Given that dCK is the only enzyme known to phosphorylate L-OddC, the difference between dCyd and L-OddC phosphorylation might be explained by substrate specificity. The reduction in L-OddC phosphorylation may represent a cellular defense mechanism to a cytotoxic agent, whereas the increase in dCyd may be a nonspecific response to a need to repair damaged DNA.

TABLE 1

dCK and dCMPK activities in KB and HepG2 cells

KB and HepG2 cells were treated with 100 nM CPT or 1 μM L-OddC or combinations for 16 h. The cytoplasmic fractions were tested for dCK and dCMPK activities with 100 μM dCyd or L-OddC or 1 mM dCMP as a substrate. Enzyme activity levels are presented as the percentages compared with the control cells (100%; no drug treatment). Each data point represents averages from three independent experiments with S.D.

Phosphorylation of	Cell Line	CPT	L-OddC	CPT + L-OddC
dCyd	KB	154.5 ± 10.0	154.2 ± 6.3	174.3 ± 8.9
	HepG2	122.5 ± 30.5	136.5 ± 21.6	136.9 ± 13.8
L-OddC	KB	105.4 ± 9.3	81.6 ± 7.0	113.6 ± 5.3
	HepG2	71.3 ± 8.3	79.7 ± 5.7	81.9 ± 7.5
dCMP	KB	97.8 ± 8.8	87.2 ± 0.8	99.7 ± 7.2
	HepG2	82.2 ± 12.5	77.2 ± 4.9	82.5 ± 6.8

dCK, by yet unknown mechanism, may be capable of differentiating between the two substrates. For our purpose, L-OddC rather than dCyd is the preferred substrate for evaluating dCK activity. Although we were unable to use L-OddC monophosphate in the dCMPK assay because of the difficulty in procuring radioactive materials, dCMPK activity using dCMP as the substrate did show different responses in the two cell lines.

By combining the dCK and dCMPK data, we show a significant difference between L-OddC treatment alone (72% of control) and in combination with CPT (113% of control) in KB cells. In keeping with our L-OddC incorporation data, this difference was apparent in synergistic KB cells and not in HepG2 cells. These observations suggest that CPT-induced enhancement of dCK and dCMPK activity is responsible for the increased incorporation of L-OddC into DNA, rendering cells more vulnerable to the drug's toxic effects. From our experiments, however, it is unclear whether the changes in enzyme activity is caused by a change in the enzyme amount, nor do we know why the responses are different between cell lines. Previous work by others have suggested that the changes in enzyme activity may be mediated by the phosphorylation of dCK (Csapo et al., 2003). Further investigation is needed to uncover detailed mechanisms.

We have completed initial work on nude mice bearing KB cell xenografts. CPT-11 and L-OddC combination treatment using two weekly doses was associated with enhanced antitumor activity without added toxicity compared with either agent alone (data not shown). We need to try various treat-

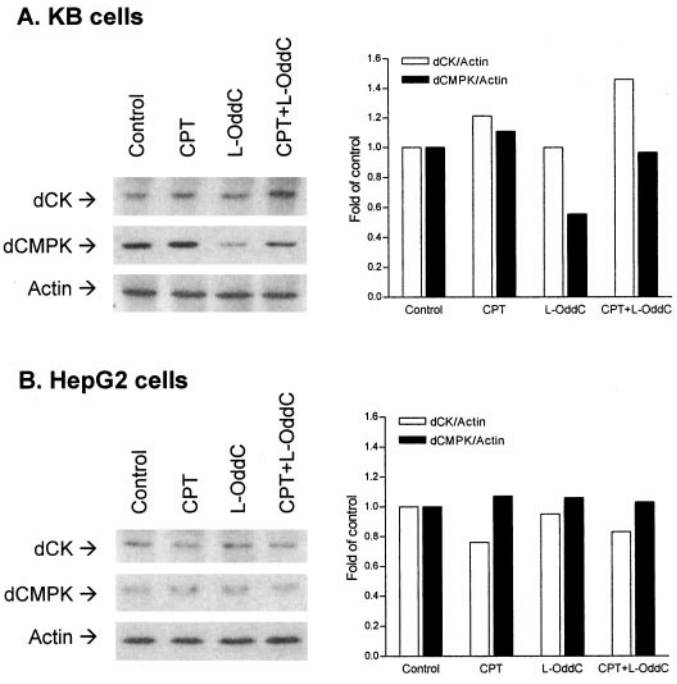

Fig. 6. Effects of CPT, L-OddC, or combination on protein expression levels of dCK and dCMPK. KB and HepG2 cells were treated with 100 nM CPT or 1 μM L-OddC or combinations for 16 h. The cells were lysed, and 50 μg of protein was separated by SDS-polyacrylamide gel electrophoresis. The membranes were then probed with antibodies against dCK protein (31 kDa) or dCMPK protein (21 kDa). The antibody to actin (42 kDa) was used as a loading control. Immunoblotting pictures shown are representative of three independent experiments. Histograms show relative intensities of protein bands from one representative immunoblotting.

ment schedules to validate and optimize the effect of this combination. It should be noted that CPT analogs do not behave the same when combined with L-OddC. In fact, a clinical study of L-OddC and TPT undertaken for the treatment of myeloid leukemias was not encouraging in terms of efficacy (5% response rate among 18 patients) and proved prohibitively toxic as well (Giles et al., 2003). Our preliminary animal data suggest that CPT-11 may be a more promising analog to be combined with L-OddC, with a potential for broad applicability against human malignancies.

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Address correspondence to: Dr. Yung-Chi Cheng, Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, SHM B226, New Haven, CT 06520. E-mail: cheng.lab@yale.edu