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Pharmacodynamic and pharmacokinetic study of chronic low-dose metronomic cyclophosphamide therapy in mice

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Abstract

Prolonged, frequently administered low-dose metronomic chemotherapy (LDM) is being explored (pre)clinically as a promising antiangiogenic antitumor strategy. Although appealing because of a favorable side effect profile and mostly oral dosing, LDM involves new challenges different from conventional maximum tolerated dose chemotherapy. These include possible altered pharmacokinetic characteristics due to long-term drug exposure potentially resulting in acquired resistance and increased risk of unfavorable drug interactions. We therefore compared the antitumor and antivascular effects of LDM cyclophosphamide (CPA) given to mice that had been pretreated with either LDM CPA or normal saline, obtained blood 4-hydroxy-CPA (activated CPA) concentrations using either gas chromatography/mass spectrometry or liquid chromatography/tandem mass spectrometry in mice treated with LDM CPA, and measured hepatic and intratumoral activity of enzymes involved in the biotransformation of CPA and many other drugs [i.e., cytochrome P450 3A4 (CYP3A4) and aldehyde dehydrogenase]. Exposure of mice to LDM CPA for $\geq$8 weeks did not compromise subsequent activity of LDM CPA therapy, and biologically active 4-hydroxy-CPA levels were maintained during long-term LDM CPA administration. Whereas the effects on CYP3A4 were complex, aldehyde dehydrogenase activity was not affected. In summary, our findings suggest that acquired resistance to LDM CPA is unlikely accounted for by altered CPA biotransformation. In the absence of reliable pharmacodynamic surrogate markers, pharmacokinetic parameters might become helpful to individualize/optimize LDM CPA therapy. LDM CPA-associated changes of CYP3A4 activity point to a potential risk of unfavorable drug interactions when compounds that are metabolized by CYP3A4 are coadministered with LDM CPA. [Mol Cancer Ther 2007;6(8):2280–9]

Introduction

After years of promising preclinical studies, the concept of antiangiogenic tumor therapy has recently been validated in various phase III clinical trials involving colorectal, breast, non–small cell lung, and kidney cancer (1, 2). Beside combinations of bevacizumab (the monoclonal anti–vascular endothelial growth factor antibody) with conventional, maximum tolerated dose (MTD) chemotherapy, and the use of small-molecule receptor tyrosine kinase inhibitors such as sunitinib and sorafenib, another promising antiangiogenic approach is emerging, namely the frequent or continuous administration of comparatively low doses of cytotoxic drugs over extended periods with no prolonged breaks [i.e., low-dose metronomic chemotherapy (LDM); ref. 3]. Although such therapy is thought to act mainly via antiangiogenic mechanisms, alternative antitumor effects might apply, such as the enhancement of immune effectors (4) or the targeting of the putative tumor stem(-like) cell subpopulation (5). Compared with MTD chemotherapy, LDM offers several advantages, such as low toxicity, increased convenience by potential oral route of drug administration, cost-efficacy when using off-patent chemotherapy drugs, and, at least theoretically, the prospect of delayed development of resistance by virtue of targeting normal host endothelial cells or their precursors (3, 6, 7).

LDM protocols have shown promising activity in many preclinical and several phase I/II human studies involving metastatic breast, prostate, and recurrent ovarian cancer as well as advanced non–Hodgkin's lymphoma (3, 8–14).

Many of the clinical studies have used oral cyclophosphamide (CPA), usually given at a fixed low dose of 50 mg/d. Compared with MTD chemotherapy combined with anti-angiogenic compounds, LDM regimens have the potential benefit that they might be combined for more extended periods with targeted antiangiogenic agents, such as anti–vascular endothelial growth factor receptor-2 antibodies (15) or sunitinib (16), due to the limited toxicity of both types of drugs. Recently, a randomized phase II trial evaluating LDM CPA in combination with the aromatase inhibitor letrozole for the neoadjuvant treatment of breast cancer in elderly patients not only showed excellent tolerance but also enhanced clinical efficacy when LDM CPA was added to letrozole (13).

Although being very promising, oral LDM regimens involve new challenges that differ from traditional i.v. MTD chemotherapy. For instance, drug absorption, distribution, biotransformation, and excretion might change over time due to the underlying malignancy, concomitant therapies, dietary factors, and metabolic interference. Such changes might account for acquired resistance eventually seen in clinical trials. In this regard, we recently showed that reimplanted variants derived from PC-3 human prostate cancer xenografts that had become resistant after 6 to 8 weeks of daily oral LDM CPA therapy responded to renewed therapy in a similar way as parental PC-3. Such results suggest the possibility of host-based rather than tumor cell–mediated mechanisms of resistance, such as altered drug metabolism (17).

The biotransformation of CPA (Fig. 1) involves a 4-hydroxylation activation step carried out by several cytochrome $P450$ isoforms, including 2B6, 3A4, and 2C9. Cytochrome $P450$ 2B6 is the most important isoform in this respect, and the liver is the main organ of this rate-limiting reaction resulting in 4-hydroxy-CPA (4-OH-CPA;

ref. 18). Although activation might occur in tumor tissues, this unlikely accounts for a significant fraction of CPA-related cytotoxicity (19). Genetic and environmental factors as well as comedications that affect cytochrome $P450$ activity are known to modify the antitumor effects of MTD (20) and, potentially also, LDM CPA. Interestingly, CPA in high doses is capable of inducing its own activation, which is mediated at least partially by cytochrome $P450$ isoform 3A4 (CYP3A4) induction (21). CYP3A4 is also involved in a competing side chain oxidation of CPA, which results in N-dechloroethylation and the formation of the neurotoxic metabolite chloroacetaldehyde (18). However, the major detoxification pathway is the class 1 aldehyde dehydrogenase (ALDH)-mediated oxidation of aldophosphamide, the tautomer of 4-OH-CPA (18, 22). Consequently, increased intratumoral ALDH activity has been reported as a mechanism of resistance to CPA (23). In other words, the activity status of ALDH and cytochrome $P450$ isoforms implicated in CPA metabolism could have a detrimental effect on the long-term biological effects of LDM CPA protocols. Moreover, it is likely that consequences of modified CYP3A4 activity would go beyond CPA because many other compounds used in cancer therapy (and potentially coadministered with CPA) are metabolized by CYP3A4 (18, 24, 25).

