Research Article (Preclinical Development)

Inhibition of OCTN2-Mediated Transport of Carnitine by Etoposide

Chaoxin Hu¹, Cynthia S. Lancaster¹, Zhili Zuo³, Shuiying Hu¹, Zhaoyuan Chen¹, Jeffrey E. Rubnitz², Sharyn D. Baker¹, and Alex Sparreboom¹

Authors’ Affiliations: ¹Department of Pharmaceutical Sciences and ²Department of Oncology, St. Jude Children’s Research Hospital, Memphis, TN; ³School of Biomedical Sciences, Curtin University, Perth, Australia. Current affiliation for C. Hu: Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD; Current affiliation for Z. Chen: TDM Pharmaceutical Research, Newark, DE.

Corresponding Author: Alex Sparreboom, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, CCC, Room I5308. Phone: (901) 595-5346; Fax: (901) 595-3125; E-mail: alex.sparreboom@stjude.org.

Keywords: OCTN2, Carnitine, Etoposide, Hypocarnitinemia, Cancer

Running title: Interference of OCTN2 function by etoposide

Note: This work was presented previously in part at the 110th annual meeting of the American Society of Clinical Pharmacology & Therapeutics (ASCPT) in 2009.

This work was supported in part by the American Lebanese Syrian Associated Charities (ALSAC), NIH/NCI grant 5R01CA151633-01 (to A.S.) and United States Public Health Service Cancer Center Support Grant 3P30CA021765 (to S.D.B.).
ABSTRACT

OCTN2 is a bifunctional transporter that reabsorbs filtered carnitine in a sodium dependent manner and secretes organic cations into urine as a proton antiport mechanism. We hypothesized that inhibition of OCTN2 by anticancer drugs can influence carnitine resorption. OCTN2-mediated transport inhibition by anticancer drugs was assessed using cells transfected with human OCTN2 (hOCTN2) or mouse Octn2 (mOctn2). Excretion of carnitine and acetylcarnitine was measured in urine collected from mice and pediatric cancer patients before and after administration of etoposide. Five of 27 tested drugs (50-100 µM) inhibited hOCTN2-mediated carnitine uptake by 42-85% (P<0.001). Of these inhibitors, etoposide was itself a transported substrate of hOCTN2 and mOctn2. Etoposide uptake by hOCTN2 was reversed in the presence of excess carnitine. This competitive inhibitory mechanism was confirmed in an in silico molecular docking analysis. In addition, etoposide inhibited the transcellular apical-to-basolateral flux of carnitine in kidney cells. Etoposide was also associated with a significant urinary loss of carnitine in mice (~1.5-fold) and cancer patients (~2.4-fold). Collectively, these findings indicate that etoposide can inhibit hOCTN2 function, potentially disturb carnitine homeostasis, and that this phenomenon can contribute to treatment-related toxicities.
INTRODUCTION

Carnitine (vitamin B₁) is a highly polar molecule that plays an essential role in the transport of long-chain fatty acids across the inner mitochondrial or peroxisomal membrane for β-oxidation. Carnitine is retained in the body by a mechanism that involves resorption of filtered carnitine in the kidney by active transport across the tubular epithelial cell membrane (1). This process is mediated by the human organic cation transporter OCTN2 (hOCTN2), a sodium-dependent, high-affinity carnitine carrier. Defects in hOCTN2 function resulting from mutations in the gene SLC22A5 can cause a potentially lethal, autosomal-recessive disease known as primary systemic carnitine deficiency (SCD). Patients with primary SCD lose most (>95%) of the filtered carnitine in their urine and even heterozygosity for SLC22A5 mutations can result in a carnitine loss that is 2-3 times higher than the normal amount (2). These prior genetic studies suggest that unintentional alteration of hOCTN2 function, for example by the use of drugs (3), can potentially lead to deleterious phenotypic changes in patients. Indeed, the principal metabolic abnormalities associated with excessive urinary carnitine loss in individuals with primary SCD are also observed following treatment with certain prescription drugs known to inhibit hOCTN2 function in vitro, such as verapamil (4). Few previous studies have identified a number of widely used anticancer drugs as inhibitors of hOCTN2, including actinomycin D (5) and vinblastine (6). However, a systematic approach to evaluate the ability of anticancer drugs to interact with OCTN2 and subsequently affect carnitine homeostasis is still lacking. In the current study, we studied the interaction of 27 anticancer drugs with hOCTN2 in vitro in cells, in silico by molecular docking simulations, and in vivo in mice and cancer patients.
MATERIALS AND METHODS

