INTERACTION OF PHARMACEUTICAL EXCIPIENTS AND CHITOSAN DERIVATIVES WITH ORGANIC CATION TRANSPORTERS AND POTENTIAL FOR THE PREVENTION OF CISPLATIN-INDUCED NEPHROTOXICITY

By
MRS. Sirima SOODVILAI

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy (PHARMACEUTICAL TECHNOLOGY)
Department of PHARMACEUTICAL TECHNOLOGY
Graduate School, Silpakorn University
Academic Year 2018

อันตรกิริยาของสารช่วยทางเภสัชกรรมและอนุพันธ์ไคโตซานต่อการทำงานของตัวขนส่งยาประจุบวกและการใช้ป้องกันความเป็นพิษต่อไตจากยาซิสพลาทิน

โดย
นางศิริมา สุดวิลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปรัชญาดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีเภสัชกรรม แบบ 1.1 ปรัชญาดุษฎีบัณฑิต
ภาควิชาเทคโนโลยีเภสัชกรรม
บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
ปีการศึกษา 2561

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Title	Interaction of pharmaceutical excipients and chitosan derivatives with organic cation transporters and potential for the prevention of cisplatin-induced nephrotoxicity
By	Sirima SOODVILAI
Field of Study	(PHARMACEUTICAL TECHNOLOGY)
Advisor	PRANEET OPANASOPIT

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57353801 : Major (PHARMACEUTICAL TECHNOLOGY)
Keyword : EXCIPIENTS, CHITOSAN DERIVATIVES, DRUG TRANSPORTER, CISPLATIN, NEPHROTOXICITY

MRS. SIRIMA SOODVILAI : INTERACTION OF PHARMACEUTICAL EXCIPIENTS AND CHITOSAN DERIVATIVES WITH ORGANIC CATION TRANSPORTERS AND POTENTIAL FOR THE PREVENTION OF CISPLATIN-INDUCED NEPHROTOXICITY THESIS ADVISOR : PROFESSOR PRANEET OPANASOPIT

Pharmaceutical excipients are pharmacologically inert, however, they have been reported to have several biological effects. They showed an effects on several transport function of uptake and efflux drug transporters, these excipients may alter the pharmacokinetic of an incorporated drug thereby affecting its intended therapeutic efficacy and/or enhancing adverse side effects. The present study uncovers whether the pharmaceutical excipients including conventional excipients and chitosan derivatives affect transport function of organic cation transporter (OCT)1 and OCT2 responsible for transport of organic drugs into hepatocytes and renal proximal tubular cells, respectively. In addition, the potential therapeutic application of the excipients on reducing nephrotoxicity of anticancer drug (cisplatin) was revealed. The present study demonstrates the inhibitory effect of surfactants frequently used pharmaceutical excipients, especially Tween20 Tween60, and Tween80, on the transport function of OCT1 and OCT2. In addition, chitosan derivatives (MDM, BSCS, NSCS and OSCS), but not chitosan inhibited transport function of OCT1 and OCT2. Co-administration of cisplatin, an OCT2 substrate, plus OSCS significantly reduced cisplatin cellular accumulation compared with receiving cisplatin alone in *in vitro* study and in animal model. This result was accompanied by decrease in nephrotoxicity without reversing activity of cisplatin in cancer cells. In addition, cisplatin-OSCS complex showed less toxicity in renal proximal tubular cells than cisplatin alone. Its anti-cancer activity was existent but less extent rather than cisplatin alone. In conclusion, the present study demonstrates the pharmaceutical excipients including Tweens and chitosan derivatives inhibit the transport function of OCT1 and OCT2 that might affect pharmacokinetic of drug and/or their efficacy and toxicity. Chitosan derivative, OSCS, might be developed as nephroprotective agent for cisplatin treatment.

ACKNOWLEDGEMENTS

This thesis would not be completed without the help, support, and the guidance of many kind people, whom I wish to thank in this section.

First of all, I would like to express my sincere appreciation to my advisors, Professor Dr. Praneet Opanasopit for excellent guidance, great suggestions and encouragement. I also would like to express my gratitude to my co-advisor, Associate Prof. Dr. Theerasak Rojanarata for valuable advice, support and kindness. I wish to thank my thesis committees, Associate Prof. Dr. Tanasait Ngawhirunpat and Dr. Promsuk Jutabha for valuable time and insightful comments and suggestion.

I am grateful to thank the Commission of Higher Education (Thailand), the Thailand Research Funds through the Royal Golden Jubilee PhD Program (Grant No.PHD/0139/2557) for financial support during my study as well as Faculty of Pharmacy, Silpakorn University for facility support and partial financial support. The acknowledgement is given to College of Pharmacy, Rangsit University for giving me a chance to study.

My special appreciation goes to Dr. Warayuth Sajomsang, National Nanotechnology Center (NANOTECH), National Science and Technology Development Agency for kindly give me the amphiphilic chitosan derivatives used in this work.

I would like to express my gratitude to Professor Jindřich Kopeček, for giving me a great opportunity to conduct a research at Center of Controlled Chemical Delivery, Department of Pharmaceutics and Pharmaceutical chemistry, School of Pharmacy, University of Utah, USA. He gave the excellent ideas, precious suggestion and supported me everything in USA. I also would like to express my sincere appreciation to Professor Pavla Kopečková, and Reseach Professor Jiyuan Yang, for excellent guidance, great suggestions and kindness. I also wish to thank my friends in Dr. Kopeček's lab and my friends in Salt Lake City for their helping and caring during my living in USA.

I would like to show my greatest appreciation to my family for their support, understanding, encouragement, and believing in me, as always, for which my mere expression of thanks likewise does not suffice.

Last but not least, I would like to thanks my friends and all PDGIG members, especially Mrs. Areerut Sripattaporn, Mr.Teeratas Kansom, and Miss Wajee Tipparos for their help, technical supports, and all unforgettable time through this program.

For any mistake or inadequate information that may still in this dissertation, certainly, the responsibility is entirely my own.

Sirima SOODVILAI

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CHAPTER 1 INTRODUCTION

Pharmaceutical excipients are important for therapeutic drug formulations in order to facilitate their preparations. They are used in formulation of drugs for improving patient acceptability. Excipients are classified into two groups including classical excipient and novel excipients. Classical excipients such as surfactants, are extensively used in pharmaceutical formulations to improve dissolution of poorly soluble drugs [1-4]. In addition, solubilizing agents which are one of the classical excipients are commonly used in pharmaceutical formulations including cosolvents (dimethyl sulfoxide; DMSO), propylene glycol (PG), polyethylene glycol (PEG)), cyclodextrins (CD), and surfactants (Cremophor, Pluronic, Tween and Span) [5]. Novel excipients are developed to use in drug derivery systems such as nanocarriers. Chitosan has been interested as novel excipients due to their properties as biocompatible and biodegradable polymers. Although, its applications as drug or gene carriers are reported, poor soluble of chitosan limits the use in biological condition. To enhance the solubility, the biological properties and application of chitosan, its derivatives have been developed [6-8].

Although, excipients have been considered to be inert, the emerging data demonstrate that many of inert excipients may directly or indirectly alter the activity of membrane-spanning proteins such as drug transporters. Previous studies have been reported that numbers of surfactants/excipients inhibit transport activity of P-glycoprotein (P-gp), a member of the human ABC transporters that actively "pumps" xenobiotics out of cells. Cremophor EL, Tween 80 and D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) inhibited P-gp subsequently increased absorption of its substrates [9]. In addition, Cremophor EL, Tween 20, Span 20, Pluronic P85 and Brij 30 increased the accumulation of [^{3}H] mitoxantrone, a substrate of breast cancer resistance protein (BCRP), in BCRP-expressing cells [9, 10]. Recently, excipients commonly used in self-emulsifying drug delivery system have been shown to interact with human multidrug resistance related protein (MRP2) [11]. In addition, the amphiphilic graft copolymer, *N*-Octyl-*O*-sulfate chitosan (NOSC), has been

investigated on P-gp inhibition. It was found that NOSC inhibited P-gp-mediate paclitaxel, a P-gp substrate, efflux in human hepatocellar carcinoma cells (HepG2) [12]. This evidence indicated that chitosan and its derivatives may have effects on other drug transporters. In last decade the chitosan derivatives have been synthesized and characterized such as trimetyl quaterniztion chitosan (TM-CS), chitosan oligosaccharide and amphiphilic chitosan. Quaternized *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan (TM-Bz-CS), a water soluble chitosan, was evaluated as a gene carrier. It showed the high gene transfection efficiency [13, 14]. Amphiphilic chitosan derivatives such as N-benzyl-N,O-succinyl chitosan (BSCS), N-naphthyl-N,O-succinyl chitosan (NSCS) and N-octyl-N-O-succinyl chitosan (OSCS) were synthesized by introducing hydrophobic (benzyl, octyl, naphthyl group) and hydrophilic moiety (succinyl group) into chitosan backbone. The critical micelles concentration (CMC) values of these polymers are lower than the CMC of low molecular weight surfactants [6, 15, 16]. Drug delivery systems using these chitosan derivatives to increase solubility of curcumin have been reported [6]. In addition, meloxicam-incorporated NSCS micelles via physical entrapment were developed to improve solubility of meloxicam [15]. Taken together, these data indicate that excipients may alter the pharmacokinetic of an incorporated drug thereby affecting its intended therapeutic efficacy and/or enhancing adverse side effects.

Renal organic cation transporters (OCTs) play a crucial role in excretion of a wide range of organic cations, including therapeutic agents and xenobiotics [17-21]. OCTs transport many drugs such as beta-blockers, quinidine, cisplatin, morphine, metformin, phenoxybenzamine, prazosin, procainamide, and cimetidine [19, 22, 23]. Thus, the renal OCTs are important determinant of drug efficacy and toxicity [19, 22-24]. Three members of the OCTs (SLC22A) family of transport proteins have been cloned and characterized, including OCT1, OCT2, and OCT3 [25, 26]. Human OCT1 and OCT2 are highly expressed in liver and kidney, respectively, whereas OCT3 is ubiquitously expressed in multiple tissues such as skeletal muscles, brain, placenta, liver, and kidney [17, 23]. OCTs play pivotal roles for pharmacokinetics and pharmacodynamic of many cationic drugs at the level of OCTs that are of high clinical relevance [27]. It has been reported that metformin fails to reduce fasting

plasma glucose in type 2 diabetes patients who have mutation of OCT1 which is explained by decreasing hepatic uptake of metformin [28]. Moreover, cimetidine which is a substrate of OCT1and OCT2 significantly increased the plasma concentration of metformin and reduced its hepatic and renal clearance [29]. These evidences indicated that OCT1 and OCT2 play a crucial role in pharmacokinetic and drug interaction.

A relationship between OCT2 and anticancer-induced nephrotoxicity has been observed [22, 30]. Cisplatin is one of a highly effective anticancer drug for many types of solid tumors including non-small cell lung, ovarian, head and neck, advance cervical, bladder, and breast cancer [31-33]. Although it is an effective anticancer drug, cisplatin's therapeutic benefits are limited by serious nephrotoxicity [34]. Cisplatin is transported into renal tubular cells by OCT2 at the basolateral membrane [35]. When cisplatin is present in cytosol, chloride dissociates from platinum becoming positively charged and pharmacologically active. Cisplatin induces cellular damage and nephrotoxicity through several mechanism including oxidative stress, DNA damage, cell apoptosis, and inflammation [36]. To improve the therapeutic efficiency of cisplatin, minimizing nephrotoxicity is very important. Therefore, new therapeutic interventions to reduce nephrotoxicity are still necessary. Inhibiting cellular accumulation of cisplatin by imatinib and cimetidine, substrates of OCT2, reduced cisplatin-induced nephrotoxicity in renal proximal tubular cells [37]. Accordingly, inhibiting OCT2 may be a treatment for cisplatin-induced nephrotoxicity.

One of the strategies to reduce the cisplatin-induced nephrotoxicity is to develop platinum-based polymeric drug delivery for the treatment of cancers. The advantages of platinum-based nanocarriers are such as enhancing drug efficacy, prevention of side effects, and increasing in cellular accumulation mediated drug resistance. Cisplatin is one of the standard chemotherapeutics drugs. However, nephrotoxicity is the major side effect and dose-limiting toxicity of cisplatin. Nanocarriers have been utilized to overcome the disadvantages of cisplatin. The carboxylic moieties on the polymer backbone could be employed to form coordinate bonds with cisplatin.

The present study investigated the effects of the pharmaceutical excipients including conventional excipients (solubilizing agents, suspending agents, and cosolvents, emulsifiers) and chitosan derivatives on transport function of OCT1 and OCT2. In addition, potential therapeutic application of pharmaceutical excipients and chitosan derivatives on reducing nephrotoxicity induced by anticancer drug (cisplatin) were evaluated. Furthermore, the platinum-chitosan complex was characterized and the nephroprotective effect of platinum-chitosan complex was investigated.

OBJECTIVES

1. To investigate the interaction of the pharmaceutical excipients and chitosan derivatives with organic cation transporters (OCT1 and OCT2).
2. To evaluate the impact of the selected compounds (pharmaceutical excipients or chitosan derivatives) on plasma concentration of OCTs substrate.
3. To evaluate the effect of the selected compounds (pharmaceutical excipients or chitosan derivatives) on *in vitro* and *in vivo* prevention of cisplatin-induced nephrotoxicity and *in vitro* anticancer activity of cisplatin.
4. To characterize the platinum-chitosan complex and to investigate the nephroprotection of platinum-chitosan complex in *in vitro* and in *vivo*

HYPOTHESIS

1. The pharmaceutical excipients can interact with organic cation transporters and influence on plasma concentration of OCTs substrate.
2. The pharmaceutical excipients showing OCT2 inhibition can reduce cisplatin-induced nephrotoxicity and do not alter anti-cancer activity of cisplatin.

CHAPTER 2 LITERATURE REVIEW

2.1 Pharmaceutical excipients

Drugs have never been administered alone but they are formulated to be the form of drug products such as capsules, solutions, and tablets. The formulations of drug contain the active ingredient in association with the excipients (usually inactive non-drug ingredients). Pharmaceutical excipients are important for therapeutic drug formulations in order to facilitate their preparations. Pharmaceutical excipients, such as surfactants, are extensively used in pharmaceutical formulations to improve dissolution of poorly soluble drugs [1-4]. In addition, the excipients are used in formulation of drugs for improving patient acceptability. The excipients commonly used pharmaceutical formulations include cosolvents (dimethyl sulfoxide (DMSO), propylene glycol (PG) and polyethylene glycol (PEG)), cyclodextrins (CD), and surfactants (Cremophor, Pluronic, Tween and Span) [1, 5].

Excipients have multiple functions in the drug formulations such as physical stabilizers, preservatives and buffers, and some of these are included in the majority of drug formulations. Other excipients are improving the characteristics of certain formulations. The folowings are excipients frequenctly used in the pharmaceutical formation including; 1) fillers or diluents which are added to solid formulations to provide bulk when the required dosage form components themselves are inadequate to give the tablet an appropriate weight and size. Fillers are also added to improve blend properties of materials such as their flowability and homogeneity as well as the solubility properties of the incorporated drug. The fillers commonly used are lactose, mannitol, calcium phosphate, starches, microcrystalline cellulose, sucrose, or sodium chloride. 2) Surfactants that are amphiphilic molecules are used to reduce surface or interfacial tension. They contain both hydrophilic and hydrophobic regions, therefore, they are commonly used as emulsifying agents, solubilizing agents, detergents, and wetting agents. Two types of surfactants, ionic and nonionic, are commonly described. The increased hydrophobicity and the absence of charge make nonionic surfactants such as TPGS, CremophorTM, Pluronic®, polysorbate and Span® less toxic to biological membranes than ionic surfactants. In addition, nonionic surfactants possess a higher capacity to dissolve poorly water-soluble drugs. In addition to

surfactants, phospholipids are used together with surfactants as emulsifying agents in the composition of liposomes. Moreover, cholesterol and phospholipid derivatives such as sphingomyelin and ceramide, are found to be efficient in the formation of liposomes because they decrease the glass transition temperature which increases the fluidity and improves the stability of the liposomes during the manufacturing process [38]. Liposomes are used to increase the oral bioavailability of poorly water-soluble active ingredients such as polypeptides [39], immunoglobulins [40] or anticancer agents [41]. Indeed, liposomes can enhance the solubility of lipophilic drugs in aqueous solution. In addition, it can promote intestinal absorption of the drugs since cholesterol and phospholipids can be dissolved in the cell membrane. 3) Polyethylene glycol (PEG) is an excipient commonly used in pharmaceutical formulations to improve the solubility of drugs in aqueous for oral administration. PEG exists in a variety of molecular weight grades, ranking from 200 to 35,000. Concentrations of PEG up to 40% (v/v) are considered safe and relatively non-toxic [42]. 4) Cyclodextrins (CDs), enzymatically modified starches, are used to increase solubility and subsequent bioavailability (Uekama et al., 1998). The molecular size of the hydrophobic molecule, the size of the cyclodextrin, and the lipophilicity of the molecule determine the inclusion capacity. The 2,6-di-O-methyl-β-cyclodextrin (DM-β-CyD) derivative is known to be one of the most effective cyclodextrins to improve the bioavailability of orally administered drugs with low aqueous solubility [43]. 5) Cellulose derivatives (HPMC) and synthetic polymers (Carbomer, Povidone) are used as suppending agents. These agents are used to increase the viscosity of the dispersion in order to avoid agglomeration and to delay the sedimentation of the particles in the formulation.

2.1.2 Chitosan and its application in pharmaceutics

Chitosan is a natural polycationic linear polysaccharide derived from chitin as shown in Fig. 2.1

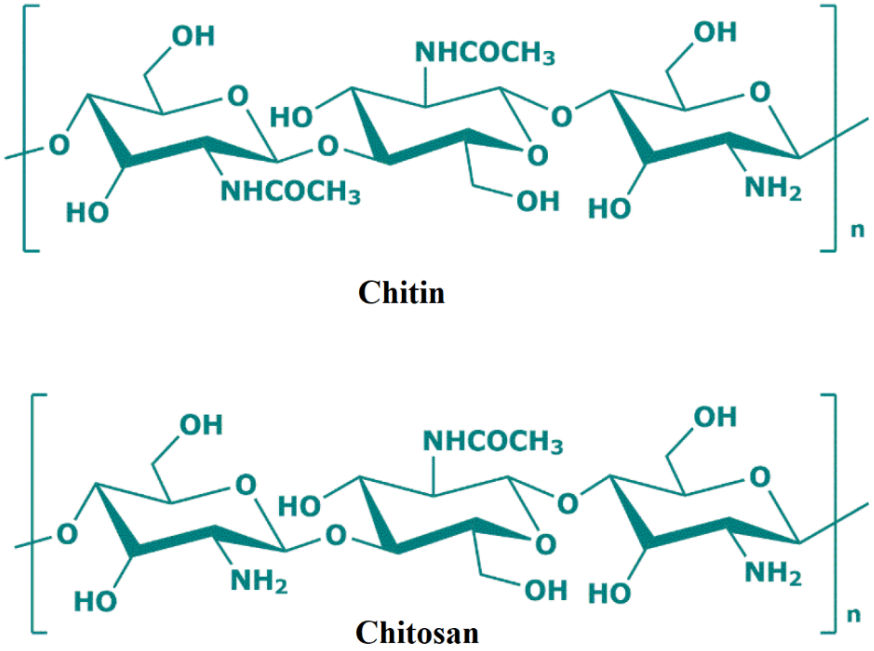

Figure 2.1 Chitina and chitosan [44]

Their characteristic includes biocompatibility, non-toxicity, low allergenicity and biodegradability. These properties of chitosan are used in various biomedical activities including antioxidant, anti-inflammatory and anti-bacterial [45] and pharmaceutical applications such as drug carrier and delivery material are revealed [46, 47]. Although, its applications as drug or gene carriers are reported, poor solubility of chitosan limits the use in biological condition. Due to chitosan contains amino groups (pKa 6.2-7.0) in the structure resulting in insoluble in water or aqueous bases.