Here, we describe the effects of LDM CPA pretreatment on the antitumor and antivascular effects in mice when subsequently using the same LDM CPA regimen for treatment purposes and present the results of an analysis of the blood levels of the CPA metabolite 4-OH-CPA in mice given LDM CPA. Although phosphoramide mustard is the ultimate alkylating agent formed from the rapidly interconverting tautomers 4-OH-CPA and aldophosphamide, it is only poorly transported into cells. Therefore, quantifications of blood levels of 4-OH-CPA/
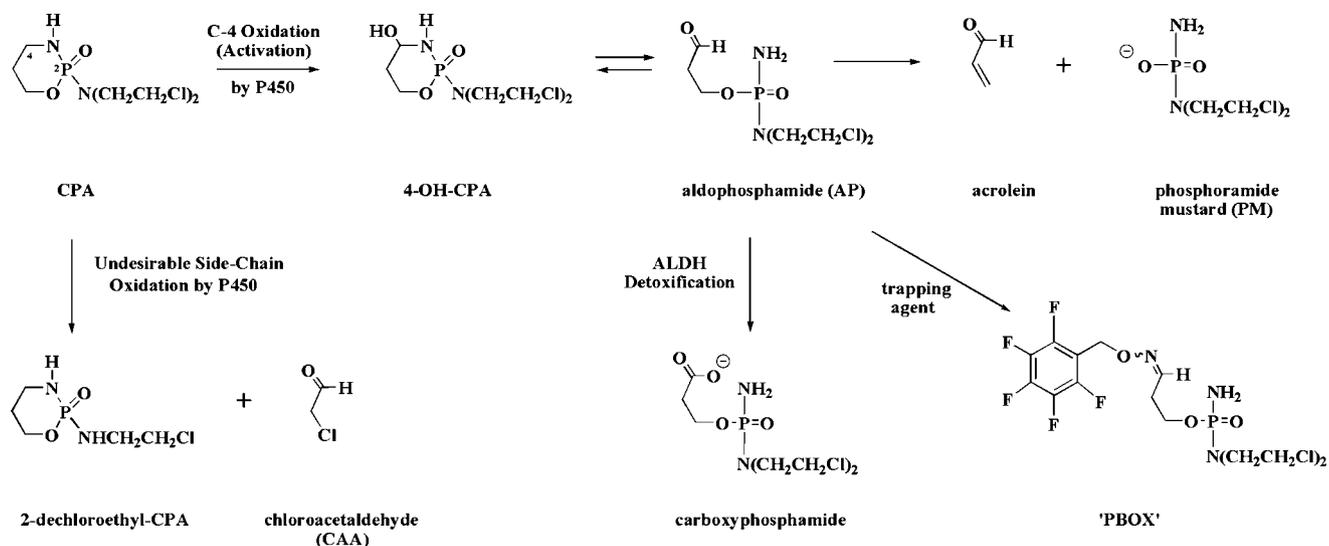

Figure 1. Important elements of the metabolism of CPA and the conversion of 4-OH-CPA to the stable oxime, PBOX, for analytic purposes.

aldophosphamide better reflect the tumor exposure to active drug than do measurements of circulating phosphoramide mustard or parent CPA (26–28). Finally, we determined CYP3A4 as well as ALDH activity in liver and tumor specimens of mice undergoing LDM CPA therapy.

We show that prolonged LDM CPA exposure does not have a negative effect on the biological effects of subsequent LDM CPA therapy. This coincides with sustained 4-OH-CPA levels over treatment periods of up to 8 weeks. The effects of LDM CPA on CYP3A4 activity are complex, whereas ALDH activity does not significantly change neither in tumor nor in liver tissue samples. No evidence was obtained that host-related mechanisms account for the acquired resistance to LDM CPA.

Materials and Methods

Animal Procedures

All animal procedures were done in accordance with institutional and national guidelines. The PC-3 human prostate cancer cell line was purchased from the American Type Culture Collection and maintained in a humidified atmosphere of 5% CO_2 at 37°C in DMEM supplemented with 5% FCS. Cells (2×10^6) were injected s.c. into the right flank of 6- to 8-week-old male NIH Swiss athymic nude mice (Taconic Laboratories), Harlan athymic nude mice (Harlan Biotech Center), or C.B-17 severe combined immunodeficient mice (Charles River Canada). Tumor size was assessed weekly by means of Vernier calipers and the formula ($w_1 \times w_2 \times w_2$) / 2, where w_1 and w_2 are the largest and the smallest tumor diameter (mm), respectively. 113/6-4L is a lung metastatic variant of the human melanoma cell line WM239,[4] which was orthotopically implanted into the dermis of female C.B-17 severe combined immunodeficient mice for this study.

Matrigel Plug Perfusion Assay and Circulating Endothelial Progenitor Cell Enumeration

The Matrigel plug perfusion assay was done as described (7). Briefly, 0.5 mL of Matrigel (BD Biosciences) supplemented with 500 ng/mL of basic fibroblast growth factor (National Cancer Institute Biological Resources Branch) was injected s.c. on day 0 into the flanks of female BALB/cJ mice 9 weeks of age (The Jackson Laboratory). After 10 days, mice were injected i.v. with 0.2 mL of 25 mg/mL FITC-dextran (Sigma-Aldrich Chemical Co.). Plasma was collected 30 min later and Matrigel plugs were removed followed by overnight digestion at 37°C with dispase (Collaborative Biomedical Products) and subsequent homogenization. Fluorescence readings were obtained using a FL600 fluorescence plate reader (Bio-Tek Instruments). The level of the angiogenic response was assessed as the ratio of Matrigel plug fluorescence to plasma fluorescence ($n = 6–7$ plugs per group). Circulating endothelial progenitor cell enumera-

tion was done by four-color flow cytometry using blood obtained from the retro-orbital venous plexus as previously described in ref. 29.

CPA Regimens

CPA (Baxter Corp.) was prepared and given as previously reported (17). Unless otherwise indicated, the LDM CPA protocol used consisted of the continuous administration of 20 mg/kg/d of CPA via the drinking water based on an average intake of 3 mL of drinking water per day by 20 g mice. The drinking water with CPA was changed twice weekly. Control animals received corresponding amounts of 0.9% normal saline (CPA diluent) added to the drinking water. The MTD CPA regimen involved the administration of 100 mg/kg of CPA i.p. on days 1, 3, and 5 of each 3-week cycle.

