Université de Montréal

The human organic cation transporter OCT1 mediates high affinity uptake of the anticancer drug daunorubicin

Emil Andreev

Microbiology, Infectious Diseases and Immunology, Université de Montréal
Faculty of Medicine

A thesis submitted in fulfill of the requirements of the degree
Magister Scientiae (M. SC.)

Microbiology and Immunology

May,2016

Université de Montréal

Faculté des études supérieures et postdoctorales

The human organic cation transporter OCT1 mediates high affinity uptake of the anticancer drug daunorubicin

Emil Andreev

Thesis examiners:

Dr. Elliot Drobetsky, president of the jury

Dr. Dindial Ramotar, thesis supervisor

Dr. Janos Filep, member of the jury

Résumé

Les anthracyclines tels que la doxorubicin et la daunorubicin sont une famille de médicaments anticancéreux hydrophiles qui doivent être transportés dans les cellules afin d'exercer leur action par intercalation à l'ADN dans le noyau cellulaire. Ceci mène à la perturbation du métabolisme de l'ADN et entraine la mort cellulaire. Les anthracyclines sont utilisés pour le traitement d'une variété de cancers incluant la leucémie, les lymphomes, le cancer du sein, le cancer des poumons et le cancer des ovaires. Étant donné que le transport actif des anthracyclines dans les cellules a partiellement été démontré, le transporteur spécifique impliqué dans ce processus n'est pas encore connu. En utilisant un modèle de cancer des ovaires, la lignée cellulaire TOV2223G, nous avons démontré que des substrats spécifiques au transporteur de cations organiques 1 (OCT1), notamment la ergothionéine, la thiamine et la phenformin, ont partiellement inhibé l'absorption de la daunorubicin en différence de la carnitine qui est un substrat de haute affinité des transporteurs CT2 et OCTN2. Ces résultats suggèrent que les transporteurs organiques spécifiques au transport de la carnitine ne sont pas impliqués dans le transport des anthracyclines. Ainsi, nos résultats ont démontré que l'absorption de la daunorubicin est orchestrée par le transporteur OCT1 dans les cellules TOV2223G (Km ~ 5 μM) et des concentrations micromolaires de choline ont complètement abolies l'absorption de la drogue. De plus, un ARN sh dirigé contre OCT1 a réprimé son expression protéique, ce qui a été confirmé par la technique d'immuno-buvardage en utilisant un anti-OCT1 anticorps. Les cellules déficientes en OCT1 n'ont pas été capables d'absorber la daunorubicin et ont été plus résistantes à l'action de la drogue par rapport aux cellules contrôle. La transfection des cellules HEK293T avec un plasmide construit de façon à faire exprimer OCT1 comme protéine de fusion avec la protéine fluorescente EYFP a montré que celle-ci est localisée dans la membrane plasmique. Les cellules transfectées ont été capables d'absorber cinq fois plus de daunorubicin comparé aux cellules contrôles. Cette étude est, selon nous, la première à démontrer que OCT1 est un transporteur de haute affinité des anthracyclines. Ainsi, nous avons émis l'hypothèse que des défauts de OCT1 peuvent contribuer à l'efficacité de la réponse des cellules cancéreuses à la chimiothérapie avec les anthracyclines.

Keywords : Transporteur de cations organiques 1 (OCT1), anthracyclines

Abstract

Anthracyclines such as doxorubicin and daunorubicin are hydrophilic anticancer agents that must be transported into cells. These drugs accumulate in the nucleus where they intercalate with DNA, thereby interfering with DNA replication in turn leading to cell death. Anthracyclines are used for treating a variety of cancers including leukemia, lymphomas, breast, lung, and ovarian. Despite evidence for active uptake of anthracyclines, the specific transporter has not been identified. Using the ovarian cancer cell line TOV2223G, we show that substrates reported for the organic cation transporter OCT1, such as ergothioneine, thiamine and phenformin, partially compete with uptake of daunorubicin, but not of L-carnitine, i.e., a high affinity substrate transported by hCT2 and OCTN2. These findings exclude the involvement of the L-carnitine organic cation family of transporters in anthracycline uptake. Moreover, we show that OCT1 actively mediates high affinity (Km ~ 5 μM) transport of daunorubicin into TOV2223G cells, whereas micromolar amounts of choline completely abolish drug uptake. shRNA-mediated downregulation of OCT1 causes defective uptake of daunorubicin, as well as significant resistance to the drug, as compared to the vector control. Transfection of HEK293T cells with a plasmid expressing OCT1 as a GFP fusion protein revealed that OCT1-EYFP was predominantly localized to the plasma membrane. These transfected cells manifested nearly 5-fold increased uptake of daunorubicin compared to the empty vector control. In summary, we show for the first time that human OCT1 is a high affinity transporter for anthracyclines. As such, we postulate that OCT1 status represents a critical determinant in the response of cancer cells to chemotherapy with anthracyclines

Keywords: Human organic cation transporter 1(OCT1), anthracyclines

Table of contents

List of tables

List of figures

Abbreviations

A

ABC- ATP-binding cassette

AML- acute myeloid leukemia

B

BCRP- breast cancer resistance protein

C

CALYPSO- Caelyx in Platinum Sensitive Ovarian patients

C_6–NBD–PC- Ethanamimium 2-hydroxy-C_6-*N*,*N*-dimethyl-*N*-[2-*N*-(2,1,3-benzoxadiazol-4-amine,-*N*-methyl,-7- nitro)-ethyl] bromide- phosphatidylcholine

CD- Carboplatin/cisplatin and paclitaxel

cDNA- complementary DNA

CHP- cyclo (his-pro)

CHT1- Choline transporter 1

CT1, 2- Carnitine transporter 1, 2

CTL-1- Choline transporter-like 1

cRNA- complementary RNA

D

DAT- Dopamine transporter

DOX- Doxorubicin

DNR- Daunorubicin

DSB- double strand break

DRG- dorsal root ganglion

E

EMA- European Medicines Agency

EMT- Extra neuronal monoamine transporter

ETT- Ergothineine transporter

EYFP- Enhanced yellow fluorescent protein

F

FACS- Fluorescence Activated Cell Sorting

FDA- Food and Drug Administration

G

GAT2- GABA transporter 2/ sodium- and chloride-dependent GABA transporter 2

H

HFN-4α- Hepatocyte nuclear factor 4 alpha

M

MDR- Multidrug resistance

MFS- Major Facilitator Superfamily

1-MPP- 1-methyl-4-phenylpyridinium

MRP- Multidrug resistance protein

MXR- Multixenobiotic resistance

N

NAPDH- Nicotinamide adenine dinucleotide phosphate

NBD-choline- Ethanamimium 2-hydroxy- *N*,*N*-dimethyl-*N*-[2-*N*-(2,1,3-benzoxadiazol-4-amine,-*N*-methyl,-7- nitro)-ethyl] bromide- choline

NCI- National Cancer Institute

NKT- Novel kidney transporter

O

OAT- Organic anion transporter

OC- Organic cations

OCT- Organic cation transporter

OCTN- Novel organic cation/zwitterion transporter

P

PAH- p-aminohippuric acid

PD- PEGylated DOX

PFA- paraformaldehyde

R

RT-qPCR- Quantitative reverse transcription polymerase chain reaction

S

SAL- Salsolinol

SLC- Solute carrier

SNc- *substantia nigra pars compacta*

T

TCGA- The Cancer Genome Atlas

TEA- Tetraethyl ammonium

Acknowledgments

First and foremost, I would like to thank my supervisor Dr. Dindial Ramotar for his mentorship and constant support throughout my master's degree study. Dr. Ramotar has given me the opportunity to be a part of his team and to develop a passion in science and in cancer research. I also would like to thank our research assistant Tara Harihar and all lab members for their help, guidance and also for their enjoyable company. I want to thank Nicolas Brosseau for his devoted work and contribution in OCT1 research. I want to thank Dr. Euridice Carmona and Dr. Anne-Marie Mes-Masson for their expertise and contribution in completing the present thesis.

Lastly, I would like to thank my family for their constant encouragement and support.

Chapter 1: Introduction

1. Chemotherapy and DNA-damaging agents

Cancer is a complex disease that is characterized by deregulated cell proliferation caused by various factors including genetic and environmental (1). Currently, cancer is responsible for more than seven million deaths worldwide according to statistics from the World Health Organization. The battle with cancer has been led for more than four decades as all began with the War on Cancer with the President Nixon's National Cancer Act of 1971 (2). Despite the increasing success of recently introduced novel cellular therapies, such as hematopoietic stem cell transplantations, treatment of cancer is still widely based on the use of chemotherapeutic agents to eradicate cancer cells, to reduce tumor growth, and to ameliorate patient's quality of life. During the World War I and II, when studying victims exposed to sulfur mustard gas, important discoveries were made using mice as a model. By injecting stable nitrogen mustard compounds, it was observed an important tumor regression. Moreover, leukemia patients treated with folic acid showed increased proliferation of acute lymphoblastic leukemia cells (1). This has led to the discovery of folate analogs such as aminopterin whose action led to successful, yet short, remissions. Nonetheless, the most important role of these compounds was finally established and that consisted of damaging DNA (1). However, it took another ten years before the discovery of the exact mechanisms by which these compounds caused DNA damage. Hence, it was established that nitrogen mustard act by directly alkylating DNA on purine bases. Based on this mechanism, derivatives such as cyclophosphamide, chlorambucil and melphalan were developed. In addition, platinum agents possessing DNA alkylating capabilities were also discovered and led to a new era of anticancer drug research. It all began with the accidental discovery of cisplatin by Rosenberg et al., (3) followed by the development of other platinum derivatives such as carboplatin and oxaliplatin (4). However, cisplatin remains the most used platinum drug and cisplatin therapy succeeds to treat more than 90% of all testicular cancer patients, and has a good efficacy in treating ovarian, bladder, and head and neck cancers (5). A second mechanism of DNA damage is the interference with DNA replication, and antimetabolites which are part of anticancer drugs, have the ability to mimic endogenous cellular molecules. Such antimetabolites are the pyrimidine analogs 5-fluorouracil

(5-FU), gemcitabine, floxuridine, and capecitabine whose action consists of incorporating purine and pyrimidine analogs into DNA during S phase which in turn prevents addition of normal nucleobases leading to abrogation of DNA replication (1). Lastly, a third mechanism of DNA damage is interference with the normal DNA function by targeting protein-DNA complexes. Topoisomerases are a class of enzymes that participate in the release of the torsional strain of the DNA double helix (1, 6). Topoisomerases act by causing transient single strand breaks (topo I) or double strand breaks (topo II) (6). Topoisomerase poisons have the ability to trap the complex formed by DNA –enzyme as intermediate which prevents religation of the break leading to multiple alterations in DNA transactions such as replication fork progression in turn causing fatal double strand breaks (DSBs) (1, 6) . Anthracyclines represent a very important class of anticancer drugs that act as topoisomerase II poison and are the most effective anticancer agents ever used in treatment of a variety of cancers (7).

1.1. Anthracyclines

The first anthracyclines, i.e., daunorubicin (DNR) and doxorubicin (DOX), were isolated in 1960 form *Streptomyces peucetius*. DNR (trade name Cerubidine) and DOX (trade names Adriamycin and Rubex) have similar structures characterized by aglyconic and sugar moieties. The aglyconic structure consists of a tetracycline backbone attached to the sugar moiety referred to as daunosamine by a glycosidic bond. DOX and DNR differ structurally only at C-14, terminating with alcohol and methyl groups respectively (figure 1, indicated with arrows) (6, 8)

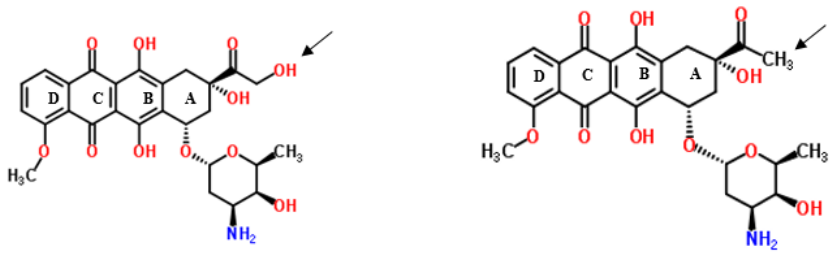


Figure1. Chemical structure of DOX and DNR. Adapted from (6)

1.2. Anthracyclines as topoisomerase inhibitors

Anthracyclines kill cancer cells by several mechanisms including (i) DNA intercalation and thus interference with DNA replication, (ii) generation of reactive oxygen species (ROS) which damage DNA and other cellular macromolecules, (iii) poisoning topoisomerase II (topo II), and (iv) interference with DNA unwinding and helicase activity (6). Interference with topo II activity consists of modifying DNA topology without affecting DNA structure or sequence. As mentioned above, topoisomerases act by causing transient single strand breaks (topo I) or double strand breaks (topo II) (6). Numerous studies have focused on drugs targeting topo II such as etoposide. This drug "traps" topo II at break sites, i.e., stabilizes the cleavage complex by blocking the DNA ligation step. Anthracyclines are believed to act similarly to etoposides; for example, topo II activity correlates with the anticancer efficiency of anthracyclines in a mouse model of lymphoma (9). However, DOX was shown to trigger cell death independently of topo II in a promyelocytic leukemic cell line where it rather resulted in histone eviction (9). Anthracyclines intercalate into DNA at sites adjacent to GC base pairs due hydrogen bond formation with guanine, leading to the formation of DNA adducts which are characterized as DNA fragments bound to an antineoplastic agent. Moreover, anthracycline-DNA interactions are stabilized by formation of covalent bonds mediated by cellular formaldehyde and generated by free radical reactions that result from carbon sources supplied by lipids and spermine. As a result, anthracyclines form a covalent bond on one strand of the DNA and a hydrogen bond with guanine on the opposite strand (9).

1.3. Anthracyclines and ROS formation

The entry of the anthracyclines in the cell is accompanied by the addition of one-electron to the quinone moiety present in ring C (figure 1) which results in the formation of semi-quinone. Regeneration of quinone is followed by reducing oxygen to superoxide anion and hydrogen peroxide (6). This formation is maintained by a number of NADPH-oxidases including mitochondrial NADPH, cytochrome P450 and others. Moreover, a further contribution of the semi-quinone is to oxidize the bond between ring A and the sugar moiety daunosamine which results in a deglycosylation followed by the formation of deoxyglycone. This process is

believed to confer more lipid solubility which allows the anthracyclines to easily incorporate into biological membranes creating reactive oxygen species (10), (6).

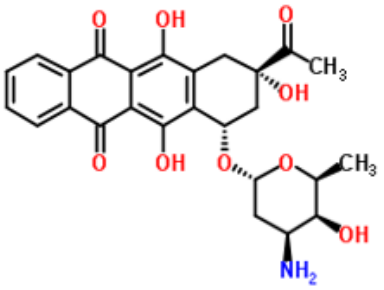
1.4. Anthracyclines and cardiotoxicity

Despite the effectiveness of anthracyclines as anticncer drugs, there are important limitations, particularly cardiac toxicity in the form of myocyte damage, reduced contractility, and total cardiac failure (11). Hefti et al., (12), reported that multiple processes are involved in cardiomyocyte dysfunction, including the implication of first stage alcohol metabolites in the disruption of iron and calcium homeostasis. This alters mitochondrial calcium release which, as a consequence, impairs mitochondrial creatine kinase activity. Overall, perturbed mitochondrial function is believed to underlie severe cardiomyocyte deregulation. Cardiomyocyte failure is further promoted by release of anthracycline-induced ROS coupled with a substantial decreases in the level of detoxifying enzymes such as superoxide dismutase and deregulation of nitric oxide production (12).

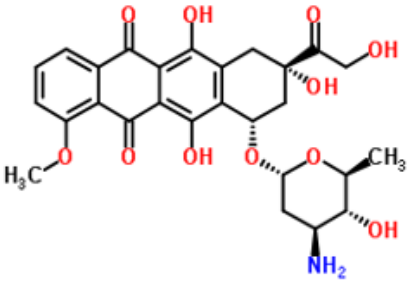
1.5. Novel generations of Anthracyclines

To counteract anthracycline toxicity, new semi-synthetic and synthetic formulations have been produced for approval by the Food and Drug Administration (FDA) and European Medicines Agency (EMA). Only few semisynthetic anthracyclines derivatives, i.e., idarubicin, epirubicin, pirarubicin, aclarubicin and mitoxanthrone, have obtained such approval (figure 2). Idarubicin, derived from DNR, appears to be more effective than DNR in several aspects including increased liposolubility and oral administration (13). Epirubicin, a close relative to DOX, has been used mostly in treatment of breast cancer with relatively good success especially when in combination with cyclophosphamide and 5-fluorouracil. However, similar to DNR and DOX, epirubicin induces significant cardiotoxicity (8). A study in rats investigated the effectiveness of pirarubicin, another close relative of DOX. The former manifested significantly lower cardiotoxicity, but cancer cell killing was also less effective compared to DOX (Adriamycin) (14). Aclarubicin and aclamycin were also shown to display only a relatively modest cardiotoxicity improvement over DNR and DOX. Mitoxanthrone which is similar to DOX has

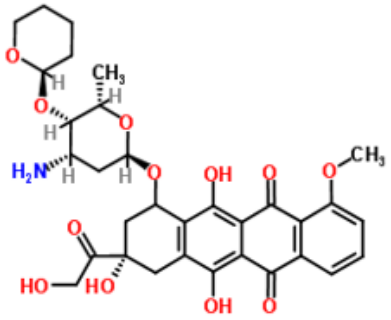
been extensively tested for treatment of breast cancer, prostate cancer and leukemia because of reduced cardiotoxicity. Nonetheless, Thomas and coworkers reported that mitoxanthrone induces cardiotoxicity levels similar to DOX and DNR (6, 15)



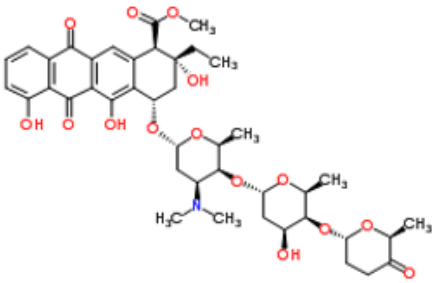
Idarubicin



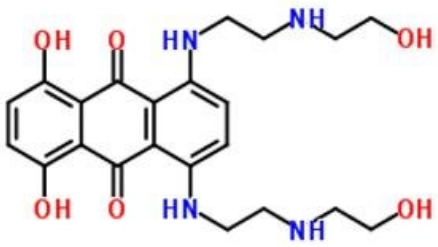
Epirubicin



Pirarubicin



Aclarubicin



Mitoxanthrone

Figure 2. Chemical structures of various anthracyclines derivatives.

2. Transporters of Anthracyclines

Anthracyclines are positively charged drugs that must be actively transported into and out of cells. Numerous studies have revealed two major families of anthracycline transporters which will be discussed here in some detail: (i) the ATP-binding cassette family (ABC) and (ii) the organic cation transporters (OCTs). In addition, the organic anion transporters (OATs) will also be reviewed briefly since they are part of SLC22 subfamily and have been reported to interact with typical OCT substrates.

2.1. ATP-binding cassette family (ABC)

The ABC transporter family has a role in tissue protection against toxic compounds including endogenous metabolites and exogenous xenobiotics. They have also garnered attention for their role in chemoresistance (16). This family of transporters consists of seven subfamily members designated A to G and are encoded by 48 genes (17). They display broad tissue distribution and are present in key physiological barriers such as the biliary canalicular membrane of hepatocytes, apical membrane of the proximal tubules of the kidney, brush border membrane of intestine and epithelium of the blood-brain barrier (17). Various studies have established that among all ABC transporters, only 12 members belonging to 4 subfamilies exert an important role in drug resistance (17).

2.1.1. The ABCB subfamily transporters

The ABCB subfamily of transporters, also known as multidrug resistance (MDR) proteins, includes the well characterized P-glycoprotein which transports mostly anionic compounds (16), but also various positively charged antineoplastic drugs such as anthracyclines, taxanes and vinca alkaloids. P-glycoprotein is expressed in various tissues such as liver, kidney and intestine, as well as specialized stem cells of the immune system (17). Moreover P-glycoprotein is expressed in the blood-brain barrier which prevents access of many xenobiotics to the central nervous system (18). Such xenobiotics have been reported to modulate P-glycoprotein. For example, it was reported that cationic (hydrophilic) compounds

can strongly compete with hydrophobic ones, thereby acting as either inhibitors or inducers of P-glycoprotein. Some drugs that specifically inhibit P-glycoprotein interact with digoxin leading to toxic accumulation of this cardiac glycoside. To prevent this accumulation, inducers of P-glycoprotein are required (18). There are two types of P-glycoprotein inhibitors: calcium channel blockers and beta blockers. Examples of the former type include nicardipine, verapamil, diltiazem, nifedipine, while the latter type includes carvedilol, bisoprolol, propranolol (18).

2.1.2. The ABCC subfamily transporters

The ABCC subfamily transporters, known as multidrug resistance-associated proteins (MRPs), differ structurally from ABCB counterparts in possessing additional domains aside from the common nucleotide binding- and transmembrane-domains. In fact, some members of the ABCC1, ABCC2, ABCC3, ABCC6 and ABCC10 subfamilies possess an N-terminal membrane domain attached to the core structure by a cytoplasmic linker, whereas ABCC4, 5, 11 and 12 lack an N-terminal domain but retain the cytoplasmic linker (17). ABCC transporters also differ in the ability to transport negatively charged compounds. This evolutionary property of the ABCC transporters confers improved protection from differently charged toxic biomolecules (17).

2.1.3. The ABCG subfamily transporters

ABCG subfamily transporters contains, among others, their most important member involved in drug resistance, i.e., ABCG2 efflux transporter also known as MXR or BCRP (17). ABCG2 was first discovered based on its ability to transport mitoxanthrone and it was reported that this substrate is not transported either by P-gp or MRP1 (19). Unlike P-glycoprotein which more selectively transports negatively charged compounds, ABCG2 can transport either negatively or positively charged compounds. It was reported that unlike P-glycoprotein, ABCG2 cannot transport key substrates such as verapamil, taxols, imatinib, *Vinca* alkaloids and anthracyclines (17). Figure 3 presents an overview of some of the ABC transporters and their localization in various tissues.

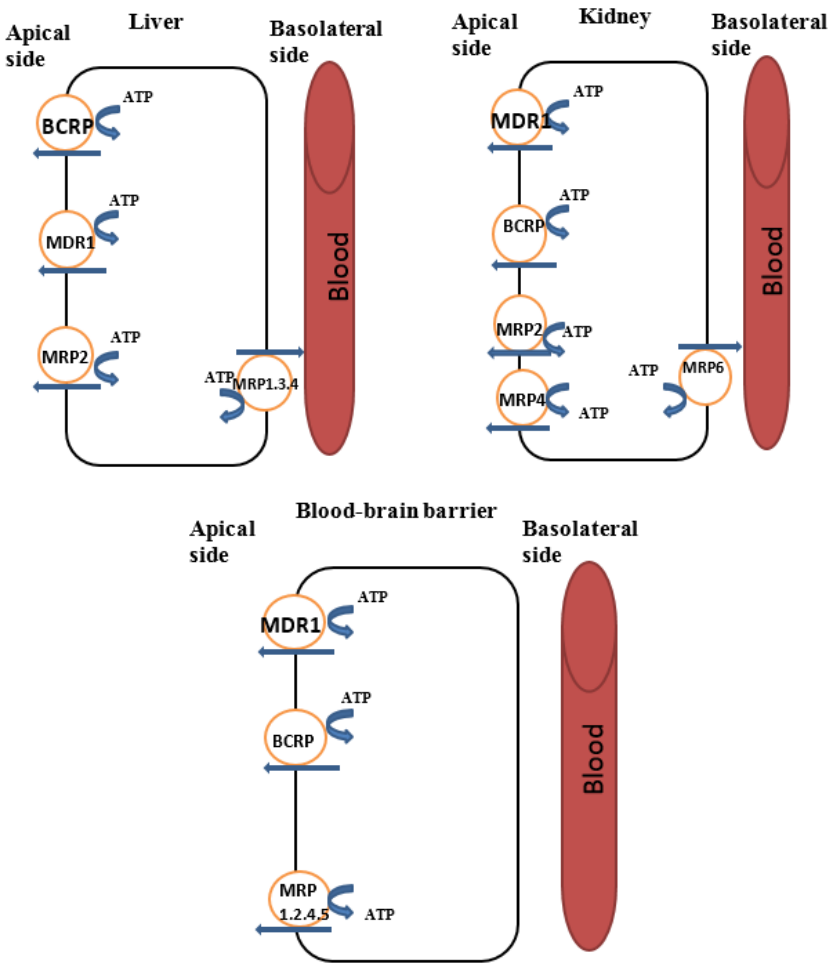


Figure 3 Schematic representation of the ABC transporter localization in liver (hepatocytes), kidney (proximal tubule cells) and blood-brain barrier (brain endothelial cells). Adapted from (20).