To enhance the solubility, the biological properties and application of chitosan, its amphiphilic derivatives have been developed [7, 8, 13]. The fabrication of nanocarriers from chitosan derivatives offers an effective drug delivery system. Amphiphilic chitosan derivatives have been synthesized such as N-benzyl-N,O-succinyl chitosan (BSCS), N-naphthyl-N,O-succinyl chitosan (NSCS) and N-octyl-N-O-succinyl chitosan (OSCS) by introducing hydrophobic (benzyl, octyl, naphthyl group) and hydrophilic moiety (succinyl group) into chitosan backbone, and these can form derivatives polymeric self-assembly (Fig. 2.2).

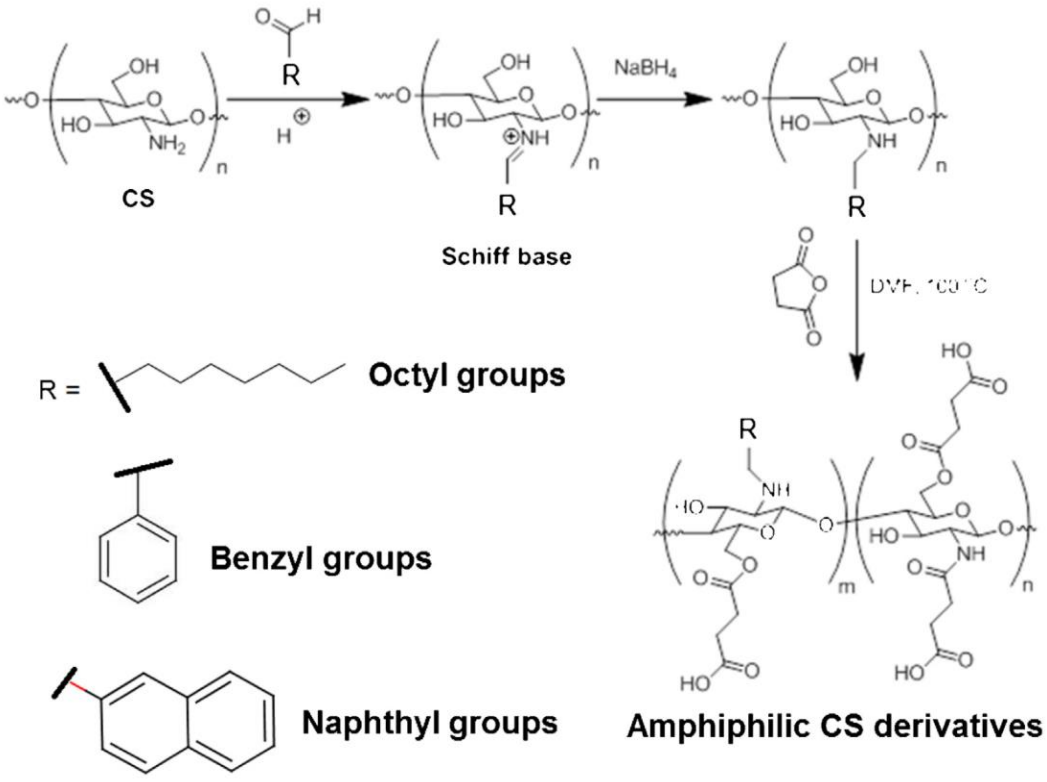

Figure 2.2 Synthesis of amphiphilic chitosan derivatives [16].

N-benzyl-*N,O*-succinyl chitosan (BSCS) was first an amphiphilic chitosan derivative by replacing OH group of chitosan backbone with *N*-benzylation and *N,O*-succinylation to have a pH-responsive characteristic property [6]. In addition to BSCS, other chitosan derivatives were developed are *N*-naphthyl-*N,O*-succinyl chitosan (NSCS) and *N*-octyl-*N*-*O*-succinyl chitosan (OSCS). These polymers can form polymeric micelles by self-aggregation in aqueous media. The CMC value was determined by fluorescence spectroscopy with pyrene probe, found to be 0.0855, 0.0678 and 0.0575 mg/mL for BSCS, NSCS and OSCS, respectively. The results showed the lower CMC value compared to low molecular weight surfactants. The atomic force microscopy (AFM) images exhibited different shape and size of the micelles in various pH medium. At pH above pKa, the micelles will be dissociating and swelling leading to the release of drug from the micelles. Self-aggregate property of BSCS that form micelles in water of is a result of the properties of hydrophilic moieties and hydrophobic moieties. The polymeric micelle of amphiphilic chitosan derivatives (BSCS, NSCS, and OSCS) were used to be a carrier of water-insoluble drugs such as curcumin and melaxicam. Curcurcumin-loaded polymeric micelles were prepared by dialysis method introduced by Sajomsang et al. [6]. The micellar size was less than 100 nm and presented in spherical shape. The release behavior of curcumin from BSCS revealed that rate of curcumin release from BSCS micelles was significantly higher than free drug at pH 5.5-7.4. These data indicated that BSCS micelles were pH-responsive polymeric micelles which were potentially drug delivery of hydrophobic drug. NSCS was used as a carrier of meloxicam for improving solubility of meloxicam [15]. Meloxicam-loaded NSCS was prepared by various physical entrapment methods including dialysis, o/w emulsion, dropping and evaporation. The release profile of meloxicam from polymeric micelles was performed in pH changed medium. meloxicam releasing from free drug and polymeric micelles were not difference in pH 1.2 whereas the higher amount of meloxicam was release from polymeric micelles compared to free drug was found in the pH 6.8. These data indicated that NSCS micelles might be a potential carrier for oral drug delivery of meloxicam carrier [15]. Interestingly, the entrapment efficiency and loading capacity was affected by different types of polymers. Meloxicam-loaded OSCS micelles which were prepared by solvent evaporation method with various

amount of initial drug (0-40%wt) showed highest entrapment efficiency and loading capacity.

Drug delivery systems using the chitosan derivatives have been introduced [12, 48, 49]. These nanocarriers could increase the dissolution of poorly soluble drug such as meloxicam, curcumin, cotrimazole [6, 16, 50]. Quaternized chitosan, a chemically modified chitosan, was investigated as a carrier to improve gene transfection. Recent study have successfully synthesized the water soluble quaternized chitosan derivatives as gene carrier by efficient DNA condensation and mediated higher level of gene transfection [13, 51]

Derivatives of the amphiphilic chitosan have been shown to influence the function of membrane drug transporters. Previous studies reported that N-Octyl-O-sulfate chitosan (NOSC) inhibited P-gp-mediate paclitaxel efflux in cancer cells [12, 52]. This evidence indicates that chitosan derivatives may affect other drug transporters. In addition, in vivo data revealed that NOSC was excreted by urinary clearance when administrated as nano-carrier of intravenous drug [53]implying chitosan derivatives can be used in biomedical and pharmaceutical applications.

2.1.3 Pharmaceutical excipients and drug transporters

Although, excipients have been considered to be inert, the emerging data demonstrate that many of inert excipients may directly or indirectly alter the activity of membrane-spanning proteins such as drug transporters. Previous studies have been reported that numbers of surfactants/excipients inhibit transport activity of P-glycoprotein (P-gp), a member of the human ABC transporters that actively “pumps” xenobiotics out of cells. Cremophor EL, Tween 80 and D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) inhibited P-gp subsequently increased absorption of its substrates [9]. In addition, Cremophor EL, Tween 20, Span 20, Pluronic P85 and Brij 30 increased the accumulation of [^{3}H] mitoxantrone, a substrate of breast cancer resistance protein (BCRP), in BCRP-expressing cells [9, 10]. Recently, excipients commonly used in self-emulsifying drug delivery system have been shown to interact with human multidrug resistance related protein (MRP2) [11]. These data indicate that

excipients may alter the pharmacokinetic of an incorporated drug thereby affecting its intended therapeutic efficacy and/or enhancing adverse side effects.

2.2 Membrane drug transporters

Membrane transporters play an important role in governing the transport of solutes (for example, drugs and other xenobiotics) into and out of cells [27]. Intestine, liver and kidney are the major organ responsible for drug absorption, distribution, metabolism and excretion in the body [21, 27, 54-56]. The two major superfamilies of membrane transporters are the ATP-biding cassette (ABC) and solute carrier (SLC) superfamilies [27]. The SLC is facilitating passive diffusion along the concentration gradient of the substrate or by co-transport and counter-transport against the concentration gradient of another solute such as organic cation transporter (OCTs) and organic anion transporters (OATs) [21, 23, 57]. The ABC transporters are typical efflux transporters using energy from the hydrolysis of ATP as the driving force to move substrates against the electrochemical gradients such as P-glycoprotein (P-gp) [11] as shown in. Fig. 2.3.

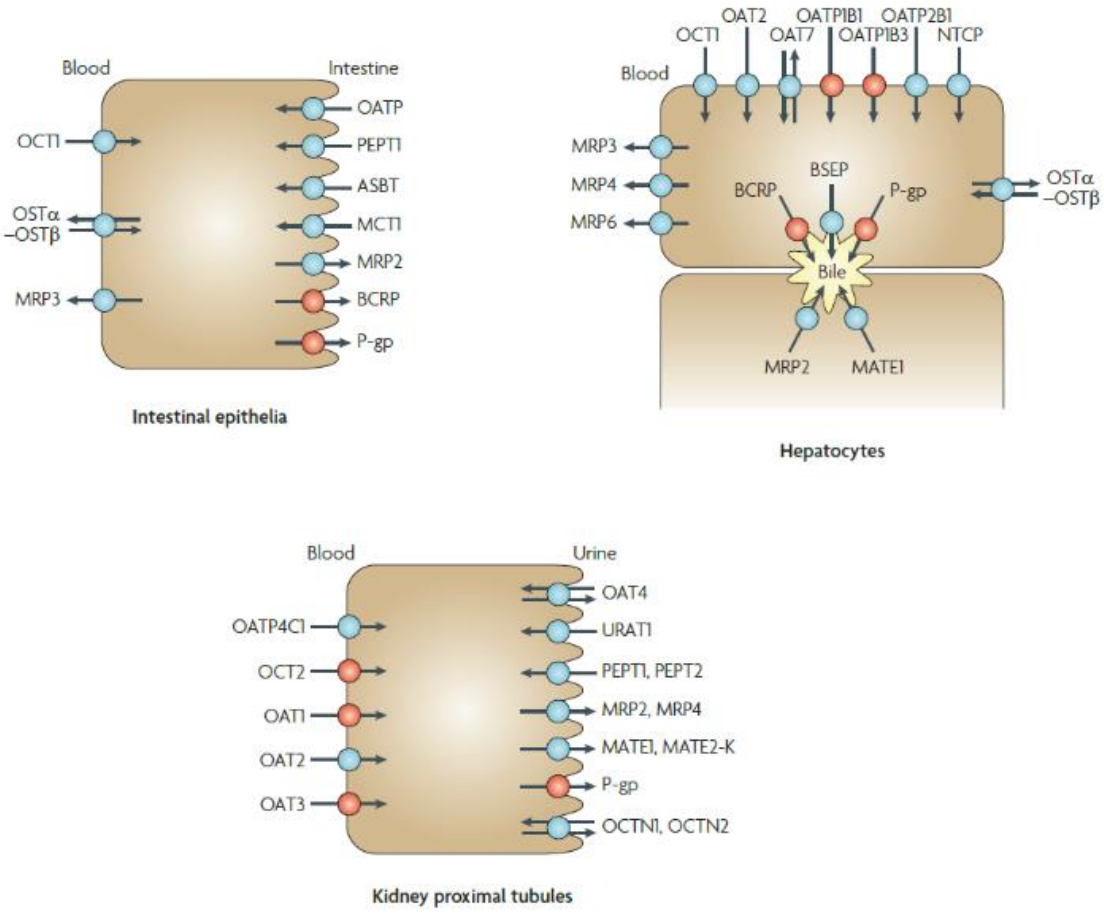

Figure 2.3 Human membrane transports expressed in enterocyte, hepatocytes, and renal proximal tubular cell [27].

This thesis focuses on the OCTs therefor the information concerning OCTs are described in the following subtopics.

2.2.1 Organic cation transporters

The transport of organic cations including endogenous and xenobiotic compounds is mediated by the organic cation transporters (OCTs) family belonging to the solute carrier family 22 (SLC22) [58]. These transporters play an essential role in the uptake of substrate, cationic compounds, at the basolateral membrane into the cells and subsequently, for further elimination by other transporters at the apical or luminal membrane. OCTs transport many endogenous substrates such as creatinine, serotonin, norepinephrine, epinephrine etc. [59-61]. For xenobiotics, OCTs transport several clinically therapeutic drugs in the treatment of certain disease such as metformin, lamivudine, cisplatin and oxaliplatin. In human, OCTs composed of three member called OCT1 (SLC22A1) and OCT2 (SLC22A2) and OCT3 (SLC22A3) [23, 54].

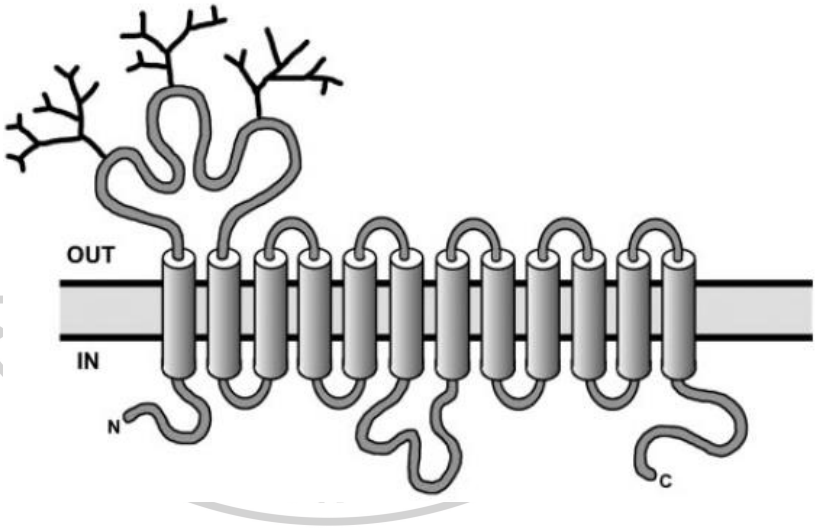

Figure 2.4. Representative the predicted secondary structure of OCT. This transporter is contained 12 transmembrane domains (TMDs) with both N and C terminus located intracellularly. A large extracellular loop, located between the first and second TMD, containing three predicted N-linked glycosylation sites [17].

OCTs are polyspecific transporters that operate as uniporters and mediated facilitated diffusion. In general, the OCTs mediate the bidirectional transport of small cationic compounds which had size smaller than 400 Da. [17, 24]. These transporter function as electrogenic cation transporters which used membrane potential-dependent transports from the action of sodium/potassium ATPase pump generates the intracellular negative membrane potential leading to uptake of organic cation into the cells [24, 57]. The structure of OCTs is generally composed of 12 transmembrane domains (TMDs) which both N and C terminus are located in the intracellular face as shown in Fig. 2.4 and the human OCTs proteins are contained around 554-556 amino acids. The large extracellular loop between the first and second TMD is consisted of three predicted N-linked glycosylation sites, and a large intracellular loop located between TMDs 6 and 7, is responsible for the phosphorylation site that plays a role in the regulations of transporter activity [21, 57, 62].

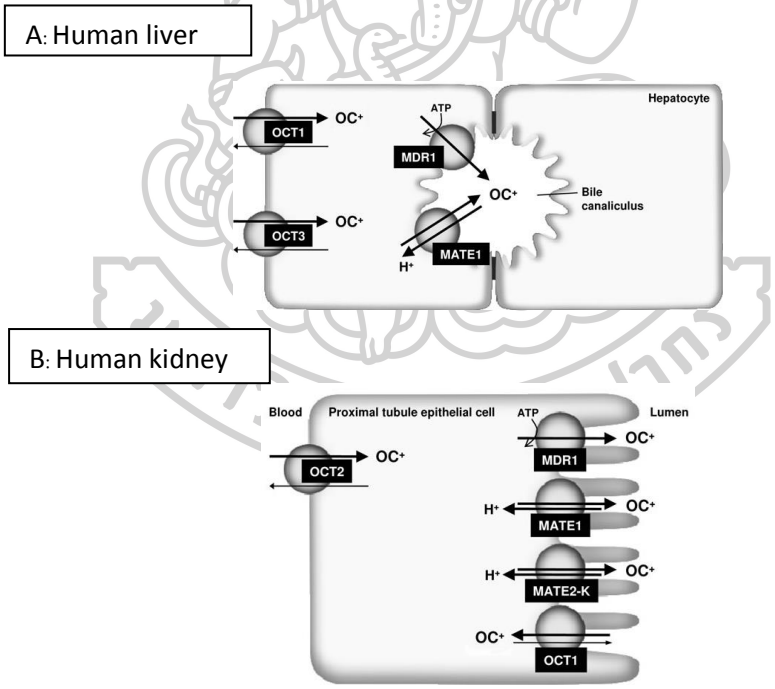

Figure 2.5 Representative the OCT localization in human (A) hepatocytes and (B) renal proximal tubular cells [58]

OCT1 (SLC22A1)

OCT1 was cloned in 1994 from a cDNA library prepared from rat kidney [63]. OCT1 is mainly expressed in liver where it is located in the sinusoidal membrane of the hepatocyte (Fig. 2.5) [58]. OCT1 exhibits a broad tissue distribution as shown in Table 2.1. Beside hepatocytes, human OCT1 is expressed in various organ including small intestine, lung, heart skeletal muscle, brain, and etc. [21, 58]. OCT1 mediates the first step in hepatic excretion of several cationic drugs, and xenobiotics (Fig. 2.4). Human lung OCT1 mediates the absorption of some drugs from the bronchi. In human brain, OCT1 facilitates passage of endogenous substrates and of drugs across the blood-brain barrier [23]. Expression and transport activity of OCT1 influences the pharmacokinetics of drugs that are transported by OCT1 [21, 27, 58] Therapeutic effects and toxicity of drugs may be reduced by co-medication of other substrates or inhibitors of OCT1.

OCT2 (SLC22A2)

Human OCT2 is mainly expressed in kidney where it is localized to the basolateral membrane of renal proximal tubules as shown in Figure. 2.3 [23, 24, 27]. The low expression of human OCT2 is also found in other organs, including small intestine, lung, placenta, thymus, brain, and the inner ear [24]. Human OCT2 transport the model cationic substrates such as TEA^+, MPP^+, ASP^+, *N'*-methylnicotinamide, and aminoguanidine [17, 21, 24]. Drugs which are transported by human OCT2 include the anti-parkison drugs (memantine and amantadine), the antineoplastic drugs (oxaliplatin, picoplatin, cisplatin, and ifosfamide), the histamine H_2 receptor antagonist (cimetidine, famotidine, and ranitidine). In human, OCT2 is located on the basolateral membrane of renal proximal tubules in kidney. In contrast, the trachea and bronchi OCT2 protein have been identified in the luminal membrane (Koepsell, 2007).