4-OH-CPA Measurements by Gas Chromatography/ Mass Spectrometry and Liquid Chromatography/ Tandem Mass Spectrometry

Because of the reactivity of 4-OH-CPA and aldophosphamide, whole blood was added to a solution of a hydroxylamine-based derivatizing ("trapping") agent, *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine-HCl (PFBHA), immediately after bleeding. PFBHA reacts rapidly and quantitatively with 4-OH-CPA/aldophosphamide to give an oxime, "PBOX" (Fig. 1; ref. 27), which is stable in the trapping solution for weeks at room temperature and indefinitely at −20°C, and can be quantified by gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/tandem mass spectrometry (LC/MS/MS) as a measure of the concentration of 4-OH-CPA in the original blood sample (26, 27). Using this methodology, "4-OH-CPA" levels refer to aggregate concentrations of 4-OH-CPA, aldophosphamide, and any other species that reversibly interconvert with these metabolites (e.g., iminophosphamide and glutathione adducts; ref. 28). The syntheses of 4-hydroperoxy-CPA (4-OOH-CPA; synthetic precursor of 4-OH-CPA) and "PBOX-d_4" (internal standard with four deuterium atoms in chloroethyl side chains) used in GC/MS and LC/MS/MS analyses have been reported (27). These materials are also available commercially: Qventas, Inc. (4-OOH-CPA) and Dr. Ulf Niemeyer, IIT, Universität Bielefeld GmbH, part NIOMECH, Bielefeld, Germany (4-OOH-CPA and PBOX-d_4).

For GC/MS analysis, blood samples were obtained from tumor-free male C57BL/6J mice (The Jackson Laboratory) after 0.5, 1, 2, 6, 12, 24, and 48 h as well as after 7 and 56 days of treatment with LDM CPA given through the drinking water. For some mice, LDM CPA administration was stopped after 7 or 56 days, respectively, and the mice were bled 1, 6, and 24 h later. Similar experiments were done in two different athymic nude mouse strains (Harlan and NIH Swiss athymic *nu/nu*) with s.c. implanted PC-3 xenografts using the following time points: 1, 2, 7, and 56 days of LDM CPA treatment and 6 h after removal of CPA-medicated drinking water in mice that had been treated for 7 or 56 days, respectively. In additional studies using LC/MS/MS, mice were bled after 7 or 56 days of LDM CPA treatment.

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[4] S. Man et al., unpublished observation.

GC/MS analysis was done as published (26), with minor modifications. Briefly, whole blood samples from five to six mice were collected by cardiac bleeding, pooled ($\sim$5 mL total volume per time point, the exact volume was measured for quantification purposes), and immediately added to a polypropylene tube containing 2 mol/L monobasic ammonium phosphate (5 mL), acetonitrile (10 mL), methanol (5 mL), 250 μL of a methanol solution containing PFBHA (50 mg/mL methanol; all chemicals from Sigma-Aldrich Chemical Co.), and the internal standard PBOX-d_4 (16 μg/mL methanol). Samples were incubated overnight at room temperature and then stored at $-20°C$. Each sample was extracted and silylated with *N*-tert-butyldimethyl-silyl-*N*-methyltrifluoroacetamide as previously described (26) but using reaction conditions of 70°C for 2 h. Sample analysis was done using an Agilent 6890/5973 GC/electron image mass spectroscopy (Agilent Technologies, Inc.); HP-5MS capillary column (30 m × 0.25 mm); and injection port at 275°C; initial oven temperature 165°C, hold for 2 min, and then raise at 2.75°C/min to 240°C and 8°C/min to 300°C, hold 10 min, and then reduce at 20°C/min to 165°C. Data were collected in single ion monitoring mode at *m/z* 241 (PBOX fragment, ^{35}Cl) and *m/z* 245 (corresponding PBOX-d_4 fragment, ^{35}Cl; ref. 26). Quantification was done using a calibration curve constructed from freshly prepared standard solutions of 4-OOH-CPA (0.05–20 μg/mL range) in human blood. An aliquot (5.0 mL) of each standard was added to a polypropylene tube containing PFBHA and processed as described above for the mouse blood samples. A single calibration curve was run along with the samples as a single sample test, giving a linear response ($r^2 = 0.999$). The lower limit of quantitation was 0.05 μg/mL (15% error criteria) and the lower limit of detection was 0.01 μg/mL (determined in a separate experiment). Values below the lower limit of quantitation were obtained by mathematical extrapolation and duly rated as such.

For LC/MS/MS analysis, the blood of individual mice was collected and added to tubes containing PFBHA and internal standard, which were processed as described above for the GC/MS procedure. An aliquot of 20 μL of the trapping tube supernatant was diluted by 200 μL of mobile phase A and 10 μL were injected into the column. LC conditions were as follows: mobile phase A: 95% aqueous 2 mmol/L formic acid, pH 3.5 adjusted by ammonium hydroxide, 5% acetonitrile; mobile phase B: 100% acetonitrile; flow rate: 0.5 mL/min; gradient, 0 to 1 min, 70% A; 1 to 3.5 min, 70% to 30% A; 3.5 to 4.5 min, 30% to 70% A, 6-min equilibration at 70% A; and column: Phenomenex C_{18}, 150 × 3 mm, 3 μm, at 45°C. MS/MS conditions were as follows: Applied Biosystems 3200 Q-Trap instrument, in multiple reaction monitoring mode, *m/z* 472/221 (PBOX-H$^+$-^{35}Cl,^{35}Cl), *m/z* 480/229 (PBOX-d_4-H$^+$-^{37}Cl,^{37}Cl); curtain gas 40 p.s.i.; ion spray voltage, 5,500 V; temperature, 300°C; ion source gas 1, 40 p.s.i.; ion source gas 2, 30 p.s.i.; declustering potential, 35 V; entrance port potential, 3 V; collision entrance potential, 26 V; collision cell exit potential, 4 V. For calibration curves, standard solutions of analyte in a 0.016 to 4 μg/mL range in blood were prepared the same way as described above for GC/MS analysis. A linear response was obtained ($r^2 = 0.999$). The lower limit of quantitation was 0.016 μg/mL (15% error criteria).