2.2. Mechanism of transport by ABC transporters

Drug transport by ABC family members consists of two stages: ATP hydrolysis which activates transport, followed by export of the compound from the cytoplasm to the extracellular space. However, the exact mechanism by which the binding and subsequent transport occur remain unclear (16). The following mechanism has been proposed: ATP-

dependent drug binding (first stage) is followed by physical translocation from the cytoplasm to the extracellular space (second stage). In the first stage, ATP binding occurs in the nucleotide binding domain, consisting of three highly conserved sequences and the signature C motif. All ABC transporters display high ATPase activity in the micromolar range, but low affinity for ATP in the millimolar range (16). The second stage is highly dependent on the structure presented by the transporter. For example, P-glycoprotein (ABCB1) has 12 transmembrane domains and 2 nucleotide binding domains while ABCG2 displays only half of both. This suggests that the latter is likely to homodimerize. Because of its function in expelling toxic compounds outside the cell, the substrate binding site is located on the cytoplasmic side of the plasma membrane and consists of transmembrane helices which form a binding pocket (16). Studies have revealed that additional binding minipockets are located adjacent to the main binding pocket, together forming a funnel-like shape with the narrowest part facing the cytoplasm. Two co-substrates can easily enter the narrow opening and determine either massive and rapid efflux of the targeted compound or its inhibited transport regulated by the binding of the inhibitor substrate (16).

3. Organic cation transporters (OCTs and OCTNs)

OCTs belonging to the SLC22 family are part of the major, highly-conserved facilitator superfamily (MFS) of transporters (20). OCTs include two subclasses based on substrate specificity:1) OCT1, OCT2, and OCT3 and 2) zwitterion/cation transporters OCTN1, OCTN2, OCTN3 and CT2/OCT6 (20, 21). The OCTs subgroup (OCT1-3) exhibits close similarity in substrate specificity and transport mechanism. They all translocate various cations across the plasma membrane. The subclass of OCTNs (OCTN1, OCTN2 and CT2) are either uniporters of cations and zwitterions, and differ from OCTs in their ability to transport carnitine to the peroxisome for beta oxidation in a sodium-dependent manner (symport mechanism) (20).

3.1. OCT1

OCT1 (gene number SLC22A1) is located on chromosome 6. The first characterization of OCT1 was carried out by Gründemann et al., (22). Using a *Xenopus* oocyte-based expression system, this group successfully isolated OCT1 cDNA from a rat kidney library. The reported clone contained an open reading frame encoding a plasma membrane protein of 61.5 kDa. Overexpression of this protein in oocytes was shown to correlate with increased uptake of tetraethyl ammonium (TEA) with Km of approximately 100μM (22). Despite evidence that TEA transport by OCT1 is potential-dependent, it was shown that inwardly or outwardly proton gradients of 1 pH unit do not alter significantly uptake of this substrate (22). In mouse and rabbit, using RT-qPCR, mRNA levels were detected in kidney and to a lesser extend in liver (20). In humans, OCT1 is predominantly expressed in liver and less so in kidney. hOCT1 mRNA levels are also detected in the immune system and various organs such as small intestine, heart, skeletal muscle, brain, eye, placenta and others (20).

3.1.1. OCT1 as metformin transporter

Metformin belongs to the class of the biguanides that are used to treat type 2 diabetes; indeed, this drug efficiently reduces gastrointestinal glucose absorption and glucose production in the liver (23) (24). Metformin is initially activated through phosphorylation by AMP-activated protein kinase which in turn greatly reduces glucose production in hepatocytes thus leading to enhanced uptake of circulating glucose by the muscle and hepatocytes (24). OCT1 is a determinant of metformin uptake and it was reported that variations in response to metformin are attributable to genetic variants of OCT1 as reported by Shu et al., (24). The authors reported that several genetic variants displayed normal uptake of the model OCT1 substrate 1-methyl-4-phenylpyridinium (1-MPP), but greatly reduced metformin uptake suggesting the determinant role of OCT1 in the transport of metformin.

3.1.2. OCT1 as thiamine transporter

Chen et al.,(23) reported that OCT1 is a high capacity thiamine transporter. Thiamine is transported by two high affinity transporters belonging to the SLC19 family, i.e., members 2 and 3 which act in sodium-dependent manner. However, using both human and mouse hepatic cells, the authors reported that OCT1 retains the ability to take up thiamine with high affinity. This seems consistent with the fact that thiamine is directed to the liver where OCT1 is predominantly expressed, and particularly in the central vein where important metabolic reactions occur.

3.1.3. OCT1 as donepezil transporter

Kim et al., (25) reported that OCT1 has an important role in transport of donepezil, a therapeutic agent for Alzheimer's disease which is characterized by progressive degeneration of cholinergic neurons. Donepezil is a cholinesterase inhibitor which acts similarly to tacrine by increasing levels of the neurotransmitter acetylcholine in the presynaptic cleft of the cholinergic neurons. It was shown in rat brain, that donepezil is taken up with high affinity by CHT1 and OCT1 and to a lesser extent by other organic cation/zwitterion transporters such as OCT2 and OCTN2 (25). CHT1 (CHoline Transporter 1), is the main transporter for acetylcholine in neurons and manifests the highest affinity for donepezil. However, Murakami et al., (26) showed that CHT1 is a close relative to OCT1 in terms of substrate specificity, and also reported that CHT1 in the blood-brain barrier might in fact be an isoform of the OCTs family (26)

3.1.4. OCT1 as choline transporter

Choline plays an important role in cell homeostasis by providing phospholipid compounds for the plasma membrane. In addition, it is involved in methionine metabolism as a methyl donor and in neurotransmission by providing acetylcholine (27). Moreover, deficiency in choline is associated with various neuronal and hepatic disorders and is also linked to various cancers (27). Choline is transported by three systems based on tissue localization and substrate affinity. The highest affinity choline transporter is CHT1, which delivers acetylcholine in cholinergic neurons. The role of the organic cation transporters OCT1 and OCT2 in choline

transport is however unclear and results vary from study to study (27). For example, overexpression of either OCT1 or OCT2 in human embryonic kidney HEK293T cells revealed increased uptake of choline by OCT1, but not by OCT2 (28). Another study showed that both OCTs were efficient in choline uptake in *Xenopus* oocytes (29). Various reports also claim considerable variability of the Michaelis-Menten affinity constant ranging from 100μM to 350 μM for either OCT1 or OCT2 (27). However, the evidence for OCT1 as a donepezil transporter in rat brain supports its role in choline and/or acetylcholine uptake in both neuronal and non-neuronal cells (25); nonetheless more studies are needed to determine whether OCT1 is indeed able to transport choline independently of CHT1 and/or OCT2.

3.1.5. OCT1 as transporter for anticancer drugs

Anticancer therapy outcome depends on the activity of the anticancer agent and the cellular machinery (30). For example, platinums such as picoplatin, oxaliplatin and cisplatin activities lead to differences in the formation of DNA adducts depending on the activity of the nucleotide excision repair mechanism to repair the resulting DNA damage. Thus, the cytotoxicity effect of this formation depends on the cellular expression of transporter proteins for these platinum drugs. More et al., (30) demonstrated that expression of OCT1, OCT2, but not OCT3 significantly enhances the uptake of picoplatin and the resulted formation of DNA adducts. Disposition of picoplatin (i.e. absorption, distribution, metabolism and excretion) was shown not to be dependent on OCT1 expression as demonstrated by pharmacokinetic studies (30). It also was shown that OCT1 expression greatly influences oxaliplatin uptake, but not carboplatin or cisplatin in two independent studies done by Zhang et al., (31) and Yokoo et al., (32). OCT1 expression levels were also demonstrated to correlate with imatinib action in treating patients with chronic myelogenous leukemia (CML) as shown by White et al., (33). Taxanes such as paclitaxel are important class of drugs whose action interferes with microtubule proper function leading to deregulation in mitosis (34). The role of OCT1 in the transport of paclitaxel was investigated by Gupta et al., (35) who demonstrated that OCT1 expression in model lymphoma cell lines and in primary chronic lymphocytic leukemia patients samples is a determinant of enhanced paclitaxel uptake. Moreover, the authors investigated the uptake of another anticancer agent- irinotecan, and found that OCT1 expression pattern influences the efficacy of treatment with this particular agent (35).

3.2. OCT2

OCT2 (gene number SLC22A2) was first cloned in 1996 by Okuda et al. (36) from rat. Functional analysis of the open reading frame revealed that rOCT2 is a membrane protein containing twelve membrane spanning helices with an extracellular loop between helices 1 and 2 and two glycosylation sites. The extracellular loop, which contains the substrate binding pocket, was reported to be highly similar to rOCT1 with 85% identity. The authors also identified four potential phosphorylation sites for protein kinase A and two phosphorylation sites for protein kinase C. By injecting OCT2 cRNA in *Xenopus* oocytes, uptake studies with TEA showed a marked increase in transport rate that was greatly decreased in the case of cimetidine, quinidine, and procainamide. By varying the pH, it was established that OCT2 acts independently of proton gradient and/or environmental pH (36). Further studies investigating the role of OCT2 revealed that this transporter mediates, among others, electrogenic transport of various organic cations such as N-methyl-nicotinamide and choline (37). A study from Mitohashi et al., (38) reported that human OCT2 is the main transporter in kidney and is similar to rat OCT2 which is localized on the basolateral membrane of proximal tubules. Moreover, immunohistochemistry studies on human OCT2 also showed basolateral membrane localization (38). However, there was a discrepancy over OCT2 localization from that study and a study from Gorboulev et al., (39) that showed OCT2 being localized on the apical membrane in distal tubules of human kidney. The marked difference was associated with the antibody which apparently displayed some cross-reactivity with closely related transporters in the same region (38). Several other studies investigating the role and behavior of OCT2 have also been conducted. In terms of substrate binding and stability, Barendt et al., (40) showed that among all organic cation transporters, interactions between substrates and OCT2 are greatly influenced by the charge in such way that when there were variations in the pH of the environment, an increase or a decrease of a protonated fraction of a substrate was observed. This, in consequence, affected the binding properties of substrates for OCT2. However, these changes did not affect the model substrates TEA and 1-MPP (40). Aside from its role in kidney, OCT2 was found in the brain where it participates in the transport of corticosterone (41). Although uptake of this steroid is mainly transported by extra neuronal monoamine transporter or EMT (see OCT3/EMT), the authors noted that OCT2 and OCT3 are not

identical and can be distinguished pharmacologically by use of O-methyllisoprenaline which appears to inhibit potently EMT, but not OCT2 (41). OCT2 can also transport several neurotransmitters, but most importantly dopamine in both kidney and brain. Evidence for OCT2 involvement in dopamine clearance came from a study reporting that proximal and distal tubules were able to express dopamine synthesizing enzyme L-aromatic amino acid decarboxylase. In support of this, cells forming the proximal tubules are known to produce dopamine which is believed to act as natriuretic hormone (41).

3.2.1. OCT2 as metformin transporter

Based on the primary localization of OCT2 in kidney, and as renal excretion is the major pathway for the elimination of xenobiotics, OCT2 plays an important role in detoxification. Among several drugs, OCT2 appears to be critical for disposition of metformin (42, 43). Numerous studies have been conducted to evaluate the relationship between OCT2 and the pharmacokinetics of metformin. Although the uptake of metformin was shown to depend on OCT1 function, Kimura et al., (44) claimed that metformin is a better substrate for OCT2 than for OCT1. Nonetheless, the role of OCT2 in metformin uptake is mostly associated with several genetic OCT2 variants such as A270S, M165I, R400C and K432Q which are nonsynonymous single nucleotide polymorphisms (SNPs) (42, 43). It was shown that metformin pharmacokinetics are deregulated in humans presenting these mutations. In addition, these mutations are ethnic-specific and the A270S variant represents the most important allele frequency for five ethnic populations including Caucasians, Afro-Americans, Asian-Americans, Mexicans and Pacific Islanders. M165I and R400C variants are present only in Afro-Americans and K432Q is present in both Afro-Americans and Mexicans (43).

3.2.2. OCT2 role in Parkinson's disease

Parkinson's disease is a neurological disorder which is characterized by loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNc) region of the brain (45, 46). In healthy individuals, normal functioning of dopaminergic neurons is maintained by a normal distribution of the dopamine transporter (DAT) whose role is to remove circulating dopamine

from presynaptic cleft in order to terminate neurological synapse (45, 46). However, Taubert et al., (47) reported an unexpected role of OCT2 in maintaining the healthy state of these neurons. They demonstrated that OCT2, besides of its role in secretion and removal of various cations in kidney, has a particular role in human brain where it localizes in SNc and where it colocalizes with DAT and tyrosine xydroxylase. In addition, OCT2 participates in the exclusive transport of two endogenous compounds, i.e., cyclo (his-pro) (CHP), and salsolinol (SAL). The first compound has a neuroprotective role by contributing to the maintenance of presynaptic transactions and by stimulating neuronal mitochondrial respiration. Inversely, SAL appears to act as a neurocytotoxic compound by inhibiting mitochondrial respiration and therefore by promoting oxidative stress (47). Moreover, OCT2 assures a strict balance in secreting CHP and SAL in such way that the ratio between the two is maintained with increased CHP levels. It was also demonstrated that the mutant OCT2 R400C loses the ability to transport CHP while maintaining SAL levels; moreover this switch may be detrimental for proper neuronal functioning and is therefore favorable for Parkinson's disease development (47)

3.2.3. OCT2 as transporter for anticancer drugs

As mentioned above, cisplatin is one of the most effective antineoplastic agents used for treating a variety of solid tumors. In a study from Filipski et al., (48), it was shown that mice deleted in OCT1 and OCT2 severely abrogated urinary excretion of cisplatin, although plasma levels were not affected. Moreover, mice deficient in both OCTs was efficiently protected from cisplatin induced renal tubular damage. However, patients presenting OCT2 variant rs316019 were less affected from cisplatin cytotoxicity compared to reference OCT2. Therefore, the authors concluded that OCT2 possesses a critical role in renal toxicity induced by cisplatin (48). The role of OCT2 in cisplatin handling was also reported by Tanihara et al.,(49) and the authors also demonstrated the protective role of imatinib on renal nephrotoxicity caused by cisplatin. As mentioned above, OCT2 also participates in oxaliplatin and picoplatin uptake (30-32).

3.3. OCT3

OCT3 (gene number SLC22A3) was independently identified in 1998 by two groups from Japan and Germany. Kekuda et al., (50) using two different expression systems, i.e., X. *laevisoocyte* and mammalian expression system (HeLa cells), reported that the newly identified protein was electrogenic cation transporter mainly expressed in fetal basal membrane of placenta and not in liver or kidney. Based on membrane localization, the authors reported that the main role of this transporter is to participate in the first line of defense against toxic xenobiotics. Data analysis also showed that interactions with OCT1 and OCT2 were negligible. OCT3 has the capacity to transport 1-MPP similarly to OCT1 and OCT2, but can also transport serotonin and desipramine (50). Transport of catecholamines occurs via two different systems: neuronal monoamine transporter (uptake1) and extra neuronal monoamine transporter (uptake2). The latter is characterized by the ability to transport catecholamines in an Na^+ and Cl^- independent manner as reported by Wu et al., (51). Moreover, the authors identified OCT3 as extra neuronal monoamine transporter (EMT) or uptake2 system. OCT3 can transport various compounds such as amphetamine and methamphetamine, serotonin and dopamine. Thus, a role in handling of neuroactive compounds in the brain was exclusively attributed to OCT3 (51). A functional study from Gründemann et al., (52) using amino acid sequence analysis revealed that OCT3 is similar to OCTs and organic anion transporters (OATs) with respectively 50% and 30% homology. The protein is composed of 556 amino acids and contains 12 putative transmembrane domains. Gene localization was mapped to 6q27 locus using fluorescence in situ hybridization (52). Regarding the distribution and the function of the organic cation transporters in the brain, both OCT2 and OCT3 are mainly expressed in cortex, thalamus, and hypothalamus. However, within these regions, OCT2 and OCT3 occupy different sub regions. Although both OCT2 and OCT3 are expressed in the central nervous system, OCT3 seems to be exclusively expressed in astrocytes that are localized in restricted areas (53). In the brain, OCT3 appears to perform a very important function by regulating osmotic pressure by salt ingestion (53). The exact mechanism by which OCT3 regulates the osmolarity is however unclear, but it is believed that it might participate in activation of secondary neuronal pathways responsible for insuring proper ways of neurotransmission which go through osmosensitive regions and other relay regions (53).

OCT3 is also believed to be strongly involved in stress, as it was labeled a corticosterone sensitive monoamine transporter. Moreover, in view of the fact that OCT3 has a high affinity for dopamine and its clearance, this transporter is strongly linked to sensitivity to psychostimulants and therefore to addiction behavior (53). These data suggest that brain transporters from the group of OCTs have a special role to play in drug transport and are targets for new drug development.

3.3.1. OCT3 as transporter for anticancer drugs

The role of OCT3 in the uptake of irinotecan, vincristine and melphalan was investigated by Shnitsar et al., (54). By stably transfecting Chinese hamster ovary (CHO) cells with human OCT3 cDNA and performing competition studies with (3) H]MPP (4-methyl-pyridinium iodide and either melphalan, irinotecan or vincristine, the authors reported a significantly inhibited transport of (3) H]MPP (4-methyl-pyridinium iodide by these three agents suggesting the important role of OCT3 in handling of these antineoplastic compounds (54).

3.4. OCTN1

In 1997, Tamai et al., (55) cloned a cDNA corresponding to the novel organic cation transporter OCTN1 (gene number SLC22A4), which was then transiently expressed in HEK293T cells. The transfected cells were evaluated for transport capacity using the model organic cation transporter substrate TEA. The results showed that transport was saturable, energy dependent, and pH-sensitive. However, based on kinetic studies, OCTN1 exhibited a higher Michaelis-Menten constant compared to both OCT1 and OCT2, i.e. 440 μM vs 20 μM -100μM. Moreover, the authors revealed that OCTN1 operates in the apical membrane of renal epithelial cells. Because of the presence of a nucleotide binding motif, OCTN1 was suggested to act as an ATP-dependent transporter and it was shown that at pH 6.0 under ATP depletion, TEA transport was greatly decreased (55). Since OCTN1 is expressed in various human cell lines, Tamai et al., (55) suggested that it may participate in the efflux of anti-cancer drugs, although functional studies needed to be done in order to identify substrate specificity. A few years later, Gründemann et al., (56) expressed OCTN1 in HEK293T cells

and cells were assayed for transport of various substrates including TEA, carnitine, the anti-oxidants ergothioneine, stachydrine (proline betaine) and betaine. Surprisingly, they reported that uptake of TEA and carnitine was negligible compared to that of ergothioneine, stachydrine and betaine and proposed OCTN1 be named ergothioneine transporter (ETT). However, despite their recommendation for the new appellation, both OCTN1 and ETT refer to gene number SLC22A4. Using RT-PCR, tissue distribution of OCTN1 was revealed to be highest in bone marrow and fetal liver (56). Consistent with other localization studies, OCTN1 was reported to be specifically expressed in monocytes (CD14) and absent in stem and blast cells. Strong OCTN1 expression in small intestine and kidney was linked to its function in initial absorption and reabsorption of ergothioneine (56). Moreover, Gründemann et al., (57) reported that the newly proposed OCTN1 is not expressed in human liver, and to a very low extent in heart, muscle and skin. OCTN1 appears to be expressed in various species including rat and mouse. Despite the reported lower affinity of OCTN1 for carnitine, Pochini et al., (58) reported that structural analysis of OCTN1 binding pocket can fit substrates like acetyl carnitine and acetylcholine. However, when carnitine and ergothioneine are present, there seems to be a different structural organization of these molecules that prevents overlapping with the cation site, which explains the noncompetitive interaction with TEA. Regarding the transport of acetylcholine, Pochini et al., (58) also reported that human OCTN1 is involved in the bidirectional transport of this substrate. As epithelial cells of the small intestine and immune system cells such as lymphocytes participate in extra neuronal cholinergic transport, and since OCTN1 is predominantly expressed in these regions, it was suggested that this transporter may participate in catalysis of neo synthesized acetylcholine in order to activate receptor pathways in autocrine and paracrine mode (58). In heart, OCTN1 seems to play a role in absorption and removal of cations thereby modulating myocardial biogenesis (58)

3.4.1. OCTN1 as transporter for anticancer drugs

The role of OCTN1 in anticancer drug uptake was investigated by two groups. Okabe et al., (59) performed a screen with 60 cancer cell lines from the National Cancer Institute (NCI-60) for anticancer substrates. This revealed that DOX and mitoxanthrone, a close DOX relative, are substrates for OCTN1. Jong et al., (60) investigated the role of OCTN1 and OCTN2 in oxaliplatin uptake and cytotoxicity. Using HEK293T cells overexpressing OCTN1 and

OCTN2 and primary dorsal root ganglion (DRG) neurons, data from this study showed an increased uptake and cytotoxicity of oxaliplatin in both model systems compared to mock. The authors also showed that uptake of ergothioneine (OCTN1) and carnitine (OCTN2) in HEK293T was decreased when competing with oxaliplatin, and as a consequence, the cytotoxicity was also reduced. In DRG neurons, the same effect was observed and the uptake was determined time, concentration, temperature and sodium dependent (60).

3.5. OCTN2

OCTN2 (gene number SLC22A5) was cloned from rat and human in 1998 by several groups including Sekine et al.,(61), Tamai et al., (62), and Wu et al., (63). OCTN2 is expressed in various tissues and organs including, among others, liver, kidney, small intestine, colon (64); skeletal muscle, lung (65); ovary, placenta and brain (66). OCTN2 is a high affinity sodium dependent carnitine transporter needed for the transport and oxidation of fatty acids in the mitochondria via a process known as β-oxidation (58), (67). Without carnitine, fatty acids cannot cross the mitochondrial inner membrane by the acyl carnitine translocase. Thus OCTN2, as a high affinity carnitine transporter, is abundantly expressed in various tissues, where it plays specific roles. For example, it was shown that in murine kidney, OCTN2 regulates glomerular reabsorption of carnitine, some of which is excreted in urine due to its inability to bind to serum protein. Immunohistochemistry studies revealed that OCTN2 is localized to the apical membrane of proximal tubular epithelial cells. Transport of carnitine by OCTN2 in kidney is sodium dependent and seems to be linked to co-expression of PDZ domain-containing proteins such as PDZK1 protein at the apical membrane, although levels of OCTN2 expression do not seem to depend on PDZK1 expression (67). PDZ domains are protein modules that regulate multiple processes including transport and signaling (68). In the intestine, OCTN2 participates in intestinal absorption of carnitine, although its role in this process is still controversial (67). In addition, although significant levels of OCTN2 are detected in small intestine and colon, there is another nutrient transporter, PEPT1, which is strongly expressed in these organs. Similarly, to kidney, OCTN2-mediated transport of carnitine in the intestine seems to be regulated by PDZK1 protein. Human and murine *in vitro* studies showed that uptake of carnitine is saturable between 10 μM and 20 μM (67). In heart,

carnitine plays an important role in preserving skeletal muscle and carnitine deficiency causes cardiomyopathy. OCTN2, which is expressed in the vascular endothelium, transports carnitine in sodium dependent manner (67). The precise localization of OCTN2 in the vascular endothelium was also suggested to indicate a role in carnitine accumulation in heart. OCTN2 is also expressed in liver where carnitine biosynthesis occurs. Tamai et al., (67) reported strong expression in liver of gamma-butyrobetaine dioxygenase which catalyzes carnitine production from gamma-butyrobetaine. In fact, OCTN2 can transport gamma-butyrobetaine in a sodium dependent manner with Km of 13 μM, although the real contribution of gamma-butyric transport comes from another transporter, i.e., GAT2 (67). Yet, based on studies using mutated OCTN2 in mice, it appears that GAT2 gamma-butyrobetaine transport prevails only when OCTN2 does not perform its function and GAT2 only transports gamma-butyrobetaine acid into the liver, where carnitine biosynthesis occurs, to supply other tissues with carnitine (67). OCTN2 is also expressed in brain capillary endothelial cells and this correlates with regulatory transport of carnitine across the blood-brain barrier. Studies have revealed that the precise localization of OCTN2 is on the brain side and not on blood side suggesting that OCTN2 might only participate in one directional transport of carnitine and acetyl carnitine from the blood to the brain (67). Moreover, OCTN2 is highly involved in carnitine and acetyl carnitine circulation in placenta. Lahjouji et al., (69) showed that OCTN2 is localized in the brush border membrane of placenta and is involved in carnitine transport in a sodium-, pH-, temperature- and osmolarity-dependent manner. They also reported that carnitine transport in placenta, mediated by OCTN2, is saturable at 11 μM which appears to be similar to the transport observed in kidney. Moreover, while in competition with TEA, valspodar and valproate, carnitine uptake is greatly inhibited suggesting that these substrates are specific for OCTN2 as well. However, in the case of verapamil, it seems that inhibition of OCTN2 is triggered by both competitive and noncompetitive modes (69). The implication of other carnitine transporters has not yet been excluded. In fact, OCTN1 is also expressed in placenta and transports carnitine in a sodium-dependent manner although the affinity of OCTN1 for carnitine has not been reported. A study from Maekawa et al., (70) has identified cartregulin which is homologous to OCTN2 in rat brain and acts by stabilizing the mRNA of OCTN2. Moreover, a novel gene encoding full length of OCTN2 was identified with the addition of 24 amino acids in the first extracellular loop. This variant, named OCTN2 N2VT, exhibited

several different features compared to wild type OCTN2 including reduced glycosylation which regulated retention of OCTN2 N2VT in the reticulum endoplasmic compared to normal glycosylation and subsequent expression on the cell surface. It was also found that while OCTN2 N2VT is expressed in HeLa cells, carnitine uptake is severely abrogated due to lack of expression on the cell surface (70).