Chemicals and reagents

[3H]carnitine (80 Ci/mmol), [3H(G)]daunorubicin (5 Ci/mmol), [3H]sunitinib (12.5 Ci/mmol), and [ethyl-1-14C]tetraethylammonium (55 mCi/mmol; TEA) were purchased from American Radiolabeled Chemicals, [3H]etoposide (0.5 Ci/mmol) and [3H]vinblastine (5 Ci/mmol) from Moravek Biochemicals, and [3H(G)]paclitaxel (25.6 Ci/mmol) from Vitrax. Etoposide-Injection solution was purchased from the pharmacy of St Jude Children’s Research Hospital. All other chemicals and drugs were purchased from Sigma and were of reagent grade or better.

Plasmids and cell culture

A pcDNA3 vector, a hOCTN2-pcDNA3 plasmid, and HEK293 cells transfected with an empty pcDNA3 vector, hOCTN2, or mOctn2 were kindly provided by Dr. Akira Tsuji. The cells were cultivated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Invitrogen), 1 mg/ml G418, 100 U/ml penicillin, and 100 µg/ml streptomycin in tissue culture dishes in a humidified incubator at 37°C under 5% CO2 and 95% humidity. The porcine kidney epithelial LLC-PK1 cell line was kindly provided by Dr. John Schuetz. LLC-PK1 cells were cultured in M199 medium supplied with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Inhibition of carnitine uptake

A total of 27 test compounds were selected as representative, FDA-approved agents from the main classes of anticancer chemotherapeutics. The classes include: (i) antifolates (methotrexate); (ii) cytidine analogues (clofarabine, cytarabine); (iii) antimetabolites/hydroxyurea (hydroxyurea); (iv) antimitotic drugs (docetaxel, paclitaxel, vinblastine, vinorelbine, vincristine); (v) alkylating agents (cyclophosphamide, ifosfamide, mechlorethamine); (vi) platinum analogs.
(carboplatin, cisplatin); (vi) antitumor antibiotics (rapamycin); (vii) topoisomerase inhibitors (daunorubicin, doxorubicin, etoposide); (ix) molecularly-targeted therapies (dasatinib, gefitinib, imatinib, sorafenib, sunitinib); and (x) corticosteroids (cortisone, dexamethasone, prednisolone, prednisone). For our initial screen we did not consider (i) immunologic agents; (ii) hormonal therapy; (iii) supportive care agents; and (iv) fluoropyrimidines.

The assays were carried out in 6-well plates (Biocoat) with cells seeded 48 hours before each experiment or till over 90% confluency was achieved. Immediately before the experiment, the cells were washed once with 2 ml PBS after removal of the incubation buffer. Next, the cells were incubated with 2 ml uptake buffer containing carnitine (10 nM) and unlabeled compound (100 µM unless specified otherwise) applied simultaneously to the cells at 37°C. The uptake buffer consisted of 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 25 mM HEPES (pH 7.4). At the end of the incubation period, cellular uptake of carnitine was terminated by removing the uptake buffer. A 5-min time point was selected based on published functional data for other OCTN2 substrates in the same cell lines, including carnitine, that suggest that OCTN2 transport activity is typically linear up to at least 8 minutes (4, 7). After washing twice with 2-ml of ice-cold PBS, the cells were lysed directly on the plate in the presence of 1 ml of 1.0 M NaOH for 2 hours. Radioactivity in each sample containing a 600-µl lysate and 5 ml scintillation liquid was determined on a Beckman LS 6500 scintillation counter. The ratio of carnitine transport in the presence and absence of the anticancer drugs was calculated after correcting for nonspecific transport observed in the vector control cells, and normalized to protein content, determined using a bicinchoninic acid protein-assay kit (Pierce Biotechnology).