Pharmacokinetic parameters were calculated with Win-Nonlin version 4.1 software (Pharsight Corp.). 4-OH-CPA steady-state concentrations were calculated as mean values of plasma levels determined at 24, 48, 168, and 1,344 h of drug administration according to Takimoto et al. (30).

Measurement of Cytochrome *P*450 CYP3A4 Activity in Liver and Xenograft Microsome Preparations

Liver or xenograft tissue (100 mg) of individual mice was homogenized in 1 mL of homogenization buffer (25 mmol/L KCl, 20 mmol/L Tris, 1 mmol/L EDTA, 10% glycerol). After centrifugation for 30 min at 4°C (2,000 × *g*), supernatants were ultracentrifuged (100,000 × *g*, 1 h at 4°C). Pellets were resuspended in PBS buffer containing 5% glycerol and stored at $-70°C$ until further use. Protein concentration was determined by the method described by Bradford (Bio-Rad protein assay, Bio-Rad Laboratories) and CYP3A4 activity measurement was done according to the manufacturer's instructions using the *P*450-Glo CYP3A4 Assay kit (Promega Corp.). Luciferin 6′ benzyl ether is debenzylated through CYP3A4 activity and the released luciferin reacts with luciferase resulting in light production that was measured with a TopCount NXT Microplate Scintillation and Luminescence Counter (Packard Instrument Co.). Specificity of the reaction was confirmed by using the highly specific cytochrome *P*450 CYP3A4 inhibitor troleandomycin (Biomol).

Measurement of ALDH Activity in Liver and Xenograft Lysates

Liver and xenograft lysates of individual mice were prepared in 100 mmol/L Tris buffer (pH 8.0). Tissue (100 mg) was homogenized and centrifuged for 5 min at 4°C (200 × *g*) followed by further centrifugation of the supernatant for 30 min at 4°C (2,000 × *g*). Aliquots were stored at $-70°C$ until analyzed. The protein concentration was determined as described above. ALDH activity was determined according to Bostian and Betts (31) with the following final assay concentrations: 103 mmol/L Tris, 0.67 mmol/L β-NAD$^+$, 100 mmol/L potassium chloride, 10 mmol/L 2-mercaptoethanol, 0.0007% (w/v) bovine serum albumin, and 2 mmol/L acetaldehyde substrate. As an internal standard, we used ALDH (*Saccharomyces cerevisiae*) obtained from Sigma-Aldrich Chemical Co.

Statistical Analysis

All statistical analyses (one-way ANOVA with Newman-Keuls multiple comparison test and *t* test, as indicated) were done with Prism version 4.00 software (GraphPad). The level of significance was set at $P < 0.05$.

Results

Pretreatment with LDM CPA Does Not Impair Its Antitumor and Antivascular Effects

To test whether long-term exposure (i.e., 8 weeks) to LDM CPA has an effect on the biological effects of the

same regimen subsequently used therapeutically, male C.B-17 severe combined immunodeficient mice were pretreated for 8 weeks with either LDM CPA or normal saline in the drinking water followed by s.c. implantation of PC-3 cells. LDM CPA pretreatment did not affect tumor take. Furthermore, no significant effect was observed on the growth kinetics of PC-3 xenografts compared with the saline control groups (Fig. 2). Most importantly, LDM CPA treatment exhibited equivalent antitumor effects in mice exposed beforehand to LDM CPA compared with mice that had received normal saline (i.e., there was no evidence of induced resistance).

Similarly, the antiangiogenic activity of the LDM CPA regimen in a Matrigel plug assay was not compromised by preceding LDM CPA exposure as shown in Fig. 3A. Compared with the rather short elimination half-life of 4-OH-CPA (Table 1), the antiangiogenic effects decline more slowly. In fact, stopping LDM CPA administration immediately before Matrigel plug injection resulted in antiangiogenic effects similar to LDM CPA treatment initiated after Matrigel injection, and plug perfusion was still reduced by ∼41% when LDM CPA was stopped 3 days before Matrigel injection. Furthermore, the number of circulating endothelial progenitor cells, which are bone marrow–derived endothelial precursors that contribute to the growth of the vascular tumor compartment (29), decreased after 1 week of LDM CPA therapy and remained suppressed for the entire study period of 8 weeks (Fig. 3B).

Pharmacokinetic Studies

Using GC/MS, the levels of activated CPA (i.e., 4-OH-CPA) were measured in the blood of three different mouse strains (tumor-free C57BL/6J and PC-3 bearing NIH Swiss and Harlan nude mice) continuously given 20 mg/kg/d of CPA through the drinking water for up to 8 weeks, with no breaks. This daily dose of CPA corresponds to the optimal biological (i.e., antiangiogenic) dose of CPA given in a metronomic manner as determined by maximum reduction in bone marrow–derived circulating endothelial progenitor cell in the absence of significant toxicity (32). Because of the very low daily CPA dose applied, blood pooling (five or more mice per time point) was necessary to achieve detectable levels of 4-OH-CPA when using the GC/MS method. The obtained pharmacokinetic parameters are summarized in Table 1. The variability in $AUC_{0→1344}$ and C_{max} values is no surprise given known interstrain heterogeneity of CPA biotransformation (33) and the documented effect of xenograft growth on enzymes involved in CPA metabolism, such as CYP3A4 (34). Similarly, the 4-OH-CPA concentrations obtained after 1 and 8 weeks of LDM CPA therapy vary in the different strains by >10-fold. Otherwise, the interstrain variation of the 4-OH-CPA elimination half-life is less pronounced (∼2-fold). Steady-state 4-OH-CPA concentrations are obtained after 1 week. Importantly, within the same experiment, the 4-OH-CPA levels measured after 8 weeks of LDM CPA are similar to those after 1 week of treatment, suggesting the maintenance of a steady-state level of active metabolite achieved by continuous ingestion of CPA (Fig. 4).