3.5.1. OCTN2 as transporter for anticancer drugs

As mentioned above, OCTN2 plays a role in oxaliplatin uptake (60). Moreover, Hu et al., (71) reported a role of OCTN2 in the uptake of the topoisomerase II poison etoposide. The study was performed in cells expressing either human or mouse OCT2 challenged with both carnitine and etoposide. The results showed a marked decrease in carnitine transport suggesting the important role of OCTN2. However, the authors were able to reverse the inhibition by adding carnitine in excess.

3.6. OCTN3

OCTN3 is another member of the SLC22 family that has not received much attention. Duran et al., (72) successfully isolated mOCTN3 cDNA from small intestine. Moreover, using commercially available antibodies against OCTN2 and OCTN3, the authors also reported that OCTN3 localizes on the basolateral membrane of the enterocytes in rat and chicken small intestine compared to OCTN2 which is localized on the apical membrane. OCTN3 was also found to be responsible for carnitine uptake in the peroxisome in mammals. Using a specific antibody 629 that binds to mouse OCTN3, Lamhonwah, et al., (73) localized OCTN3 on the peroxisomes and the cisternae of the rough reticulum endoplasmic. As already pointed out, carnitine is essential for β-oxidation in mitochondria, but is also important for β-oxidation in the peroxisome. The role of carnitine in the peroxisome is to shuttle short-sided hexacosanoid acid and pristanic acid which are products of β-oxidation in the peroxisome and exports them into the mitochondria as carnitine esters (73). The evidence for the existence of carnitine acyltransferases and carnitine translocase in the peroxisome further supports the importance of carnitine transport to this organelle. mOCTN3 transports carnitine in the peroxisome with

affinity of 20μM (73). The authors, based on comparison with mOCTN3, have also predicted that, similarly to OCTN1 and OCTN2 which are mapped at 5q31 locus, OCTN3 may also be localized at the same region in humans and may play an important role in carnitine homeostasis. They also predicted that if OCTN3 is mutated, this should confer susceptibility to several diseases related to OCTNs such as Crohn's disease (73).

3.7. CT2 (OCT6)

OCT6 or CT2, for carnitine transporter 2 (gene number SLC22A16), has been expressed in *Xenopus laevis* followed by characterization of the corresponding protein by Enomoto et al., (74). Structural analysis studies performed by the authors allowed to identify a CT2 cDNA encoding 543 amino acid protein that shared up to 40% identity with the other carnitine transporter members such as OCTN1 and OCTN2 (reported to be CT1 for carnitine transporter 1). Carnitine transport was found to be saturable at 50 μM and the affinity for this substrate was evaluated to 20 μM. It was shown that uptake of carnitine is not entirely sodium dependent. Competition studies with other carnitine derivatives and betaine greatly inhibited CT2, but not choline and other selected substrates. Tissue distribution analysis revealed that CT2 is expressed in human testis, and not in any other tissues including brain, placenta, heart, ovary, small intestine, and colon (74). Nonetheless Sato et al., (75) found that CT2 was strongly expressed in normal human endometrium and that this expression was regulated by progesterone. Analysis of CT2 gene also revealed that there were no identical sequences in the 5' regions upstream of progesterone responsive elements suggesting that, in endometrial cancer, CT2 expression should not depend on progesterone (75). In another study, Okabe et al., (76) characterized CT2 from normal vs tissues from patients with acute leukemia and reported that CT2 is predominantly expressed in bone marrow and testis and that high levels of CT2 are observed in fetal liver, but not in normal liver (76). Moreover, CT2 is expressed in a variety of leukemia and its expression is tightly related to hematopoiesis. CT2 was reported to transport typical OCTs substrates such as TEA (76). CT2 seems to have an important role in lung cancer and Kunii et al., (77) reported that its expression correlates with cisplatin uptake. The authors also reported that OCT2 is not in fact the main platinum drug uptake transporter, but is more likely to be CT2 (OCT6). Studies from our laboratory involving the role of CT2

have demonstrated that this protein is involved in the transport of the anti-cancer polyamine analogue Bleomycin- A5 (78). Bleomycin is currently used as antineoplastic agent for treating various cancers such as lymphomas and carcinomas. By overexpressing CT2 in various cancer cell lines, Aouida et al., (78) showed that these cells are extremely sensitive to Bleomycin- A5 compared to control cells. The results correlated with the previously shown increased uptake of polyamines mediated by Agp2 which is a high affinity carnitine transporter in *Saccharomyces cerevisiae* (79).

3.7.1. CT2 as anthracycline transporter

By injecting CT2 cRNA in *Xenopus* oocytes and stably transfecting Jurkat cells, Okabe et al., (76) tested the behavior of this transporter towards DOX. The authors reported that transport of DOX was saturable, dose dependent and uptake was independent of sodium with apparent affinity constant of 5 μM. A slight decrease in cell viability in control cells vs ones overexpressing CT2 was observed. Nonetheless, it was claimed that CT2 is a DOX importer. In a study involving the role of CT2 in human epithelial ovarian cancer, Ota et al., (80) reported that CT2 transports DOX in clear cell adenocarcinoma cell lines. Data from this study suggested that anthracyclines might be used in combination chemotherapy with paclitaxel and other platinum drugs used in ovarian cancer. Moreover, they predicted that clear cell adenocarcinoma expressing CT2 might display a better response to combinational treatment with platinum drugs and anthracyclines compared to other ovarian cancer cell lines (80). Correlation between CT2 expression and anthracyclines action was reported by Lal et al., (81) in a study investigating the genetic variants of this transporter versus DOX and its metabolite doxorubicinol in Asian breast cancer patients. Moreover, by studying different ethnic groups, the authors revealed that CT2 presented at least four different polymorphisms. Comparison between Asian and Caucasian groups showed differences in allele and genotype frequencies and data suggested that CT2 variations present the hallmark of DOX sensitivity and even other drugs candidates. CT2 c.146G allele was reported to harbor increased exposure levels to DOX and its metabolite doxorubicinol and has, as a consequence, shown better outcome of cancer treatment (81).

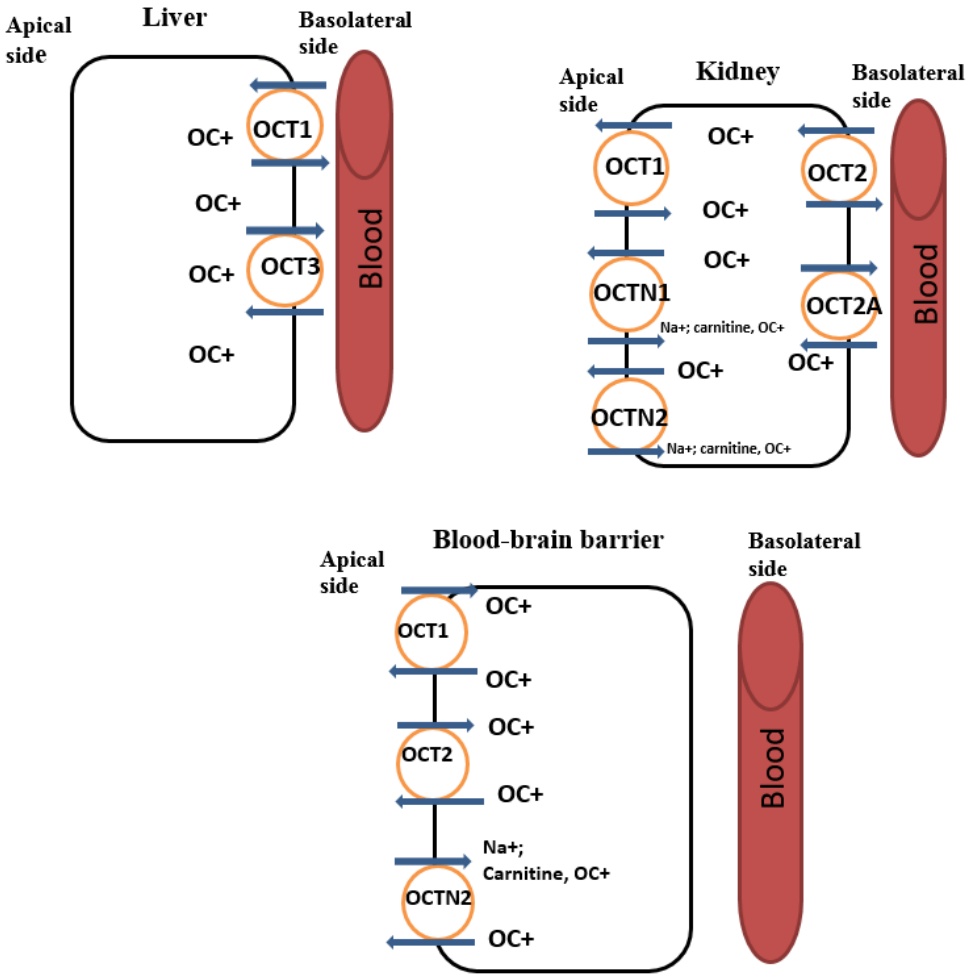


Figure 4. Schematic representation of the OCTs and OCTNs transporters localization in liver (hepatocytes), kidney (proximal tubule cells) and blood-brain barrier (brain endothelial cells). OC+- organic cations. Adapted from (20).

Table 1. SLC22 organic cations/carnitine transporters. Adapted from (20, 21, 82)

Gene number	Protein	Tissue localization	Substrate specificity	Drug transport implication
SLC22A1	OCT1	liver; kidney; small intestine; lung; heart; skeletal muscle; brain; placenta; immune cells	TEA; MPP; thiamine; choline; dopenezil	metformin; paclitaxel; oxaliplatin; irinotecan; ganciclovir; acyclovir
SLC22A1	OCT2	Kidney; brain; small intestine; lung; placenta; thymus; brain	TEA; MPP; N-methyl-nicotineamide; cyclo (his-pro); salsolinol choline; dopamine; epinephrine; norepinephrine; acetylcholine	oxaliplatin; picoplatin; memantine; amantadine; cisplatin; lamivudine
SLC22A3	OCT3	Heart; skeletal muscle; brain; small intestine; liver; lung; kidney	MPP; serotonin; desipramine, epinephrine; norepinephrine, dopamine	Lidocaine; metformin, oxaliplatine; lamivudine
SLC22A4	OCTN1	Kidney; small intestine; colon; spleen; heart; skeletal muscle; brain	Ergothioneine; TEA; carnitine; acetyl carnitine;	Ipratropium; gapentin; mitoxanthrone; verapamil
SLC22A5	OCTN2	Liver; kidney; small intestine; colon; skeletal muscle; heart; ovary; placenta; brain	Carnitine; acetyl carnitine;	Oxaliplatin; ipratropium; tiludronate; verapamil
SLC22A21	OCTN3	Small intestine; peroxisome	Carnitine; acetyl carnitine	----------
SLC22A16	CT2/OCT6	Testis; heart; skeletal muscle; kidney; liver; placenta; brain	Carnitine; acetyl carnitine;	Bleomycin; doxorubicin;

4. Organic anion transporters

Organic anion transporters (OATs) include more than ten transporter members (figure 4) and are part of the SLC22 subfamily of transporters (83). Localized on almost all epithelial physiological barriers, OATs are involved in the transport of numerous anionic compounds. OATs are mostly facilitator transporters, but it was shown that anionic substrates are also imported with counter transport of α-ketoglutarate. Thus, OATs that operate by such mechanism form part of a rate-limiting «tertiary» transport system as transport is driven by concentration gradient created from the sodium-decarboxylate cotransporter (NaDC3) and Na^+, K^+, ATPase (83). The first cloned OAT was the novel kidney transporter (NKT) which was designated OAT1 (SLC22A6). It was demonstrated that the hippuric acid derivative, the p-aminohippuric acid (PAH) is a typical OAT substrate. Moreover, OATs have the ability to transport xenobiotics such as antibiotics, antivirals, anti-inflammatory drugs, diuretics, and non-steroidals (83). A study conducted by Ahn et al., (84) demonstrated that typical OCT substrates can interact with OATs, specifically with mOAT1, mOAT3 and mOAT6. These substrates include TEA, 1-MPP, cimetidine, metformin, verapamil procainamide, clonidine and others. Moreover, some of these substrates appear to be member specific as procainamide, clonidine and metoclopramide can bind to mOAT3, but not to mOAT6. It was also demonstrated that 1-MPP can bind to mOAT1 and mOAT6 although with low affinity (Km of ~1mM and ~2mM). The authors also reported that the assayed cationic substrates have the ability to equally inhibit the cationic transport system (N^1- methyl nicotinamide) and the anionic transport system (PAH) (84).

Table 2. SLC22 organic anion transporters. Adapted from (83)

Gene number	Protein	Tissue localization	Substrate specificity
SLC22A6	OAT1	kidney, choroid plexus	Prostaglandin E_2, PAH, indoxyl sulfate
SLC22A7	OAT2	liver, kidney	cGMP, Prostaglandin E_2, salicylate, antivirals
SLC22A8	OAT3	kidney, brain, choroid plexus, retina, testis	Carnitine, Prostaglandin E_2, plant-derived metabolites, vitamins
SLC22A11	OAT4	placenta, kidney, retina	Estrone sulfate, Prostaglandin E_2, ochratoxine A, urate
N/D	OAT5	kidney	Estrone sulfate, ochratoxine A
SLC22A20	OAT6	nasal mucosa, testis, liver	Estrone sulfate, odorants
SLC22A9	OAT7	liver	Estone sulfate, butyrate, ochratoxine A
Rat SLC22a9	OAT8	kidney	Estrone sulfate, ochratoxine A
SLC22A27	OAT9	liver	Carnitine, xenobiotics
SLC22A13	OAT10	kidney, brain, small intestine, colon	Nicotine, urate
SLC22A12	URAT1	kidney	Urate

N/D- not documented

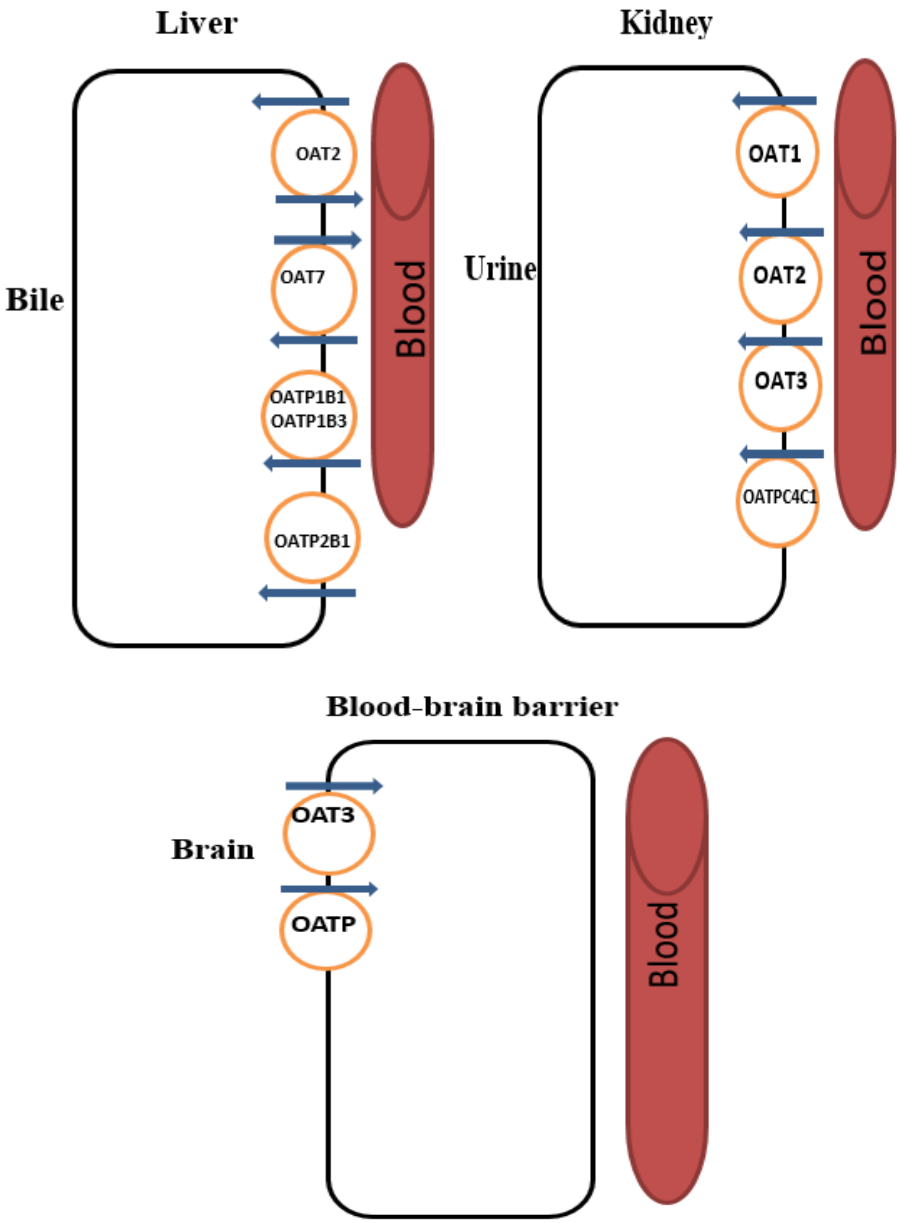


Figure 5. Schematic representation of the OATs and OATPs transporters localization in liver (hepatocytes), kidney (proximal tubule cells) and blood-brain barrier (brain endothelial cells). Adapted from (20, 83, 85).

5. Hypothesis and Objectives

As discussed above in the Introduction and despite numerous studies aimed at investigating the role of OCTs transporters in the uptake of various anticancer agents including anthracyclines, the implication of these transporters in anthracyclines uptake has yet not been clearly demonstrated. It is noteworthy that anthracyclines are still believed to undergo cellular penetration by passive diffusion and cellular transport of the anthracycline DOX by facilitated transport exerted by CT2 was only demonstrated by Okabe et al., (76). However, as the authors only showed a slight decrease in cell viability in cells overexpressing CT2 protein compared to control, and as down regulation of CT2 was never done, the role of this transporter in anthracyclines transport remains highly inconclusive. We propose that the anthracycline DNR, a close relative to DOX, enters cells by means of facilitated transport mechanism mediated specifically by OCT1. Thus, screening of patients currently treated with anthracyclines, for OCT1 status might constitute a useful predictor of responsiveness towards DNR.

In view of these considerations, our general objective is to demonstrate that OCT1 is the specific transporter for DNR and we intend to accomplish this by:

- Evaluating DNR accumulation in various cell lines
- Evaluating OCT1 expression in various cell lines
- Performing competition studies with DNR and key OCTs substrates
- Downregulating OCT1 and subsequent evaluation of DNR transport
- Overexpressing OCT1 and subsequent evaluation of DNR transport

Chapter 2. The human organic cation transporter OCT1 mediates high affinity uptake of the anticancer drug daunorubicin

Emil Andreev[1], Nicolas Brosseau[1], Euridice Carmona[2], Anne-Marie Mes-Masson[2], and Dindial Ramotar[1*]

[1]Maisonneuve-Rosemont Hospital, Research Center, Université de Montréal, Department of Medicine, 5415 Boul. de l'Assomption, Montréal, Québec, Canada, H1T 2M4,

[2]Institut du cancer de Montréal/Centre de recherche du Centre hospitalier de l'Université de Montréal (CRCHUM), 900 rue Saint-Denis, Montréal, Québec, Canada, H2X 0X9

Running title: The human transporter of anthracyclines

Keywords: organic cation transporters, SLC22A1/OCT1, mammalian cells, anticancer drug resistance, daunorubicin, patients survival,

Background: Anthracyclines are antitumor agents used for treating various cancers.

Results: Human OCT1 displayed a high affinity for the uptake of the anthracycline daunorubicin. Downregulation of OCT1 blocked the uptake of daunorubicin and conferred cellular resistance to the drug.

Conclusion: Human OCT1 possesses the ability to transport anthracyclines into cancer cells.

Significance: Defects in OCT1 function are likely to cause drug resistance.

ABSTRACT

Anthracyclines such as daunorubicin are anticancer agents that are transported into cells, and exert cytotoxicity by blocking DNA metabolism. Although there is evidence for active uptake of anthracyclines into cells, the specific transporter involved in this process has not been identified. Using the high-grade serous ovarian cancer cell line TOV2223G, we show that OCT1 mediated the high affinity (K_m ~ 5 μM) uptake of daunorubicin into the cells, and that micromolar amounts of choline completely abolished the drug entry. OCT1 downregulation by shRNA impaired daunorubicin uptake into the TOV2223G cells, and these cells were significantly more resistant to the drug in comparison to the control shRNA. Transfection of HEK293T cells, which accommodated the ectopic expression of OCT1, with a plasmid expressing OCT1-EYFP showed that the transporter was predominantly localized to the plasma membrane. These transfected cells exhibited an increase in the uptake of daunorubicin in comparison to control cells transfected with an empty EYFP vector. Furthermore, a variant of OCT1, OCT1-D474C-EYFP, failed to enhance daunorubicin uptake. This is the first report demonstrating that human OCT1 is involved in the high affinity transport of anthracyclines. We postulate that OCT1 defects may contribute to the resistance of cancer cells treated with anthracyclines.

INTRODUCTION

Members of the anthracycline family such as doxorubicin (DOX) and daunorubicin (DNR) are hydrophilic anticancer agents that are transported into cells where they intercalate into the DNA, disrupt the function of DNA polymerase, and promote cell death (86). Anthracyclines exhibit high tissue-penetration and retention in nucleated cells, and are used for treating a variety of cancers that include leukemia and lymphomas, as well as breast, lung, ovarian, gastric and thyroid cancers (87). However, a significant fraction of these cancer types is resistant to anthracyclines. Notably, over 50 % of older patients (> 60 yrs) with acute myeloid leukemia (AML) do not achieve complete remission after induction chemotherapy with DNR, and in some cases, remission is short-lived due to drug resistance (88-90).

A characteristic mechanism associated with drug resistance is the elevated levels of plasma membrane ABC transporters (91). These include the multidrug resistant efflux pump, MDR1/ABCB1, and the multidrug resistant-associated protein, MRP1, which are known to increase efflux of chemotherapeutic agents, allowing tumor (and normal) cells to evade drug-induced cytotoxicity (91, 92). Drug efflux transporters are known to be upregulated in some AML patients, and there is evidence suggesting that ABCB1 can expel DNR from the cells (93). However, inhibition of ABCB1 with valspodar did not improve outcomes for/treatment in drug-resistant AML patients (93). This suggests that other alternative mechanisms are involved in anthracycline resistance. These mechanisms are likely to involve (i) defects in DNR entry into cancer cells via uptake transporter(s), (ii) enhanced sequestration of DNR by lysosomes for detoxification and (iii) increased repair of DNR-induced DNA lesions. To date, the uptake transporter allowing entry of DNR into cells has not been identified (76). Such a transporter could represent a critical pathway that, when defective, leads to drug refractory disease.