**Inhibition of transcellular carnitine transport**
Empty vector pcDNA3 and hOCTN2-pcDNA3 were stably transfected into LLC-PK1 cells using a commercial kit (InvitroGen) according to the manufacturers’ instructions. The transfected cell lines were selected using a medium containing G418 (1.0 mg/ml). Transcellular transport in the LLC-PK1 cell lines was evaluated using 5×10⁵ cells per well, seeded on a 12-mm Transwell plate with a 3.0-μm pore size (Costar). The cells were grown for 3 days in complete medium, which was changed daily. The medium at both the apical and the basolateral side of the monolayer was replaced with 0.5 ml fresh medium about 1 hour prior to starting the experiment. In select experiments, cells were permeabilized at the basolateral side with 100 µg/ml Staphylococcus aureus α-toxin (EMD4Biosciences) for 30 min at 37°C without affecting the functional integrity of the apical membrane, according to an established protocol (8). The experiment was initiated by replacing the medium at either the apical or basolateral side with 0.5-ml uptake buffer containing carnitine (50 nM) in the presence or absence of etoposide (100 µM). The cells were incubated at 37°C, and 40-µl aliquots were taken from each compartment. The appearance of radioactivity in the opposite compartment was measured and presented as the fraction of total radioactivity added at the beginning of the experiment.

Drug uptake assays

Uptake of radiolabeled anticancer drugs in cells transfected with hOCTN2 or mOctn2 were carried out as described above for carnitine, with minor modification. The contribution of hOCTN2 or mOctn2 to intracellular drug uptake was established by comparing data obtained in HEK293 cells overexpressing the transporter and HEK293 cells transfected with an empty vector. To evaluate sodium-dependence of transport, experiments were repeated in medium where sodium chloride was replaced with isotonic N-methylglucamine chloride. Buffer pH was adjusted with HCl, NaOH, or KOH when uptake was measured at an acidic or alkaline pH.
Computational docking studies

Development and optimization of a computational model of hOCTN2 have been reported in detail elsewhere (9). Briefly, the putative active sites of hOCTN2 were indicated based on our experimental data obtained in hOCTN2-overexpressed HEK293 cells. The TMD1-7 were found to be responsible for organic cation transport and for sodium dependence in carnitine transport, and carnitine transport by hOCTN2 requires the linkage between TMD1-7 and TMD11 (10). Furthermore, the residues of Q180, Q207, S467, and P478 are known to be critical for the function of hOCTN2 (11). Collectively, these prior studies have indicated that the putative active site of hOCTN2 for carnitine recognition is identified as the cave between TMD1-7 and TMD11, which is near to the residues mentioned above. As a next step, automated docking studies were performed with GOLD 3.0 (Genetic Optimisation for Ligand Docking). A radius of 10 Å from the residue of S467, which is critical to the activity of the hOCTN2, was used to design the binding site. 30 GA runs were performed in each docking calculation. For each of the genetic algorithm runs, a maximum number of 150,000 operations were performed on a population of 100 individuals with a selection pressure of 1.1. Operator weights for crossover, mutation, and migration were set to 95, 95, and 10, respectively.

Animal experiments

Male FVB mice, aged 7-9 weeks (18-22 g in weight), obtained from Charles River Laboratories, were used in all experiments. The experimental protocols were reviewed and approved by the St Jude Children’s Research Hospital Animal Care Committee in accordance with the “Guide for the Care and Use of Laboratory Animals”. The animals were acclimatized for at least 1 week before experimentation, fed with a standard National Institute of Nutrition diet, and allowed tap water ad libitum. Etoposide was administered by intraperitoneal bolus injection at a dose of 10 mg/kg and saline was used as a control. While the average systemic
exposure to etoposide in humans is higher (90 µg·h/mL for 100 mg/m²) (12) compared with that observed in FVB mice (5.37 µg·h/mL for 10 mg/kg) (13), the luminal (urinary) concentrations within the first 24 hours at these respective doses are very similar, namely ~89 µM in patients and 71 µM in FVB mice. Therefore, the mouse studies as performed in our experiments have direct relevance to the clinical situation.