OCT3 (SLC22A3)

OCT3 is very broad the expression of tissue distribution. In human, the strongest expression was found in skeletal muscle, liver, placenta, and weaker signals in the lung, intestinal cells, heart, and brain. In liver, kidney, and placenta, OCT3 was localized on the basolateral membrane of epithelial cells (Fig. 2.3) whereas, OCT3 was localized on luminal membranes of bronchial epithelial cells and small intestinal enterocytes [64, 65].

Substrate of OCTs

Most substrates of OCTs are low molecular weight (<400 Da) and hydrophilic organic cations exhibiting molecular structures [21]. In addition to organic cations, OCTs also transport non-charged compounds [17]. Transportation endogenous substrates of OCTs include monoamine neurotransmitters, steroid hormone, and other compounds such as creatinine, choline, guanidine, L-carnitine and bile acids. Various clinically used drugs were identified as substrate of OCTs including antidiabetic drug (metformin), chemotherapeutics (cisplatin and oxaliplatin), and H2-antagonists (cimetidine and ranitidine). The endogenous and exogenous substrates of OCTs are summarized in Table 2.1

Table 2.1 Transporter-distributed tissues and endogenous and exogenous organic cation substrates of human OCTs family [54, 58].

Transporter	Tissues distribution		Endogenous substrates		Drug substrates	
OCT1	liver	brain	epinephrine	choline	Metformin	lamuvidine
	kidney	trachea	creatinine	serotonin	acyclovir	ganciclovir
	lung	skeletal	histamine	dopamin	pentamidin	oxalipatin
	intestine	muscle	acetylcholine		furamidine	paclitacel
	stomach	spleen	corticosterone		berberine	irinotecan
	placenta	heart	prostaglandin		picoplantin	cisdiammin
OCT2	kidney	lung	dopamine	choline	memantine	amantadine
	spleen	brain	guanidine	creatinine	oxalipatin	picoplatin
	intestine	neurons	serotonin	salsolinol	ifosfamide	cimetidine
	placenta		β-estradiol	histamine	famotidine	ranitidine
			acetylcholine		zalcitabine	lamuvidine
			epinephrine		amiloride	metformin
			nor-epinephrine		cisplatin	varenicline
OCT3	vessels	lung	epinephrine		cimetidine	verapamil
	skeletal-muscle	urinary-bladder	nor-epinephrine		prazosin	memantine
			acetylcholine		etilefrine	dizocilpine
	brain	neurons	L-Carnitine		citalopram	clonidine
	placenta	heart	histamine		metformin	ranitidine
	small-intestine	liver	salsolinol		diphenylhydramine	
		kidney	guanidine		phenoxybenzamine	
			dopamine		amantadineketamine	

2.2.2 OCT-mediated drug interaction

The mechanism underlying OCT-mediated drug interactions was first described more than 20 years ago by Somogyi et al. They reported the interaction between 0.25 g metformin daily and cimetidine (0.4 g twice daily) in man. The results showed that cimetidine significantly increased the area under the plasma metformin concentration-time curve (AUC) and reduced its renal clearance over by an average of 50% and 27%, respectively. This study suggests that cimetidine inhibits the renal tubular secretion of metformin in man, resulting in higher circulating plasma concentrations [23, 27, 29, 55, 66].

2.2.3 OCT2 and drug-induced nephrotoxicity

A relationship between OCT2 and anticancer-induced nephrotoxicity has been observed [22, 30, 67]. Cisplatin is one of a highly effective anticancer drug for many types of solid tumors including non-small cell lung, ovarian, head and neck, advance cervical, bladder, and breast cancer [31-33]. Although it is an effective anticancer drug, cisplatin's therapeutic benefits are limited by serious nephrotoxicity [34]. Cisplatin is transported into renal tubular cells by OCT2 at the basolateral membrane [35, 67]. When cisplatin is present in cytosol, chloride dissociates from platinum becoming positively charged and pharmacologically active. Cisplatin induces cellular damage and nephrotoxicity through several mechanism including oxidative stress, DNA damage, cell apoptosis, and inflammation [36]. To improve the therapeutic efficiency of cisplatin, minimizing nephrotoxicity is very important. Therefore, new therapeutic interventions to reduce nephrotoxicity are still necessary. Inhibiting cellular accumulation of cisplatin by imatinib and cimetidine, substrates of OCT2, reduced cisplatin-induced nephrotoxicity in renal proximal tubular cells [37]. Accordingly, inhibiting OCT2 may be a treatment for cisplatin-induced nephrotoxicity.

2.3 Cisplatin and its nephrotoxicity

2.3.1 Cisplatin

Cisplatin or *cis*-diamminedichloroplatinum(II) (CDDP) was first discovered and exerted the anticancer effects [68]. Cisplatin is a first platinum containing compound which has been widely used as chemotherapeutic agent [69]. The structure of cisplatin consists of platinum molecule that is responsible for cytotoxic activity (Fig. 2.4) The structure of cisplatin is composed of two chloride and two amine groups and this form is hydrolyzed in aqueous solution [70] as shown in Fig. 2.6.

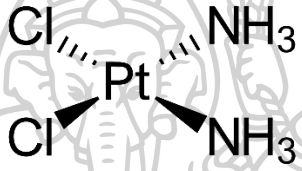

Figure 2.6 A schematic represents the chemical structure of cisplatin $Pt(NH_3)_2 Cl_2$.

High concentration of chloride (~103 mEq/L) in plasma can prevent cisplatin hydrolysis but after cisplatin uptake into the cell, chloride concentration drops down to 4 mEq/L which can induce cisplatin hydrolysis. In acquiesce condition , cisplatin is a potential electrophile and interacts with nucleotides such as nucleic acids of DNA or sulfhydryl groups of proteins [70] then induces cytotoxicity. Cisplatin has been approved by the Food and Drug Administration in 1978. After that, cisplatin became to be one of the most effective chemotherapeutic drug that is widely used in the treatment of various cancers. Cisplatin has been used for treatment of numerous human cancers including bladder, head and neck, lung, ovarian, and testicular cancers [69] or used as an adjuvant chemotherapy with other chemotherapeutic drugs [71]. In addition, cisplatin is also widely used as an additional cancer therapy following surgery or radiation [70, 71]. In general, cisplatin is considered as cytotoxic drugs which kills cancer cells by damaging DNA, inhibiting DNA synthesis and mitosis, and inducing apoptotic cell death [69]. Several molecular mechanisms of action are

revealed including induction of oxidative stress as characterized by reactive oxygen species production and lipid peroxidation, induction of p53 signaling and cell cycle arrest, down-regulation of proto-oncogenes and anti-apoptotic proteins, and activation of both intrinsic and extrinsic pathway of apoptosis [69].

2.3.2 Cisplatin-induced nephrotoxicity

The treatment of cisplatin is usually limited by its complications such as nausea and vomiting, bone marrow suppression, peripheral neuropathy, auditory impairment, anaphylaxis, and the most severe complication of cisplatin treatment is still nephrotoxicity [36, 72-74]. Acute kidney injury is a clinical symptom classified by a rapid decreasing in renal function. Cisplatin is transported into kidney cells by passive diffusion or active transport. Cisplatin uptake depends on the cellular content and transporters which are involved in cisplatin accumulation. It has been reported that cisplatin is mainly transported by two major transporter families, plasma membrane copper transporter 1 (CTR1) and the organic cation transporter 2 (OCT2) [34, 70]. It has been reported that knock out of CTR1 can reduce cisplatin accumulation in renal cells [75]. However, The role of CTR1 in cisplatin nephrotoxicity in animal model has not been investigated [36]. OCT2 is located at basolateral membrane in renal proximal tubules and participated in cisplatin influx in kidney cells. After cisplatin gets into the cells, chloride ions dissociate from platinum in cisplatin complex. The positive charged platinum ions can bind to nucleotides in DNA, RNA, and proteins [76]. Binding of platinum ions and DNA, platinum-DNA adducts, is a defect factor which can modulate multiple signaling pathways in kidney cells [77].

In kidney cells, cisplatin is conjugated to glutathione and then metabolized through a gamma-glutamyl transpeptidase and cysteine S-conjugate beta-lyase-dependent pathways to be a reactive thiol which is a potent nephrotoxin. Gamma-glutamyl transpeptidase is located on the cell surface, whereas cysteine-*S*-conjugate beta-lyase is an intracellular enzyme [36, 78]. Gamma-glutamyl group of the glutathione-conjugate is cleaved by gamma-glutamyl transpeptidase and cysteinyl-glycine bond is cleaved by aminodipeptidase-N, leading to a platinum-cysteine-conjugate [71, 78]. Finally, a platinum-cysteine-conjugate is metabolized by cysteine-

S-conjugate beta-lyase to be a reactive thiol which contributes to renal cell injury [73].

Cisplatin induces nephrotoxicity by multiples pathways including the activation of oxidative stress [79, 80], inflammation [80, 81], and MAPKs [82-84]. Inside renal cells, cisplatin binds to DNA, leading to DNA replication and synthesis arrest [34, 70]. Cisplatin-DNA complex or DNA adduct is recognized by various damage recognition proteins [76]. The activation of these recognition proteins results in the modulation of multiple factors which involve in renal cell apoptosis. The factors involving cell apoptosis include tumor protein p53(p-53) [85-89], mitochondrial-caspase [90-93], ROS [79, 80, 94], and MAPKs [82-84, 95, 96]. The summary of cisplatin-induced cytotoxicity of renal proximal tubular cells is shown in Fig.2.7.

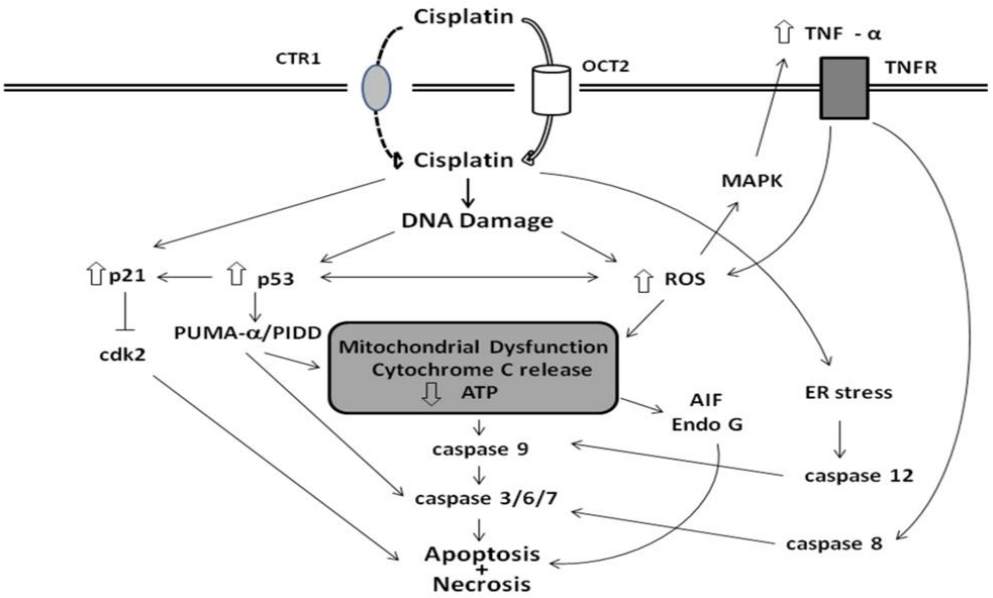

Figure 2.7 Pathways of cisplatin-induced epithelial cell death. Cisplatin enters renal epithelial cells via the OCT2 and Ctr1 transporters then damages DNA and induces ROS production [36].

2.3.3 Treatment of cisplatin nephrotoxicity

Cisplatin nephrotoxicity is reduced by diuretics, hydration, and reduction of cisplatin metabolism [97]. The principle of diuretics and hydration treatment is reducing cisplatin concentration and exposure time with tubular epithelium [98]. Hydration with normal saline and subsequently administration of diuretic drug such as mannitol before, during, and after cisplatin treatment can reduce cisplatin nephrotoxicity [98-100] and this protocol is used as the standard treatment of cisplatin nephrotoxicity [101]. Another study reported that normal saline alone or combination with furosemide is more effective than normal saline with subsequently administration of mannitol [97, 100]. Volume expansion with normal saline may increase the excretion rate of cisplatin [102] or the use of salt reagents also provide a high concentration of chloride ions, leading to the dissociation of the chloride ions from the platinum molecule, resulting in the reduction of reactive forms of cisplatin [103]. In addition, Ozakok *at al*. recently reported that sodium ions could reduce cisplatin nephrotoxicity [70]. However, the mechanism of salt reagent protection is still unclear. Recently, it has been report that normal saline dose not reduce cisplatin accumulation but instead induces a stress response [70, 97]. The osmotic stress response reduces the potential of cisplatin binding to DNA, provides renal proximal tubule cell against to apoptosis, and modulates the metabolic stimulation of nephrotoxins [34, 98]. However, hydration treatment is effective only one-third of patients treated with cisplatin and the treatment can interfere anticancer effect of cisplatin [71].

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Drug and pharmaceutical excipients

- Cisplatin (Sigma Aldrich®, St. Louis, MO, USA)
- Tween (Sigma Aldrich®, St. Louis, MO, USA)
- Span (Sigma Aldrich®, St. Louis, MO, USA)
- Chitosan (Degree of deacetylation; DDA 96%, MW 10-13 kDa) (OilZac Technologies Co., Ltd., Bangkok, Thailand)
- Quaternized chitosan (QC) (degree of quatenization (DQ) = 65 %) was a gift from National Nanotechnology Center (NANOTEC), Thailand.
- Methylated N-(4-N,N-dimethylaminobenzyl) chitosan (MDM) (DQ = 75 %, the extent of N-substitution (ES=60 %)) was gift from National Nanotechnology Center (NANOTEC), Thailand.
- N-benzyl-N,O-succinyl chitosan (BSCS) (degree of benzyl substitution (DS) =0.69, degree of N,O-succinylation (DSS) =1.07) was a gift from National Nanotechnology Center (NANOTEC), Thailand.
- N-naphthyl-N,O-succinyl chitosan (NSCS) (DS=0.54, DSS =0.52), was a gift from National Nanotechnology Center (NANOTEC), Thailand.
- N-octyl-N,O-succinyl chitosan (OSCS) (DS=0.47, DSS =1.13) was a gift from National Nanotechnology Center (NANOTEC), Thailand.

3.1.2 Cell line

- Chinese hamster ovary (CHO-K1) cells stably transfected with rbOCT1 or rbOCT2 (rbOCT1-CHO-K1 and rbOCT2-CHO-K1 cells) were given from Professor Stephen H Right (University of Arizona, USA)
- Human hepatocellular carcinoma cells; HepG2 (American Type Culture Collection (ATCC), Manassas, VA)

- Human renal proximal tubular cells; RPTEC/TERT1 (American Type Culture Collection (ATCC), Manassas, VA)
- Head and Neck cells; HN22 was provided from Faculty of Dentistry, Naresuan University (Phisanulok, Thailand)
- Human breast adenocarcinoma cells; MCF-7 (American Type Culture Collection (ATCC), Manassas, VA)

3.1.3 Tissue culture reagents

- DMEM (GIBCO®, Grand Island, NY, USA)
- DMEM/F12 (GIBCO®, Grand Island, NY, USA)
- Fetal bovine serum (GIBCO®, Grand Island, NY, USA)
- Penicillin/Streptomycin (GIBCO®, Grand Island, NY, USA)
- Human Apotranferin (Sigma Aldrich®, St. Louis, MO, USA)
- Human insulin
- Selenite sodium (Sigma Aldrich®, St. Louis, MO, USA)
- Epidermal Growth Factor (Sigma Aldrich®, St. Louis, MO, USA)
- Hydrocortisone (Sigma Aldrich®, St. Louis, MO, USA)
- Glutamax (GIBCO®, Grand Island, NY, USA)
- Non-essential amino acid (GIBCO®, Grand Island, NY, USA)
- Trypsin–EDTA (0.25 %) solution (GIBCO®, Grand Island, NY, USA)

3.1.4 Chemical for transport function experiments

- 3H-MPP+ (N-[methyl-3H]-4-phenylpyridinium acetate) (80 Ci/mmol) was purchased from American Radiolabeled Chemical Inc. (MO, USA).

3.1.5 Chemical for immunoblotting

- NGAL
- B-actin

3.1.6 All other chemicals

a. Annexin V-FITC apoptosis kit

3.2 Equipments

- 1.5 mL microcentifuge tube (Eppendorf®, Corning Incorporated, NY, USA)
- 15, 50 mL centrifuge tubes-sterile (Biologix Research Company, KS, USA)
- Analytical balance (Sartorious CP224S; Scientific Promotion Co., Ltd., Bangkok, Thailand)
- Beaker (Pyrex, USA)
- Centrifuge tube (Biologix Research Company, KS, USA)
- CO_2 incubator (Heraeus HERA Cell 240, Heraeus Holding GmbH., Germany)
- Dialysis bag (CelluSep® (6000–8000 MWCO) Membrane Filtration Products, USA)
- Duran bottle 500, 1,000 mL
- Freeze-dryer (Model: Freezone 2.5, LABCONCO, USA)
- Freezer/Refrigerator -20 °C, -80 °C, 5°C
- Incubated shaker (Model: KBLee 1001, Daiki sciences, Bio-Active, Bangkok,
- Incubator Model 3111 Thermo Scienctific (Forma Series II water jacket CO_2 Incubator, USA)
- Laminar air flow (BIO-II-A, Telstar Life Science Solutions, Spain)
- Liquid scintillation Counter Model 1214 Rackerbeter (LKB Wallac, Sweden)
- Magnetic stirrer (Framo, Germany) and magnetic bar
- Microcentifuge tube (Eppendorf®, Corning Incorporated, NY, USA)
- Microcentrifuge (Microfuge 16®, Model: A46473, Beckman Coulter Inc.,
- Micropipette 0.1-2.5 µL, 2–20 µL, 20–200 µL, 100–1000 µL, 1–5 mL, and micropipettetip
- Microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA)
- Nylon membrane filter (diameter 47 mm, pore size 0.45 µm)

- pH meter (Mettler Toledo, Switzerland)
- Probe-type sonicator (model CV 244, Sonics VibraCellTM, USA)
- Vortex mixer (VX100, Model: Labnet, NJ, USA)
- Water bath (WiseBath with digital Fuzzy Control System, Korea)
- Well-plate (96 Well plate) (Corning Incorporated, NY, USA)
- ZetasizerNano ZS (Malvern Instruments, Malvern, UK)

3.3 Methods

3.3.1 Cell culture and cultivation

3.3.2 Uptake experiments

3.3.3 Inhibitory potency (IC_{50}) study

3.3.4 Mechanism of action study

3.3.5 *In vitro* cytotoxicity assay

3.3.6 *In vivo* determination of the effect of the pharmaceutical excipients on plasma concentration of OCTs substrate

3.3.7 Preparation of OSCS polymeric micelles (PMs) and cisplatin –OSCS PMs complexes

3.3.8 Characterization of cisplatin -OSCS PMs complexes

3.3.8.1 Loading efficiency and loading capacity

3.3.8.2 pH

3.3.8.3 Osmolality

3.3.8.4 Particle size and zeta potential

3.3.9 *In vitro* protective effect of selected compounds on cisplatin-induced nephrotoxicity

3.3.9.1 Cytotoxicity of cisplatin on RPTEC/TETR1 cells

3.3.9.2 Protective effects

3.3.9.2.1 MTT assay

3.3.9.2.2 cell counting method

3.3.9.2.3 flow cytometry method

3.3.9.3 Intracellular accumulation of platinum in RPTEC/TETR1 cells

3.3.10 *In vivo* protective effect of the selected compounds on cisplatin-induced nephrotoxicity

3.3.10.1 Immunoblotting

3.3.10.1.1 Western-blot analysis in RPTEC/TETR1 cells

3.3.10.1.2 Western blot analysis in murine kidney

3.3.10.2 Accumulation of platinum in murine kidney

3.3.10.3 Determination of renal function

3.3.10.4 Histological evaluation of cisplatin-induced toxicity

3.3.11 Effect of the selected compounds on anticancer activity of cisplatin

3.3.1 Cell culture and cultivation

1) rbOCT1-CHO-K1 and rbOCT2-CHO-K1 cells

The rbOCT1-CHO-K1 and rbOCT2-CO-K1 cells were cultured in F-12 Ham Kaighn's Modification containing 10% fetal bovine serum with 100 U/ml penicillin and 100 µg/ml streptomycin under 5% CO_2/ 95% air at 37˚C.