Human cells possess three high affinity (OCTN1/SLC22A4, OCTN2/SLC22A5 and hCT2/SLC22A16), as well as three low affinity (OCT1/SLC22A1, OCT2/SLC22A2 and OCT3/SLC22A3) L-carnitine transporters (21). These are versatile organic cation transporters that possess varying affinities for a range of substrates, including several anticancer drugs such as bleomycin and oxaliplatin (31, 78). Okabe *et al* (2005) initially reported that hCT2 has the ability to transport DOX, although the evidence remains circumstantial (76). In their study, the authors injected hCT2 cRNA into the oocytes of *Xenopus laevis* and determined a high affinity for the uptake of DOX with an apparent K_m of 5.2 μM (76). However, the characterization of hCT2 as a DOX-transporter was limited as competition assays were not performed with the known substrate L-carnitine and knockdown of hCT2 expression was not analyzed for effects on anthracycline resistance. It is noteworthy that the authors examined a few paraffin embedded samples derived from AML patients who were either responsive or unresponsive to anthracyclines in order to determine whether a correlation exists with hCT2 gene expression level, but the results were inconclusive (76). We subsequently discovered that hCT2 is involved in the uptake of the anticancer drug bleomycin, using cancer cell lines that either expressed or did not express hCT2 (78, 94, 95). Moreover, we demonstrated that L-carnitine can effectively block bleomycin uptake and protect cancer cells expressing hCT2 from the genotoxic effects of the drug (78). In addition to hCT2, OCT1 is another organic cation transporter involved in the uptake of anticancer agents such as platinum drugs (31, 96). OCT1 has also been implicated in the transport of the anti-diabetic drug metformin, underscoring the wide range of substrate recognition by these organic cation transporters (97). Together with the work by Okabe et al (2005) and the above findings, it appears that one or more transporter(s) may be involved in the uptake of anthracyclines into cells.

In this study, we used the high-grade serous ovarian cancer cell line TOV2223G and show for the first time that OCT1 is a high affinity transporter for DNR. Uptake of DNR was effectively blocked by low concentrations of choline, which was previously reported to be a high affinity substrate for OCT1 (98). We also show that shRNA knockdown of OCT1 significantly diminished the expression of the transporter as determined by Western blot analysis probed with anti-OCT1 antibody and greatly reduced DNR uptake in TOV2223G cells. Downregulation of OCT1 enhanced the resistance of these cells to DNR. In support of these observations, expression of OCT1 as an EYFP fusion protein, OCT1-EYFP, revealed that the primary cellular localization of the transporter is on the plasma membrane. These OCT1-EYFP expressing cells increased the uptake of DNR. Collectively, our findings provide compelling evidence that suggests OCT1 functional levels may have a vital role in modulating the response to chemotherapy with anthracyclines.

RESULTS

DNR accumulates in several cancer cell lines in a time- and concentration-dependent manner—Once suitable assay conditions were established (see Materials and Methods), DNR (see chemical structure in Fig. 1A) uptake into cancer cell lines was monitored by three independent methods using either (i) FACS, (ii) a fluorometric microplate reader equipped with a tandem filter set with excitation at 544 nm and emission at 590 nm, or (iii) an epifluorescent microscope. We analyzed DNR uptake in five cell lines that included two of ovarian cancer (TOV2223G and OV866(2)), one of embryonic kidney (HEK293T), and two of leukemia (K562 and HL60). We monitored DNR uptake when the concentration was fixed at 5 μM, a concentration that was used in a study involving ceramide potentiating multidrug resistance (99) and, in particular, since it was near the estimated concentration of 4 μM given to cancer patients (100). Each of these cancer cell lines showed a different rate of uptake of DNR with K562 showing the highest level when the concentration was fixed at 5 μM as demonstrated by FACS or fluorometric analysis (Fig. 1B and 1C, respectively). In addition, DNR entered TOV2223G and HL60 cells in a concentration-dependent manner, with nearly 2-fold more drug accumulating in the TOV2223G cells (Fig. 1D). These data indicate that DNR uptake cannot be by diffusion, otherwise each cell line examined would show the same level of drug uptake (see discussion). Instead, our data suggest that there is an active process to mediate the entry of the drug into these cell lines, which could vary between cell lines.

In parallel, these cells were examined by epifluorescent microscopy which revealed that DNR entered and accumulated in the nucleus (Fig. 1E, and Supplemental data Fig. S1), consistent with the action of the drug in damaging DNA (86). Accumulation of the drug was not influenced by the cell size as the drug does not stain the cytoplasm, and in addition TOV2223G is significantly

larger that K562, which showed the highest level of DNR accumulation. All three methods showed similar results for DNR uptake in a given cell type, although quantitative assessment was achieved by FACS or the fluoro spectrometer.

A high-affinity transporter mediates DNR uptake in TOV2223G cells—We selected TOV2223G cells as the model cancer cell line (Fig. 1B) to determine whether DNR transport would be mediated by a low or high affinity transporter. We chose to study the TOV2223G cell line as it showed an intermediate uptake and responded to DNR toxicity (see below), as well as the fact that ovarian cancer patients are also treated with anthracyclines. Kinetic analysis showed that DNR uptake was saturable at ~ 15 μM (V_{max} of 29 $\pm$ 0.41 pmol/2 x 10^4/min) with an apparent K_m of 3.0 $\pm$ 0.4 μM (Fig. 2). Comparable to the kinetic values reported for L-carnitine transport by the high affinity L-carnitine transporter hCT2 (101), these results indicate that there exists at least one high affinity component for DNR transport into TOV2223G cells, again countering previous arguments that DNR diffuses into cells. Similar kinetics were observed with HEK293T or HL60 cells, yielding apparent K_m of ~ 5 μM, suggesting that the high affinity DNR transporter also exits in these cancer cell lines. As such, we conducted all subsequent experiments with low concentrations (5 μM) of DNR. In a similar manner, we measured the kinetics for the uptake of another anthracycline, DOX. DOX uptake was also saturable with an apparent K_m of 10 $\pm$ 3 μM, suggesting that DNR is a better substrate than DOX for the transporter. In addition, DNR or DOX uptake was not influenced by changes in the pH ranging between 6.5 to 8.0.

The organic cation transporters hCT2, OCTN1 and OCTN2 are not involved in DNR uptake—hCT2 and OCTN2 have been discovered as high affinity transporters for L-carnitine, while OCTN1 is reported to be a low affinity transporter for this substrate (21, 56, 62, 101, 102). Subsequent studies by Okabe et al., raised the possibility that hCT2 and OCTN1 could be transporters for DOX, although no direct experiments were performed to test these assertions (59, 76). To determine whether any of these three organic cation transporters are involved in DNR uptake, we examined whether the known substrates for these transporters could compete for the drug uptake. Briefly, a fixed concentration of DNR (5 μM) was added together with a 200-fold excess of L-carnitine (1 mM) to cultures of the TOV2223G and OV866(2) cells preincubated in uptake buffer. As shown in Fig. 3, L-carnitine did not block the uptake of DNR into either of the ovarian cancer cell lines (see also Supplemental data Fig. S2). If indeed hCT2 and OCTN1 were involved in DNR uptake, as previously implicated, we would expect that the excess of L-carnitine to at least cause a significant reduction in DNR uptake. These results indicate that the presence of excess L-carnitine does not affect DNR uptake, suggesting that the three transporters OCTN1, OCTN2 and hCT2 do not mediate DNR uptake. Progesterone, which has been shown to induce hCT2 expression by several fold (75), did not cause an influx of DNR when cells were pre-cultured in the presence of the hormone for 3 h (Fig. 3D). This is consistent with the above observation that hCT2 does not have a major role in the uptake of DNR.

Since many transporters are known to be Na^+-dependent or -independent, we examined if omission of NaCl from the uptake buffer would alter DNR uptake in cells. Replacing NaCl with *N*-methyl-*D*-glucamine that maintains the ionic strength in the uptake buffer did not affect DNR entry into cells (Fig. 3E), suggesting that transport of DNR into the cells is not coupled with the movement of Na^+ ions.

Choline effectively blocks DNR uptake—We next examined whether the OCT family members of polyspecific organic cation transporters would be involved in mediating DNR uptake. OCT1, 2 and 3 are reported to have different affinities for a wide range of cationic substrates that include choline, ergothioneine, phenformin (a structurally related form of metformin), thiamine and polyamines (23, 98, 103). Incubation of the TOV2223G cells with DNR together with the indicated cationic substrate, revealed that choline effectively competed with DNR for uptake, whereas ergothioneine (100 μM), phenformin (50 μM), and thiamine (50 μM), partially ($p < 0.01$) inhibited DNR uptake at the concentrations used (Fig. 3 F and G and Fig. 4, respectively). In contrast, a high concentration (1 mM) of polyamine spermidine (SPD) had only a modest effect on the uptake of DNR (Fig. 4). This is consistent with OCT transporters that function as low affinity permeases for polyamines (104). Since choline is a potent competitor in DNR uptake and OCT1 has been previously described as a high affinity transporter of choline, our results suggest that OCT1 may play a role in the high affinity uptake of DNR.

shRNA-OCT1 knockdown in TOV2223G cells reduces DNR uptake and enhances resistance to the drug—It has been shown that OCT1 is expressed predominantly in the liver, the organ that metabolizes DNR, whereas expression levels of OCT2 and OCT3 are not detected(20). To test the hypothesis that OCT1 is a DNR transporter, we designed two shRNA constructs to specifically downregulate the transporter in the TOV2223G cells. These constructs targeted the 5`-untranslated and the C-terminus of the mRNA sequence of the OCT1 gene. Stable clones carrying the shRNA construct against the 5`-untranslated region was effective in down-regulating OCT1 expression levels by more than 95% when compared to the control

shRNA LMP vector, as determined by immunoblot analysis probed with polyclonal antibody against OCT1 and quantified by Image J (Fig. 5A). The shRNA construct against the C-terminus of OCT1 had no effect in downregulating OCT1 expression (data not shown). Consistent with the downregulation of OCT1, fluorometric analysis revealed that DNR uptake was significantly reduced (3- to 5-fold) in the shRNA-OCT1 knockdown cells compared to cells carrying the control shRNA (Fig. 5B). The low level of DNR uptake observed with the shRNA-OCT1 knockdown cells demonstrates that downregulation by shRNA-OCT1 was not sufficient to abolish DNR transport or that gene knockdown was incomplete, although other redundant transporter may exist to mediate DNR uptake. In a parallel experiment, epifluorescent microscopy showed that the accumulation of DNR was nearly undetectable in the shRNA-OCT1 knockdown cells compared to the shRNA LMP vector control cells (Fig. 5C). There was no difference in DNR uptake between the shRNA LMP vector control and the TOV2223G cells alone (data not shown), suggesting that the slightly higher OCT1 protein level detected in the LMP vector control vs the TOV2223G cells could be due to susceptibility to extraction (Fig. 5A). In another control experiment, the uptake of rhodamine was not altered in the shRNA-OCT1 knockdown cells compared to the shRNA control cells (see also below). These results strongly support a role for OCT1 in mediating DNR uptake into TOV2223G cells.

To investigate the effect of OCT1 knockdown with respect to cell sensitivity to DNR, we performed a viability assay to monitor surviving cells, following exposure to the drug for 72 hours. Under these conditions, the normal TOV2223G cells carrying the LMP vector showed a survival of nearly 40% (Fig. 5D). However, OCT1 knockdown TOV2223G cells were protected from DNR toxicity and showing nearly 100 % survival (Fig. 5D). This finding strongly supports

a role for OCT1 in the transport of DNR, and excluding any possibility of drug diffusion into the cells.

OCT1-EYFP overexpression enhances DNR uptake in HEK293T cells—To determine whether OCT1 overexpression could enhance DNR uptake in cells, we designed an expression system to drive OCT1 expression as a OCT1-EYFP fusion protein in the vector pEYFP-N1 (105). Transient transfection with pOCT1-EYFP into the TOV2223G cell line yielded very few cells that expressed the OCT1-EYFP fusion protein (< 1 % in the transfected population). Therefore, we used HEK293T cells as an alternative host cell since it was reported to accommodate the expression of plasma membrane transporters (23, 106). At least 5 to 10 % of the pOCT1-EYFP transfected HEK293T cells expressed the OCT-1 EYFP fusion protein. Epifluorescent microscopy revealed that the OCT1-EYFP protein was specifically present on the plasma membrane, consistent with a previous report that used immunopurified antibodies against OCT1 (23, 106), while the EYFP alone showed non-specific cellular localization (Fig. 6A). Because HEK293T cells can accommodate the ectopic expression of the OCT1-EYFP fusion protein, we monitored the uptake level of DNR into these cells. After 24 hours of transient transfection of HEK293T cells with the empty pEYFP-N1 vector or the pOCT1-EYFP plasmid, the cells were preincubated in uptake buffer followed by the addition of DNR (5 μM) for 1 hour and uptake was determined by FACS analysis only in the fraction of EYFP expressing cells (see Materials and Methods). The OCT1-EYFP expressing cells showed nearly 50 % increase in DNR uptake, compared to the cells that were transfected with the empty vector alone (Fig. 6B). In contrast, OCT1-EYFP expression did not stimulate rhodamine uptake in HEK293T cells (Fig.

6C). These findings show that the expression of OCT1-EYFP fusion protein correlates with enhanced DNR uptake, and strongly reinforces the role of OCT1 as a transporter of DNR.

The variant OCT1-D474C-EYFP is unable to increase DNR uptake in HEK293T cells—Molecular models have predicted that the transmembrane helix domain 11 of the rat OCT1 is involved in substrate binding (107). Several mutations have been created in this region of the rat OCT1 gene (107). In particular, the replacement of Asp475 with cysteine resulted in nearly complete reduction (98%) of the transport of the model substrate tetraethylammonium in oocytes expressing the rOCT1-Asp475Cys variant (107). The same mutation was created in the OCT1-EYFP construct by site-directed mutagenesis; Asp474, which corresponded to the amino acid residue of the human OCT1, was substituted with cysteine generating the variant OCT1-D474C-EYFP. Transient expression of this variant in HEK293T cells demonstrated its localization to the plasma membrane (Supplemental data Fig. S4). However, this OCT1-D474C variant did not enhance DNR uptake in HEK293T cells, as observed with the native OCT1-EYFP construct (Fig. 6B). We conclude that the transport function of OCT1 is required for the uptake of DNR and exclude the possibility that OCT1 might act *via* another transporter through protein-protein interaction.

High levels of OCT1 mRNA correlates with increased survival in patients with high-grade serous epithelial ovarian cancer—To explore the potential significance of OCT1 in the context of chemotherapy, we used the Affymetrix gene expression to analyze 469 cases of high-grade serous epithelial ovarian cancer from The Cancer Genome Atlas (TCGA) dataset (108). The dataset represented patients in different stages of the cancer and were treated with a combination

chemotherapy that included platinum and taxol reagents. Using the online tool Kaplan-Meier Plotter (109, 110), we observed that patients with a high level of OCT1 mRNA have significantly better overall survival ($p = 0.013$) than those with low expression levels of this gene (Fig. 7). This difference accounts for an average of 10 months increased life expectancy for patients with higher OCT1 expression levels (Fig. 7). Since OCT1 has the ability to transport derivatives of cisplatin, as well as paclitaxel (31, 35), it is possible that ovarian cancer patients expressing reduced levels of OCT1 are associated with poor outcome following chemotherapy.

DISCUSSION

In this study, we demonstrate that the human organic cation transporter OCT1 is involved in the high affinity transport of DNR, a member of the anthracycline family of anticancer drugs. This conclusion was derived from the following findings: (i) several cell lines displayed saturable transport of DNR; (ii) the kinetics for DNR transport is comparable for the uptake of the model substrate tetraethylammonium and the neurotoxin methyl-4-phenylpyridinium with apparent K_m ranging between 5 to 50 μM by HEK293 cells designed to express the human OCT1 (107, 111); (iii) the rate of DNR uptake varies between cell lines and may be dependent upon the expression level of the OCT1 transporter (see Supplemental data Fig. S3); (iv) choline at low concentrations effectively competed with DNR uptake, underscoring the participation of a high affinity choline uptake system in the transport of DNR; (v) downregulation of OCT1 reduced the transport of DNR into the cancer cell line TOV2223G, as well as causing DNR resistance; and (vi) expression of OCT1 as an EYFP fusion protein revealed that it is localized to the plasma membrane consistent with a previous report (23, 106), and enhanced the uptake of DNR, but not rhodamine. In this latter finding, not all of the expressed OCT1-EYFP protein might be functionally active to yield substantially higher level of DNR uptake. The overexpression of OCT1-EYFP might displace resident plasma membrane protein that could have adverse effects on the functioning of the transporter such as causing the eviction of accessory proteins that are necessary for the proper transport function of OCT1 (112). On the basis of our findings, we exclude the possibility that DNR uptake can be explained by the drug diffusing into the cells. In fact, there is growing evidence precluding a diffusion process by which DNR enters into cells. An earlier study demonstrated that DOX can penetrate artificial membranes, but it cannot readily go through natural membranes (113). Moreover, yeast cells lacking the plasma membrane

regular Agp2 that controls the expression of the uptake transporters Sam3 and Dur3 do not take up DOX (114). Uptake of DOX into the *agp2Δ* mutant can be rescued by the expression of either of the active transporters Sam3 or Dur3, which provides strong support for transporter-mediated uptake of anthracyclines into cells as the principal process (114).

In the OCT family of transporters, OCT1 shares 75 and 50 % identity at the amino acid level with OCT2 and OCT3, respectively. We propose that OCT1 has a major role as a transporter for anthracyclines. The deletion of the OCT1 gene in mice caused significant upregulation of OCT2 and OCT3 (23, 98, 103), suggesting that if a similar mechanism occurs with OCT1-shRNA downregulation in human cells then these two later transporters are unlikely to be involved in DNR uptake since they cannot compensate for OCT1 role in the drug uptake. Notwithstanding, we have not directly tested whether OCT2 and OCT3 could perform a role in DNR transport. While these three transporters share the ability to transport metformin and the model substrate tetraethylammonium, they also mediate the uptake of distinct substrates (23, 106). OCT2 has been shown to transport platinum anticancer drugs and both OCT2 and OCT3 secrete small organic cations in the kidney and placenta. OCT2 and OCT3 also play important roles in the brain by transporting neurostimulants such as dopamine, corticosterone, amphetamine and methamphetamine (20, 53). Thus, these two transporters have an important role in the neurological response to psychostimulants, and their expression is strongly linked to addiction behavior (20, 115). Since anthracyclines undergo metabolism in the liver where OCT1 is expressed, as well as OCT3, but not OCT2 (116), raises the possibility that at least OCT2 may not be involved in the uptake of anthracyclines and that substrate specificity may be governed by the site of action. Thus, the residual level of DNR uptake in the TOV2223G cells knockdown for OCT1 could be accounted for if there is at least one other DNR transporter.

OCT1 also shares nearly 30% identity at the amino acid level with transporters that belong to the OCTN family, which include OCTN1, OCTN2 and OCT6/CT2 (20). OCTN1 is a low affinity transporter for L-carnitine, but a high affinity transporter for ergothioneine, while both OCTN2 and CT2 are high affinity transporters for L-carnitine (56, 62, 101). L-carnitine in excess of 200-fold did not block DNR uptake, thereby excluding these three transporters in the uptake of anthracyclines. This is in stark contrast to the previous report by Okabe *et al* which described CT2 as a high affinity transporter for DOX, based on limited data from *X. laevis* oocytes injected with hCT2 cRNA, and K_m measurements for DOX uptake (76). It remains inconclusive whether hCT2 cRNA produced a modified protein that indirectly promoted high affinity uptake of DOX since inhibition of DOX uptake and CT2 knockdowns were not conducted in their study (76). However, there is additional evidence that excludes the OCTN family as transporters of anthracyclines. The leukemia cell line K562 does not express CT2 (Supplemental Fig. S5), yet these cells allow entry of DNR, thus eliminating the possibility that CT2 is involved in the transport of this drug (117). Moreover, the HL60 cells expressed the CT2 transporter, but exhibited the slowest rate of DNR uptake. It is noteworthy that the HL60 cells weakly expressed the OCT1 protein, while it is highly expressed in the K562 cells (Supplemental Fig. S3). This observation is consistent with the notion that DNR uptake is directly proportional to the expression level of the OCT1 transporter.

We note that OCTN1 was described previously as a high affinity transporter of ergothioneine (56) and subsequently, as a potential transporter of DOX based on gene expression analysis data from the NCI-60 panel of cell lines (59). However, when the TOV2223G cells were challenged with a 200-fold excess of ergothioneine, there was only a partial effect on the uptake of DNR. Therefore, OCTN1 does not appear to have a major role in anthracycline uptake. Our

observation of partial inhibition of DNR uptake by ergothioneine may be explained if human OCT1 also has a role in the uptake of this antioxidant. This is consistent with reports describing *C. elegans* OCT-1 as a transporter for ergothioneine, and more recently, as a transporter for DOX (103, 114).

Of the various competitors tested in this study, choline, an important component for the biogenesis of the plasma membrane and synthesis of the neurotransmitter acetylcholine (27), was the most effective in abolishing DNR uptake. This is consistent with a previous report demonstrating that OCT1 displayed the ability to transport choline (98). This raises the issue whether high affinity choline transporters in general have the ability to transport DNR. Three different transport systems participate in choline uptake: the high affinity choline transporter 1, CHT1; the choline transporter like 1, CTL1; and the OCTs such as OCT1 and OCT2 (27). CHT1 has the highest affinity (K_m of 1 μM) for choline uptake, while CTL1 is in the range of 25-50 μM and OCT1 at nearly 42 μM (98). The CHT1 transporter acts exclusively in neurons where it transports acetylcholine in a sodium-dependent manner. Since the uptake of DNR is independent of sodium, it suggests that CHT1 is unlikely to be involved in DNR uptake. CTL-1 primarily transports choline in non-neuronal cells independent of sodium (118). As such, both CTL-1 and OCT1 share common properties, however we did not examine whether shRNA against CTL-1 would further diminish the uptake of DNR in TOV2223G shRNA-OCT1 cells that were downregulated for OCT1 expression. Other possibilities might exist to account for our finding that very low concentrations (0.5 to 5 μM) of choline can strongly compete for DNR uptake, if indeed both CTL-1 and OCT1 operates at low K_m. One possibility is that a high affinity sensor could sense the low level of choline and in turn negatively regulates the activity of

the lower affinity choline transporters such as OCT1 in a manner that has been reported for transceptors (119).

Our findings could have direct impact on the chemotherapy regimen that is given to ovarian cancer patients. There are four major types of primary ovarian adenocarcinoma and these are categorized as serous, mucinous, endometrioid, and clear-cell. Serous adenocarcinoma comprises about one half of all ovarian cancers. Currently, the combination of paclitaxel and cisplatin is the standard regimen for ovarian cancers with a response rate of nearly 70% (120). This combination of paclitaxel-platinum chemotherapy appears to improve overall and disease-free survival not only for patients with primary ovarian cancer, but also for patients with relapsed disease (121). Although treatment of this disease has improved, the 5-year survival of patients with advanced-stage ovarian cancer is less than 20%, and there is an ongoing effort to improve the outcome of treatment of ovarian cancer. There is evidence that addition of DOX to the paclitaxel-platinum combination regimen can significantly improve survival of ovarian cancer patients (122-124). However, the actual molecular mechanism leading to improved survival is not known. Here, we propose that the expression level of OCT1 could dictate the added benefits for ovarian cancer patients receiving DOX together with the paclitaxel-platinum regimen, or to substitute paclitaxel with DOX in light of the observation that OCT1 can also transport paclitaxel (35). This is in accordance with our results showing a better overall survival in high-grade serous ovarian cancer patients having high OCT1 mRNA levels, which could mediate increasing uptake of both platinum and paclitaxel (Fig. 7) (31, 35, 96). OCT1 could also play a pivotal role in the outcome of other diseases that are treated with anthracyclines. For example, patients with AML, a major cause of mortality from hematological malignancies in adults, are given a standard induction chemotherapy consisting of an anthracycline, such as DNR (125).

Importantly, a significant fraction (> 50 %) of older AML patients (> 60 yrs) do not achieve complete remission after the induction chemotherapy with DNR, and in some cases, remission lasts for a short duration due to drug resistance (88-90). The underlying mechanisms that are responsible for DNR resistance in these AML non-responding patients are not known, although it is possible that one of the mechanisms could be due to a defect in the entry of the drug into the cancer cells. Whether there are mutations that disrupt the function of OCT1 in a fraction of the AML non-responders, remains to be explored. It should be noted that there are genetic polymorphisms in the OCT1 gene that altered, e.g., metformin uptake (https://www.pharmgkb.org/). However, it is cumbersome to recreate the various mutations to test for alteration in DNR uptake, and instead we are screening DNR unresponsive AML patients for defective drug uptake and then establish whether these patients have mutations in the OCT1 gene.