Metabolic cages were used to collect the urine of each mouse at 24-hour intervals, starting the day before drug administration, for up to 4 days post treatment. The urine samples were collected into 5-ml tubes containing 1.0 M HCl to prevent continuing degradation of carnitines ex vivo. All the samples were stored at -20°C before analysis for carnitine and acetylcarnitine by a validated method based on liquid chromatography with tandem mass spectrometric detection (9). Urinary levels of glucose, BUN, total protein, and creatinine were determined using kits from Roche Diagnostics.

**Patient samples**

Urine samples were obtained from 5 pediatric patients with acute myeloid leukemia treated with an induction regimen comprising of etoposide (100 mg/m² i.v. over 4 hours on days 1-5, cytarabine (100 mg/m² i.v. over 30 minutes every 12 hours on days 1-10), and daunorubicin (50 mg/m² i.v. over 6 hours on days 2, 4, and 6 (1.67 mg/kg for patients less than 10 kg). Eligibility criteria have been documented previously (14). The study protocol was approved by the St. Jude Children’s Research Hospital review board, and a separate informed consent related to urine collection for the purpose of this study was obtained. Samples were collected from each patient for a total of 24 hours before the start of induction therapy, then for one 24-hour collection period on day 6. Urine was collected in plastic containers containing 1.0 M HCl, and kept in a refrigerator until the end of collection. The total urine volume was recorded and 5-ml aliquots stored frozen until analysis as described above for mouse samples. A partial
revalidation of this method was performed by analyzing in quintuplicate, on 4 separate occasions, human urine samples spiked with carnitine (1.0, 3.0, 15, 150, or 1000 ng/ml) and acetylcarnitine (0.25, 0.75, 3.75, 37.5, or 250 ng/ml) on calibration curves constructed in mouse urine. The percent deviation from nominal values, within-run precision, and between-run precision for each analyte were always within ±3.9%, ≤5.9%, and ≤5.0%, respectively.

**Statistical analysis**

All *in vitro* and *in vivo* experiments were performed on 3 separate occasions at least in triplicate, and all data are presented as mean and standard error, unless otherwise stated. Statistical analyses were done using a two-tailed *t* test (for 2 groups) or a one-way ANOVA (for multiple groups), and *P*<0.05 was considered statistically significant. All statistical calculations were performed using the software package NCSS v2004 (Number Cruncher Statistical System).
RESULTS

Inhibition of OCTN2 function by anticancer drugs

In an effort to understand the selectivity of hOCTN2 and its mouse ortholog mOctn2, we assessed the inhibitory potential of 27 anticancer drugs toward hOCTN2 by evaluating changes in the transport of carnitine relative to cells transfected with an empty vector (**Supplementary Fig. S1**). These results indicate that several classes of agents, including taxanes (eg, paclitaxel), anthracyclines (eg, daunorubicin), epipodophyllotoxins (eg, etoposide), and *Vinca* alkaloids (eg, vinblastine) can significantly inhibit OCTN2 function in a mammalian system (**Table 1**). A subsequent examination revealed that mOctn2-mediated carnitine transport was also sensitive to inhibitory actions by several of the same compounds (**Table 1**).

Identification of etoposide as an hOCTN2 substrate

Because hOCTN2 transports cationic compounds as well as zwitterions, we next examined whether 5 representative xenobiotics that inhibit hOCTN2 are also transported substrates. The uptake of etoposide was significantly increased in hOCTN2-transfected HEK293 cells (**Fig. 1A**), but this was not noted for daunorubicin, paclitaxel, the tyrosine-kinase inhibitor sunitinib, or vinblastine (**Fig. 1A**), regardless of the tested concentrations (**Supplementary Fig. S2**). This result suggests that inhibitors of hOCTN2-mediated carnitine transport are not necessarily transported substrates. The net transport of etoposide by hOCTN2 (uptake coefficient, 2.85±0.313 µL/mg/5 min) was not dependent on pH (**Fig. 1B**) or sodium (**Fig. 1C**) at the initial times, and was reversed in the presence of excess carnitine (**Fig. 1D**). These findings support the hypothesis that the structure of OCTN2 includes a binding surface comprised of multiple, distinct, but probably overlapping binding sites (see below).