2) RPTEC/TERT1 cells

The RPTEC/TERT1 and HepG2 cell lines expressing human OCT2 and OCT1, respectively, were cultured as recommended by ATCC. RPTEC/TERT1 cells were cultured in a mixture of DMEM and Ham's F-12 (1:1) supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 10 ng/ml epithelial growth factor, 36 ng/ml hydrocortisone, and 1% penicillin- streptomycin antibiotic. Cells were stored in humidified incubator under 5% CO2 air at 37˚C.

3) HN22 MCF-7 and HepG2 cells

The HN22 and MCF-7 cells were grown in DMEM medium containing 10% FBS, 2mM L-glutamine, 1% non-essential amino acids, and 0.1% penicillin-streptomycin. All cells were stored in humidified incubator under 5% CO2 air at 37˚C.

3.3.2 Uptake experiments

This study was to determine the effect of pharmaceutical excipients and chitosan derivatives on transport function of OCT1 and OCT2 expressed in single transfected OCT1 or OCT2 (rbOCT1-CHO-K1 or rbOCT2-CHO-K1) and endogenous expressing OCT1 or OCT2 such as human hepatocellular carcinoma cell (HepG2) or human renal proximal tubular cell (RPTEC/TERT1). The transport function of OCT1 or OCT2 was determined by the measurement the uptake of ^{3}H-MPP^{+}, a prototypic substrate of OCTs. The cells were cultured in 24-well plates until confluent, and subsequently washed twice with Waymouth buffer (WB) then incubated for 15 min. Uptake experiment was performed by exposing the cell monolayers with 200 µl of WB containing ~10 nM ^{3}H-MPP^{+} for 5 min at 37ºC. At the end of incubation period, the cell monolayers were three-times washed with ice-cold WB to terminate the uptake. Cells were then lysed by adding 200 µl of 0.4 N NaOH in 10% sodium dodecyl sulfate (SDS) for at least 3 h. The samples were neutralized by 1 N HCl and

then transferred into scintillation vials for measurement of accumulated ^{3}H-MPP^{+} using liquid scintillation counter (1214 Rackbeta, Wallac, Sweden). The transporter-mediated uptakes were calculated by subtracting the total uptake with the uptake of ^{3}H-MPP^{+} in the presence of tetrapentylammonium (TPeA), an OCTs inhibitor. The ^{3}H-MPP^{+} uptakes were calculated as fmol/min/cm^{2} and expressed as percentage of control as previous described [104, 105].

3.3.3 Inhibitory potency (IC_{50}) study

In this study, the pharmaceutical excipients and chitosan derivatives which affect the transport function of OCT1 or OCT2 were selected to determine the inhibitory potency (IC_{50}) in rbOCT1-CHO-K1, rbOCT2-CHO-K1, HepG2 and RPTEC/TERT1 cells. The confluent cell monolayers were incubated with WB containing ^{3}H-MPP^{+} plus varying concentration of samples (0 – 5000 µg/ml) for 5 min. After the end of incubation, the cell monolayers were washed and lysed by the same method as mention in section 2. Then cells were transferred into scintillation vials for measurement of accumulated ^{3}H-MPP^{+} using liquid scintillation counter (1214 Rackbeta, Wallac, Sweden). The ^{3}H-MPP^{+} uptakes were calculated as fmol/min/cm^{2} and expressed as percentage of control. IC_{50} values were estimated using nonlinear regression analysis.

3.3.4 Mechanism of action study

The pharmaceutical excipients and chitosan derivatives which affect the transport function of OCT1 or OCT2 were selected to investigate the inhibiting OCTs-mediated ^{3}H-MPP^{+} transport mechanism in rbOCT1-CHO-K1 and rbOCT2-CHO-K1. The cells were divided into 2 groups including control (vehicle) and selected compounds (pharmaceutical excipients or chitosan derivatives). Confluent cells were washed twice with WB buffer and incubated with WB for 15 min. After the end of incubation period, the cells were incubated with WB containing 10 nM ^{3}H-MPP^{+} and varying concentrations of unlabeled MPP^{+} for 5 min for control group. For selected compound, the concentration at IC_{50} was added to WB containing 10 nM ^{3}H-MPP^{+} and varying concentrations of unlabeled MPP^{+} for 5 min. The uptake was stopped by removing transport buffer then rinsing the cells with three successive 1 ml

washes of ice-cold WB. The cells were lysed by adding 10% SDS in 0.4 N NaOH for overnight, neutralized with 1 N HCl. The accumulated radioactivity was measured by liquid scintillation beta counter (1214 Rackbeta, Wallac, Sweden). The transport of ^{3}H-MPP^{+} was calculated as fmol/cm^{2} of the confluent monolayer surface.

The hyperbolic inhibition of ^{3}H-MPP^{+} transport described by Michaelis-Menten kinetic of competitive interaction of unlabeled and labeled MPP^{+} is defined by the following equation [106];

$$J = (J_{max} [*T]) / (Kt+[*T]+T) + C$$

where J is the rate of ^{3}H-MPP^{+} uptake from the concentration of labeled substance [*T]; J_{max} is the maximum rate of MPP^{+} uptake; Kt is the MPP^{+} concentration that results in half-maximum transport (Michaelis constant); [T] is the concentration of unlabeled MPP^{+} in the transport reaction; and *C* is a constant that represents the component of total MPP^{+} uptake that is not saturated (over the range of substrate concentration tested) and presumably reflects the combined influence of diffusive flux, nonspecific binding, and/or incomplete rinsing of the cell layer.

3.3.5 Cytotoxicity assay

Cell viability was determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on RPTEC/TERT1 cells. The cells were seeded on 96-well plate, and then were incubated with selected compounds (pharmaceutical excipients or chitosan derivatives) for 5 min, 24 h, 48 h, and 72 h in RPTEC/TERT1 cells. At the end of the incubation, cells were washed three times with serum-free media, removed and MTT was added to each well, and cells were incubated for another 1 h at 37°C. Then, MTT medium was replaced with 100 µl of 100% DMSO. Cell viability was examined by measuring the absorbance at 570 nm using microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard Bio-Science, CT, U.S.A.). Results were expressed as a percentage cell viability of the control.

3.3.6 In vivo determination of the effect of the pharmaceutical excipients on plasma concentration of OCTs substrate

This study aimed to determine whether selected compounds (pharmaceutical excipients interacting OCTs and subsequently affect plasma concentration of organic cation compound. C57BL/6 mice (20 g body weight, n=3 animals per group) were obtained from National Laboratory Animal Center, Bangkok, Thailand. They were fed with standard laboratory chow and water ad libitum and housed maintained on a 12-h dark/light cycle (in an air-controlled room; 25±1°C and 55 ± 5% humidity). They were acclimatization for 7 days before using. Mice were received a bolus (200 µl) intraperitoneal injection of saline solution containing ^{3}H-MPP^{+} (1 µM) alone or saline solution containing ^{3}H-MPP^{+} plus selected compounds. Blood samples (10 µl) were collected from tail vein using capillary tube at 5, 15, 30, 60, 120, and 180 min after administration. The radioactivity in blood samples were measured by a liquid scintillation counter (1214 Rackbeta, Wallac, Sweden).

3.3.7 Preparation of chitosan derivatives polymeric micelles

The synthesis of BSCS, NSCS, and OSCS was performed by Dr. Warayuth Sajomsnag, NANOTEC, Thailand. The self-assembled polymeric micelles were produced by a dialysis method. In brief, the BSCS, NSCS, or OSCS (5 mg) were separately dissolved by DMSO (2 ml) at room temperature and transferred into dialysis bag (CelluStep®, 6000-8000 MWCO, Texas, USA). Then the dialysis bag was subsequently immersed in distilled water for 48 h (distilled water was changed every 4 h).

OSCS blank micelles were produced by dialysis method as mentioned in above (part 3.3.7). After the dialysis, the volume was measured and the concentration of OSCS was calculated. The pH of OSCS blank micelles was controlled at 8.5 using diluted NaOH. Then the OSCS blank micelles and cisplatin were mixed at the weight ratio 1:1.5 and stirred in the dark for 12 h to allow the formation of complex. After that, the OSCS-platinum complex was dialyzed against distilled water for 3 h (distilled water was changed every 30 min) to remove free cisplatin.

3.3.8 Characterization of blank micelles and platinum-loaded OSCS complex

3.3.8.1 Loading efficiency and loading capacity

Platinum-loaded OSCS complex (100 µl) were mixed with concentrated nitric acid (1 ml), then the mixture was heated at 90°C for 90 min. Ultrapure water was used for adjusting the mixture volume to 10 ml. The amount of platinum was determined using inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies, CA, USA). The percent loading efficiency (% LE) and loading capacity (LC) were calculated according to following equation:

% LE = (amount of cisplatin on the polymeric complex / theoretical amount of cisplatin) x 100

LC = amount of cisplatin on the polymeric complex / weight of polymer

3.3.8.2 pH measurement

The pH values were measured at room temperature using a pH meter (Metler Toledo). All measurements were performed in triplicate.

3.3.8.3 Osmolality measurement

The osmolality of blank micelles and platinum-OSCS complex were determined using the *Advanced*™ Model *3320* Micro-Osmometer. All samples were analyzed in triplicate.

3.3.8.4 Particle size and zeta potential

The sizes and zeta potentials of blank micelles and platinum-OSCS complex were measured using a DLS particles size analyser (Zetasizer Nano ZS, Malvern Instrument, Malvern, UK) at 25°C. Each sample was measured in triplicate.

3.3.9 *In vitro* protective effect of the selected compounds on cisplatin-induced nephrotoxicity

This study aimed to investigate the optimal dose and time exposure of cisplatin to produce cytotoxicity on RPTEC/TERT1 cells. Then the protective effects of the selected compounds (pharmaceutical excipients or chitosan derivatives) on cisplatin-induced cytotoxicity were also determined in order to find the best compound to reduce cisplatin toxicity.

3.3.9.1 Cytotoxicity of cisplatin on renal proximal tubular cell

The effect of cisplatin on cell viability of RPTEC/TERT1 cells was determined by MTT assay. The cells were seeded in 96-well plate for 14 days until cells reached 100% confluency. Then, the cells were treated with cisplatin at various dose 10, 20, 50, 100, 200 and 500 µM and incubated at various times 24, 48, 72 h. At the end of the incubation, cells were washed three times with serum-free media, removed and MTT is added to each well, and cells were incubated for another 1 h at 37°C. Then, MTT medium was replaced with 100 µl of 100% DMSO. Cell viability was examined by measuring the absorbance at 570 nm by using microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard Bio-Science, CT, U.S.A.). The percent cell viability is expressed as a percentage of the control value.

3.3.9.2 Protective effects

3.3.9.2.1 Determination by MTT assay

To determine whether the selected compounds reduce cytotoxicity of cisplatin, the cell viability was measured by MTT assay. In this experiment, RPTEC/TERT1 cells were seeded in 96-well plate for 14 days. Cells were pretreated with or without the selected compounds for 1 h and then cultured in the presence or absence of cisplatin (optimal concentration; IC_{50}) for 72 h. At the end of the incubation, cells were washed three times with serum-free media, removed and MTT is added to each well, and cells were incubated for another 1 h at 37°C. Then, MTT medium was replaced with 100 µl of 100% DMSO. Cell viability was examined by measuring the

absorbance at 570 nm by using microplate reader. The percent cell viability was expressed as a percentage of the control value.

3.3.9.2.2 Determination by cell counting method

In this study, the cell counting method was performed to confirm the data obtained from MTT assay. RPTEC/TERT1 cells were seeded in 24-well plate for 14 days until cells reached 100% confluency. Cells were pretreated with or without the selected compounds for 1 h and then cultured with or without cisplatin for 72 h. After treatment cells were washed with PBS for 2 times and removed by incubation with 0.25% trypsin for 20 min at 37 °C. Then cells were added with medium containing 1% *fetal bovine serum* (FBS) and mixed with equal volumes of 0.4% trypan blue stain. Cells were counted by using hemocytometer under inverted microscope. Live cells appear colorless and dead cells stain blue. Cell viability was calculated from equation as shown below and expressed as total number of living cell/ml. The living cells of treated cell were compared with control (vehicle).

$$\frac{Cells}{ml} = \frac{N}{f} \times d \times c$$

Where N = mean cell number per field count

f = number of field count = 4

d = dilution factor

c = hemocytometer constant = 10^4

3.3.9.2.3 Determination by flow cytometry method

The apoptotic cells were evaluated by Annexin V and propidium iodide (PI) staining analysis. Cells were treated with the vehicle or test compounds for 48 h. After the incubation period, cells were suspended in 500 µl of Annexin V binding buffer and incubated with Annexin V-fluorescein conjugate and PI for 15 min at 4 °C before analysis by flow cytometry (BD Biosciences, CA, USA). Apoptotic cells were counted and expressed as percentage of total cells (30,000 cells).

3.3.9.3 Intracellular accumulation of platinum in RPTEC/TERT1 cells

This experiment investigated the mechanism of the selected compounds via OCT2-mediated transport of cisplatin. The platinum accumulation in RPTEC/TERT1 cells was determined using atomic absorption. RPTEC/TERT1 cells were seeded in 6-well plate for 14 days until cells reached 100% confluency. Cells were pretreated with the selected compounds for 1 h and then cultured with or without cisplatin for 4 h. After treatment, cells were washed with PBS for 2 times and removed by incubation with 0.25% trypsin for 20 min at 37 °C and then were centrifuged at 1500 rpm for 10 min. Subsequently, cells were solubilized overnight in 0.5 ml of concentrated nitric acid. Samples were subjected to flame atomic absorption spectrophotometer; FAAS (AAnalyst 200, Perkin Elmer) to determine platinum content. Data were expressed as ng of platinum/mg of total protein. OCT2-mediated platinum accumulation was calculated by subtracting platinum accumulation in the presence of 1 mM TPeA, an inhibitor of OCT, from the total platinum accumulation.

3.3.10 *In vivo* protective effect of the selected compounds on cisplatin-induced nephrotoxicity

In this study, nephrotoxicity was induced by intraperitoneal injection of 15 mg/kgBW of cisplatin. Male C57BL/6 mice (20-25g, 6-8 weeks, n=5 animal per group) were housed at least 7 days before treatment. At day 0, mice were randomly divided as followings

I. PBS pH 7.4 (i.p.)

II. 15 mg/kgBW of cisplatin (i.p.)

III. 15 mg/kgBW of cisplatin (i.p.) plus 5 mg/kgBW of OSCS-polymer (i.v.)

IV. 15 mg/kgBW of cisplatin (i.p.) plus 5 mg/kgBW of OSCS-micelle (i.v.)

For group III, and IV, mice were pretreated with 5 mg/kgBW OSCS-polymer or OSCS-micelle via i.v. injection for 24 h., then each group of animals were treated for 72 h. After treatment periods, all mice were scarified, blood was collected for kidney function test and kidneys were collected for histology, measurement of platinum accumulation and immunoblotting.

3.3.10.1 Immunoblotting

3.3.10.1.1 Western-blot analysis in RPTEC/TETR1 cells

Cells grown in 6-well plates were lyzed with RIPA buffer and protease inhibitor cocktail for 20 min at 4 °C. Kidneys from mice were homogenized and extracted in ice-cold buffer containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, and protease inhibitor cocktail. Samples were centrifuged at 12,000 rpm for 30 min. Protein was separated in 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Millipore, Thailand). Membranes were 1 h blocked with 5% of non-fat dry milk and were incubated overnight with monoclonal antibodies against NGAL. The secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG antibodies. The immunoblot was visualized using a chemiluminescence detection method. The intensity was measured by Image J and calculated using β-actin as a loading control.

3.3.10.1.2 Western blot analysis in kidney tissue

The expression of acute kidney injury biomarker, Ngal, was determined by Western blot analysis. Neutrophil gelatinase-associated lipocalin (Ngal), a 25 kDa protein produced by injured nephron epithelia, is one of the most promising new markers of renal epithelial injury. Representative Western blots of kidney lysates from mice treated with cisplatin or cisplatin plus selected compound were probed with a polyclonal antibody to NGAL or a monoclonal antibody to β-actin (loading protein). Total protein samples in kidney tissues were extracted using a lysis buffer, and the concentrations were measured using Nano Drop instrument. Equal amounts of tissue protein were denatured in 10 min at 95°C. Then the protein samples were separated by 10%SDS-PAGE, and then the subsequent procedure was mentioned above. The data were shown as interested protein and loading protein ratio.

3.3.10.2 Accumulation of platinum in kidney

The platinum accumulated in kidney was measured using ICP-MS. Each sample was weighed and then added 1 ml of concentrated nitric acid for digestion. After that, the samples were diluted with ultrapure water up to 10 ml and analyzed by ICP-MS.

3.3.10.3 Determination of renal function

Mouse blood was collected and centrifuged at 4°C 10000 rpm for 20 min. The serum samples were kept at -80° C until used. 20 µl of plasma were diluted with deionized water then the serum creatinine was measure using Automatic Chemistry Analyzer (BT1000, Biotecnica Instrument).

3.3.10.4 Histological evaluation of cisplatin-induced toxicity

The pathophysiology of cisplatin-induced renal injury can be classified into 4 types of toxicity including tubular toxicity, vascular damage, glomerular injury, and interstitial injury. To examine cisplatin-induced renal injury and the potential protective effect of compounds, kidney specimens were stained with hematoxylin and eosin (H&E). Kidney tissues were fixed in 4% paraformaldehyde and subjected to H&E. Morphology was analyzed using a light microscope (Zeiss Axio Observer A1). Tubular damage in HE-stained kidney section was evaluated and scored based on the percentage of cortical tubular necrosis as following 0=no damage, 1=0-10%, 2=11-25%, 3=26-45%, 4=46-75%, 5=more than 75%. Slides were blind scored by pathologist. Mean scores were calculated by counting 10 different fields for each group.