ACKNOWLEDGMENTS:

The authors thank J. Chung for the critical reading and editing of the manuscript. This work was supported by research grants (MOP-93573 and RGPIN/202432-2012) to D. R. from the Canadian Institute of Health Research and the Natural Science and Engineering Research Council of Canada. The Banque de tissus et de données of the Réseau de recherche sur le cancer (RRCancer) was supported by the Fonds de recherche Québec - Santé (FRQS) in affiliation with the Canadian Tumor Repository Network (CTRNet).

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Competing financial interests: The authors declare that there are no competing financial interests associated with this work.

Author contributions: EA, NB and DR conceived the experiments, EA and NB conducted most of the experiments, EA, NB, DR analyzed the results and wrote most of the paper. EC and A-MM-M analyzed the patients data and wrote the corresponding sections.

MATERIALS AND METHODS

Cell lines and cell culture—TOV2223G and OV866(2) were obtained from the RR Cancer-Biobanque de cancer de l'ovaire du CHUM and are spontaneously immortalized cell lines derived from high-grade serous ovarian cancer patients' solid tumor or ascites, respectively, and previously described (126)(Fleury et al., *in press*). Cell lines were grown in OSE growth medium (Wisent, St-Bruno, QC) supplemented with 10% FBS, 2 mM L-glutamine, 0.5 μg/mL amphotericin B and 50 μg/mL gentamycin at 37°C, 5% CO_2, 95% Air. The leukemia cell lines HL60 and K562 were purchased from ATCC and kindly provided by Dr. Denis-Claude Roy (Maisonneuve-Rosemont Hospital research center) were grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100U/mL penicillin and 100μg/mL streptomycin at 37°C with 5% CO_2. The embryonic human kidney cell line HEK293T was kindly provided by Dr. El Bachir Affar (Maisonneuve-Rosemont Hospital research center) and grown in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 100U/mL penicillin and 100μg/mL streptomycin at 37°C with 5% CO_2.

Antibodies, cDNA, vectors, and drugs—The SLC22A1 (NBP-59464) and β-Actin (AC-15) antibodies were purchased from Novus and Santa Cruz, CA, USA, respectively. hSLC22A1 cDNA (RDC0425) was purchased from R&D Systems. The MSCV-LTRmiR30-PIG (LMP) vector was purchased from Thermo Scientific and pEYFP-N1 (6006-1) was purchased from Clontech. Daunorubicin was purchased from Maisonneuve-Rosement Hospital, Montreal, Canada.

Generation of hOCT1 shRNA for OCT1 knock down in TOV2223G cells—hOCT1 knock down was generated by two shRNA sequences obtained from the RNAi Central- RNAi Oligo

Retriever (http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA; *hannonlab.cshl.edu/GH_shRNA.html;*) and listed in supplemental Table S1. Among the two generated sequences, sequence 1 was able to most efficiently silence hOCT1 protein expression and was chosen for all subsequent studies. The sense and antisense strands were linked with miR30 loop sequence and the miR-30 styled sequence was synthesized as a single stranded DNA oligonucleotide with one part corresponding to the endogenous miR-30 miRNA flanking sequence. The resulting 97-mer was PCR amplified using common miR30 primers listed in supplemental Table S2 and subcloned into the EcoRI/ XhoI sites of MSCV-LTRmiR30-PIG (LMP). This generated the hOCT1 shRNA plasmid.

Generation of hOCT1-EYFP fusion protein and the D474C variant—Human SLC22A1 cDNA plasmid (RDC0425, R&D Systems, USA) containing the hOCT1 cDNA isoform 1 (1678 bps) was used as template for PCR amplification with the primers listed in supplemental Table S2. The PCR product was subcloned into the XhoI/EcoRI sites of the mammalian expression vector, pEYFP-N1. The resulting hOCT1-EYFP construct was amplified and positive clones were sequenced using sequencing primer. The hOCT1-D474C-EYFP variant was created by site-directed mutagenesis using the primers listed in supplemental Table S2.

DNR uptake assay—The day of the experiment, media was removed; cells were then washed with 2 mL Uptake buffer (125 mM NaCl, 10 mM HEPES pH 7.4, 5.6 mM glucose, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2mM $CaCl_2$, 1.2 mM $MgSO_4$) for 5 min at gentle shaking and incubated in 1.9 mL of the same buffer for 20 min. Drug uptake was initiated by the addition of 5 μM DNR. DNR (5 mg/mL equivalent to 8.86 mM) was kept as a stock solution in water at -20 ^{o}C and diluted to 100 μM in uptake buffer. Uptake was monitored over time, most often for 60 min at 37°C. For competition studies, the competitor was mixed together with 5 μM

DNR and then added to the cells in the uptake buffer. Uptake was stopped by directly adding 2 mL ice cold uptake buffer to the drug-buffer complex or the drug-competitor-buffer complex which was immediately removed, followed by three successive washes with 2 mL of ice cold uptake buffer with gentle shaking for 5 min each. Cells were then collected by adding 0.25% Trypsin-EDTA to detach adherent cells (TOV2223G, OV866(2) and HEK293T) and centrifuged at 1000 x g for 5 min.

Fluoroskan analysis–Cells treated as described were resuspended in 1 mL Uptake buffer prior to cell count or directly spin in the case of suspension cells HL60. Cells (2×10^5) were resuspended in 1 mL of Uptake buffer and 100 µL of resuspension ($2x10^4$) was added to each well in triplicate in a 96-well black well with optical bottom (Fisher Scientific). DNR cellular uptake was monitored with a microplate fluorometer (Fluoroskan Ascent, Thermo Scientific, USA) using tandem filter 544 nm excitation and 590 nm emission.

Flow cytometry analysis–Cells were fixed with 100 µL of 4% paraformaldehyde (PFA) for 10 min. Total cells were centrifuged to be resuspended in PBS (300 to 500 µl depending on the pellet size) then DNR cellular uptake was measured by FACScalibur, Becton-Dickinson (San Jose, CA). In the case of simple DNR uptake assessment, 10 000 cells were analyzed with FL2 (585/42) as described previously. In the case of overexpression with EYFP tag, 55 000 cells (or total sample) were analyzed and cells expressing EYFP fluorescence were gated and the DNR fluorescence level was measured only for these EYFP expressing cells. For EYFP FL1 (530/30) was used and FL4 (670LP) was used for DNR fluorescence detection.

Epifluorescence analysis for DNR uptake—Cells were grown in 6-well plates containing 18 x 18-1 coverslips in 2 mL OSE complete medium and left to recover for 16 to 24 h. The cells were treated with DNR as previously described then fixed with 100 µL of 4% PFA for 10 min.

After fixation, cells were washed three times as described previously and mounted with 5 µL of mounting media (Vectashield, Vector Laboratories Inc., CA, USA) containing 1.5 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) on a microscope slide, and sealed with nail polish. Images were photographed with the Zeiss Z2 imager or the Olympus BX53 fluorescent microscope at 63 X and 60 X magnification, respectively, using DAPI/Texas Red filters. Images were then processed with Axio Vision (Zeiss Z2) or ImageJ (Olympus BX53) software.

Epifluorescence analysis for hOCT1-EYFP overexpression—After overnight transfection in 6 well plate, the media was removed and HEK293T were briefly washed in PBS then collected by adding 0.25% Trypsin-EDTA. Cells were centrifuged and wash once in 1 ml PBS then fixed with 100 µL of 4% PFA for 10 min. After fixation, cells were washed once in PBS and resuspended in 30 µL of Vectashield mounting media containing 1.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., CA, USA). 7,5 µl of the suspension were put on a microscope slide. Images were photographed with Zeiss Z2 imager at 63 X magnification using DAPI/ EYFP filters. Images were then processed with Axio Vision software.

shRNA transfection—TOV2223G cells were grown in 6-well plate in OSE complete media containing 10% FBS and antibiotics. When cells reached 60-70% confluence, media was replaced with fresh OSE media containing 10% FBS, but no antibiotics. 2.5 µg hOCT1 shRNA pDNA were mixed with 7.5 µL Lipofectamine 2000(Life Technologies) in ratio 1:3. LMP shRNA pDNA was used as control vector. Cells were selected with Puromycin (2µg/mL) for stable clones for 10 days before analysis.

Transfection of HEK293T cells for epifluorescence analysis—HEK293T cells at 60-70% confluence on coverslips in a 6-well plate were transfected overnight with one of three plasmids EYFP-N1, hOCT1-EYFP, hOCT1-D474C-EYFP and Lipofectamine 3000, as per manufacturer`s instructions. Next day, they were processed for either DNR uptake assay analyzed by flow cytometry or fluorescence microscopy analysis.

Rhodamine B (RhB) uptake assay—Transfected HEK293T cells were processed the same way as for DNR uptake assay but the 5 μM DNR were replaced by 50 μM RhB and FL2(585/42) was used to detect RhB fluorescence in FACS analysis of the samples

Western Blot Analysis—Equal amounts of whole cell extract protein (30 μg) were separated by sodium dodecyl-polyacrylamide (SDS) polyacrylamide gel electrophoresis. Proteins were then transferred onto PVDF membrane and immunoblotted with anti-hOCT1 antibody at a 1:1000 dilution in PBST buffer (137 mM NaCl, 2,7 mM KCl, 10 mM Na2HPO4, 1,8 mM KH_2PO_4, 0,1% Tween 20). Equal protein loading was confirmed by reprobing the membrane with anti-β-Actin.

MTT Assay—TOV2223G pLMP control vector and TOV2223G hOCT1 shRNA were seeded in a 96-well plate. Each well contained 7 x 10^3 cells per 100 μl with 5 μM DNR. After 72 hours of incubation, 20 μL of Methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, Ltd, Ontario, Canada) concentrated at 5 mg/mL in uptake buffer, was added to each well and incubated for another 3 h at 37°C. The reaction was stopped by adding DMSO (Sigma lifesciences, Canada) in each well to dissolve the MTT crystals. Conversion of tetrazole into purple formazan product was detected at 540 nm, using the ELx808 Absorbance Microplate Reader (Biotek, Winooski, USA). All experiments were performed in triplicate.

Kinetic studies—TOV2223G cells were seeded at 2 x 10^4 cells per 100 μL with increasing concentrations of DNR. Initial rates were obtained by measuring uptake at 2.5 min, using the Fluoroskan Ascent Microplate reader set at 544 nm excitation/590 nm emission tandem filter. Measurements were expressed in relative fluorescence units (RFU). To estimate the kinetic parameters, uptake rates were fitted to the equation y =Vmax*x/(Km+x), by means of nonlinear regression using GraphPad Prism(GraphPad Software Inc, CA, USA) in which Km and Vmax are Michaelis-Menten constant and maximum velocity respectively. The recorded RFUs were first multiplied by 1 x 10^{-4} L (100 μL), the total volume of buffer used to stop uptake, and then divided by 2.5 (time for uptake), and finally multiplied by 10^6 to give pmole DNR/2 x 10^4cells/min.

Kaplan-Meier plotter analysis—The prognostic value of the SLC22A1 gene (hOCT1 protein) in high-grade serous epithelial ovarian cancer was analyzed using the online tool Kaplan-Meier Plotter (http:/kmplot.com/analysis/), a database that integrates gene expression data and clinical information of breast, ovarian, lung and gastric cancers (109, 110). Kaplan-Meier Plotter uses three versions of the Affymetrix HG-U133 datasets (with 22,277 probe sets in common), and clinical data from Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) datasets. The expression of SLC22A1 in the TCGA dataset for high-grade serous epithelial ovarian cancer was verified with the best specific probe (JetSet probes) of this gene (207201_s_at). A total of 469 patients were available for analysis on overall survival. Patient samples were split into two groups according to the median value, using the query parameter of auto-select best cutoff. The signal range of the SLC22A1 probe was 1-150, and the cutoff value was 19. The two patient groups (high and low expression levels)

were compared using a Kaplan-Meier survival plot. The hazard ratio with 95% confidence intervals and log rank p value was calculated, and significance was set at $p < 0.05$.

REFERENCES

1. Westmoreland, T.J.*, et al.* Comparative genome-wide screening identifies a conserved doxorubicin repair network that is diploid specific in Saccharomyces cerevisiae. *PLoS One* **4**, e5830 (2009).
2. Lown, J.W. Anthracycline and anthraquinone anticancer agents: current status and recent developments. *Pharmacol Ther* **60**, 185-214 (1993).
3. Chauncey, T.R. Drug resistance mechanisms in acute leukemia. *Curr Opin Oncol* **13**, 21-26 (2001).
4. Marie, J.P. Drug resistance in hematologic malignancies. *Curr Opin Oncol* **13**, 463-469 (2001).
5. Byrd, J.C.*, et al.* Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood* **100**, 4325-4336 (2002).
6. Gillet, J.P. & Gottesman, M.M. Mechanisms of multidrug resistance in cancer. *Methods Mol Biol* **596**, 47-76 (2010).
7. Dean, M., Hamon, Y. & Chimini, G. The human ATP-binding cassette (ABC) transporter superfamily. *J Lipid Res* **42**, 1007-1017. (2001).
8. Kolitz, J.E.*, et al.* P-glycoprotein inhibition using valspodar (PSC-833) does not improve outcomes for patients younger than age 60 years with newly diagnosed acute myeloid leukemia: Cancer and Leukemia Group B study 19808. *Blood* **116**, 1413-1421 (2010).
9. Okabe, M.*, et al.* Characterization of the organic cation transporter SLC22A16: a doxorubicin importer. *Biochem Biophys Res Commun* **333**, 754-762 (2005).
10. Koepsell, H., Lips, K. & Volk, C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm Res* **24**, 1227-1251 (2007).
11. Aouida, M., Poulin, R. & Ramotar, D. The human carnitine transporter SLC22A16 mediates high affinity uptake of the anticancer polyamine analogue bleomycin-A5. *J Biol Chem* **285**, 6275-6284 (2010).
12. Zhang, S.*, et al.* Organic cation transporters are determinants of oxaliplatin cytotoxicity. *Cancer Res* **66**, 8847-8857 (2006).
13. Aouida, M., Leduc, A., Wang, H. & Ramotar, D. Characterization of a transport and detoxification pathway for the antitumour drug bleomycin in Saccharomyces cerevisiae. *Biochem J* **384**, 47-58 (2004).
14. Aouida, M., Page, N., Leduc, A., Peter, M. & Ramotar, D. A Genome-Wide Screen in Saccharomyces cerevisiae Reveals Altered Transport As a Mechanism of Resistance to the Anticancer Drug Bleomycin. *Cancer Res* **64**, 1102-1109. (2004).
15. Yonezawa, A., Masuda, S., Yokoo, S., Katsura, T. & Inui, K. Cisplatin and oxaliplatin, but not carboplatin and nedaplatin, are substrates for human organic cation transporters (SLC22A1-3 and multidrug and toxin extrusion family). *J Pharmacol Exp Ther* **319**, 879-886 (2006).

16. Becker, M.L.*, et al.* Genetic variation in the organic cation transporter 1 is associated with metformin response in patients with diabetes mellitus. *Pharmacogenomics J* **9**, 242-247 (2009).
17. Sinclair, C.J., Chi, K.D., Subramanian, V., Ward, K.L. & Green, R.M. Functional expression of a high affinity mammalian hepatic choline/organic cation transporter. *J Lipid Res* **41**, 1841-1848 (2000).
18. Liu, Y.Y., Han, T.Y., Giuliano, A.E. & Cabot, M.C. Ceramide glycosylation potentiates cellular multidrug resistance. *FASEB J* **15**, 719-730 (2001).
19. de Bruijn, P.*, et al.* Determination of doxorubicin and doxorubicinol in plasma of cancer patients by high-performance liquid chromatography. *Anal Biochem* **266**, 216-221 (1999).
20. Enomoto, A.*, et al.* Molecular identification of a novel carnitine transporter specific to human testis. Insights into the mechanism of carnitine recognition. *J Biol Chem* **277**, 36262-36271. (2002).
21. Tamai, I.*, et al.* Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. *J Biol Chem* **273**, 20378-20382. (1998).
22. Nezu, J.*, et al.* Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter. *Nat Genet* **21**, 91-94 (1999).
23. Grundemann, D.*, et al.* Discovery of the ergothioneine transporter. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 5256-5261 (2005).
24. Okabe, M.*, et al.* Profiling SLCO and SLC22 genes in the NCI-60 cancer cell lines to identify drug uptake transporters. *Mol Cancer Ther* **7**, 3081-3091 (2008).
25. Sato, N.*, et al.* Expression of organic cation transporter SLC22A16 in human endometria. *Int J Gynecol Pathol* **26**, 53-60 (2007).
26. Cheah, I.K.*, et al.* Knockout of a putative ergothioneine transporter in Caenorhabditis elegans decreases lifespan and increases susceptibility to oxidative damage. *Free Radic Res* **47**, 1036-1045 (2013).
27. Chen, L.*, et al.* OCT1 is a high-capacity thiamine transporter that regulates hepatic steatosis and is a target of metformin. *Proc Natl Acad Sci U S A* **111**, 9983-9988 (2014).
28. Sala-Rabanal, M.*, et al.* Polyamine transport by the polyspecific organic cation transporters OCT1, OCT2, and OCT3. *Mol Pharm* **10**, 1450-1458 (2013).
29. Koepsell, H. The SLC22 family with transporters of organic cations, anions and zwitterions. *Mol Aspects Med* **34**, 413-435 (2013).
30. Azam, S., Drobetsky, E. & Ramotar, D. Overexpression of the cis/trans isomerase PTPA triggers caspase 3-dependent apoptosis. *Apoptosis* **12**, 1243-1255 (2007).
31. Nies, A.T.*, et al.* Proton pump inhibitors inhibit metformin uptake by organic cation transporters (OCTs). *PLoS One* **6**, e22163 (2011).
32. Gorbunov, D.*, et al.* High-affinity cation binding to organic cation transporter 1 induces movement of helix 11 and blocks transport after mutations in a modeled interaction domain between two helices. *Mol Pharmacol* **73**, 50-61 (2008).
33. Integrated genomic analyses of ovarian carcinoma. *Nature* **474**, 609-615 (2011).
34. Gyorffy, B.*, et al.* An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treat* **123**, 725-731 (2010).

35. Gyorffy, B., Lanczky, A. & Szallasi, Z. Implementing an online tool for genome-wide validation of survival-associated biomarkers in ovarian-cancer using microarray data from 1287 patients. *Endocr Relat Cancer* **19**, 197-208 (2012).
36. Gupta, S.*, et al.* Human organic cation transporter 1 is expressed in lymphoma cells and increases susceptibility to irinotecan and paclitaxel. *J Pharmacol Exp Ther* **341**, 16-23 (2012).
37. Nies, A.T., Koepsell, H., Damme, K. & Schwab, M. Organic cation transporters (OCTs, MATEs), in vitro and in vivo evidence for the importance in drug therapy. *Handb Exp Pharmacol*, 105-167 (2011).
38. Ahlin, G.*, et al.* Structural requirements for drug inhibition of the liver specific human organic cation transport protein 1. *J Med Chem* **51**, 5932-5942 (2008).
39. Dupou-Cezanne, L., Sautereau, A.M. & Tocanne, J.F. Localization of adriamycin in model and natural membranes. Influence of lipid molecular packing. *Eur J Biochem* **181**, 695-702 (1989).
40. Brosseau, N., Andreev, E. & Ramotar, D. Complementation of the Yeast Model System Reveals that Caenorhabditis elegans OCT-1 Is a Functional Transporter of Anthracyclines. *PLoS One* **10**, e0133182 (2015).
41. Courousse, T. & Gautron, S. Role of organic cation transporters (OCTs) in the brain. *Pharmacol Ther* **146**, 94-103 (2015).
42. Courousse, T.*, et al.* Brain organic cation transporter 2 controls response and vulnerability to stress and GSK3beta signaling. *Mol Psychiatry* **20**, 889-900 (2015).
43. Nies, A.T.*, et al.* Expression of organic cation transporters OCT1 (SLC22A1) and OCT3 (SLC22A3) is affected by genetic factors and cholestasis in human liver. *Hepatology* **50**, 1227-1240 (2009).
44. Fadili, K.E., Andreev, E., Wang, Z. & Ramotar, D. Cellular Responses to Anthracyclines Identify Ku70, a DNA Repair Factor that Changes Compartment and Remains Stable in Leukemic Cells. *American Journal of Cancer Science* **2**, 73-87 (2013).
45. Michel, V., Yuan, Z., Ramsubir, S. & Bakovic, M. Choline transport for phospholipid synthesis. *Experimental biology and medicine (Maywood, N.J.)* **231**, 490-504 (2006).
46. Taguchi, C.*, et al.* Functional analysis of [methyl-(3)H]choline uptake in glioblastoma cells: Influence of anti-cancer and central nervous system drugs. *Biochem Pharmacol* **88**, 303-312 (2014).
47. Kriel, J., Haesendonckx, S., Rubio-Texeira, M., Van Zeebroeck, G. & Thevelein, J.M. From transporter to transceptor: signaling from transporters provokes re-evaluation of complex trafficking and regulatory controls: endocytic internalization and intracellular trafficking of nutrient transceptors may, at least in part, be governed by their signaling function. *Bioessays* **33**, 870-879 (2011).
48. van der Burg, M.E.*, et al.* Long-term results of a randomised phase III trial of weekly versus three-weekly paclitaxel/platinum induction therapy followed by standard or extended three-weekly paclitaxel/platinum in European patients with advanced epithelial ovarian cancer. *Eur J Cancer* **50**, 2592-2601 (2014).
49. Parmar, M.K.*, et al.* Paclitaxel plus platinum-based chemotherapy versus conventional platinum-based chemotherapy in women with relapsed ovarian cancer: the ICON4/AGO-OVAR-2.2 trial. *Lancet* **361**, 2099-2106 (2003).

50. Kim, J.E., *et al.* Paclitaxel-exposed ovarian cancer cells induce cancerspecific CD4+ T cells after doxorubicin exposure through regulation of MyD88 expression. *Int J Oncol* **44**, 1716-1726 (2014).
51. A'Hern, R.P. & Gore, M.E. Impact of doxorubicin on survival in advanced ovarian cancer. *J Clin Oncol* **13**, 726-732 (1995).
52. Hess, V., *et al.* Phase I study of carboplatin, doxorubicin and weekly paclitaxel in patients with advanced ovarian carcinoma. *Ann Oncol* **14**, 638-642 (2003).
53. Dohner, H., *et al.* Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **115**, 453-474 (2010).
54. Ouellet, V., *et al.* Characterization of three new serous epithelial ovarian cancer cell lines. *BMC Cancer* **8**, 152 (2008).

FIGURE LEGENDS

FIGURE 1. Assessment of DNR uptake in cancer cell lines. After addition of DNR (5 μM) to cells, samples were harvested at the indicated time to monitor for the level of drug uptake. **A**, Chemical structure of daunorubicin (DNR) obtained from https://www.ncbi.nlm.nih.gov/pccompound?term=daunorubicin. **B**, Comparison of the relative uptake of DNR into four cancer cells using FACS analysis. Once the drug uptake was stopped, the washed cells were processed for FACS analysis. A total of 10,000 cells were analyzed for DNR fluorescence using FL2 (585/42). Results are expressed as the mean ± S.D. from three separate experiments. **C**, Fluorescent spectrometry analysis of DNR uptake into TOV2223G, OV866(2), and HL60 cells. The control (Ctrl) are TOV2223G cells without DNR. Increased fluorescence denoting drug uptake was detected with Fluoroskan Ascent Microplate reader set at 544 nm excitation/590 nm emission. The relative fluorescent unit (RFU) representing the measurement of drug uptake was expressed as pmol DNR/2 $x10^4$ cells/ min (see Material and Methods). Results are expressed as the mean ± S.D. from three separate experiments. **D**, Comparison of the concentration-dependent uptake of DNR by TOV2223G and HL60 cells. Cells were incubated with indicated concentrations of DNR then analyzed by the Fluoroskan Ascent Microplate reader. Results are expressed as the mean ± S.D. from three separate experiments. **E**, Epifluorescent microscopy showing intracellular colocalization of DAPI and DNR in the TOV2223G cell line. Comparisons were made between TOV2223G cells treated with or without DNR, followed by staining of nuclear DNA. Following DNR uptake (5 μM), the cells were washed, fixed with paraformaldehyde and processed for microscopy using Vectashield mounting medium containing 1.5 μg/ml DAPI to detect the nuclear DNA. Images were captured with an Olympus BX51 epifluorescent microscope using

Texas Red and DAPI-UV filters at 100 X magnification, and then processed with ImageJ software. Merged images overlapped DAPI-stained nucleus (blue) with DNR staining (red). Results are representative of two separate experiments.