The Michaelis-Menten constant (Km) of etoposide for hOCTN2 and mOctn2 was in the same order of magnitude (Km, 150 ± 34.1 versus 76.4 ± 24.8 µM), whereas the maximum rate
of transport was about 4-fold higher for hOCTN2 compared with mOcn2 (V\text{max}, 172 ± 21.4 versus 49.8 ± 4.96 pmol/mg/min) (Fig. 1E). Of note, the intracellular uptake of etoposide was also facilitated, but to a lesser extent, by the related transporter hOCTN1 (Supplementary Fig. S3) that shares 77% identity and 88% similarity with hOCTN2 at the level of amino acid sequence.

**Characterization of the interaction of etoposide with hOCTN2**

The ability of etoposide to interfere with carnitine transport by hOCTN2 was found to be strongly dependent on inhibitor concentration, with an IC50 value of 55.0 µM (95%CI, 46.6-65.0 µM) (Fig. 2A). This value is of the same order of magnitude as the average luminal (urinary) concentration of etoposide in a cancer patients within the first 24 hours of receiving an intravenous dose of 100 mg/m², namely ~89 µM in an adult with a body-surface area of 1.86 m² and ~92 µM in a 50-kg child (12).

In order to identify the mechanism of this interaction, kinetic analyses were performed with and without etoposide using varying concentrations of carnitine. The resulting Eadie-Hofstee plot revealed that etoposide caused an increase in Km (4.21 ± 0.14 µM versus 5.63 ± 0.17 µM; P<0.0001) without a concurrent shift in V\text{max} (215 ± 2.56 versus 225 ± 3.93 pmol/mg/min; P>0.05), suggesting a competitive inhibitory mechanism (Fig. 2B). The inhibitory potential of etoposide toward hOCTN2 function was further confirmed using carnitine as a test substrate in a secondary hOCTN2-overexpressing cell line grown as a polarized monolayer. These results indicated that etoposide, following permeabilization of the basolateral membrane with α-toxin, causes substantially diminished vectorial translocation of carnitine from the apical to basolateral side (Fig. 2C).

Next, our previously generated homology model for hOCTN2 (9) was used to gain further insight into the site of interaction between etoposide and carnitine. This prior study indicated that the putative active site on hOCTN2 for carnitine recognition is the cave between transmembrane domains TMD1-7 and TMD11 (Fig. 2D). As predicted from the experimental data, the computational
modeling suggested that carnitine and etoposide have a predicted overlapping binding site within hOCTN2 (Fig. 2E).

**Influence of etoposide on urinary carnitine excretion**

Experiments performed in adult mice housed in metabolic cages indicated that even a single i.p. bolus administration of etoposide at a nontoxic dose of 10 mg/kg already resulted in a statistically significant increase in the excretion of carnitine and total carnitines within the first day after dosing (Fig. 3A-B). The time-course for the net loss of carnitine indicates that etoposide causes an increase in carnitine excretion compared with the normal loss observed before drug administration or following saline (Fig. 3A) without simultaneously affecting urinary excretion of nonspecific metabolites such as creatinine (Supplementary Fig. S4). The reversible effect of the etoposide treatment on the increases in urinary carnitine, with a trend toward normalization on day 3, is in line with the notion that the urinary excretion of etoposide in mice continues to at least 60 hours after i.v. administration (15). This suggests that sufficiently high etoposide levels in urine, capable of inhibiting Octn2-mediated carnitine resorption, can likely be maintained for time periods exceeding 24 hours.

Consistent with the murine studies, an analysis performed in pediatric patients indicated that, compared to baseline levels, etoposide-based chemotherapy increases the average recovery of carnitine in urine by 2.4-fold (18.9 versus 45.4 µmol·24 h⁻¹; P=0.033) and total carnitines by about 3-fold (48.6 versus 197 µmol·24 h⁻¹; P=0.043) (Fig. 3C-D).
DISCUSSION

This study demonstrates that the anticancer agent etoposide has the potential to dramatically affect urinary levels of carnitine, an essential cofactor for mitochondrial fatty acid oxidation. Using a variety of in vitro, in silico, and in vivo model systems, we found that etoposide-induced changes in urinary carnitine is mediated via a process that involves, at least in part, direct competitive inhibition of hOCTN2, a luminal organic cation transporter regulating tubular resorption of filtered carnitine. The current study complements previous knowledge on the interaction of anticancer drugs with renal organic cation transporters, and provides further mechanistic insight into the role these proteins may play in etoposide-related side effect profiles originating downstream of disturbances in carnitine homeostasis.