The results obtained with GC/MS were complemented with measurements using LC/MS/MS showing 4-OH-CPA levels in a similar range (Table 1). The LC/MS/MS method was more sensitive than GC/MS, and therefore, smaller blood volumes were required. Consequently, 4-OH-CPA concentrations could be determined in blood samples from individual mice (Table 1). Of note, the presence of PC-3 xenografts resulted in decreased 4-OH-CPA concentrations in Harlan nude mice compared with tumor-free animals of the same strain.
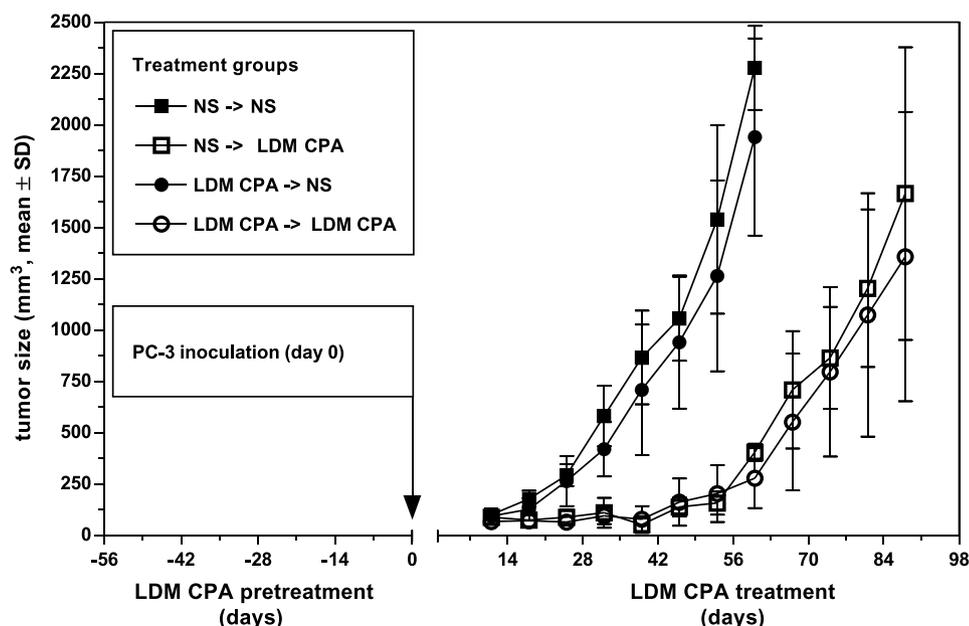
Figure 2. Administration of LDM CPA for 8 wks preceding s.c. implantation of PC-3 human prostate cancer cells has no significant effect on tumor take, xenograft growth kinetics, and the antitumor effects of LDM CPA. Male C.B-17 severe combined immunodeficient mice ($n = 7-8$ mice per group) were given either LDM CPA or normal saline through the drinking water for 8 wks. Six hours before s.c. tumor cell implantation, treatment was stopped and reinitiated after another 42 h. *NS,* normal saline.

Figure 3. **A,** pretreatment with LDM CPA does not affect its antiangiogenic properties as assessed by the Matrigel plug perfusi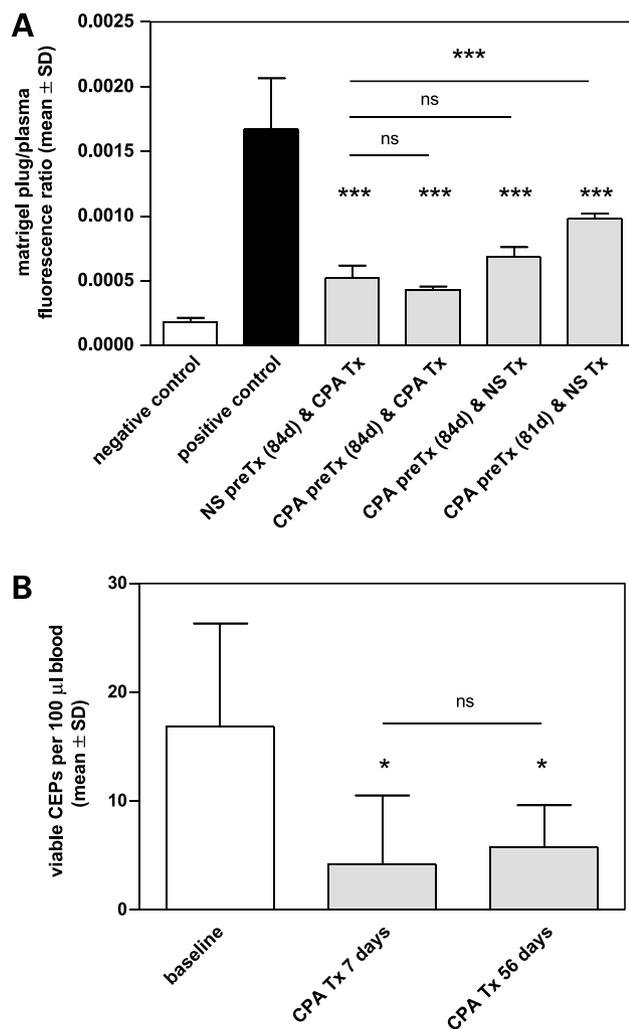on assay. Female BALB/cJ mice were exposed to either LDM CPA or normal saline given through the drinking water for 12 wks. Six hours (in one group 3 d) before Matrigel injection ($n = 6–7$ plugs per group), therapy was interrupted and reinitiated 3 d after Matrigel injection as indicated. One week thereafter, the Matrigel plug/plasma fluorescence was obtained. **B,** pretreatment with LDM CPA does not affect its antivasculogenic properties as assessed by enumeration of viable circulating endothelial progenitor cells (*CEPs*). Retro-orbital blood of PC-3 xenograft-bearing male Harlan nude mice was obtained before treatment initiation (baseline) and after 7 and 56 d of LDM CPA therapy ($n = 4–5$ mice per time point). Negative control: Matrigel without basic fibroblast growth factor; positive control: Matrigel with basic fibroblast growth factor. *preTx*, pretreatment; *Tx,* treatment, *ns,* not significant. *, $P < 0.05$; ***, $P < 0.001$, one-way ANOVA with Newman-Keuls multiple comparison test.

In clinical trials, LDM CPA is given mostly as a single oral dose of 50 mg/d. We therefore measured the trough levels of 4-OH-CPA in Harlan nude mice after 7 days of daily CPA gavage (20 mg/kg/d) compared with mice receiving the same daily dose of CPA via the drinking water. One day after the last CPA dose given by gavage, 4-OH-CPA was detectable in all mice ($n = 5$), albeit at lower

levels than seen in mice given CPA via the drinking water (0.007 ± 0.0005 μg/mL versus 0.034 ± 0.008 μg/mL). By comparison, 4-OH-CPA levels 1 day after the last 100 mg dose of a MTD CPA cycle are several fold higher (0.138 ± 0.155 μg/mL).