FIGURE 2. Kinetic analysis reveals a high affinity uptake for DNR into the TOV2223G cells. Uptake rates were recorded for 60 min and quantified with the Fluoroskan Ascent microplate reader. The data were fitted to the following equation y =Vmax*x/(Km+x) by means of nonlinear regression using GraphPad Prism (GraphPad Software Inc, CA, USA) to determine Michaelis-Menten constant and maximum velocity. Results are representative of two separate experiments with standard errors.

FIGURE 3. Choline, but not L-carnitine or ergothioneine, serves as a potent competitor in DNR uptake into the TOV2223G cells. The cells were first seeded in 6-well plate onto coverslips at 5 x 10^5 cells in growth media and allowed to grow overnight. The next day the media was removed and the attached cells were pre-incubated with uptake buffer for 20 min at 37˚C. DNR (5 μM) and selected competitors at the indicated concentrations were mixed together in uptake buffer and added to cells on coverslips, followed by incubation for 1h. Cells were fixed and images were captured and processed as in Fig. 1E. For panel E, the sodium chloride in the uptake buffer was substituted with N-methyl-D-glucamine. Choline denoted as CHL. Results are representative of at least three separate experiments.

FIGURE 4. Phenformin and thiamine partially inhibit DNR uptake in TOV2223G cells. Cells were seeded in 96-well plates and DNR uptake was monitored as in Fig. 1C, but when added

together with either choline (CHL), ergothioneine (ERGO), phenoformin (PHEN), thiamine (THIA), spermidine (SPD) or L-carnitine (L-car). Cells without DNR were designated as the control (Ctrl). Results are expressed as the mean ± S.D. from three separate experiments.

FIGURE 5. shRNA-OCT1 downregulation in TOV2223G cells diminishes DNR uptake and confers resistance to the drug. A, Western blot analysis. Total cell extracts were prepared from stable clones of TOV2223G cells carrying either the empty vector pLMP or the plasmid pshRNA-OCT1 and processed for Western Blot analysis using anti-hOCT1 antibody. Each lane contained 30 μg of total protein extract. Actin (ACT1) was used as the internal control. WT is the TOV2223G cells alone. Results are representative of three separate experiments. **B**, Comparison of the concentration-dependent uptake of DNR into stable clones carrying either pLMP or pshRNA-OCT1. DNR uptake was monitored using the Fluoroskan Ascent microplate reader as above. Results are expressed as the mean ± S.D. from three separate experiments. **C**, Epifluorescent microscopy reveals that OCT1 downregulation severely inhibits DNR uptake into TOV2223G cells. The stable clones of TOV2223G carrying either pLMP or the knockdown plasmid pshRNA-OCT1 were fixed with PFA, and processed for microscopy, using Axio Imager Z2 with Texas Red and DAPI-UV filters at 63 X magnification. Images were processed with AxioVision software. Results are representative of three separate experiments. **D**, Cell survival upon exposure to DNR. The TOV2223G cells and stable clones carrying either the empty vector pLMP or pshRNA-OCT1 were exposed to DNR (5 μM) for 72 h, and the viability of the cells was monitored using MTT assay. Results are expressed as the mean ± S.D. from two separate experiments.

FIGURE 6. Expression of OCT1-EYFP, but not the OCT1-D474C variant, increases DNR uptake in HEK293T cells. The plasmid pOCT1-EYFP was used to create the OCT1-D474C

mutation by site-directed mutagenesis. The plasmids pEYFP (empty), pOCT1-EYFP and pOCT1-D474C-EYFP were transiently transfected into HEK293T cells and exposed to DNR. Cells expressing EYFP were sorted to quantify the level of DNR uptake using FACS analysis using FL1 530/30 to detect EYFP fluorescence and FL4 670LP for DNR fluorescence. **A**, Epifluorescent microscopy showing localization of the OCT1-EYFP fusion protein. Pictures were taken with Ziess Z2 fluorescent microcope using DAPI filter set and EYFP filter set. Results are representative of three separate experiments. **B**, DNR uptake in HEK293T cells carrying either the plasmid pOCT1-EYFP, the variant, or the empty EYFP vector. Results are expressed as the mean ± S.D. from three separate experiments. **C**, Comparison of the rhodamine uptake level in HEK293T cells carrying the empty EYFP vector, or either the plasmid pOCT1-EYFP or pOCT1-D474C-EYFP. Results are expressed as the mean ± S.D. from three separate experiments.

FIGURE 7. High OCT1 mRNA levels are associated with better overall survival of high-grade serous epithelial ovarian cancer patients. Overall survival plot was generated online using the Kaplan-Meier Plotter based on signal intensity of the SLC22A1 probe (207201_s_at) in Affymetrix microarray gene expression data from high-grade serous epithelial ovarian cancer patients of The Cancer Genome Atlas. Auto select best cutoff was chosen in the analysis; cutoff value was 19 and expression range of the probe was1-150. A total of 469 patients were available and samples were split in two groups (high and low) according to the cutoff value. The hazard ratio with 95% confidence intervals and log rank p value was calculated and significance was set at $p < 0.05$.

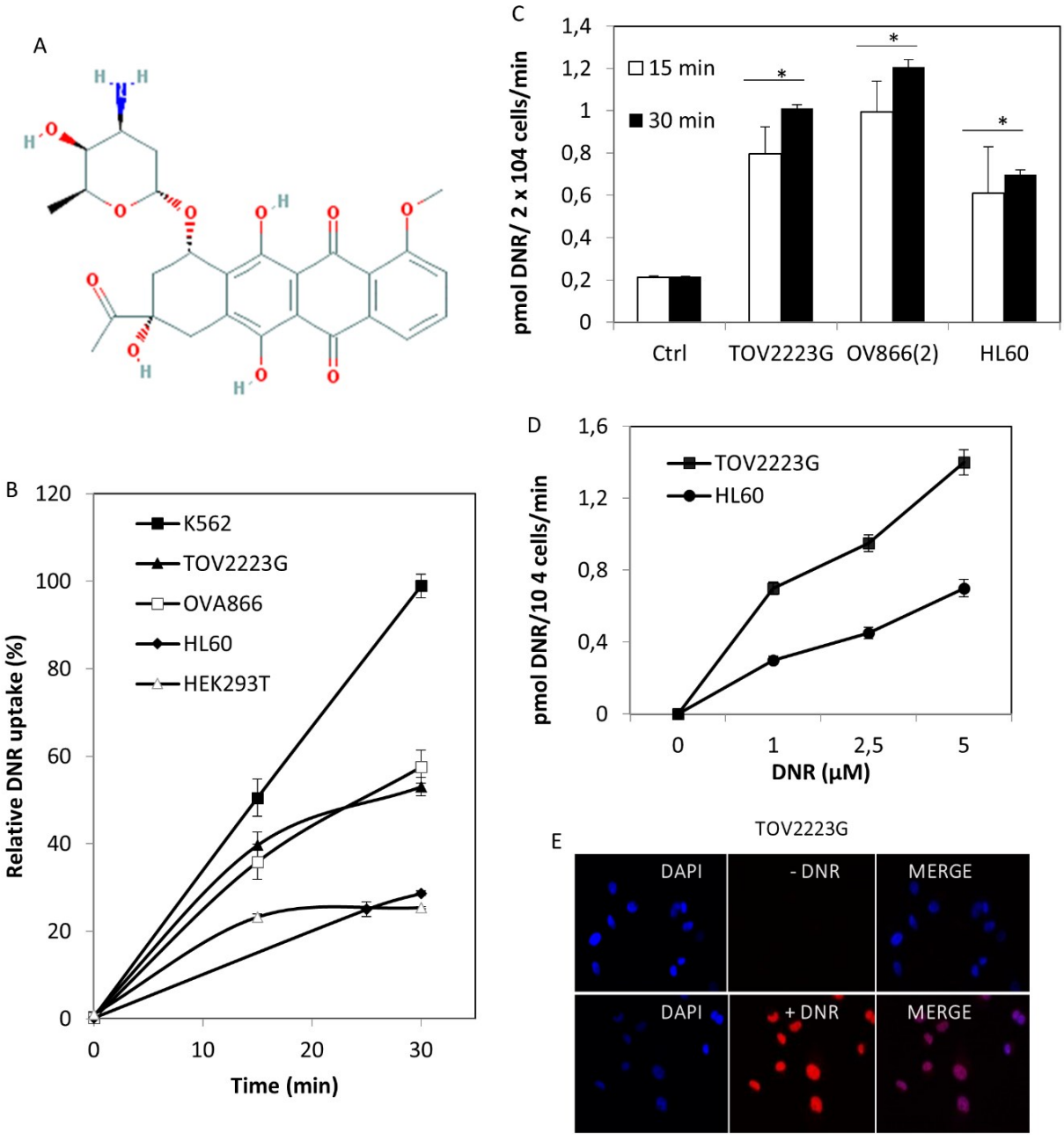


FIGURE 1. Assessment of DNR uptake in cancer cell lines.

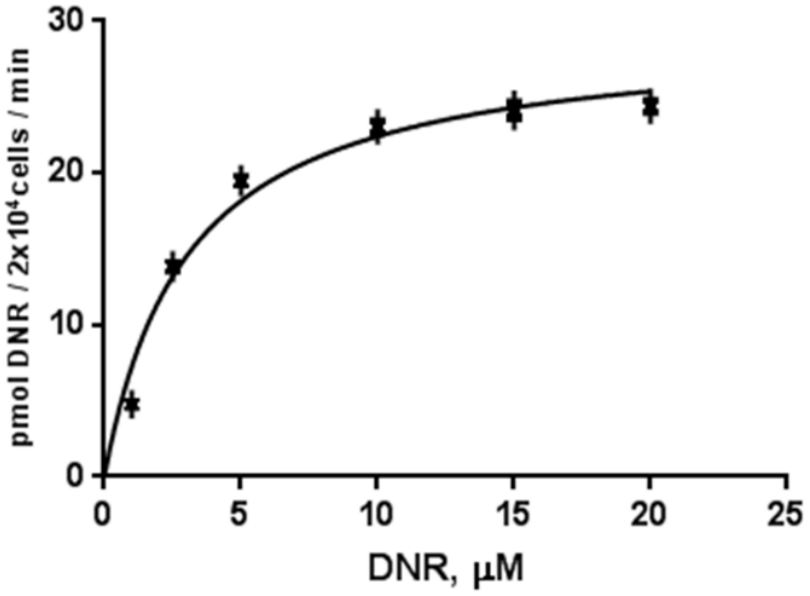


FIGURE 2. Kinetic analysis reveals a high affinity uptake for DNR into the TOV2223G cells.

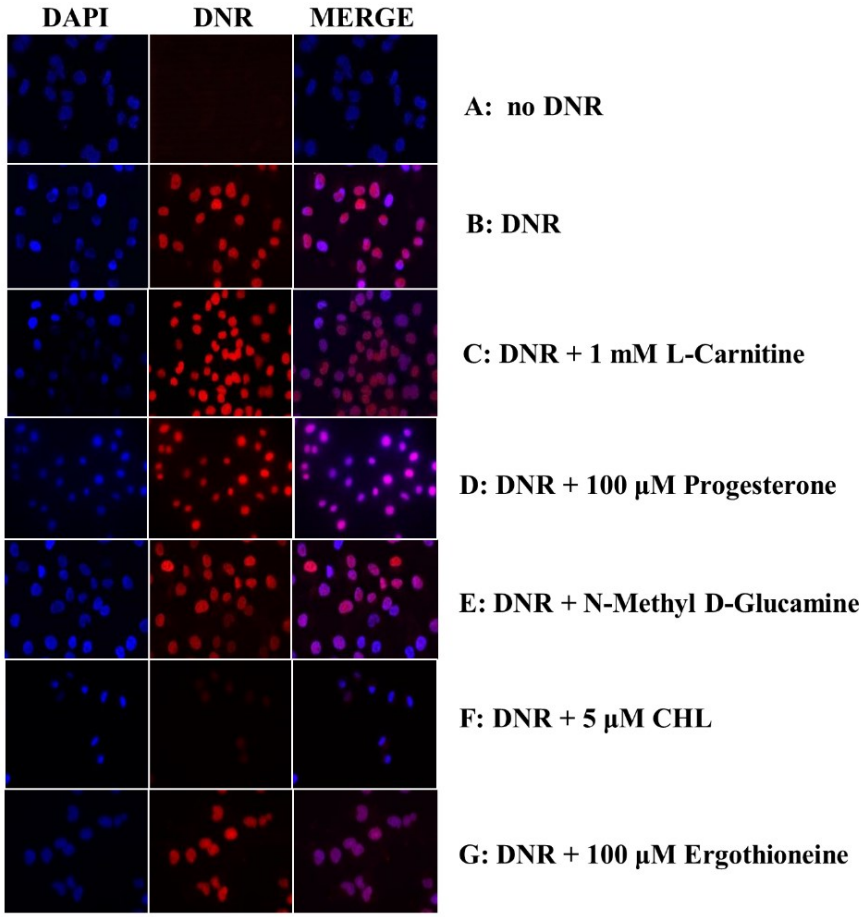


FIGURE 3. Choline, but not L-carnitine or ergothioneine, serves as a potent competitor in DNR uptake into the TOV2223G cells.

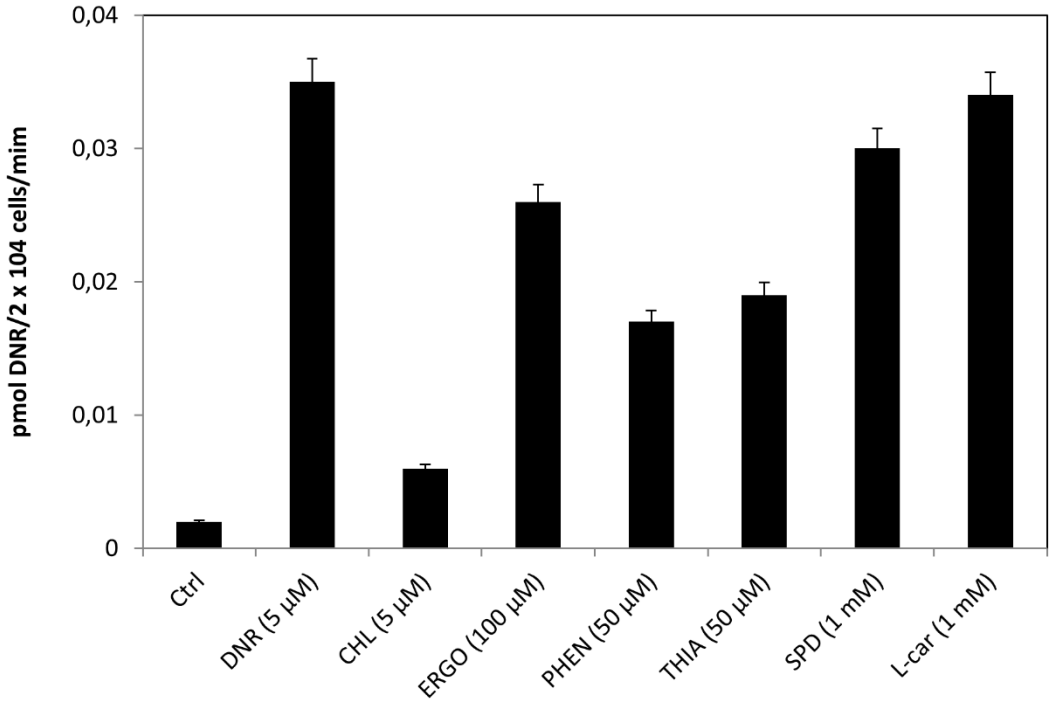


FIGURE 4. Phenformin and thiamine partially inhibit DNR uptake in TOV2223G cells.

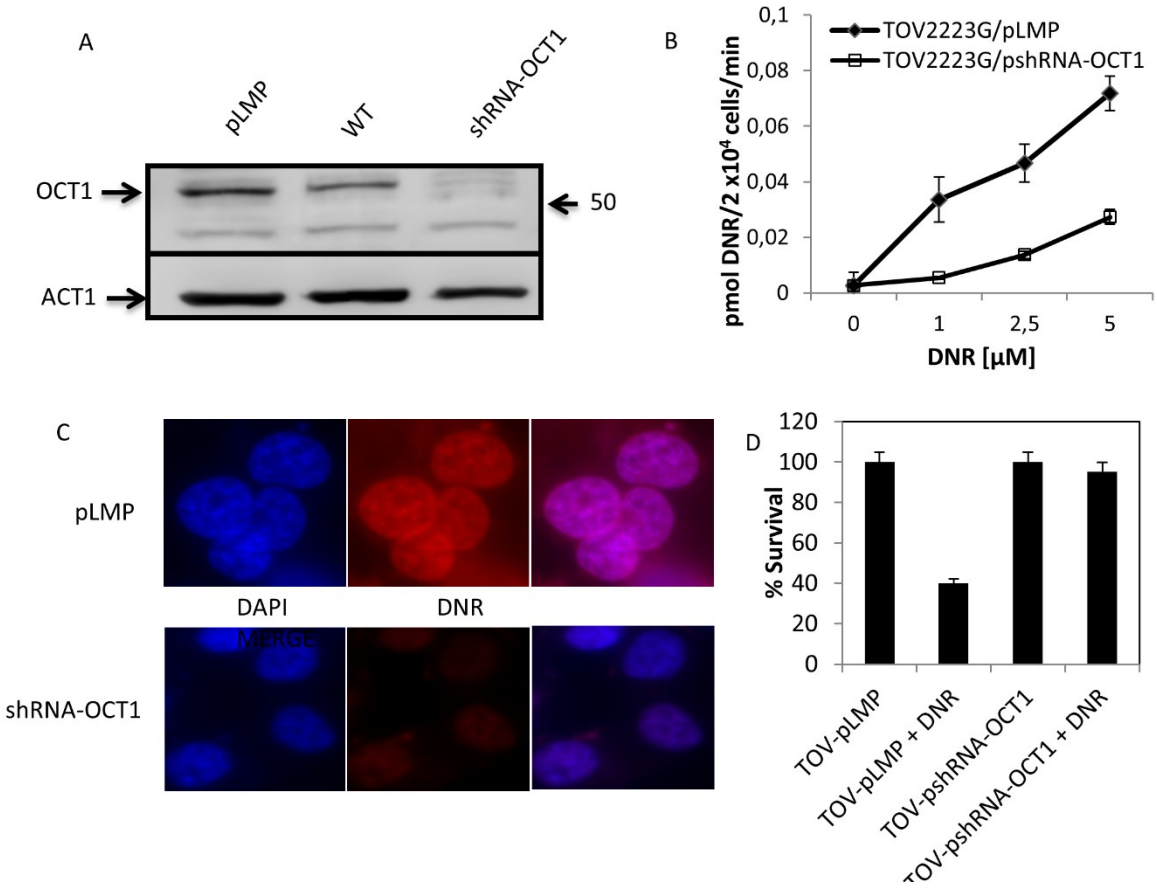


FIGURE 5. shRNA-OCT1 downregulation in TOV2223G cells diminishes DNR uptake and confers resistance to the drug.

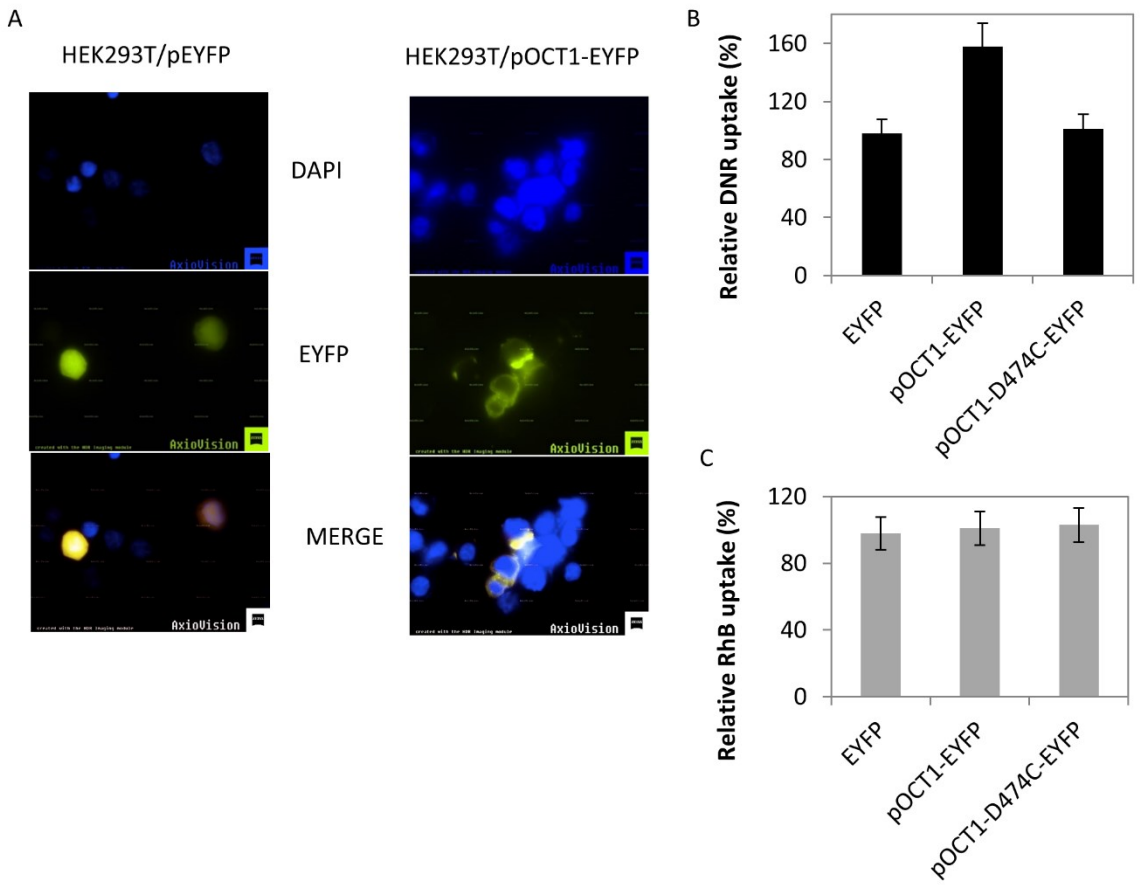


FIGURE 6. Expression of OCT1-EYFP, but not the OCT1-D474C variant, increases DNR uptake in HEK293T cells.

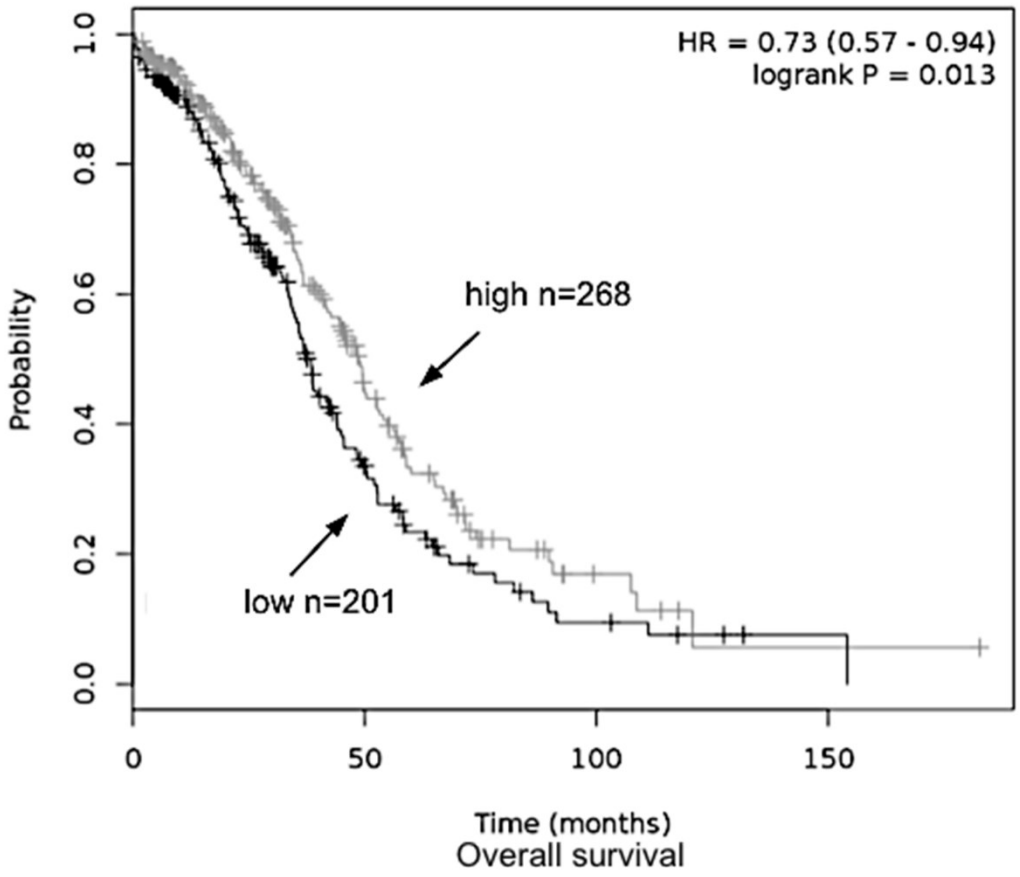


FIGURE 7. High OCT1 mRNA levels are associated with better overall survival of high-grade serous epithelial ovarian cancer patients.