The identification of etoposide as a transported substrate of hOCTN2 supports the possibility that this protein plays a role in the absorption and disposition of etoposide in vivo. For example, hOCTN2 is highly expressed in the intestine (16), where it may be involved as an active transport system in the intestinal uptake of etoposide after oral administration (17). This would be consistent with previous reports indicating that the absorption of etoposide is saturable (18), and that etoposide exhibits high (>50%) oral bioavailability in humans (19), despite its unfavorable physicochemical characteristics that predict poor absorption (20). In particular, etoposide breaks at least two of Lipinski’s “rule-of-five” rules, including (i) the molecular weight being >500 (namely 588.6), and (ii) the number of H-bond acceptors being >10 (namely 13). In addition, etoposide has a dynamic polar molecular surface area of ≥140 Å² (namely 160 Å²) with 5 rotatable bonds (21), and a basic pKa of 9.8, further suggesting that etoposide would exhibit poor absorption and/or membrane penetration properties. In order to confirm the in vivo importance of OCTN2 as a transporter of etoposide, we are currently investigating the absorption and disposition kinetics of etoposide in the jvs mouse, which carries a missense
mutation (1114T>G; L352R) in the mOctn2 gene Slc22a5, resulting in dysfunctional Octn2-related transport activity (22).

Interestingly, whereas the hOCTN2-mediated transport of carnitine itself is dependent on pH and sodium (5), no pH dependence was found for the transport of etoposide by hOCTN2, although the absolute intracellular drug uptake was increased in the absence of sodium (Supplementary Fig. S5). A similar increase in etoposide uptake was observed in vector control cells in the absence of sodium, indicating that the net hOCTN2-mediated uptake is independent of sodium. This unusual phenomenon has been reported previously for pyrilamine and verapamil in cells transfected with hOCTN2 (5), although the mechanism underlying this phenomenon is still unclear.

Studies performed in rats have recently demonstrated that the administration of etoposide during the pre-pubertal phase causes disturbances in several morphometric parameters in Sertoli cells, and that exogenous carnitine supplementation partially protects the testis against these toxicities (23). It has been suggested that the protective effects of carnitine occur through its action on stem spermatogonia by minimizing DNA damage provoked by etoposide on cells expressing high levels of topoisomerase II, including intermediary and type B spermatogonia. Our current studies, however, provide support for an alternate mechanism by which carnitine can reduce testicular toxicities caused by etoposide. In particular, we found that carnitine can inhibit OCTN2-mediated cellular uptake of etoposide, and this interaction might subsequently ameliorate etoposide-induced toxicity in cells expressing this transporter. Support for this possibility comes from prior studies indicating that OCTN2 is present on the Sertoli cell membrane (24), and that testicular damage caused by etoposide is mediated, at least in part, by Sertoli cells (25).

The reported extent of urinary carnitine loss in patients undergoing etoposide-based chemotherapy, as well as the occurrence of peak changes on the first day after drug
administration, closely matches our current observations in mice. This finding supports the contention that the mouse is an appropriate animal model to further study the mechanisms and therapeutic implications of this phenomenon. It should be pointed out that the ability of etoposide to inhibit hOCTN2 and mOctn2 showed an apparent species dependence. The mechanism underlying this finding is not entirely clear but might be associated with the possibility that this agent is a comparatively large molecule with potentially poor accessibility to critical residues in the mOctn2 structure. Furthermore, although the mOctn2 protein is more than 70% homologous to hOCTN2, the mouse transporter is much less efficient (about 3- to 4-fold) at transporting carnitine compared with hOCTN2 (Supplementary Fig. S1).