3.3.11 Effect of the selected compounds on cisplatin anticancer activity

In this experiment, the effect of the selected compounds on cisplatin's effect in HN22, HepG2, and MCF-7 cells were determined by MTT assay. The cells were seeded in 96-well plates for 1 day until cells reached 40-50% confluence. Cells were pretreated with or without the selected compounds for 1 h and then cultured in the presence or absence of cisplatin for 72 h. At the end of the incubation, cells were washed three times with serum-free media. MTT was added to each well, and cells were incubated for another 1 h at 37°C. Then, MTT medium was replaced with 100 µl of 100% DMSO. Cell viability was examined by measuring the absorbance at 570 nm by using microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard Bio-Science, CT, U.S.A.). The percent cell viability was expressed as a percentage of the control value.

4. Data analysis

All data are reported as the mean±standard error (S.E.). The significance of the differences is evaluated using a one-way analysis of variance (ANOVA) or student t-test. The significance level is set at a *p* value of 0.05.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Effect of pharmaceutical excipients on transport function of OCT1 and OCT2

4.1.1 Interaction of pharmaceutical excipients with OCT1

4.1.1.1 Interaction of pharmaceutical excipients in rbOCT1-CHO-K1 cells

4.1.1.2 Inhibitory potencies of Tweens on OCT1-mediated ^{3}H-MPP^{+} uptake

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4.2.2.4 Effect of chitosan derivatives on cationic drug accumulation in human renal proximal tubular cells

4.3 Chitosan derivatives for prevention of cisplatin-induced nephrotoxicity and anticancer activity of cisplatin.

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4.3.3 Effect OSCS on anticancer activity of cisplatin in cancer cell lines

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4.4.2 Anti-cancer activity of cisplatin-OSCS complex in HN22 cells

4.4.3 Effect of cisplatin-OSCS complex in renal proximal tubular cells

4.4.3.1 Cytotoxity of cisplatin-OSCS complex in renal proximal tubular cells

4.4.3.2 Cisplatin-OSCS complex induced apoptosis in renal proximal tubular cells

4.4.4 Protective effect of cisplatin-OSCS complex in mice

4.1 Effect of pharmaceutical excipients on transport function of OCT1 and OCT2

4.1.1 Interaction of pharmaceutical excipients with OCT1

4.1.1.1 Interaction of pharmaceutical excipients in rbOCT1-CHO-K1 cells

To determine whether pharmaceutical excipients potentially inhibited transport function of OCT, inhibitory effects of excipients on OCT1 were investigated in CHO-K1 cells expressing rbOCT1. The monolayers of rbOCT1-CHO-K1cells were incubated with transport buffer containing ^{3}H-MPP^{+} alone or in the present of 100 µg/ml of 14 kinds of excipients for 5 min. After incubation period, the cellular accumulation of ^{3}H-MPP^{+} was determined. As shown in Fig.4.1, 5 of 14 excipients significantly decreased the cellular accumulation of ^{3}H-MPP^{+} in rbOCT1-CHO-K1 cells indicating these excipients might interact with OCT1.

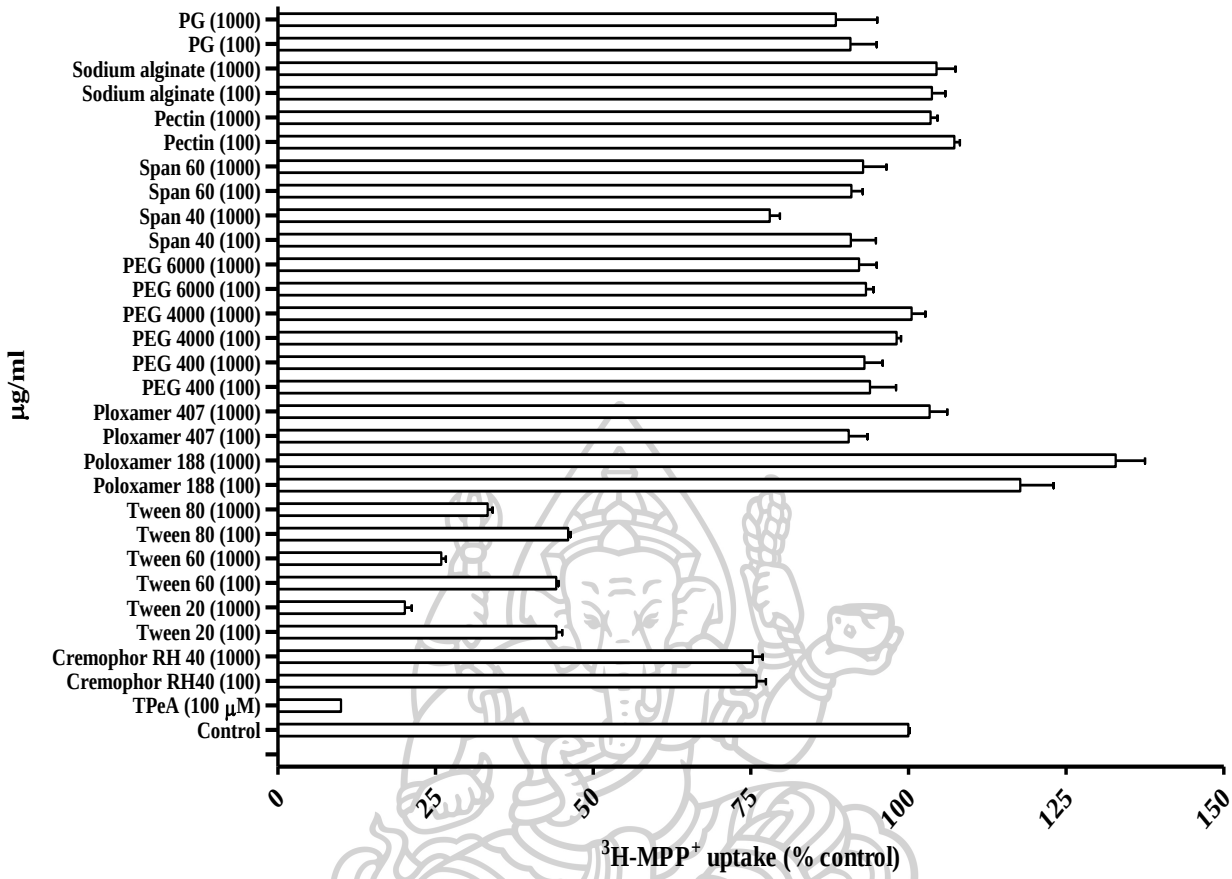


Figure 4.1 Inhibitory effects of various excipients on the uptake of ^{3}H-MPP^{+} in rbOCT1-CHO-K1 cells. Uptake of ^{3}H-MPP^{+} (10 nM) was measured in the presence or absence of excipients. TPeA (100 μM) was used as positive control. The data are expressed as mean±S.E. from three independent experiments, expressed as percentage of control.

These results suggested that these excipients have potential to inhibit OCT1 transport function. Next, the excipients showing the strong inhibitory effects (> 50% of control) were selected for further investigation. Tweens had more potent effect on the inhibition of OCT1 transport function as compared with Cremophor RH40. On the other hand, PEG400, a co-solvent, had no effect on the activity of OCT1. These data indicated that surfactants might influence the transport function of OCT1 and might subsequently alter pharmacokinetic of therapeutic drugs.

4.1.1.2 Inhibitory potencies of Tweens on OCT1-mediated ^{3}H-MPP^{+} uptake

Surfactants showed more potent inhibition than that of other group of excipients as the results showing that Tweens (20, 60, and 80) markedly inhibited OCT1-mediated ^{3}H-MPP^{+} uptake (Fig. 4.1), next, the inhibitory potencies of Tweens (20, 60, and 80) were further determined. As shown in Fig. 4.2, Tween 20, Tween 60, and Tween 80 inhibited OCT1-mediated ^{3}H-MPP^{+} uptake in concentration-dependent manners in the range of 0-1,000 µg/ml. The IC_{50} values of Tween 20, Tween 60, and Tween 80 for OCT1 were 85±1.12, 50±1.26, and 106.00±1.20 µg/ml, respectively. Among the Tweens, Tween 60 displayed higher inhibitory potency on transport activity of OCT1 than that of Tween 20 and Tween 80. The different inhibitory potencies may be described by the different structure of Tweens. Tween 20, Tween 60, and Tween 80 contain lauric acid, stearic acid, and oleic acid, respectively. It is possible that the type of fatty acid contained in the structure of Tweens may affect the inhibitory potencies. However, this notion needs to be further elucidated.

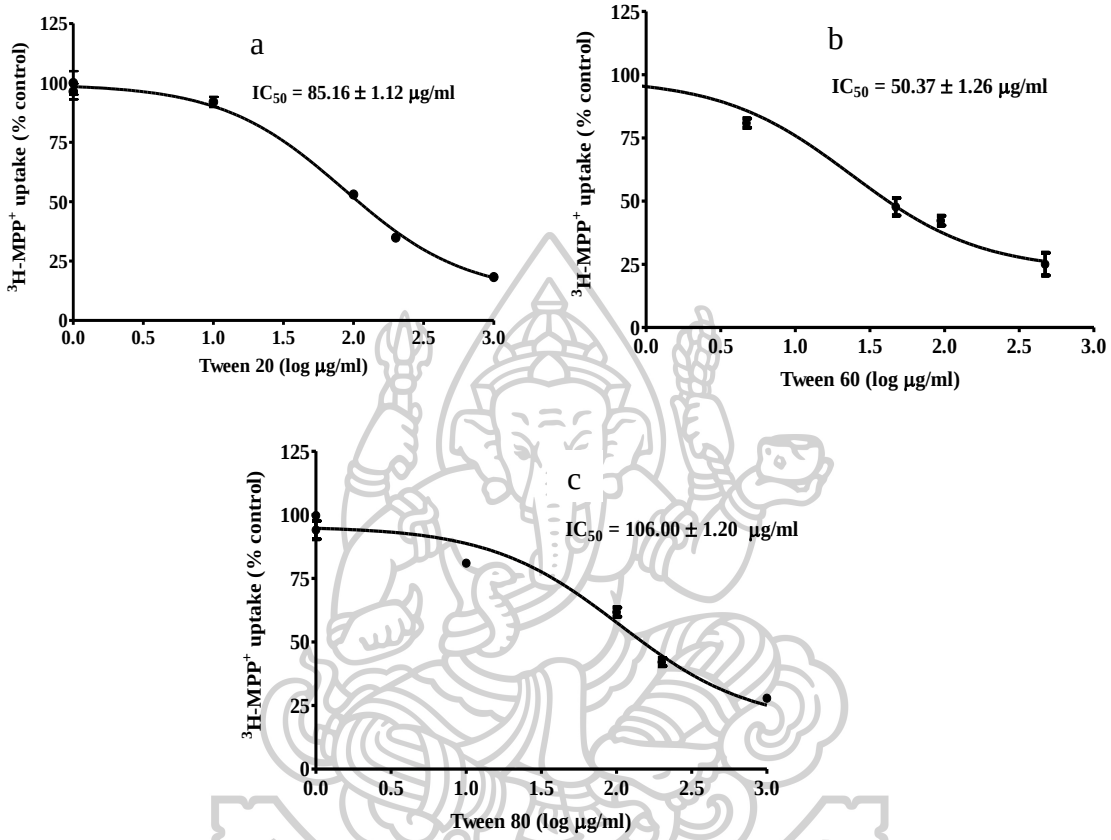
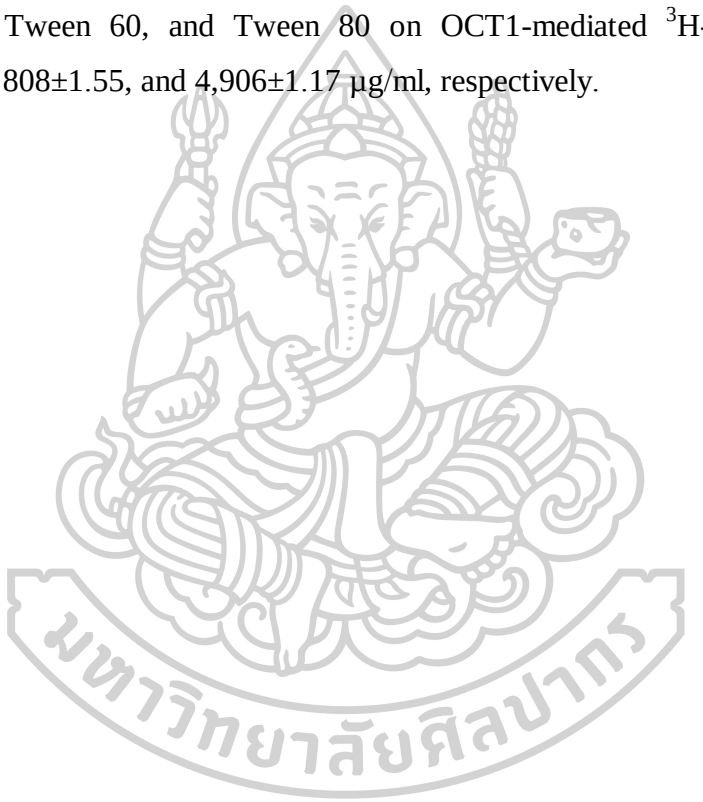


Figure 4.2 Inhibitory potency (IC_{50}) of (a) Tween 20, (b) Tween 60, (c) Tween 80 in rbOCT1-mediated $^{3}H\text{-}MPP^{+}$. CHO-K1 cells singly expressed rbOCT1 were incubated with $^{3}H\text{-}MPP^{+}$ alone, or in the presence of various concentrations of Tween 20, 60, 80 for 5 min, then radioactivity accumulation was determined. The IC_{50} values were calculated from uptake measured in triplicate from 3 independent experiments and mean±S.E.

4.1.1.3 Effect of Tweens on OCT1-mediated ^{3}H-MPP^{+} uptake in human hepatocyte

OCT1 is found to express in hepatocytes and plays an important role in hepatic cationic drug clearance [23]. The inhibitory effect of Tween 20 and Tween 80 in human hepatic cells that endogenously express OCT1 was investigated. HepG2 cells were incubated with medium containing ^{3}H-MPP^{+} alone or ^{3}H-MPP^{+} plus Tweens at the concentration range of 0-2,000 µg/ml for 5 min, then the cellular accumulations of ^{3}H-MPP^{+} were measured. As shown in Fig. 4.3, the IC_{50} values of Tween 20, Tween 60, and Tween 80 on OCT1-mediated ^{3}H-MPP^{+} uptake was 1,619±1.95, 808±1.55, and 4,906±1.17 µg/ml, respectively.



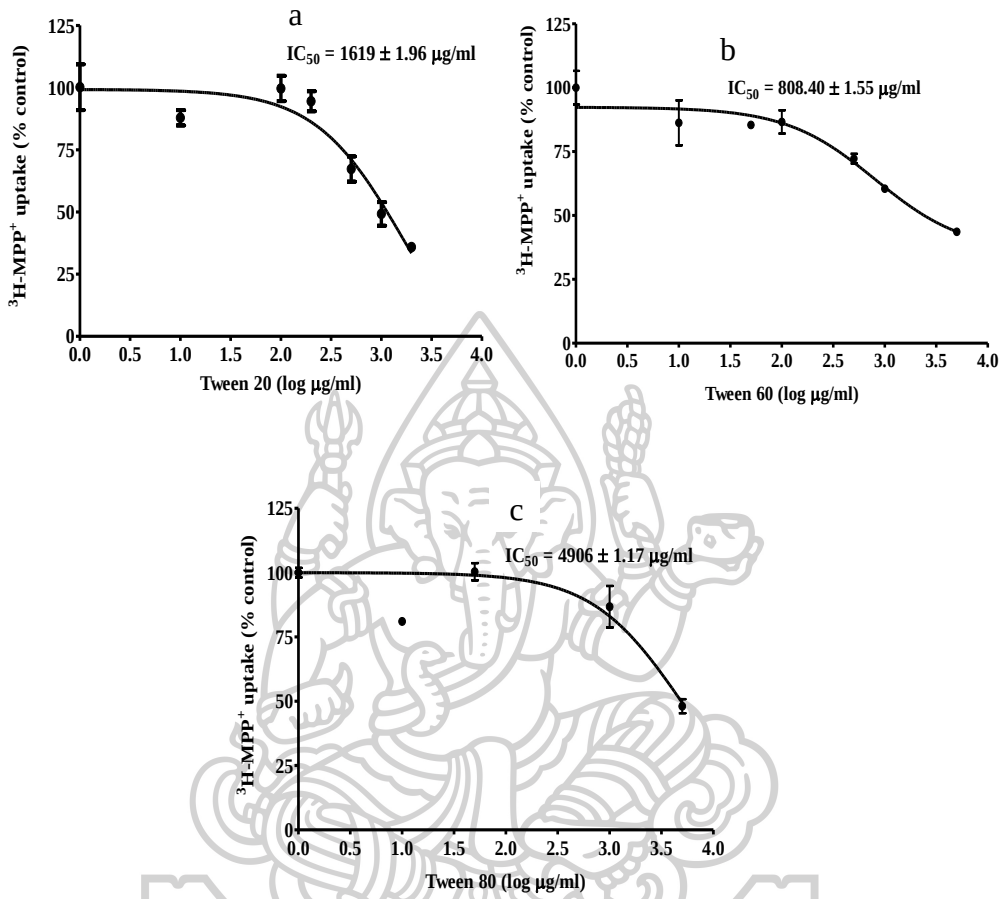


Figure 4.3 Inhibitory potency (IC_{50}) of (a) Tween 20, (b) Tween 60, (c) Tween 80 on OCT1-mediated ^{3}H-MPP^{+} in HepG2 cells, HepG2 cells were incubated with ^{3}H-MPP^{+} alone, or in the presence of various concentrations of Tween 20, 60, 80 for 5 min, then radioactivity accumulation was determined. The IC_{50} values were calculated and expressed as mean±S.E. of 3 independent experiments.

Interestingly, Tween 20, Tween 60, and Tween 80 exhibited less inhibitory effect in OCT1 expressing human hepatic cells compared with heterologous OCT1 expressing cells. HepG2 cell line used as hepatic cell model is hepatocarcinoma containing several drug transporters [107]. It is possible that ^{3}H-MPP^{+} not only is taken into the cell by OCT1, but also is taken by other transporters that are not inhibited by Tween 20, Tween 60, and Tween 80. Moreover, the possibility that rabbit and human OCT1 orthologue might have different affinity site for theses also need to be taken into account.

4.1.2 Interaction of pharmaceutical excipients with OCT2

4.1.2.1 Interaction of pharmaceutical excipients in rbOCT2-CHO-K1 cells

In addition to OCT1, OCT2 expressing in renal proximal tubular cells plays the crucial roles in renal clearance of cationic drug such as cisplatin, metformin, and beta-blockers [23, 27, 66]. The present study investigated whether the pharmaceutical excipients interact with OCT2 and might subsequently inhibit renal excretion of cationic drugs. CHO-K1 cells expressing OCT2 were exposure to transport buffer containing ^{3}H-MPP^{+} alone, or in the presence of various kinds of the excipients for 5 min. followed by measurement ^{3}H-MPP^{+} accumulation. As shown in Fig. 4.4, surfactants Tweens produced more an inhibitory effect on OCT2-mediated ^{3}H-MPP^{+} uptake in OCT2-CHO-K1 cells compared with Span and Cremophore RH. The effects of these excipients on OCT2 results were similar as found in OCT1. These results suggested that Tweens might have the potential to inhibit OCT2 transport function.