SUPPLEMENTAL DATA:

Supplemental Table 1. The short hairpin sequences used in this study

Sequence no	sh sense sequence 5'-3'	sh entire sequence 5'-3'
1	UGGCCACUGCCU GAUUUCAAAC	TGCTGTTGACAGTGAGCG**(C)GGCCACTGCCTGA TTTCAAAC**TAGTGAAGCCACAGATGTA**GTTTGA AATCAGGCAGTGGCCA**TGCCTACTGCCTCGGA
2	ACCUCUUUCAGU CCUGUUUGAA	TGCTGTTGACAGTGAGCG**CCCTCTTTCAGTCCT GTTTGAA**TAGTGAAGCCACAGATGTA**TTCAAAC AGGACTGAAAGAGGT**TGCCTACTGCCTCGGA

Supplemental Table 2. Oligonucleotides primers used in this study

Primers	Purpose	Sequence 5'-3'
miR30-F	Knockdown of OCT-1	CAGAAGGCTCGAGAAGGTATATT GCTGTTGACAGTGAGCG
miR30-R	Knockdown of OCT-1	CTAAAGTAGCCCCTTGAATTCCG AGGCAGTAGGCA
hOCT-1-F	hOCT-1-EYFP over expression	AAATCAGATCTCGAGGCCGCCAC CATGCCCACCGTGGATGAC
hOCT-1-R2	hOCT-1-EYFP over expression	ACCGTCGACTGCAGAATTCGGGT GCCCGAGGGTTCAGAGGTTTGG
Sequencing	hOCT-1-EYFP construct sequencing	CAAATGGGCGGTAGGCGTTG
hOCT-1-EYFP_D474C-F	hOCT-1-EYFP site-directed mutagenesis	GTGTTCCTCCCTGTGTTGCATAGG TGGGATAATCA
hOCT-1-EYFP_D474C-R	hOCT-1-EYFP site-directed mutagenesis	TTATCCCACCTATGCAACACAGG GAGGAACACACC

FIGURE LEGENDS TO SUPPLEMENTAL DATA

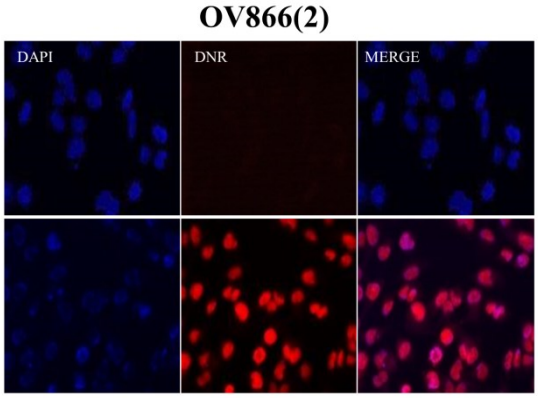
Figure S1. Epifluorescent microscopy showing DNR uptake into the nucleus of the ovarian cancer cell line OV866(2).

Figure S2. Epifluorescent microscopy showing DNR uptake under different conditions in the ovarian cancer cell line OV866(2).

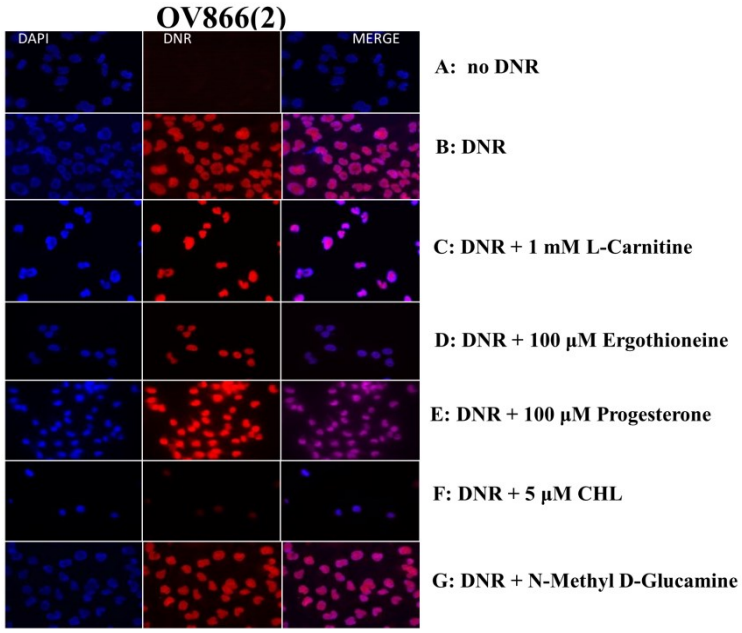
Figure S3. Western blot analysis showing the expression level of OCT1 in various cancer cell lines.

Figure S4. Plasma membrane localization of native OCT1-EYFP and its variant OCT1-D474C-EYFP.

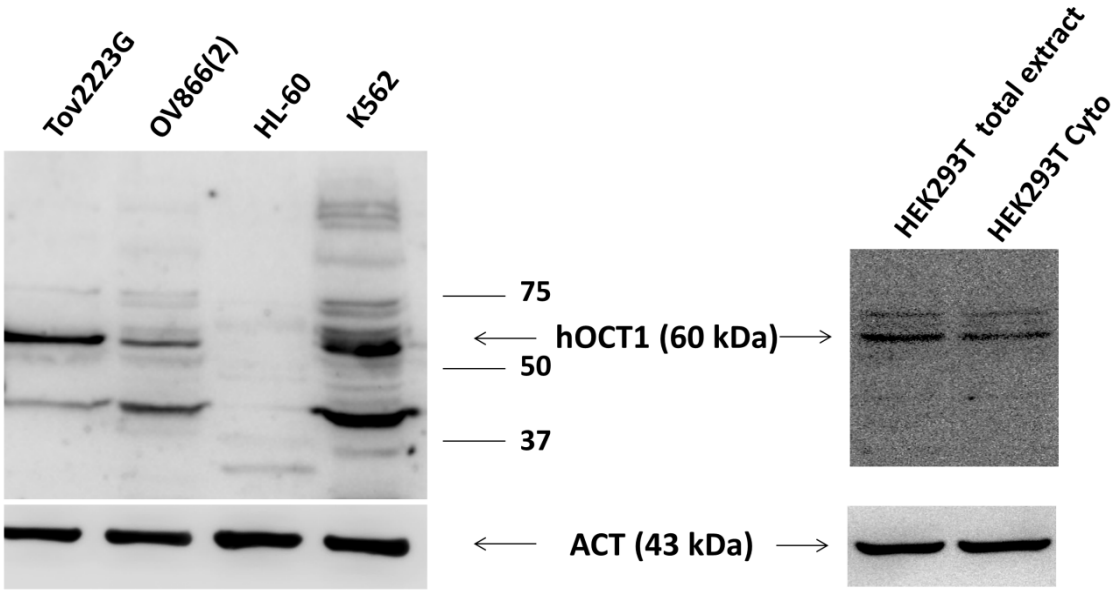
Figure S5. RT-PCR analysis showing the expression level of CT2 (panel A) and OCTN2 (panel B) in various leukemia cell lines and when cells were untreated and treated with DNR.



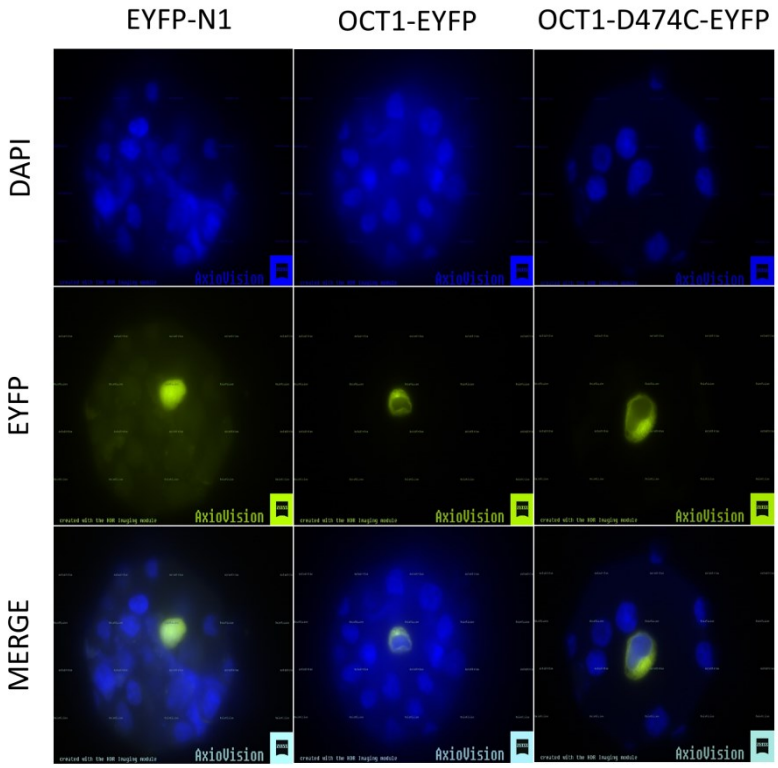
Supplemental data Fig. S1. Epifluorescent microscopy showing DNR uptake into the nucleus of the ovarian cancer cell line OV866(2)



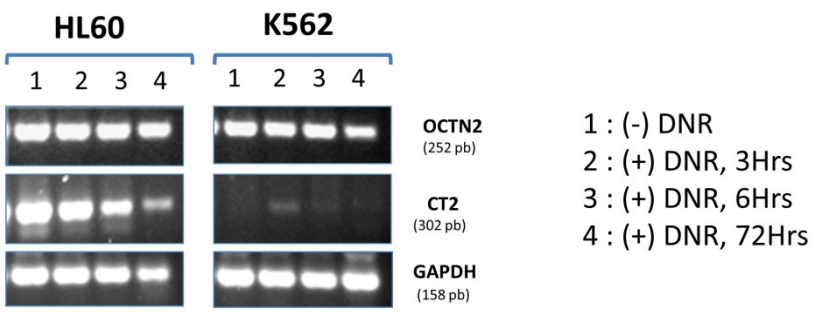
Supplemental data Fig. S2. Epifluorescent microscopy showing DNR uptake under different conditions in the ovarian cancer cell line OV866(2).



Supplemental data Fig. S3. Western blot analysis showing the expression level of OCT1 in various cancer cell lines.



Supplemental data Fig. S4. Plasma membrane localization of native OCT1-EYFP and its variant OCT1-D474C-EYFP.



Supplemental data Fig. S5. RT-PCR analysis showing the expression level of CT2 (panel A) and OCTN2 (panel B) in various leukemia cell lines and when cells were untreated and treated with DNR.

Chapter 3: Discussion

Anthracyclines: between passive diffusion, passive flip flop and facilitated transport mechanism.

Interactions between the cellular plasma membrane and anticancer drugs are of great importance and determine the proper drug delivery in cancer cells. A majority of anticancer drugs are negatively charged and lipophilic, and it was therefore proposed that the transport of these drugs occurs by passive diffusion down a drug gradient or by a slow flip-flop mechanism which is defined as transmembrane lipid translocation (113). Indeed, biological membranes possess the ability to translocate (flip flop) lipid molecules across the outer and the inner leaflets of the plasma membrane and vice versa. The flipping, also referred as trans bilayer translocation is, however, energetically unfavorable for the cell because it would consume an enormous amount of energy (up to 50 kcal/mol) in order to overcome the energy barrier needed for phospholipid displacement (127). It was shown that very slow spontaneous flipping with a half-life of hours to days does not occur in biogenic membranes; rather this process occurs very fast with rates from tens of seconds to several minutes, suggesting the involvement of specific transport systems (127). One possible way for this to occur is via specialized proteins called flippases, but the identification of such « flipping helpers» for anthracyclines translocation has not yet been discovered. Members of the ABC transporters such P-glycoprotein are believed to act as flippases and there is an ongoing debate that the resistance vs sensitivity of cancer cells towards anthracyclines depends on MDR protein expression, but a recent study using the specific P-glycoprotein inhibitor valspodar (PSC-833) clearly showed that anthracyclines entry is independent of MDR expression pattern or action (93). Thus DNR, DOX or other anthracyclines cannot cross the plasma membrane, as they would be exclusively located at the cell surface. Indeed, our results (figure 1) showing a differential uptake of DNR in various cell lines supports this notion and, although there is no additional evidence, uptake of DNR by passive diffusion or flip-flop should otherwise be seen equally in every cell line tested. In the present study we show that DNR transport depends on OCT1 protein expression and TOV2223G cells deficient in OCT1 expression (figure 5) display a substantially decreased DNR transport. Whether OCT1 is physically responsible for

further delivering anthracyclines to their final destination, the nucleus, remains to be explored. Hence, the discovery of OCT1 as functional and high affinity transporter for the anthracycline DNR would be a valuable asset for screening patients cytogenetic responses towards this chemotherapeutic drug.

OCT1 is critical for DNR uptake and may be used as prognostic marker for responsiveness to therapy with anthracyclines.

Chemoresistance to anticancer drugs is an actual challenge for the successful outcome of therapy and OCT1 has an important role to play in this regard. More et al., (30) demonstrated that functional OCT1 transporter is needed for a proper transport of picoplatin in HEK293T cells. Moreover, it was shown that structural differences in platinum derivatives have a great impact on drug interactions with OCT1, and variations in protein composition appear to affect the uptake of platinum compounds such as oxaliplatin. Therefore, it was suggested that OCT1 plays an important role in chemoresistance in various cancers including colon cancer (30). Another group working on OCT1 and metformin transport reported that this transporter is crucial for uptake of the drug in OVCAR-3 and SKOV-3 ovarian cell lines (30). It was demonstrated that metformin combined with cisplatin seems to have a synergic effect on apoptosis. The authors of this study also demonstrated that differential expression of OCT1 is a strong determinant of sensitivity to the metformin therapy and suggested the use of phenformin (128). Our *in vitro* results showing a direct role of OCT1 in the transport of DNR in TOV2223G may have an important clinical impact in terms of predicating patient response to anthracyclines. Indeed, data obtained from our *in vivo* study (figure 7) investigating the role of OCT1 in overall survival of relapsed/recurrent ovarian cancer patients comparing 469 patients samples treated with combinational chemotherapy with cisplatin and paclitaxel and differing by the expression level of OCT1, clearly show that patients overexpressing OCT1 display an increased survival probability compared to control which suggests that overexpression of this transporter favors tumor eradication. TOV2223G is a serous type, solid tumor ovarian cancer cell line model and this type of cancer accounts for one half of all ovarian cancers including mucinous, endometria and clear-cell. Serous type ovarian cancer patients are initially subjected to surgery and as these are mostly diagnosed with advanced

stage of tumor growth and spread, its removal is extremely difficult and mostly inefficient. When surgery fails, patients are then subjected to a first line chemotherapy of standard regimen including combinational treatment with carboplatin/cisplatin and paclitaxel (CP) (129). PEGylated DOX (PD) is currently used in clinical trials such as CALYPSO (Caelyx in Platinum Sensitive Ovarian patients) (130, 131). In this study, PD in combination with carboplatin (investigated arm) was compared to CP alone (control arm) and the results showed a statistically significant improvement in progression free survival (PFS) in the investigated arm (131). Although the study did not profile patients for OCT1 expression, a possible role of OCT1 should be considered as it can transport DNR and paclitaxel. Therefore, we propose that OCT1 expression levels should be monitored in order to predict better responsiveness to PD treatment. PD, which was approved in both Europe and USA (Caelyx, EU and Doxil, USA) has garnered particular attention with respect to treatment of ovarian cancer, breast cancer and multiple myelomas (data from EMA). It is noteworthy that the efficiency of the liposomal PD formulation may suggest that entry of the drug occurs via passive diffusion or by flip flop mechanism. As DOX-HCl in PD is encapsulated by a surface-bound methoxy polyethylene glycol (mPEG), the hydrophilic shell of the latter appears to contribute to a prolonged blood circulation half-life of the drug and as a consequence is less targeted to mononuclear cells such as macrophages compared to the conventional DOX-HCl formulation (132). Moreover, it was shown that PD locates at the extracellular fluid surrounding the tumor cell without entering it, where the liposomes release their cargo DOX (132). Therefore, the entry of the free drug must occur via a facilitated transport mechanism, and based on our findings, we believe that this occurs via the transporter OCT1.

Conclusion and future directions

Further studies are needed in order to demonstrate that OCT1 is indeed crucial for DNR transport. Although we clearly show that DNR is a high affinity substrate for OCT1, we do not exclude the contribution of low affinity DNR transporters such as OCT2 and OCT3 as these share 75%-85% and 50% identity in protein sequence respectively. In support of this is the residual uptake of DNR that we observed in TOV2223G down regulated for OCT1. Moreover, as DNR uptake was blocked substantially with choline, and because of the reported role of OCT2 in choline transport, a role for OCT2 in DNR transport may also be suggested.

However, if OCT2 was also involved in choline transport, down regulation of OCT1 in TOV2223G should not be sufficient for efficient inhibition of DNR transport, as the latter should occur predominantly via OCT2 and therefore we would be able to observe fluorescent DNR inside the cell. Since we did not observe DNR uptake in TOV2223G cells lacking OCT1, we believe that OCT2 is not involved in choline and DNR transport. We also exclude the contribution of CHT1 transporter in DNR transport because choline transport in TOV2223G is sodium independent (data not shown) and sodium import is absolute requirement for CHT1 to operate. It is noteworthy that although the lack of OCT1 greatly prevented the entry of DNR, we cannot rule out the possibility that choline transport was available for uptake by another transporter. Unfortunately, either in control cells or in cells with down regulated OCT1, we could not quantify choline uptake by fluorescence microscopy or fluorometric micro plate reader. To overcome this, one possibility would be the use of the fluorescent choline derivatives ethanamimium 2-hydroxy-*N*,*N*-dimethyl-*N*-[2-*N*-(2,1,3-benzoxadiazol-4-amine,-*N*-methyl,-7- nitro)-ethyl] bromide (NBD-choline) and C_6–NBD–phosphatidylcholine (C_6–NBD–PC) as suggested by Vila et al., (133). The authors reported that these two derivatives have the ability to only enter cancer cells and not normal cells, which might be an important asset for screening deregulation of choline transport. Nevertheless, we do not exclude the possibility of inefficient knock down of OCT1 and we believe that an absolute depletion of this transporter will be needed in order to confirm its particular role in the transport of DNR. Thus, we intend to knock out OCT1 using the CRISPR-Cas9 system in TOV2223G which, we believe, will confirm the role of OCT1 as high affinity transporter of DNR. Moreover, as OCT2 and OCT3 might also be involved in DNR uptake as supportive DNR transporters, knock down of both transporters might not be indicative of their contribution in DNR transport, but might be indicative of increased OCT1 protein expression on the cell surface. We exclude the contribution of OCTN1, OCTN2 and CT2 as DNR transporters, because high affinity substrates for these transporters did not block DNR uptake either in TOV2223G (figure 4) or in other tested cell lines (data not shown). In support of this is the low protein identity with OCT1 (~30%) which suggests a different role of these transporters other than in the transport of anthracyclines.

References

1. Cheung-Ong K, Giaever G, Nislow C. DNA-damaging agents in cancer chemotherapy: serendipity and chemical biology. Chemistry & biology. 2013;20(5):648-59.
2. Oronsky BT, Carter CA, Oronsky AL, Salacz ME, Reid T. "No patient left behind": an alternative to "the War on Cancer" metaphor. Medical oncology (Northwood, London, England). 2016;33(6):55.
3. Rosenberg B, Vancamp L, Krigas T. INHIBITION OF CELL DIVISION IN ESCHERICHIA COLI BY ELECTROLYSIS PRODUCTS FROM A PLATINUM ELECTRODE. Nature. 1965;205:698-9.
4. Wheate NJ, Walker S, Craig GE, Oun R. The status of platinum anticancer drugs in the clinic and in clinical trials. Dalton transactions (Cambridge, England : 2003). 2010;39(35):8113-27.
5. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nature reviews Cancer. 2007;7(8):573-84.
6. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacological reviews. 2004;56(2):185-229.
7. Weiss RB. The anthracyclines: will we ever find a better doxorubicin? Seminars in oncology. 1992;19(6):670-86.
8. Danesi R, Fogli S, Gennari A, Conte P, Del Tacca M. Pharmacokinetic-pharmacodynamic relationships of the anthracycline anticancer drugs. Clinical pharmacokinetics. 2002;41(6):431-44.
9. Yang F, Teves SS, Kemp CJ, Henikoff S. Doxorubicin, DNA torsion, and chromatin dynamics. Biochimica et biophysica acta. 2014;1845(1):84-9.
10. Gille L, Nohl H. Analyses of the molecular mechanism of adriamycin-induced cardiotoxicity. Free radical biology & medicine. 1997;23(5):775-82.
11. Steinherz LJ, Steinherz PG, Tan CT, Heller G, Murphy ML. Cardiac toxicity 4 to 20 years after completing anthracycline therapy. Jama. 1991;266(12):1672-7.
12. Hefti E, Blanco JG. Anthracycline-Related Cardiotoxicity in Patients with Acute Myeloid Leukemia and Down Syndrome: A Literature Review. Cardiovascular toxicology. 2015.
13. Borchmann P, Hubel K, Schnell R, Engert A. Idarubicin: a brief overview on pharmacology and clinical use. International journal of clinical pharmacology and therapeutics. 1997;35(2):80-3.
14. Koh E, Ueda Y, Nakamura T, Kobayashi A, Katsuta S, Takahashi H. Apoptosis in young rats with adriamycin-induced cardiomyopathy--comparison with pirarubicin, a new anthracycline derivative. Pediatric research. 2002;51(2):256-9.
15. Thomas X, Le QH, Fiere D. Anthracycline-related toxicity requiring cardiac transplantation in long-term disease-free survivors with acute promyelocytic leukemia. Annals of hematology. 2002;81(9):504-7.
16. Sharom FJ. ABC multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics. 2008;9(1):105-27.
17. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov. 2006;5(3):219-34.

18. Wessler JD, Grip LT, Mendell J, Giugliano RP. The P-glycoprotein transport system and cardiovascular drugs. Journal of the American College of Cardiology. 2013;61(25):2495-502.
19. Homolya L, Orban TI, Csanady L, Sarkadi B. Mitoxantrone is expelled by the ABCG2 multidrug transporter directly from the plasma membrane. Biochimica et biophysica acta. 2011;1808(1):154-63.
20. Koepsell H. The SLC22 family with transporters of organic cations, anions and zwitterions. Molecular aspects of medicine. 2013;34(2-3):413-35.
21. Koepsell H, Lips K, Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharmaceutical research. 2007;24(7):1227-51.
22. Grundemann D, Gorboulev V, Gambaryan S, Veyhl M, Koepsell H. Drug excretion mediated by a new prototype of polyspecific transporter. Nature. 1994;372(6506):549-52.
23. Chen L, Shu Y, Liang X, Chen EC, Yee SW, Zur AA, et al. OCT1 is a high-capacity thiamine transporter that regulates hepatic steatosis and is a target of metformin. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(27):9983-8.
24. Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, et al. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. The Journal of clinical investigation. 2007;117(5):1422-31.
25. Kim MH, Maeng HJ, Yu KH, Lee KR, Tsuruo T, Kim DD, et al. Evidence of carrier-mediated transport in the penetration of donepezil into the rat brain. Journal of pharmaceutical sciences. 2010;99(3):1548-66.
26. Murakami H, Sawada N, Koyabu N, Ohtani H, Sawada Y. Characteristics of Choline Transport Across the Blood-Brain Barrier in Mice: Correlation with In Vitro Data. Pharmaceutical research. 2000;17(12):1526-30.
27. Michel V, Yuan Z, Ramsubir S, Bakovic M. Choline transport for phospholipid synthesis. Experimental biology and medicine (Maywood, NJ). 2006;231(5):490-504.
28. Grundemann D, Liebich G, Kiefer N, Koster S, Schomig E. Selective substrates for non-neuronal monoamine transporters. Molecular pharmacology. 1999;56(1):1-10.
29. Sweet DH, Miller DS, Pritchard JB. Ventricular choline transport: a role for organic cation transporter 2 expressed in choroid plexus. The Journal of biological chemistry. 2001;276(45):41611-9.
30. More SS, Li S, Yee SW, Chen L, Xu Z, Jablons DM, et al. Organic cation transporters modulate the uptake and cytotoxicity of picoplatin, a third-generation platinum analogue. Molecular cancer therapeutics. 2010;9(4):1058-69.
31. Zhang S, Lovejoy KS, Shima JE, Lagpacan LL, Shu Y, Lapuk A, et al. Organic cation transporters are determinants of oxaliplatin cytotoxicity. Cancer Res. 2006;66(17):8847-57.
32. Yokoo S, Masuda S, Yonezawa A, Terada T, Katsura T, Inui K. Significance of organic cation transporter 3 (SLC22A3) expression for the cytotoxic effect of oxaliplatin in colorectal cancer. Drug metabolism and disposition: the biological fate of chemicals. 2008;36(11):2299-306.
33. White DL, Saunders VA, Dang P, Engler J, Zannettino AC, Cambareri AC, et al. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. Blood. 2006;108(2):697-704.