Although theoretically etoposide could increase renal excretion of carnitine as a result of extensive tissue damage, the observed extent of additional total carnitine loss associated with drug treatment (about 0.5 μmol) is unlikely to have occurred only through this mechanism. Furthermore, an additional semi-quantitative analysis of the same urine samples indicated that the administration of etoposide was not associated with substantial concurrent changes in urinary excretion of glucose, blood urea nitrogen, total protein, and creatinine. This finding, combined with the observed time course showing a rapid increase in carnitine excretion after starting drug administration and a trend toward normalization supports the possibility of inhibition of a process specifically involving carnitine resorption in the kidney. Nonetheless, it cannot be excluded that additional mechanisms beyond the scope of this investigation might have contributed to our findings. These include potential effects of etoposide resulting in increased absorption of carnitine, increased production of carnitine, and/or reduced metabolism of carnitine.

In the context of the current findings, it is noteworthy that urinary carnitine wasting has also recently been reported in patients receiving paclitaxel-carboplatin or vinorelbine-carboplatin duplets (26). Our current results provide support for the possibility that the underlying
mechanism involved in these agents’ effects on carnitine homeostasis is related to an inhibition of hOCTN2 function by taxanes and Vinca alkaloids, rather than an interaction caused by carboplatin, as suggested previously (26). Over the last few decades, a number of studies have established that non-cytotoxic drug-induced alteration of the function of proteins involved in carnitine homeostasis can also lead to deleterious phenotypic changes. An example of this has been recently reported for the antibiotic, cephaloridine (27). In particular, these studies have demonstrated that cephaloridine increases the fractional renal excretion of carnitine, presumably due to interference with a resorption process in the kidney. Similar phenomena have been described with usage of the antiepileptic valproate (28) and the antibiotic pivampicillin (29).

In conclusion, we report that etoposide treatment is associated with excessive urinary loss of carnitines, and that this process is likely associated with direct competitive inhibition of renal tubular resorption of carnitine by the luminal transporter OCTN2. Additional studies are in progress to further understand the toxicological implications of this phenomenon as well as a potential role of OCTN2 in the uptake of etoposide into cancer cells, and the influence of carnitine supplementation on such process.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declared no conflict of interests.

ACKNOWLEDGMENTS

We thank John Killmar and Kelly Filipski for assistance with experiments and data analysis, and Akira Tsuji for providing the OCTN-overexpressing HEK293 cells.
REFERENCES


### Table 1. Influence of anticancer drug on carnitine transport by hOCTN2 and mOctn2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
<th>% of control (mean ± SE) hOCTN2</th>
<th>% of control (mean ± SE) mOctn2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100 ± 4.1</td>
<td>100 ± 4.1</td>
</tr>
<tr>
<td>Prednisone</td>
<td>100</td>
<td>96.2 ± 4.3</td>
<td>n.d.</td>
</tr>
<tr>
<td>Mechlorethamine</td>
<td>100</td>
<td>90.0 ± 4.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>Imatinib</td>
<td>100</td>
<td>89.5 ± 0.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>100</td>
<td>89.0 ± 4.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>100</td>
<td>88.4 ± 5.2*</td>
<td>95.8 ± 7.3*</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>100</td>
<td>87.6 ± 2.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>100</td>
<td>86.7 ± 2.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ifosfamide</td>
<td>100</td>
<td>84.4 ± 4.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>100</td>
<td>83.4 ± 5.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>100</td>
<td>82.0 ± 5.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>100</td>
<td>81.7 ± 1.7*</td>
<td>68.3 ± 1.4*</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>100</td>
<td>81.5 ± 0.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>Clofarabine</td>
<td>100</td>
<td>81.0 ± 0.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cortisone</td>
<td>100</td>
<td>81.0 ± 0.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>100</td>
<td>78.4 ± 4.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>100</td>
<td>76.8 ± 1.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>100</td>
<td>76.6 ± 2.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>100</td>
<td>75.4 ± 1.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>100</td>
<td>73.0 ± 4.7</td>
<td>69.4 ± 8.9</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>100</td>
<td>70.2 ± 5.7</td>
<td>75.2 ± 6.7</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>100</td>
<td>64.9 ± 1.8</td>
<td>29.3 ± 8.1</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>50</td>
<td>62.3 ± 4.5</td>
<td>57.8 ± 2.2</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>100</td>
<td>58.4 ± 1.1</td>
<td>79.9 ± 10.4</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>100</td>
<td>46.2 ± 1.4</td>
<td>63.6 ± 9.5</td>
</tr>
<tr>
<td>Etoposide</td>
<td>100</td>
<td>41.2 ± 4.5</td>
<td>64.3 ± 4.1</td>
</tr>
<tr>
<td>Vincristine</td>
<td>100</td>
<td>33.6 ± 0.1</td>
<td>101 ± 7.3</td>
</tr>
<tr>
<td>Vinorelbine</td>
<td>100</td>
<td>15.3 ± 1.3</td>
<td>65.6 ± 12.1</td>
</tr>
</tbody>
</table>