Liver and Xenograft CYP3A4 as well as ALDH Activity in Mice Treated with LDM CPA

Given its important role in the biotransformation of CPA and many other drugs, we next evaluated CYP3A4 activity in liver and tumor specimens of mice treated with LDM CPA and corresponding controls. In tumor-free C57BL/6J male mice, 8 weeks of LDM CPA resulted in a significant reduction of CYP3A4 activity measured in liver microsome preparations (Fig. 5A). The opposite is true for hepatic specimens of PC-3–bearing NIH Swiss nude mice with size-matched tumors (~ 200 mm^3 before treatment initiation and after 8 weeks of LDM CPA therapy; Fig. 5B). Although no universal pattern of liver CYP3A4 activity changes was observed, in neither of the two mouse strains studied these changes resulted in reduced 4-OH-CPA levels after 8 weeks compared with 1 week of LDM CPA therapy (Fig. 4).

In contrast to the findings in the liver samples of NIH Swiss nude mice, CYP3A4 activity is reduced in xenograft tissue preparations after 8 weeks of LDM CPA therapy compared with size-matched (~ 200 mm^3) tumor specimens obtained before treatment initiation (Fig. 5C).

ALDH activity is not induced in lysates obtained from PC-3 xenograft tumors recurring under LDM CPA compared with similarly sized treatment-naive tumors (Fig. 5F). In addition, liver ALDH activity is not altered by LDM CPA therapy in the two mouse strains (C57BL/6J and NIH Swiss *nu/nu*) analyzed irrespective of the presence or absence of a tumor (Fig. 5D and E).

Discussion

Antiangiogenic therapies can now be regarded as an established antitumor strategy and LDM represents a promising approach in this respect (1), especially when it is combined with targeted antiangiogenic drugs for long-term ''maintenance-type'' regimens (9, 10, 15). LDM frequently involves daily oral cytotoxic drug administration, as it is the case with CPA, the clinically most commonly used drug in this treatment setting (1, 3, 8–14). Although the oral route is very practical and appealing, it also raises new challenges, such as treatment compliance by patients and pharmacokinetic issues that potentially differ from conventional, cyclic MTD chemotherapy administration. The findings of our study shed light on several important issues about the clinical translation of the LDM concept.

Previous studies with the PC-3 model suggested that host-mediated mechanisms, such as altered CPA biotransformation, might contribute to acquired resistance to LDM CPA (17). We now show that neither tumor growth inhibition nor antivascular activities of LDM CPA are compromised when mice are pretreated with LDM CPA for

Table 1. Pharmacokinetic parameters for 4-OH-CPA

Mouse strain	Xenograft	Detection method	C_{D7} (µg/mL)	C_{D56} (µg/mL)	$AUC_{0\to1344}$ (h × µg/mL)	C_{max} (µg/mL)	T_{max} (h)	C_{ss} (µg/mL)	$t_{1/2\ D7}$ (h)	$t_{1/2\ D56}$ (h)
C57BL/6J	—	GC/MS*	0.084	0.144	150.7	0.214	24	0.112	1.147	1.295
Harlan *nu/nu*	PC-3	GC/MS*	0.014	0.013	24.2	0.083	24	0.046	1.349	1.739
NIH Swiss *nu/nu*	PC-3	GC/MS*	0.020	0.013	44.6	0.332	24	0.148	2.210	1.751
Harlan *nu/nu*	—	LC/MS/MS†	0.067 ± 0.038	—						
Harlan *nu/nu*	PC-3	LC/MS/MS†	0.034 ± 0.008	—						
C.B-17 SCID	113/6-4L	LC/MS/MS‡	—	0.023						

Abbreviations: C_{D7}, concentration after 7 d of treatment; C_{D56}, concentration after 56 d of treatment; $AUC_{0\to1344}$, area under the curve from time 0 to 1,344 h; C_{max}, maximal concentration; T_{max}, time of C_{max}; C_{ss}, steady-state concentration (according to Takimoto et al. (30); $t_{1/2\ D7}$, elimination half-life after 7 d of treatment; $t_{1/2\ D56}$, elimination half-life after 56 d of treatment; SCID, severe combined immunodeficient.
*Pooled blood sample from five to six mice per time point.
†Four to five blood samples from individual mice per time point, results expressed as mean ± SD.
‡Pooled blood from eight mice.

extended periods. Furthermore, the blood concentration of the active CPA metabolite 4-OH-CPA after 1 and 8 weeks of treatment is comparable in all three mouse strains tested. The observed strain differences in the absolute 4-OH-CPA levels and various pharmacokinetic parameters are not completely unexpected given preclinical and clinical evidence of (epi)genetic heterogeneity of the enzymes involved in CPA metabolism (20, 33). Although differences exist between human and murine CPA biotransformation (35), our results are in accordance with a study by Mouridsen et al. (36). Using radioactively labeled CPA, the authors did not observe a change in the pharmacokinetic properties of CPA in patients presenting with advanced malignancies given 2 mg/kg/d of CPA for a period of 13 to 33 days. Otherwise, D'Incalci et al. (37)

Figure 4. Comparable levels of 4-OH-CPA are found in the blood after 7 and 56 d, respectively, of LDM CPA treatment via the drinking water in tumor-free male C57BL/6J mice and two different athymic nude mouse strains (Harlan and NIH Swiss athymic *nu/nu*) with s.c. implanted PC-3 human prostate cancer xenografts. Results were obtained from pooled blood of *n* = 5 to 6 mice per time point.

described increased CPA clearance after oral administration of 100 mg/d of CPA for 6 to 13 months to cancer patients using GC. Unfortunately, the authors did not provide parameters such as the area under the curve of CPA at the beginning and after long-term CPA administration. Furthermore, neither of these studies involved 4-OH-CPA measurements and the daily CPA doses used were rather high compared with the 50 mg/d commonly used in clinical trials that show antitumor activity of LDM CPA-based regimens (1, 3, 8–14). Finally, our finding of maintained 4-OH-CPA levels and biological effects is in line with long-term beneficial effects of oral CPA protocols used for the treatment of immunologic disorders (38).