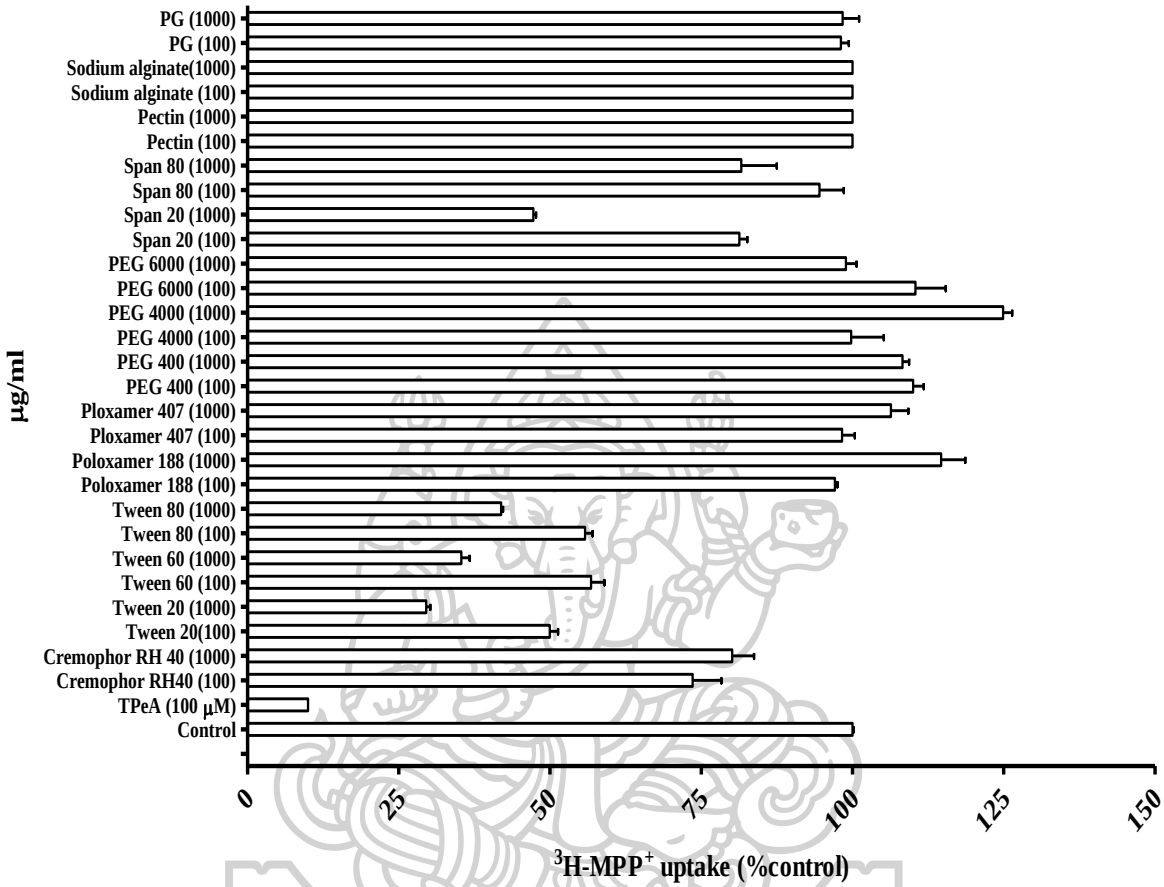
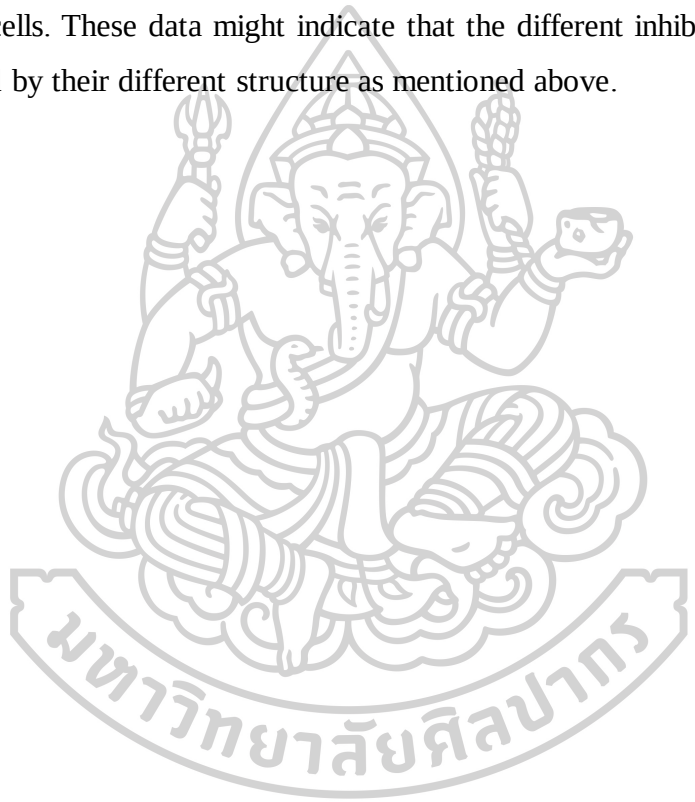


Figure 4.4 Inhibitory effects of various excipients on OCT2-mediated ^{3}H-MPP^{+} uptake in rbOCT2-CHO-K1. The data are expressed as percentage of control and showed as mean±S.E. from three independent experiments.

4.1.2.2 Inhibitory potencies of Tweens on OCT2-mediated ^{3}H-MPP^{+} uptake

Since, Tween 20, 60, and 80 showed the marked inhibition on OCT2 transport function, next, the inhibitory potencies of each were determined. As shown in Fig. 4.5, the IC_{50} of Tween 20, 60, and 80 on OCT2-mediated ^{3}H-MPP^{+} uptake was 295±1.48, 42±1.15, and 185±1.20 µg/ml, respectively. The results showed that Tween 60 showed the highest inhibitory potency compared with Tween 20 and 80. The results of Tween conducted in OCT2 expressing cells were similar as found in OCT1 expressing cells. These data might indicate that the different inhibitory potencies may be described by their different structure as mentioned above.



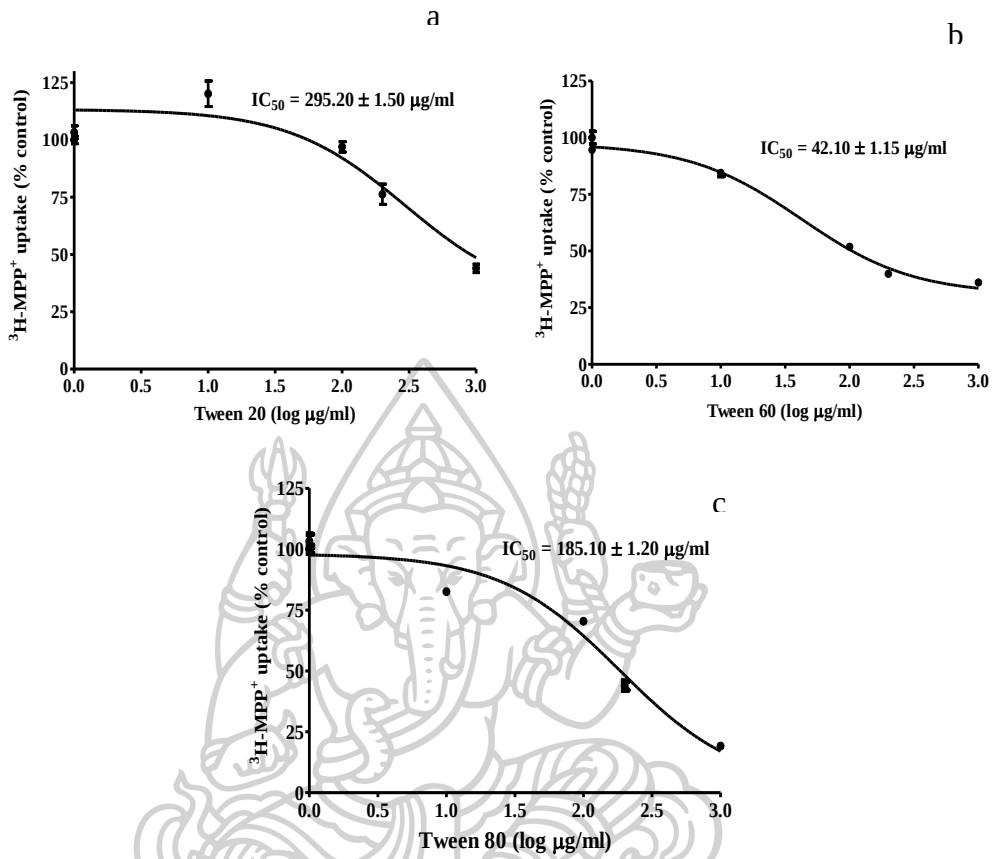


Figure 4.5 Effect of (a) Tween 20, (b) Tween 60, (c) Tween 80 in rbOCT2-mediated ^{3}H-MPP$^+$. CHO-K1 cells singly expressed rbOCT2 were exposed to ^{3}H-MPP$^+$ alone, or in the presence of various concentrations of Tween 20, 60, 80 for 5 min followed by measurement of ^{3}H-MPP$^+$ accumulation. The IC_{50} values were calculated expressed as mean±S.E from 3 independent experiments.

4.1.2.3 Effect of Tweens on OCT2-mediated ^{3}H-MPP^{+} uptake in human renal proximal tubular cells

To determine whether the inhibition of Tweens on OCT2 transport function in OCT2 expressing CHO-K1 cells was found in renal proximal tubular cells that endogenously express hOCT2, the inhibitory effects of Tweens on function of OCT2 were confirmed in RPTECT/TERT1 cells which are human renal proximal tubular cells express OCT2 [108, 109]. Co-exposing the cells with ^{3}H-MPP^{+} and Tween 20, Tween 60, or Tween 80 for 5 min significantly reduced accumulation of ^{3}H-MPP^{+} uptake compared with control. The IC_{50} values of Tween 20, Tween 60, and Tween 80 on OCT2-mediated ^{3}H-MPP^{+} uptake was determined and the results revealed that the IC_{50} of Tween 20, Tween 60, and Tween 80 were 182±1.12, 28±1.20 and 118±1.15 mg/ml, respectively (Fig. 4.6). Among Tween, Tween60 showed the highest inhibitory potency on OCT2 transport function.

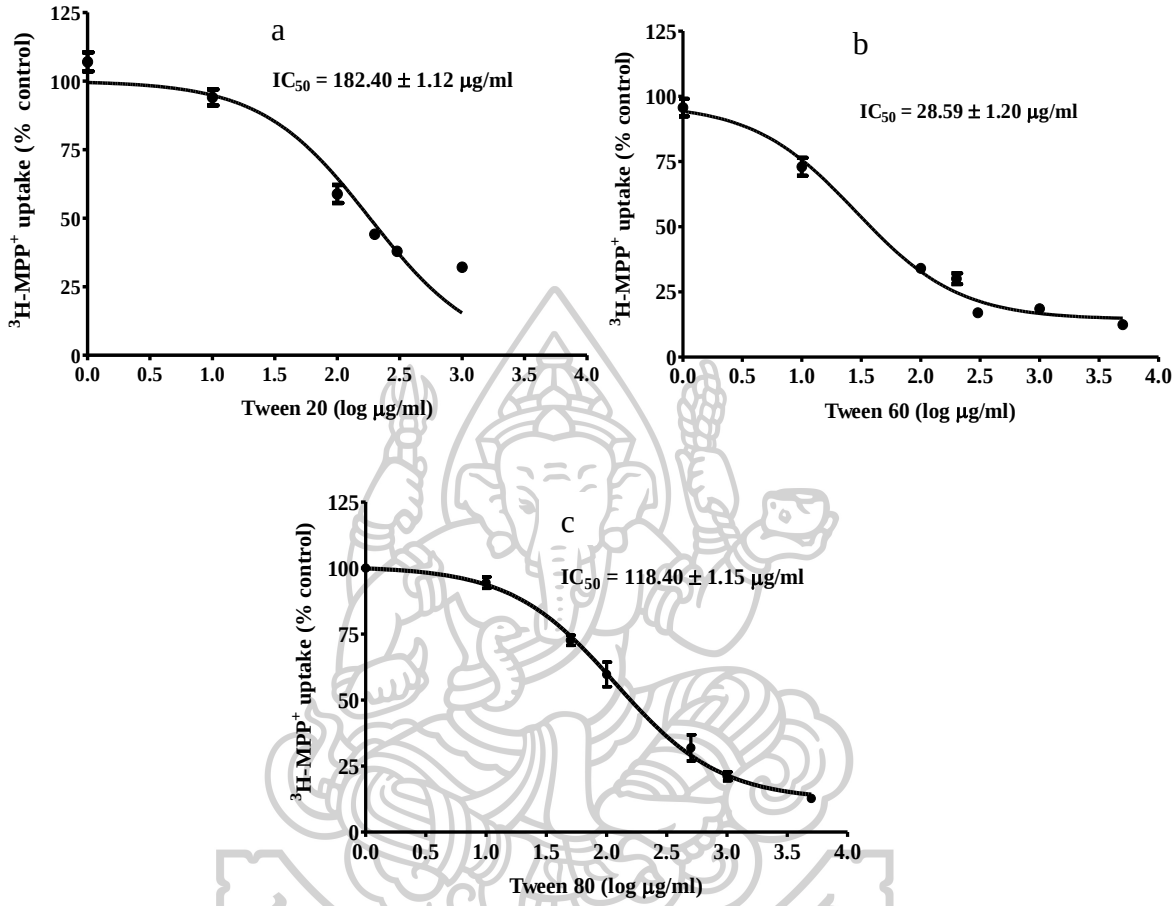


Figure 4.6 Inhibitory effects of (a) Tween 20, (b) Tween 60, (c) Tween 80 on the uptake of ^{3}H-MPP^{+} in RPTEC/TERT1 cells. Uptake of ^{3}H-MPP^{+} was measured for 5 min in the presence or absence of Tweens. The data are expressed as mean±S.E. from three independent experiments, expressed as percentage of control. The IC_{50} values were calculated from uptake measured in triplicate from 3 independent experiments and expressed as mean±S.E.

To determine whether the inhibitory effect of Tweens was not caused by their cytotoxicity, the cytotoxicity test using MTT assay was performed with Tween 20, Tween 60, and Tween 80. As shown in Fig. 4.7, Tweens at concentration of 0–500 µg/ml showed no significant effect on RPTEC/TERT1 cell viability following 5 min incubation. Therefore, inhibitory effect of Tweens was not a result of cytotoxicity.

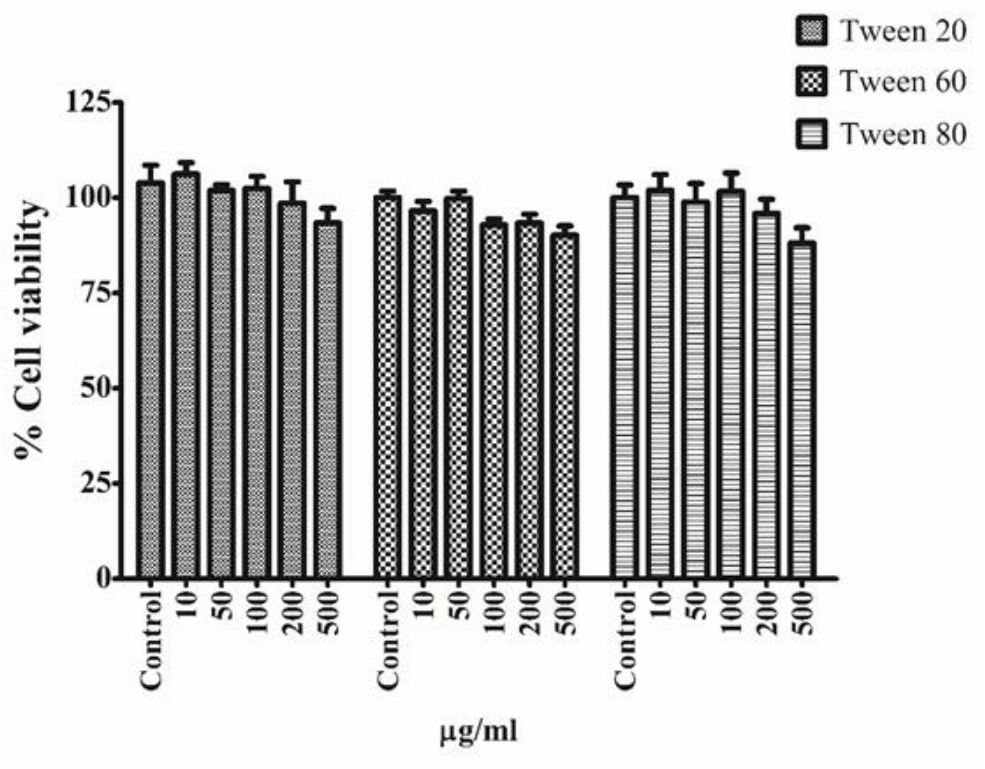


Figure 4.7 Effect of Tweens on cell viability. Confluence cell monolayers were incubated with serum free media containing Tweens (1–500 µg/ml) for 5 min at 37° C followed by MTT assay. Cell viability data are expressed as percentage of control from 3 independent experiments (mean±S.E.).

The data obtained from the present study demonstrated the similar inhibitory effect of Tween 20, 60, 80 on OCT1- and OCT2-mediated organic cation transport in the endogenously express system and heterologous expressing cells. Furthermore, to compare inhibitory effect of Tween 20, 60, and 80 on human OCT1 and human OCT2 activities, the IC_{50} values of $^{3}H\text{-}MPP^{+}$ were evaluated. All Tween inhibited human OCT2 more effectively than human OCT1. The different inhibitory potential may be described by the structure activity relation between excipients and transporters.

4.1.3 Effect of Tween 20 on plasma concentration of $^{3}H\text{-}MPP^{+}$

Tween20 and Tween 80 are widely used in injectable formulation. The present study clearly demonstrated that Tween 20 and Tween 80 could inhibit OCT1 and OCT2 transport function in human hepatocytes and renal proximal tubular cells. It is interesting whether the inhibitory effects of Tween 20 and Tween 80 affect the clinical relevance of OCT2-mediated renal clearance of therapeutic drugs. Based on average effective circulating volume (plasma volume) of an adult (5 liters), approximately 500 mg of Tween 80 are required for intravenous administration to reach the IC_{50} for OCT2 (100 µg/ml). Tween 80 containing therapeutic drugs formulation for intravenous administration such as etoposide, docetaxel, and chlordiazepoxide [4] might not reach the IC_{50} values for OCT1 and OCT2 when patients receive a single dose. Therefore, the effect of Tween 20 was selected to test whether it affected kinetic of cationic compound in vivo. To determine the effect of Tween 20 on OCT1 and OCT2 transport function, the plasma concentration of $^{3}H\text{-}MPP^{+}$ (a substrate of these transporters) in the presence and absence of Tween 20 was investigated. Mice were intraperitoneal injections of 200 µl $^{3}H\text{-}MPP^{+}$ dissolved in saline alone (control) or in concert with Tween 20 at 1 mg/ml. Blood samples were collected. As shown in Fig. 4.8, plasma concentration of $^{3}H\text{-}MPP^{+}$ in control group was increased from 5 min to 30 min and then plateaued up to 120 min. After that plasma concentration of $^{3}H\text{-}MPP^{+}$ was decreased. In the presence of Tween 20, plasma concentration of $^{3}H\text{-}MPP^{+}$ was slightly higher than that of control at matched-time points and reached significant difference at 60 min. These results implied that Tween 20 might reduce clearance of cationic compounds from body.