**Abbreviation:** n.d., not done.

Uptake of carnitine (10 nM) in the presence of various anticancer drug was measured for 30 min at 37°C in uptake buffer (pH 7.4). Each value represents mean ± SE of three determinations. Data were obtained by subtraction of uptake by empty vector pcDNA3 transfected HEK293 cells from that by hOCTN2-transfected HEK293 cells. *Data previously reported in Lancaster et al. (9).
Figure Legends

**Figure 1.** Transport of anticancer drugs by hOCTN2. (A) Transport of daunorubicin, etoposide, paclitaxel, sunitinib, and vinblastine (10 µM; 5-min incubations) in HEK293 cells transfected with an empty vector (VC) or hOCTN2. Influence of pH (B), sodium (C) and carnitine (D) on hOCTN2-mediated transport of etoposide and of etoposide concentration (E) on hOCTN2- or mOctn2-mediated transport of etoposide (10 µM, unless stated otherwise). *, P<0.05 vs VC. All data represent mean (bars) and standard error (error bars) of 3 experiments performed in triplicate.

**Figure 2.** Inhibition of hOCTN2 and mOctn2 function by etoposide. (A) Etoposide-concentration dependent inhibition (10 nM; 5-min incubations) of hOCTN2- (IC50, 55.0 µM; 95%CI, 46.6-65.0 µM) and mOctn2-mediated carnitine uptake (IC50, 148 µM; 95%CI, 122-179 µM) in transfected HEK293 cells corrected for non-specific transport in vector control (VC) cells. (B) Eadie-Hofstee plot showing velocity (V) divided by carnitine concentration (S) versus velocity in the absence or presence of etoposide (I) at a concentration of 100 µM. (C) Influence of etoposide on apical-to-basolateral (AB) and basolateral-to-apical (BA) flux of carnitine (50 nM) in vector control (VC) cells and LLC-PK1 cells transfected with hOCTN2 in the absence or presence of α-toxin to permeabilize the basolateral membrane. All data represent mean (bars) and standard error (error bars) of 3 experiments performed in triplicate. (D) Predicted binding site of carnitine (shown in blue) and (E) predicted binding site of etoposide (shown in yellow) to hOCTN2, showing partial overlap.

**Figure 3.** Influence of etoposide on carnitine homeostasis. Effect of etoposide (10 mg/kg, i.p.) compared with saline on urinary carnitine (A) and total carnitines (carnitine+acylcarnitines) (B).
loss in adult male FVB mice (n=8) within the first 4 days after treatment. Data represent mean (bars) and standard error (error bars). ** P<0.01 versus day -1 (baseline), one-way ANOVA with post hoc analysis. Effect of etoposide-based chemotherapy on urinary loss of carnitine (C) and total carnitines (D) in 5 pediatric patients with acute myeloid leukemia within the first 6 days after initiation of treatment. Each set of symbols connected with a line represent an individual patient.
# Molecular Cancer Therapeutics

## Inhibition of OCTN2-Mediated Transport of Carnitine by Etoposide

Chaoxin Hu, Cynthia S Lancaster, Zhili Zhou, et al.

*Mol Cancer Ther* Published OnlineFirst March 2, 2012.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-11-0980</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://mct.aacrjournals.org/content/suppl/2012/03/08/1535-7163.MCT-11-0980.DC1">http://mct.aacrjournals.org/content/suppl/2012/03/08/1535-7163.MCT-11-0980.DC1</a></td>
</tr>
<tr>
<td>Author Manuscript</td>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
</tr>
</tbody>
</table>

| E-mail alerts | Sign up to receive free email-alerts related to this article or journal. |
| Reprints and Subscriptions | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |
| Permissions | To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org. |