Together with the results of a recently published study (39), our data are consistent with the possibility of reduced vascular dependence of tumor cells (40) rather than host-mediated mechanisms as a major reason for acquired resistance to LDM CPA. If so, from a pharmacologic point of view, there may be no need to switch between CPA and other cytotoxic drugs (e.g., etoposide) to delay resistance as has been suggested by others (41). Moreover, stopping LDM CPA because of tumor recurrence due to reduced vascular dependence might fuel an unchecked ''proangiogenic rebound'' and consequently resumption of more rapid tumor growth as recently observed after termination of antitumor treatment using antiangiogenic receptor tyrosine kinase inhibitors (42).

By using 20 mg/kg/d of CPA (i.e., the optimal ''metronomic'' dose of CPA for various mouse strains and across several tumor models tested; ref. 32), the 4-OH-CPA levels obtained after prolonged LDM CPA therapy are comparable with the IC_{50} for proliferation inhibition of human umbilical vascular endothelial cells by 4-OOH-CPA in a 6-day *in vitro* assay (~0.013 µg/mL) yet far below the IC_{50} of PC-3 (39, 43). Trough levels after 1 week of 20 mg/kg of CPA given once daily by gavage (which likely more closely mimics the human situation where CPA is given as a single daily oral dose) are below the *in vitro* IC_{50}. However, the half-life of 4-OH-CPA would suggest that blood levels >0.013 µg/mL prevail for the majority of a
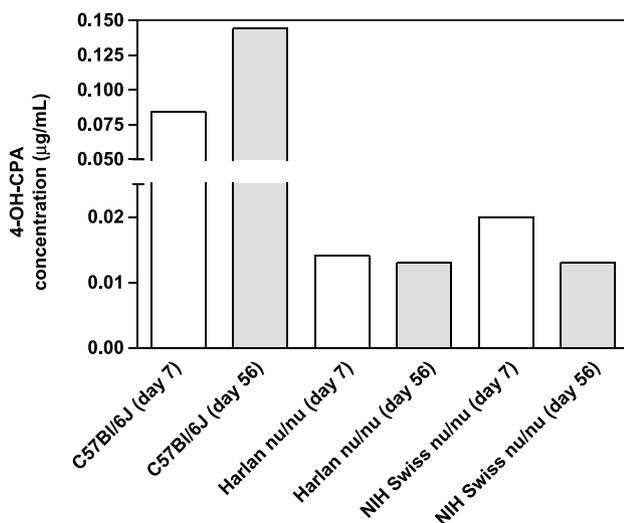
dosing interval. Similarly to our findings, Klink et al. (44) detected trofosfamide 4-OH metabolite blood concentrations in the same range as the IC_{50} for proliferation inhibition of human umbilical vascular endothelial cell in mice receiving LDM trofosfamide via the drinking water. However, only early time points (up to 96 h) were analyzed. In another study, the plasma and tumor levels of the oral alkylating agent temozolomide, given in a LDM regimen to rats, are very similar at days 1 and 28 of dosing (45). This is not entirely unexpected because temozolomide undergoes spontaneous hydrolysis at physiologic pH to form its active metabolite instead of being metabolized, as is the case for CPA.

Because the comparison of *in vitro* with *in vivo* drug effects is notoriously difficult to evaluate, and in our study mouse rather than human vasculature was targeted, clinical data will be required to establish the antiangiogenic 4-OH-CPA concentration range in humans. Thus far, a pilot pharmacokinetic study of LDM CPA in pediatric solid tumor patients was restricted to the analysis of parent CPA from blood samples obtained 24 h after the first dose (46). It will be important to see whether 4-OH-CPA levels obtained from human blood samples mirror our preclinical findings. Eventually, pharmacokinetic parameters could become a means to individualize/optimize LDM CPA therapy as opposed to the currently applied fixed 50 mg/d
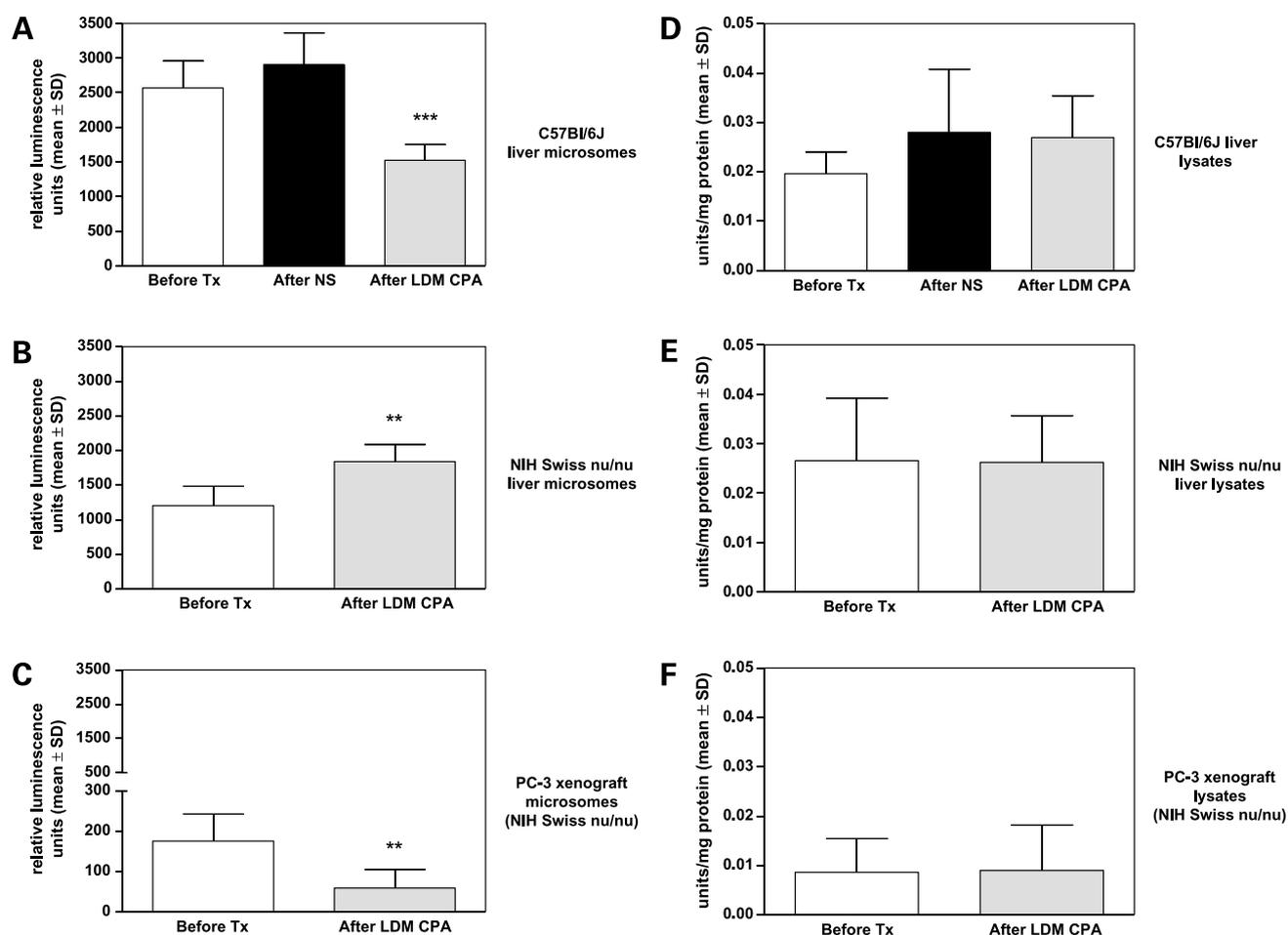