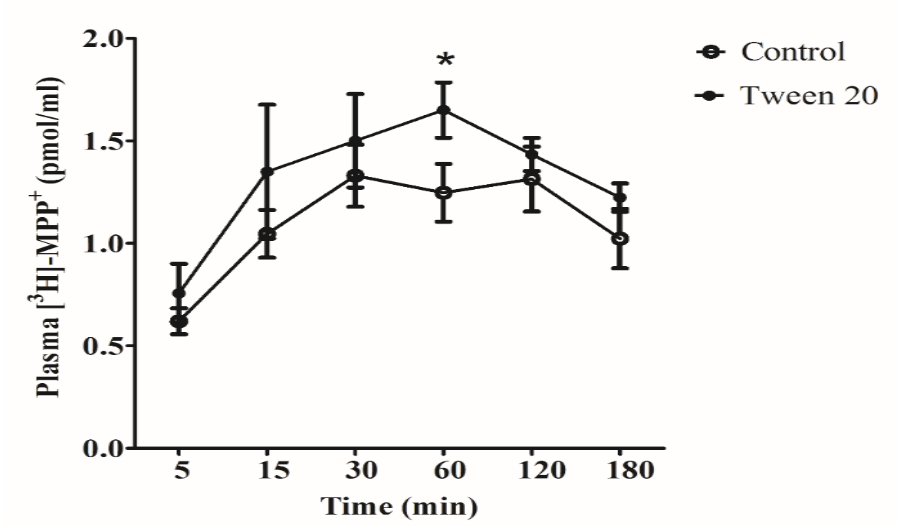


Figure 4.8 Effect of Tween 20 on plasma concentration of ^{3}H-MPP^{+}. Mice were received 200 µl of ^{3}H-MPP^{+} (1 µM) dissolved in saline buffer or in the presence of 1 mg/ml Tween 20 via intraperitoneal injected. Plasma concentration of ^{3}H-MPP^{+} was determined at 5, 15, 30, 60, 120, and 180 min. The data are expressed as mean±S.E. from 3 mice and * indicates a significant difference from the control (Student's t-test; P <0.05).

4.2 Effect of chitosan and chitosan derivatives on OCT1 and OCT2

4.2.1 Interaction of chitosan and its derivatives with OCT1

4.2.1.1 Interaction of chitosan and its derivatives in rbOCT1-CHO-K1 cells

The interaction of chitosan and its derivatives was determined by *cis*-inhibitory effect assay. The OCT1-CHO-K1 cells were incubated with transport buffer containing ^{3}H-MPP^{+} alone or with test compounds at 100 and 1,000 µg/ml for 5 min and followed by measurement of ^{3}H-MPP^{+} accumulation. As shown in Fig. 4.9, chitosan and QC had no effect on OCT1-mediated ^{3}H-MPP^{+} uptake whereas MDM, BSCS, OSCS, and NSCS showed the significant inhibitions. Test compounds that showed the inhibition on OCT1 transport function including MDM, BSCS, OSCS, and NSCS were selected for determining the inhibitory potencies.

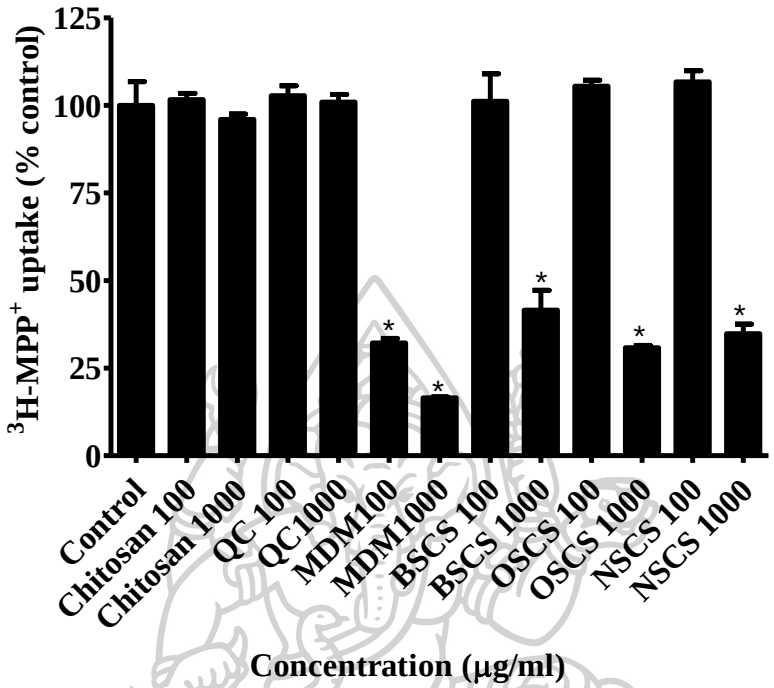


Figure 4.9 *cis*-Inhibitory effects of chitosan and its derivatives on OCT1-mediated ^{3}H-MPP^{+} uptake in rbOCT1-CHO-K1 cells. Five minutes' uptake of ^{3}H-MPP^{+} was measured in the presence or absence of test compounds. The uptakes were calculated as percentage of control and expressed as mean±S.E. from three independent experiments. $^{*}P<0.05$ compared with control.

4.2.1.2 Inhibitory potencies of chitosan derivatives on OCT1-mediated ^{3}H-MPP^{+} uptake

The inhibitory potency of chitosan derivatives (MDM, BSCS, OSCS, and NSCS) were further delineated by concentration-dependent effects. The cells were incubated with transport buffer containing ^{3}H-MPP^{+} plus increasing various concentrations of the derivatives followed by measurement of ^{3}H-MPP^{+} accumulation. As shown in Fig. 4.10, MDM, BSCS, OSBS, and NSCS inhibited OCT1-mediated ^{3}H-MPP^{+} uptake in concentration-dependent manners. The IC_{50} of MDM, BSCS, NSCS, and OSCS for OCT1 were 73±5, 1,410±15, 2,420±26, and 1.55±0.022 µg/ml, respectively. To investigate the mechanism by which MDM, BSCS, NSCS, and OSCS inhibited OCT1-mediated ^{3}H-MPP^{+} uptake, the kinetic study was performed. As shown in Fig. 4.11, MDM, BSCS, OSBS, and NSCS decreased J*max* of OCT1-mediated ^{3}H-MPP^{+} uptake without changing of Kt or Km. These data indicated that the inhibitory effect of these compounds was via non-competitive mechanism.

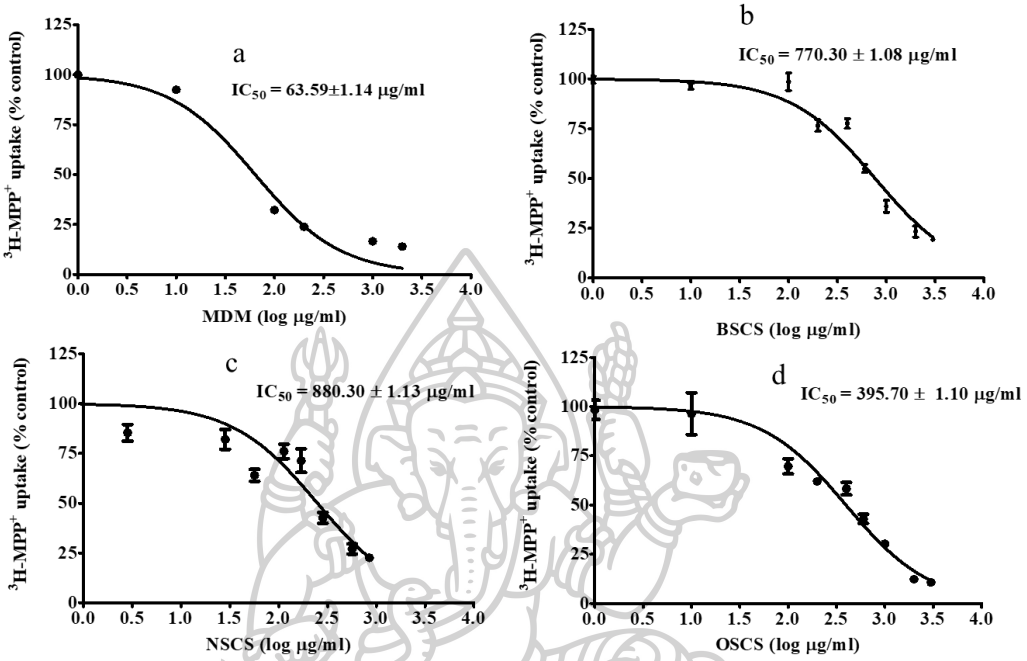


Figure 4.10 Inhibitory potency (IC_{50}) of (a) MDM, (b) BSCS, (c) NSCS, and (d) OSCS on OCT1-mediated ^{3}H-MPP^{+} uptakes in CHO-K1 cells; CHO-K1 cells singly expressed OCT1 were incubated with ^{3}H-MPP^{+} alone, or in the presence of various concentrations of MDM, BSCS, NSCS and OSCS for 5 min, then radioactivity accumulation was determined. The IC_{50} values were calculated from n=3 and expressed as mean±S.E.

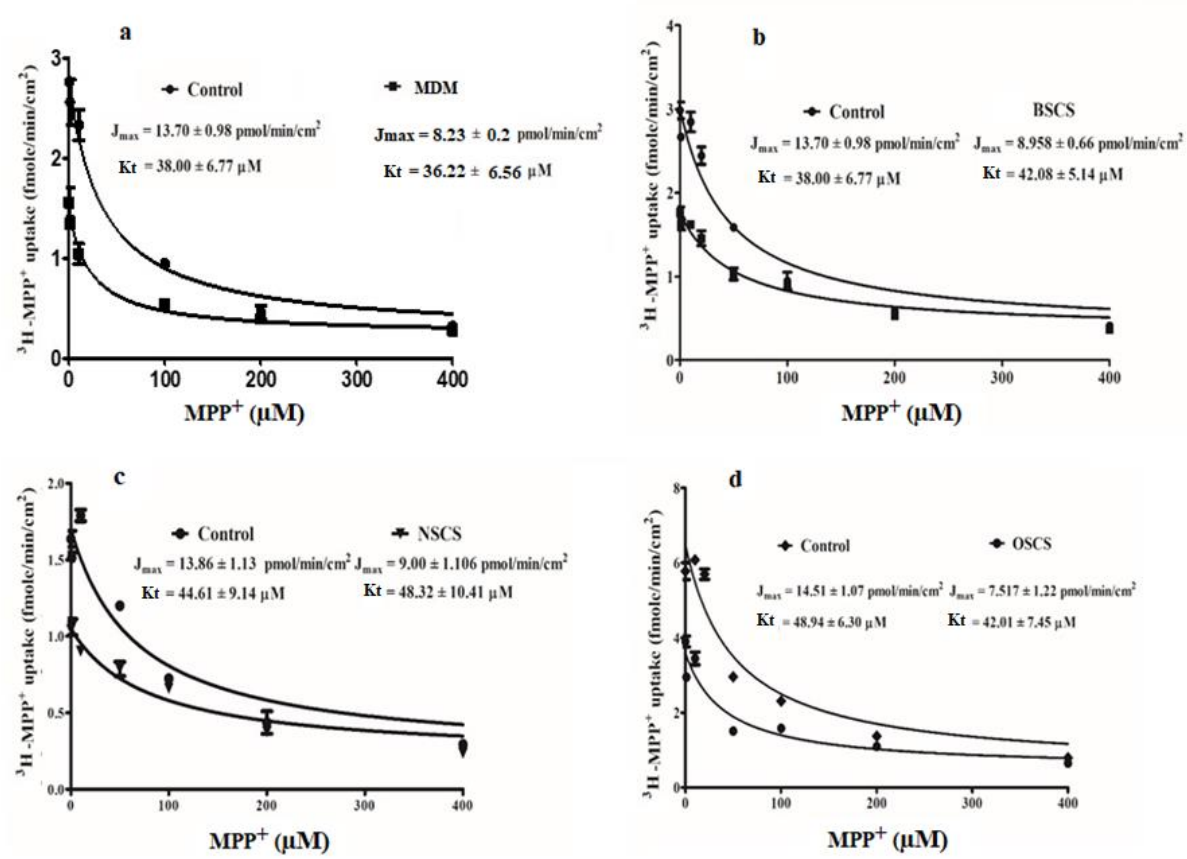


Figure 4.11 Kinetic studies of OCT1-mediated MPP^+ uptake in vehicle- and chitosan derivative-(a) MDM, (b) BSCS, (c) NSCS, and (d) OSCS treated rbOCT1-CHO-K1 cells. *Jmax*, maximum rate of MPP^+ transport; Kt, affinity of the transporter. rbOCT1-CHO-K1 cells were treated with chitosan derivative plus ^3H-MPP^+ or vehicle plus ^3H-MPP^+ for 5 min. uptake was determined in the presence of various concentrations of unlabeled MPP^+ ranging from 1 to 400 µM. Values are means±S.E. (n=3). *Significant difference from control ($P < 0.05$).

4.2.1.3 Effect of chitosan derivatives on OCT1-mediated ^{3}H-MPP^{+} uptake in human hepatocyte

The effect of the chitosan derivatives that inhibited OCT1 transport activity in OCT1-CHO-K1 cells was confirmed in endogenous expressing cells. Transport function of OCT1 was measured in HepG2 cell. As shown in Fig.12, BSCS, NSCS, and OSCS reduced cellular accumulation of ^{3}H-MPP^{+} in concentration-dependent manners as found in HegG2 cells with the IC_{50} of 1,884$\pm$6, 969$\pm$3, and 1,208$\pm$10 µg/ml, respectively (Fig. 12). Although MDM significantly inhibited ^{3}H-MPP^{+}uptake at low concentration (10 µg/ml), the IC_{50} of MDM could not be calculated because it did not inhibit ^{3}H-MPP^{+} in concentration-dependent manner in HepG2 cells. The explanation of this event was not clear. It is possible that high concentration of MDM might also inhibit efflux transporters responsible for removing of ^{3}H-MPP^{+} such as P-glycoprotein that expressed in several normal and cancer cells [107].

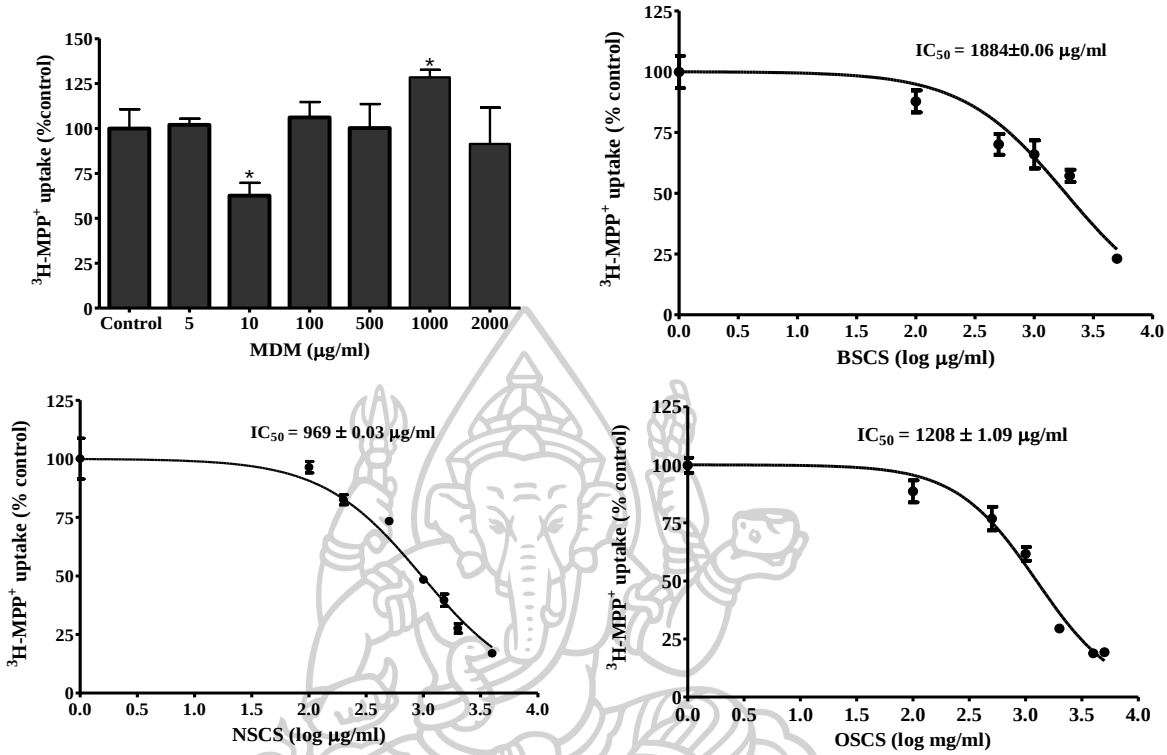


Figure 4.12 Inhibitory potency (IC_{50}) of (a) MDM, (b) BSCS, (c) NSCS, and (d) OSCS on OCT1-mediated ^{3}H-MPP$^+$ in HepG2 cells. HepG2 cells were incubated with ^{3}H-MPP$^+$ alone, or in the presence of various concentrations of BSCS, NSCS, and OSCS for 5 min, then accumulation of ^{3}H-MPP$^+$ was determined. The IC_{50} values were calculated and expressed as mean±S.E. of 3 independent experiments.

4.2.2 Interaction of chitosan and its derivatives with OCT2

4.2.2.1 Interaction of chitosan and its derivatives in rbOCT2-CHO-K1 cells

The interaction of chitosan and its derivatives was determined by *cis*-inhibitory effect assay in heterologous expressing cells. OCT2-CHO-K1 cells were incubated with transport buffer containing ^{3}H-MPP^{+} alone or plus test compounds at 1,000 µg/ml for 5 min. As shown in Fig. 4.13, chitosan and QC produced no effect on OCT2-mediated ^{3}H-MPP^{+} uptake. Interestingly, chitosan derivatives including MDM, BSCS, OSCS, and NSCS significantly inhibited OCT2-mediated ^{3}H-MPP^{+} uptake.