34. Yared JA, Tkaczuk KH. Update on taxane development: new analogs and new formulations. Drug design, development and therapy. 2012;6:371-84.
35. Gupta S, Wulf G, Henjakovic M, Koepsell H, Burckhardt G, Hagos Y. Human organic cation transporter 1 is expressed in lymphoma cells and increases susceptibility to irinotecan and paclitaxel. J Pharmacol Exp Ther. 2012;341(1):16-23.
36. Okuda M, Saito H, Urakami Y, Takano M, Inui K. cDNA cloning and functional expression of a novel rat kidney organic cation transporter, OCT2. Biochemical and biophysical research communications. 1996;224(2):500-7.
37. Cetinkaya I, Ciarimboli G, Yalcinkaya G, Mehrens T, Velic A, Hirsch JR, et al. Regulation of human organic cation transporter hOCT2 by PKA, PI3K, and calmodulin-dependent kinases. American journal of physiology Renal physiology. 2003;284(2):F293-302.
38. Motohashi H, Sakurai Y, Saito H, Masuda S, Urakami Y, Goto M, et al. Gene expression levels and immunolocalization of organic ion transporters in the human kidney. Journal of the American Society of Nephrology : JASN. 2002;13(4):866-74.
39. Gorboulev V, Ulzheimer JC, Akhoundova A, Ulzheimer-Teuber I, Karbach U, Quester S, et al. Cloning and characterization of two human polyspecific organic cation transporters. DNA and cell biology. 1997;16(7):871-81.
40. Barendt WM, Wright SH. The human organic cation transporter (hOCT2) recognizes the degree of substrate ionization. The Journal of biological chemistry. 2002;277(25):22491-6.
41. Grundemann D, Schechinger B, Rappold GA, Schomig E. Molecular identification of the corticosterone-sensitive extraneuronal catecholamine transporter. Nature neuroscience. 1998;1(5):349-51.
42. Song IS, Shin HJ, Shim EJ, Jung IS, Kim WY, Shon JH, et al. Genetic variants of the organic cation transporter 2 influence the disposition of metformin. Clinical pharmacology and therapeutics. 2008;84(5):559-62.
43. Leabman MK, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE, et al. Polymorphisms in a human kidney xenobiotic transporter, OCT2, exhibit altered function. Pharmacogenetics. 2002;12(5):395-405.
44. Kimura N, Masuda S, Tanihara Y, Ueo H, Okuda M, Katsura T, et al. Metformin is a superior substrate for renal organic cation transporter OCT2 rather than hepatic OCT1. Drug metabolism and pharmacokinetics. 2005;20(5):379-86.
45. Shih MC, Hoexter MQ, Andrade LA, Bressan RA. Parkinson's disease and dopamine transporter neuroimaging: a critical review. Sao Paulo medical journal = Revista paulista de medicina. 2006;124(3):168-75.
46. Sossi V, de la Fuente-Fernandez R, Schulzer M, Troiano AR, Ruth TJ, Stoessl AJ. Dopamine transporter relation to dopamine turnover in Parkinson's disease: a positron emission tomography study. Annals of neurology. 2007;62(5):468-74.
47. Taubert D, Grimberg G, Stenzel W, Schömig E. Identification of the Endogenous Key Substrates of the Human Organic Cation Transporter OCT2 and Their Implication in Function of Dopaminergic Neurons. PLoS ONE. 2007;2(4):e385.
48. Filipski KK, Mathijssen RH, Mikkelsen TS, Schinkel AH, Sparreboom A. Contribution of organic cation transporter 2 (OCT2) to cisplatin-induced nephrotoxicity. Clinical pharmacology and therapeutics. 2009;86(4):396-402.
49. Tanihara Y, Masuda S, Katsura T, Inui K. Protective effect of concomitant administration of imatinib on cisplatin-induced nephrotoxicity focusing on renal organic cation transporter OCT2. Biochem Pharmacol. 2009;78(9):1263-71.

50. Kekuda R, Prasad PD, Wu X, Wang H, Fei YJ, Leibach FH, et al. Cloning and functional characterization of a potential-sensitive, polyspecific organic cation transporter (OCT3) most abundantly expressed in placenta. The Journal of biological chemistry. 1998;273(26):15971-9.
51. Wu X, Kekuda R, Huang W, Fei YJ, Leibach FH, Chen J, et al. Identity of the organic cation transporter OCT3 as the extraneuronal monoamine transporter (uptake2) and evidence for the expression of the transporter in the brain. The Journal of biological chemistry. 1998;273(49):32776-86.
52. Grundemann D, Koster S, Kiefer N, Breidert T, Engelhardt M, Spitzenberger F, et al. Transport of monoamine transmitters by the organic cation transporter type 2, OCT2. The Journal of biological chemistry. 1998;273(47):30915-20.
53. Courousse T, Gautron S. Role of organic cation transporters (OCTs) in the brain. Pharmacology & therapeutics. 2015;146:94-103.
54. Shnitsar V, Eckardt R, Gupta S, Grottker J, Muller GA, Koepsell H, et al. Expression of human organic cation transporter 3 in kidney carcinoma cell lines increases chemosensitivity to melphalan, irinotecan, and vincristine. Cancer Res. 2009;69(4):1494-501.
55. Tamai I, Yabuuchi H, Nezu J, Sai Y, Oku A, Shimane M, et al. Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1. FEBS letters. 1997;419(1):107-11.
56. Grundemann D, Harlfinger S, Golz S, Geerts A, Lazar A, Berkels R, et al. Discovery of the ergothioneine transporter. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(14):5256-61.
57. Grundemann D. The ergothioneine transporter controls and indicates ergothioneine activity--a review. Preventive medicine. 2012;54 Suppl:S71-4.
58. Pochini L, Scalise M, Galluccio M, Indiveri C. OCTN cation transporters in health and disease: role as drug targets and assay development. Journal of biomolecular screening. 2013;18(8):851-67.
59. Okabe M, Szakacs G, Reimers MA, Suzuki T, Hall MD, Abe T, et al. Profiling SLCO and SLC22 genes in the NCI-60 cancer cell lines to identify drug uptake transporters. Molecular cancer therapeutics. 2008;7(9):3081-91.
60. Jong NN, Nakanishi T, Liu JJ, Tamai I, McKeage MJ. Oxaliplatin transport mediated by organic cation/carnitine transporters OCTN1 and OCTN2 in overexpressing human embryonic kidney 293 cells and rat dorsal root ganglion neurons. J Pharmacol Exp Ther. 2011;338(2):537-47.
61. Sekine T, Kusuhara H, Utsunomiya-Tate N, Tsuda M, Sugiyama Y, Kanai Y, et al. Molecular cloning and characterization of high-affinity carnitine transporter from rat intestine. Biochemical and biophysical research communications. 1998;251(2):586-91.
62. Tamai I, Ohashi R, Nezu J, Yabuuchi H, Oku A, Shimane M, et al. Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. The Journal of biological chemistry. 1998;273(32):20378-82.
63. Wu X, Prasad PD, Leibach FH, Ganapathy V. cDNA sequence, transport function, and genomic organization of human OCTN2, a new member of the organic cation transporter family. Biochemical and biophysical research communications. 1998;246(3):589-95.
64. Meier Y, Eloranta JJ, Darimont J, Ismair MG, Hiller C, Fried M, et al. Regional distribution of solute carrier mRNA expression along the human intestinal tract. Drug metabolism and disposition: the biological fate of chemicals. 2007;35(4):590-4.

65. Horvath G, Schmid N, Fragoso MA, Schmid A, Conner GE, Salathe M, et al. Epithelial organic cation transporters ensure pH-dependent drug absorption in the airway. American journal of respiratory cell and molecular biology. 2007;36(1):53-60.
66. Garrett Q, Xu S, Simmons PA, Vehige J, Flanagan JL, Willcox MD. Expression and localization of carnitine/organic cation transporter OCTN1 and OCTN2 in ocular epithelium. Investigative ophthalmology & visual science. 2008;49(11):4844-9.
67. Tamai I. Pharmacological and pathophysiological roles of carnitine/organic cation transporters (OCTNs: SLC22A4, SLC22A5 and Slc22a21). Biopharmaceutics & drug disposition. 2013;34(1):29-44.
68. Lee H-J, Zheng JJ. PDZ domains and their binding partners: structure, specificity, and modification. Cell Communication and Signaling : CCS. 2010;8:8-.
69. Lahjouji K, Elimrani I, Lafond J, Leduc L, Qureshi IA, Mitchell GA. L-Carnitine transport in human placental brush-border membranes is mediated by the sodium-dependent organic cation transporter OCTN2. American journal of physiology Cell physiology. 2004;287(2):C263-9.
70. Maekawa S, Mori D, Nishiya T, Takikawa O, Horinouchi T, Nishimoto A, et al. OCTN2VT, a splice variant of OCTN2, does not transport carnitine because of the retention in the endoplasmic reticulum caused by insertion of 24 amino acids in the first extracellular loop of OCTN2. Biochimica et biophysica acta. 2007;1773(6):1000-6.
71. Hu C, Lancaster CS, Zuo Z, Hu S, Chen Z, Rubnitz JE, et al. Inhibition of OCTN2-mediated transport of carnitine by etoposide. Molecular cancer therapeutics. 2012;11(4):921-9.
72. Duran JM, Peral MJ, Calonge ML, Ilundain AA. OCTN3: A Na+-independent L-carnitine transporter in enterocytes basolateral membrane. Journal of cellular physiology. 2005;202(3):929-35.
73. Lamhonwah AM, Ackerley CA, Tilups A, Edwards VD, Wanders RJ, Tein I. OCTN3 is a mammalian peroxisomal membrane carnitine transporter. Biochemical and biophysical research communications. 2005;338(4):1966-72.
74. Enomoto A, Wempe MF, Tsuchida H, Shin HJ, Cha SH, Anzai N, et al. Molecular identification of a novel carnitine transporter specific to human testis. Insights into the mechanism of carnitine recognition. The Journal of biological chemistry. 2002;277(39):36262-71.
75. Sato N, Ito K, Onogawa T, Akahira J, Unno M, Abe T, et al. Expression of organic cation transporter SLC22A16 in human endometria. Int J Gynecol Pathol. 2007;26(1):53-60.
76. Okabe M, Unno M, Harigae H, Kaku M, Okitsu Y, Sasaki T, et al. Characterization of the organic cation transporter SLC22A16: a doxorubicin importer. Biochemical and biophysical research communications. 2005;333(3):754-62.
77. Kunii E, Oguri T, Kasai D, Ozasa H, Uemura T, Takakuwa O, et al. Organic cation transporter OCT6 mediates cisplatin uptake and resistance to cisplatin in lung cancer. Cancer chemotherapy and pharmacology. 2015.
78. Aouida M, Poulin R, Ramotar D. The human carnitine transporter SLC22A16 mediates high affinity uptake of the anticancer polyamine analogue bleomycin-A5. The Journal of biological chemistry. 2010;285(9):6275-84.
79. Aouida M, Leduc A, Poulin R, Ramotar D. AGP2 encodes the major permease for high affinity polyamine import in Saccharomyces cerevisiae. The Journal of biological chemistry. 2005;280(25):24267-76.

80. Ota K, Ito K, Akahira J, Sato N, Onogawa T, Moriya T, et al. Expression of organic cation transporter SLC22A16 in human epithelial ovarian cancer: a possible role of the adriamycin importer. Int J Gynecol Pathol. 2007;26(3):334-40.
81. Lal S, Wong ZW, Jada SR, Xiang X, Chen Shu X, Ang PC, et al. Novel SLC22A16 polymorphisms and influence on doxorubicin pharmacokinetics in Asian breast cancer patients. Pharmacogenomics. 2007;8(6):567-75.
82. Koepsell H, Schmitt BM, Gorboulev V. Organic cation transporters. Reviews of physiology, biochemistry and pharmacology. 2003;150:36-90.
83. Nigam SK, Bush KT, Martovetsky G, Ahn SY, Liu HC, Richard E, et al. The organic anion transporter (OAT) family: a systems biology perspective. Physiological reviews. 2015;95(1):83-123.
84. Ahn SY, Eraly SA, Tsigelny I, Nigam SK. Interaction of organic cations with organic anion transporters. The Journal of biological chemistry. 2009;284(45):31422-30.
85. Nigam SK. What do drug transporters really do? Nat Rev Drug Discov. 2015;14(1):29-44.
86. Westmoreland TJ, Wickramasekara SM, Guo AY, Selim AL, Winsor TS, Greenleaf AL, et al. Comparative genome-wide screening identifies a conserved doxorubicin repair network that is diploid specific in Saccharomyces cerevisiae. PLoS One. 2009;4(6):e5830.
87. Lown JW. Anthracycline and anthraquinone anticancer agents: current status and recent developments. Pharmacology & therapeutics. 1993;60(2):185-214.
88. Chauncey TR. Drug resistance mechanisms in acute leukemia. Curr Opin Oncol. 2001;13(1):21-6.
89. Marie JP. Drug resistance in hematologic malignancies. Curr Opin Oncol. 2001;13(6):463-9.
90. Byrd JC, Mrozek K, Dodge RK, Carroll AJ, Edwards CG, Arthur DC, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). Blood. 2002;100(13):4325-36.
91. Gillet JP, Gottesman MM. Mechanisms of multidrug resistance in cancer. Methods Mol Biol. 2010;596:47-76.
92. Dean M, Hamon Y, Chimini G. The human ATP-binding cassette (ABC) transporter superfamily. Journal of lipid research. 2001;42(7):1007-17.
93. Kolitz JE, George SL, Marcucci G, Vij R, Powell BL, Allen SL, et al. P-glycoprotein inhibition using valspodar (PSC-833) does not improve outcomes for patients younger than age 60 years with newly diagnosed acute myeloid leukemia: Cancer and Leukemia Group B study 19808. Blood. 2010;116(9):1413-21.
94. Aouida M, Leduc A, Wang H, Ramotar D. Characterization of a transport and detoxification pathway for the antitumour drug bleomycin in Saccharomyces cerevisiae. Biochem J. 2004;384(Pt 1):47-58.
95. Aouida M, Page N, Leduc A, Peter M, Ramotar D. A genome-wide screen in Saccharomyces cerevisiae reveals altered transport as a mechanism of resistance to the anticancer drug bleomycin. Cancer Res. 2004;64(3):1102-9.
96. Yonezawa A, Masuda S, Yokoo S, Katsura T, Inui K. Cisplatin and oxaliplatin, but not carboplatin and nedaplatin, are substrates for human organic cation transporters (SLC22A1-3 and multidrug and toxin extrusion family). J Pharmacol Exp Ther. 2006;319(2):879-86.

97. Becker ML, Visser LE, van Schaik RH, Hofman A, Uitterlinden AG, Stricker BH. Genetic variation in the organic cation transporter 1 is associated with metformin response in patients with diabetes mellitus. Pharmacogenomics J. 2009;9(4):242-7.
98. Sinclair CJ, Chi KD, Subramanian V, Ward KL, Green RM. Functional expression of a high affinity mammalian hepatic choline/organic cation transporter. Journal of lipid research. 2000;41(11):1841-8.
99. Liu YY, Han TY, Giuliano AE, Cabot MC. Ceramide glycosylation potentiates cellular multidrug resistance. FASEB J. 2001;15(3):719-30.
100. de Bruijn P, Verweij J, Loos WJ, Kolker HJ, Planting AS, Nooter K, et al. Determination of doxorubicin and doxorubicinol in plasma of cancer patients by high-performance liquid chromatography. Anal Biochem. 1999;266(2):216-21.
101. Enomoto A, Wempe MF, Tsuchida H, Shin HJ, Cha SH, Anzai N, et al. Molecular identification of a novel carnitine transporter specific to human testis. Insights into the mechanism of carnitine recognition. J Biol Chem. 2002;277(39):36262-71.
102. Nezu J, Tamai I, Oku A, Ohashi R, Yabuuchi H, Hashimoto N, et al. Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter. Nat Genet. 1999;21(1):91-4.
103. Cheah IK, Ong RL, Gruber J, Yew TS, Ng LF, Chen CB, et al. Knockout of a putative ergothioneine transporter in Caenorhabditis elegans decreases lifespan and increases susceptibility to oxidative damage. Free Radic Res. 2013;47(12):1036-45.
104. Sala-Rabanal M, Li DC, Dake GR, Kurata HT, Inyushin M, Skatchkov SN, et al. Polyamine transport by the polyspecific organic cation transporters OCT1, OCT2, and OCT3. Mol Pharm. 2013;10(4):1450-8.
105. Azam S, Drobetsky E, Ramotar D. Overexpression of the cis/trans isomerase PTPA triggers caspase 3-dependent apoptosis. Apoptosis. 2007;12(7):1243-55.
106. Nies AT, Hofmann U, Resch C, Schaeffeler E, Rius M, Schwab M. Proton pump inhibitors inhibit metformin uptake by organic cation transporters (OCTs). PLoS One. 2011;6(7):e22163.
107. Gorbunov D, Gorboulev V, Shatskaya N, Mueller T, Bamberg E, Friedrich T, et al. High-affinity cation binding to organic cation transporter 1 induces movement of helix 11 and blocks transport after mutations in a modeled interaction domain between two helices. Mol Pharmacol. 2008;73(1):50-61.
108. Integrated genomic analyses of ovarian carcinoma. Nature. 2011;474(7353):609-15.
109. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat. 2010;123(3):725-31.
110. Gyorffy B, Lanczky A, Szallasi Z. Implementing an online tool for genome-wide validation of survival-associated biomarkers in ovarian-cancer using microarray data from 1287 patients. Endocr Relat Cancer. 2012;19(2):197-208.
111. Nies AT, Koepsell H, Damme K, Schwab M. Organic cation transporters (OCTs, MATEs), in vitro and in vivo evidence for the importance in drug therapy. Handb Exp Pharmacol. 2011(201):105-67.
112. Ahlin G, Karlsson J, Pedersen JM, Gustavsson L, Larsson R, Matsson P, et al. Structural requirements for drug inhibition of the liver specific human organic cation transport protein 1. J Med Chem. 2008;51(19):5932-42.

113. Dupou-Cezanne L, Sautereau AM, Tocanne JF. Localization of adriamycin in model and natural membranes. Influence of lipid molecular packing. Eur J Biochem. 1989;181(3):695-702.
114. Brosseau N, Andreev E, Ramotar D. Complementation of the Yeast Model System Reveals that Caenorhabditis elegans OCT-1 Is a Functional Transporter of Anthracyclines. PLoS One. 2015;10(7):e0133182.
115. Courousse T, Bacq A, Belzung C, Guiard B, Balasse L, Louis F, et al. Brain organic cation transporter 2 controls response and vulnerability to stress and GSK3beta signaling. Mol Psychiatry. 2015;20(7):889-900.
116. Nies AT, Koepsell H, Winter S, Burk O, Klein K, Kerb R, et al. Expression of organic cation transporters OCT1 (SLC22A1) and OCT3 (SLC22A3) is affected by genetic factors and cholestasis in human liver. Hepatology. 2009;50(4):1227-40.
117. Fadili KE, Andreev E, Wang Z, Ramotar D. Cellular Responses to Anthracyclines Identify Ku70, a DNA Repair Factor that Changes Compartment and Remains Stable in Leukemic Cells. American Journal of Cancer Science. 2013;2:73-87.
118. Taguchi C, Inazu M, Saiki I, Yara M, Hara N, Yamanaka T, et al. Functional analysis of [methyl-(3)H]choline uptake in glioblastoma cells: Influence of anti-cancer and central nervous system drugs. Biochem Pharmacol. 2014;88(3):303-12.
119. Kriel J, Haesendonckx S, Rubio-Texeira M, Van Zeebroeck G, Thevelein JM. From transporter to transceptor: signaling from transporters provokes re-evaluation of complex trafficking and regulatory controls: endocytic internalization and intracellular trafficking of nutrient transceptors may, at least in part, be governed by their signaling function. Bioessays. 2011;33(11):870-9.
120. van der Burg ME, Onstenk W, Boere IA, Look M, Ottevanger PB, de Gooyer D, et al. Long-term results of a randomised phase III trial of weekly versus three-weekly paclitaxel/platinum induction therapy followed by standard or extended three-weekly paclitaxel/platinum in European patients with advanced epithelial ovarian cancer. Eur J Cancer. 2014;50(15):2592-601.
121. Parmar MK, Ledermann JA, Colombo N, du Bois A, Delaloye JF, Kristensen GB, et al. Paclitaxel plus platinum-based chemotherapy versus conventional platinum-based chemotherapy in women with relapsed ovarian cancer: the ICON4/AGO-OVAR-2.2 trial. Lancet. 2003;361(9375):2099-106.
122. Kim JE, Jang MJ, Jin DH, Chung YH, Choi BS, Park GB, et al. Paclitaxel-exposed ovarian cancer cells induce cancerspecific CD4+ T cells after doxorubicin exposure through regulation of MyD88 expression. Int J Oncol. 2014;44(5):1716-26.
123. A'Hern RP, Gore ME. Impact of doxorubicin on survival in advanced ovarian cancer. J Clin Oncol. 1995;13(3):726-32.
124. Hess V, Verrill MW, Bomphray CC, Vaughan MM, Allen M, Gore ME. Phase I study of carboplatin, doxorubicin and weekly paclitaxel in patients with advanced ovarian carcinoma. Ann Oncol. 2003;14(4):638-42.
125. Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115(3):453-74.

126. Ouellet V, Zietarska M, Portelance L, Lafontaine J, Madore J, Puiffe ML, et al. Characterization of three new serous epithelial ovarian cancer cell lines. BMC Cancer. 2008;8:152.
127. Pomorski T, Menon AK. Lipid flippases and their biological functions. Cellular and molecular life sciences : CMLS. 2006;63(24):2908-21.
128. Segal ED, Yasmeen A, Beauchamp MC, Rosenblatt J, Pollak M, Gotlieb WH. Relevance of the OCT1 transporter to the antineoplastic effect of biguanides. Biochemical and biophysical research communications. 2011;414(4):694-9.
129. Pujade-Lauraine E, Wagner U, Aavall-Lundqvist E, Gebski V, Heywood M, Vasey PA, et al. Pegylated liposomal Doxorubicin and Carboplatin compared with Paclitaxel and Carboplatin for patients with platinum-sensitive ovarian cancer in late relapse. J Clin Oncol. 2010;28(20):3323-9.
130. Gladieff L, Ferrero A, De Rauglaudre G, Brown C, Vasey P, Reinthaller A, et al. Carboplatin and pegylated liposomal doxorubicin versus carboplatin and paclitaxel in partially platinum-sensitive ovarian cancer patients: results from a subset analysis of the CALYPSO phase III trial. Ann Oncol. 2012;23(5):1185-9.
131. Wagner U, Marth C, Largillier R, Kaern J, Brown C, Heywood M, et al. Final overall survival results of phase III GCIG CALYPSO trial of pegylated liposomal doxorubicin and carboplatin vs paclitaxel and carboplatin in platinum-sensitive ovarian cancer patients. British journal of cancer. 2012;107(4):588-91.
132. Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. International journal of nanomedicine. 2006;1(3):297-315.
133. Villa AM, Caporizzo E, Papagni A, Miozzo L, Del Buttero P, Grilli MD, et al. Choline and phosphatidylcholine fluorescent derivatives localization in carcinoma cells studied by laser scanning confocal fluorescence microscopy. Eur J Cancer. 2005;41(10):1453-9.