Figure 5. **A,** CYP3A4 activity is reduced (-41%) in liver microsome preparations of tumor-free C57BL/6J mice treated with LDM CPA for 8 wks (compared with samples obtained before treatment initiation or from animals undergoing normal saline administration for 8 wks). **B,** CYP3A4 activity increased ($+53\%$) in liver samples of PC-3 xenograft bearing NIH Swiss nude mice after 8 wks of LDM CPA treatment. The tumors were size matched (~ 200 mm^3 at treatment initiation and after 8 wks of LDM CPA therapy, respectively). **C,** compared with liver microsomes, CYP3A4 activity of microsomes obtained from PC-3 tumors is minor. Contrary to the liver samples of the host mice, tumor microsomes display reduced (-76%) CYP3A4 activity after 8 wks of LDM CPA compared with size-matched tumors removed before treatment initiation. **D,** ALDH activity does not change significantly ($P > 0.05$, one-way ANOVA with Newman-Keuls multiple comparison test) in liver samples of C57BL/6J mice treated with LDM CPA for 8 wks compared with samples obtained before therapy initiation or from control mice. **E,** similarly, liver samples of PC-3 xenograft (size matched) bearing NIH Swiss nude mice undergoing LDM CPA therapy show no treatment-induced variation of ALDH, as it is the case for tumor lysates ($P > 0.05$, *t* test; **F**). *Before Tx,* samples obtained before therapy initiation; *After NS/LDM CPA,* samples obtained after normal saline/LDM CPA administration through the drinking water for a duration of 8 wks ($n = 5$ liver/tumor samples of individual mice per group). **, $P < 0.01$, compared with control (*t* test); ***, $P < 0.001$, compared with control (one-way ANOVA with Newman-Keuls multiple comparison test).

dosing in adults that does not account for individual variation in CPA biotransformation, a question also raised by others (47). In this regard, it needs to be pointed out that the dosing of LDM and other antiangiogenic therapies remains largely empirical in the absence of validated clinical surrogate markers for antiangiogenic treatment effects (1). Such markers are emerging in preclinical studies [e.g., circulating endothelial progenitor cells (29)], but their value in clinical studies remains problematic (48, 49).

CYP3A4 induction was observed in the liver of PC-3–bearing NIH Swiss nude mice, contrasting with liver cytochrome activity commonly being decreased due to the tumor-associated inflammatory host response (34). Because a wide array of immunomodulatory effects of CPA has been described (pre)clinically (4), one could therefore speculate that LDM CPA might induce CYP3A4 indirectly via modification of the inflammatory tumor-host reaction. However, this would not explain the findings in tumor-free C57BL/6J, where liver CYP3A4 activity decreased after prolonged LDM CPA administration. Importantly, despite altered CYP3A4 activity in both models tested, circulating 4-OH-CPA levels after 7 and 56 days of treatment are comparable. A possible explanation for this lack of correlation is that the daily CPA dose applied in our regimen might be well below the saturation threshold for CYP3A4. Alternatively, other cytochrome $P450$ isoforms could substitute for CYP3A4 activity. Nevertheless, the observed changes raise concern about the risk of unfavorable interactions of LDM CPA with drugs potentially coadministered that are CYP3A4 substrates, such as other cytotoxics (e.g. taxanes), oncogene-targeting drugs (imatinib, gefitinib, tamoxifen, and letrozole; refs. 24, 25), antiangiogenic receptor tyrosine kinase inhibitors such as sunitinib (50), and supportive care drugs such as fluconazole, allopurinol, ranitidine, and prednisolone (18). In this regard, it is perhaps reassuring that a preclinical study undertaken by Pietras and Hanahan (16) showed outstanding antitumor activity of a combination of daily oral LDM CPA and sunitinib for the treatment of late-stage, large islet cell pancreatic carcinoma. Similarly, the combination of letrozole and LDM CPA was both beneficial and devoid of severe or unexpected side effects in the neoadjuvant treatment of breast cancer in elderly patients (13). It also is reassuring that the liver activity of ALDH, the major enzyme involved in the detoxification of CPA and many other compounds and implicated in the resistance to CPA given in a conventional way (23), is not affected by LDM CPA.

Although LDM CPA is supposed to target primarily the tumor vasculature as opposed to the parenchyma (3), we tested for enzymatic changes in tumor tissue preparations. The reduced CYP3A4 activity in PC-3 xenograft microsomes is most likely of limited biological significance because the extrahepatic biotransformation of CPA to 4-OH-CPA is very minor (19). Tumor ALDH activity does not change in association with LDM CPA treatment, suggesting that increased CPA detoxification via ALDH activity is unlikely to be implicated in the resistance observed.

In summary, our analyses suggest that the biological effects of LDM CPA are maintained over prolonged periods. Moreover, acquired resistance to LDM CPA is unlikely related to altered CPA biotransformation. Long-term LDM CPA therapy results in altered CYP3A4 activity, which could conceivably affect the activity and side effect profile of a wide array of comedications, including other anticancer compounds. If our data are confirmed in human trials, pharmacokinetic parameters might become a way to individualize LDM CPA therapy and reduce the empiricism related to CPA dosing in this context.

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