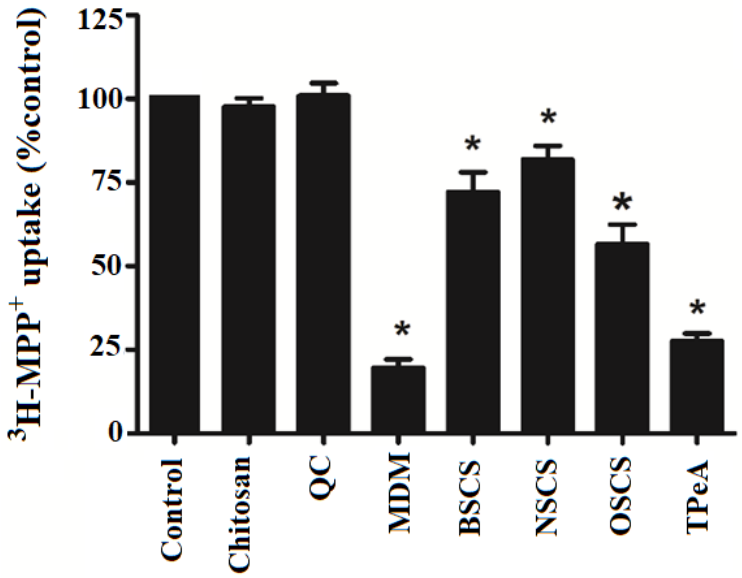


Figure 4.13 Inhibitory effect of chitosan and its derivatives on the uptake of ^{3}H-MPP$^+$ in rbOCT2-CHO-K1 cells; Uptake of ^{3}H-MPP$^+$ was measured in the presence or absence of chitosan, quaternized chitosan (QC), MDM, BSCS, NSCS, or OSCS at 1,000 µg/ml. TPeA (100 µM), an inhibitor of OCTs was used as positive control. The data are expressed as mean±S.E. from three independent experiments, expressed as percentage of control. *$P < 0.05$ compared with control.

4.2.2.2 Inhibitory potencies of chitosan derivatives on OCT2-mediated ^{3}H-MPP^{+} uptake

Since, chitosan derivatives were found to inhibit OCT2 transport function, next, the inhibitory potency of the chitosan derivatives (MDM, BSCS, OSCS, and NSCS) was further determined by testing the concentration-dependent effects. The OCT2-CHO-K1 cells were incubated with buffer containing ^{3}H-MPP^{+} plus increasing various concentrations of the derivative followed by measurement of ^{3}H-MPP^{+} accumulation. As shown in Fig. 4.14, MDM, BSCS, OSBS, and NSCS inhibited OCT2-mediated ^{3}H-MPP^{+} uptake in concentration-dependent manners. The IC_{50} of MDM, BSCS, NSCS, and OSCS for OCT2 were 73±5, 1,410±15, 2,420±26, and 1,550±22 µg/ml, respectively. These data indicated that MDM showed the highest inhibition potency on OCT2 compared with other derivatives and the results were similar as found in OCT1.

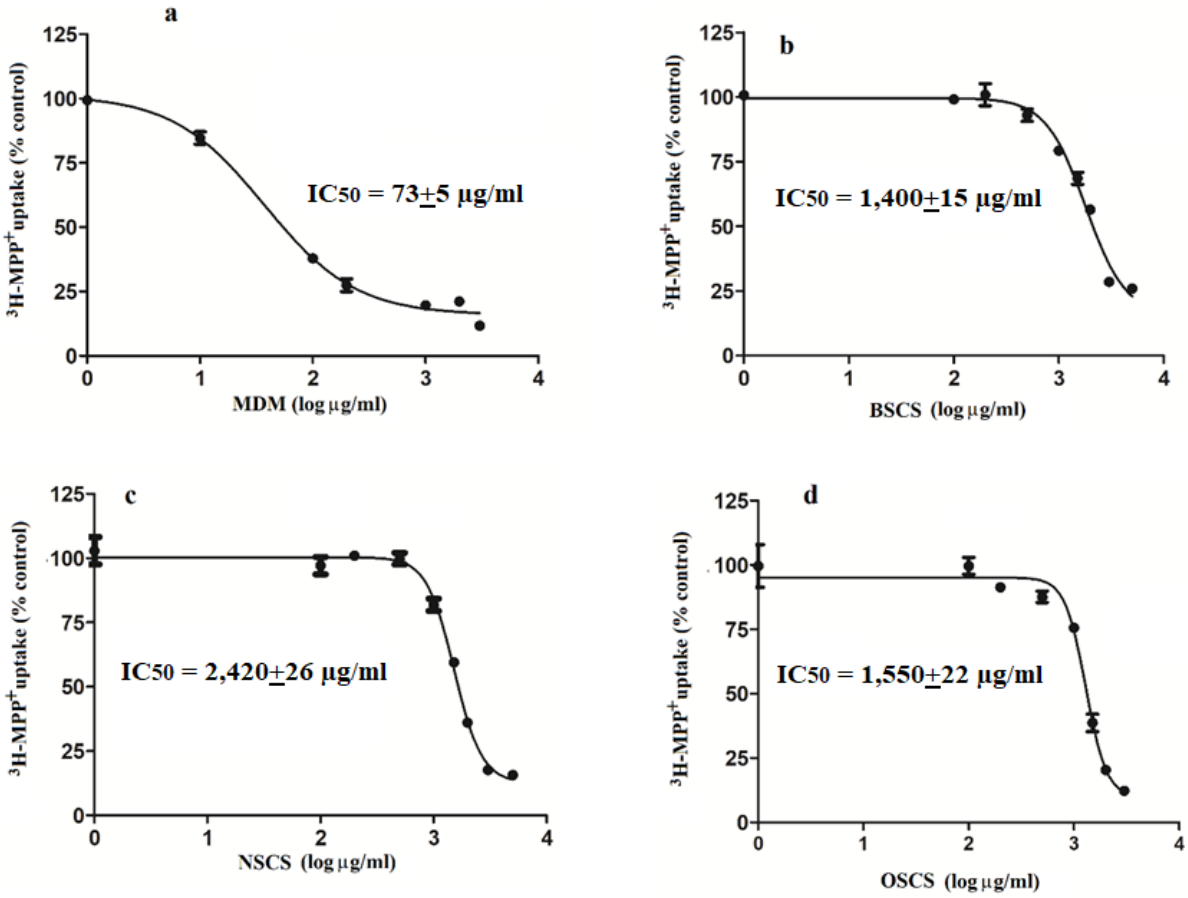


Figure 4.14 Inhibitory potency (IC_{50}) of (a) MDM, (b) BSCS, (c) NSCS, and (d) OSCS on OCT2-mediated ^{3}H-MPP^{+} uptakes in CHO-K1 cells; CHO-K1 cells singly expressed OCT2 were incubated with ^{3}H-MPP^{+} alone, or in the presence of various concentrations of MDM, BSCS, NSCS and OSCS for 5 min, then radioactivity accumulation was determined. The IC_{50} values were calculated from n=3 and expressed as mean±S.E.

Next, the question how the chitosan derivatives inhibit OCT2 transport function, and the mechanism by which MDM, BSCS, NSCS, and OSCS inhibited OCT2-mediated ^{3}H-MPP^{+} uptake was determined using the kinetic study. As shown in Fig. 4.15, MDM, BSCS, OSBS, and NSCS decreased J*max* of OCT2-mediated ^{3}H-MPP^{+} uptake without changing of Kt. These data indicated that the inhibitory effect of these compounds was via non-competitive mechanism. These results indicated that the chitosan derivatives might bind to OCT2 at different binding site of MPP^{+} binding site. Interestingly, the mechanism responsible of the chitosan derivatives for OCT2 inhibition was similar with OCT1.

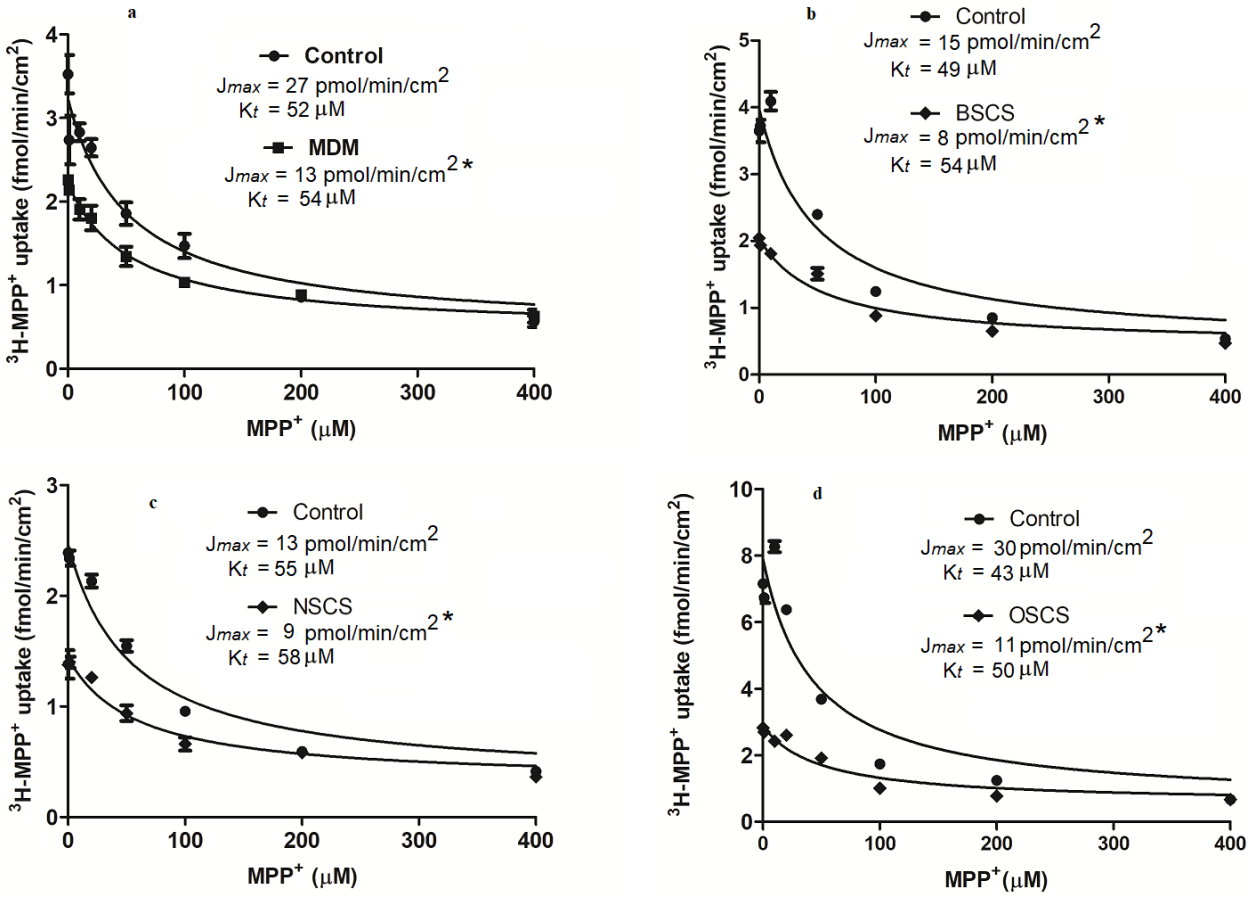


Figure 4.15 Kinetic studies of OCT2-mediated MPP^+ uptake of the chitosan derivatives: (a) MDM, (b) BSCS, (c) NSCS, and (d) OSCS in OCT2-CHO-K1 cells; Maximal rate transport (*Jmax*) and K*t*, the MPP^+ concentration that results in half-maximum transport, were calculated from 3 experiments. Values are means±S.E. * Significant difference from control (*P<0.05).

4.2.2.3 Effect of chitosan derivatives on OCT2-mediated ^{3}H-MPP^{+} uptake in human renal proximal tubular cells

In renal proximal tubular cells, OCT2 is expressed along with other transporters [21, 23]. The present study further determined whether the inhibitions of chitosan derivatives with OCT2 found in heterologous expressing cell was revealed in human renal proximal tubular cells, the RPTEC/TERT1 cells, that endogenously express OCT2 [108, 109]. The effect of the chitosan derivatives (MDM, BSCS, NSCS, and OSCS) on OCT2 transport activity was confirmed in endogenous OCT2-expressing cells. As shown in Fig. 4.16, BSCS, NSCS, and OSCS reduced accumulation of ^{3}H-MPP^{+} in concentration-dependent manners with the IC_{50} of 990±12, 790±15 and 1,050±20 µg/ml, respectively. Interestingly, MDM could not completely inhibit ^{3}H-MPP^{+} accumulation in RPTEC/TERT1 cells. The data demonstrated that chitosan derivatives inhibited ^{3}H-MPP^{+} transport in the endogenously expressing system of OCT2 with higher inhibitory potencies compared with OCT2-CHO-K1 cells. Several factors might contribute the differences; 1) species difference between rabbit and human; 2) others transporters expressed in RPTEC/TERT1 cells; 3) physiological environment difference between heterologous and endogenous OCT2-expressing cell lines and human renal proximal tubular cells might interfere the transport function of OCT2. To determine whether the inhibitory effects of the chitosan derivatives were results of reduced cell viability, the effect of these compounds on cell viability was determined. Incubation RPTEC/TERT1 cells with the BSCS, NSCS or OSOS (2,000 µg/ml) for 4 h had no significant effect on cell viability compared with vehicle treatment (Fig. 4.17).

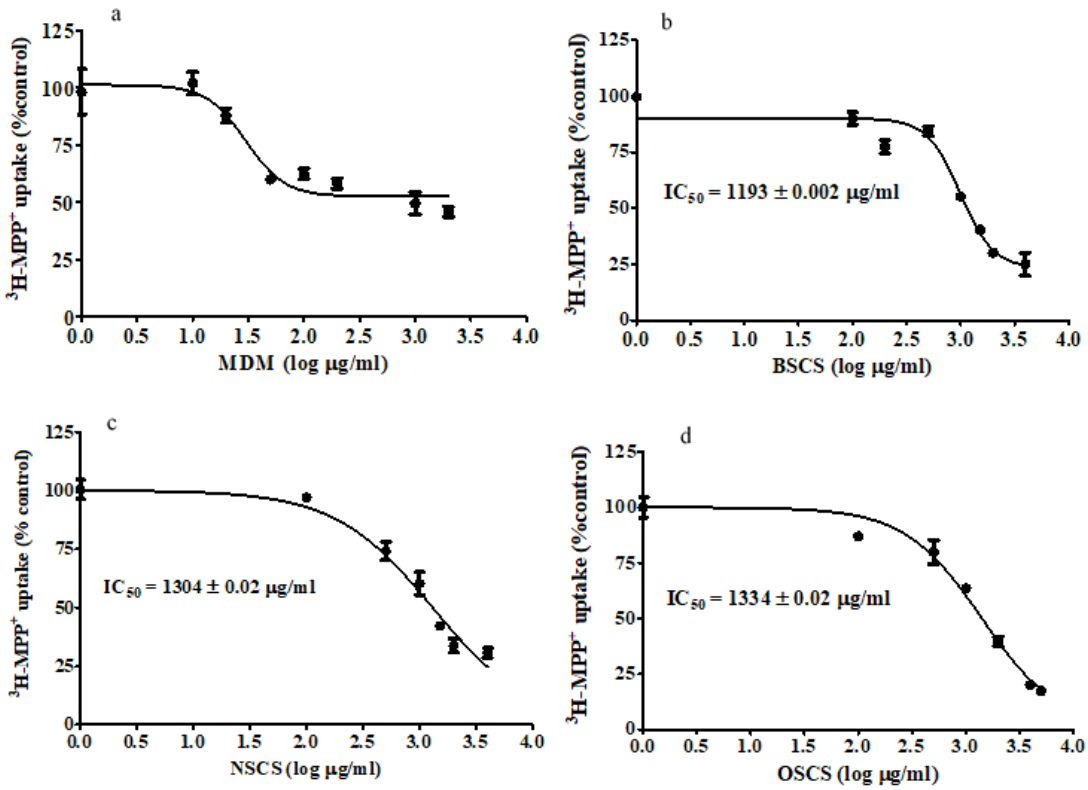


Figure 4.16 Inhibitory effects of the chitosan derivatives (a) MDM, (b) BSCS, (c) NSCS, and (d) OSCS on OCT2-mediated ^{3}H-MPP$^+$ in human renal proximal tubular cells; RPTEC/TERT1 cells were incubated with ^{3}H-MPP$^+$ alone, or in the presence of various concentrations of MDM, BSCS, NSCS, and OSCS for 5 min, after that accumulation of ^{3}H-MPP$^+$ was determined. The IC_{50} values were calculated and expressed as mean±S.E. of n=3.

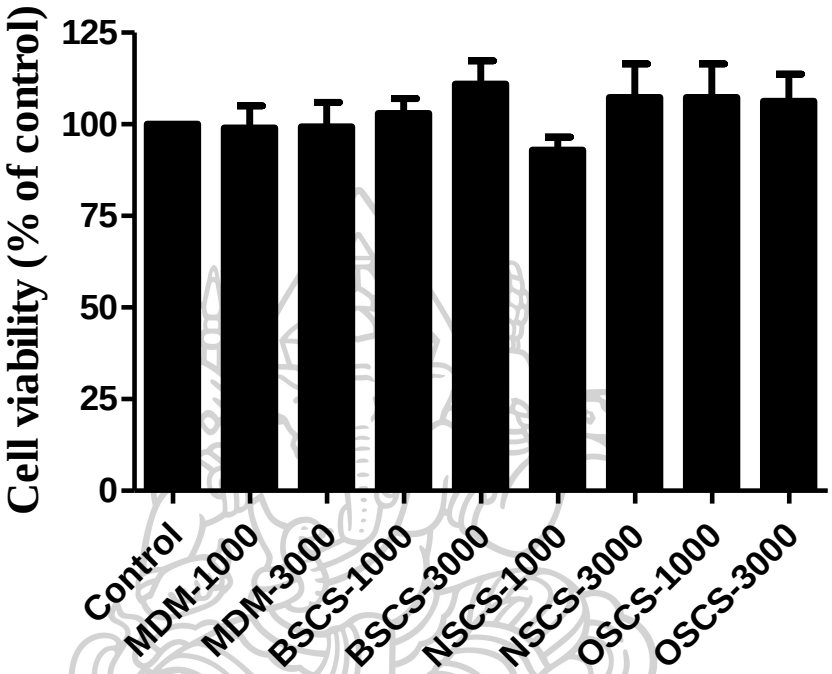


Figure 4.17 Cell viability of RPTEC/TERT1 cells following exposure to the chitosan derivative for 4 h. Data are shown as mean±S.E. n= 3.

4.2.2.4 Effect of chitosan derivatives on cationic drug accumulation in human renal proximal tubular cells

Since OCT2 is the major determinant of renal clearance of cationic drugs [23, 24, 66], inhibition of OCT2 activity by the chitosan derivatives could decrease renal clearance of these drugs. The impact of the chitosan derivatives on renal cationic drug transport was revealed in this study. The present study determined whether chitosan derivatives inhibited cisplatin, a substrate of OCT2, uptake into human renal proximal tubular cells. Confluent RPTEC/TERT1 cell monolayers were incubated with serum-free media containing 100 µM cisplatin alone or in the presence of the chitosan derivatives (1 and 2 mg/ml) or 1 mM TPeA (an OCT inhibitor)[105] for 4 h. At the end of incubation, cellular accumulation of cisplatin was determined by measurement of platinum (Pt) accumulation. As shown in Fig. 4.18, co-treatment the cells with cisplatin plus OSCS at 1 mg/ml significantly reduced accumulation of Pt compared with cisplatin alone. Interestingly, BSCS and NSCS at 1 mg/ml showed no significant effect on. Although, BSCS and NSCS inhibited OCT2-mediated ^{3}H-MPP^{+} uptake in RPTECT/TERT1 cells, they could not produce the significant inhibition on Pt accumulation. The explanation of the differences is uncertain. These results indicated that OSCS showed more potent effect on cisplatin accumulation than other derivatives.

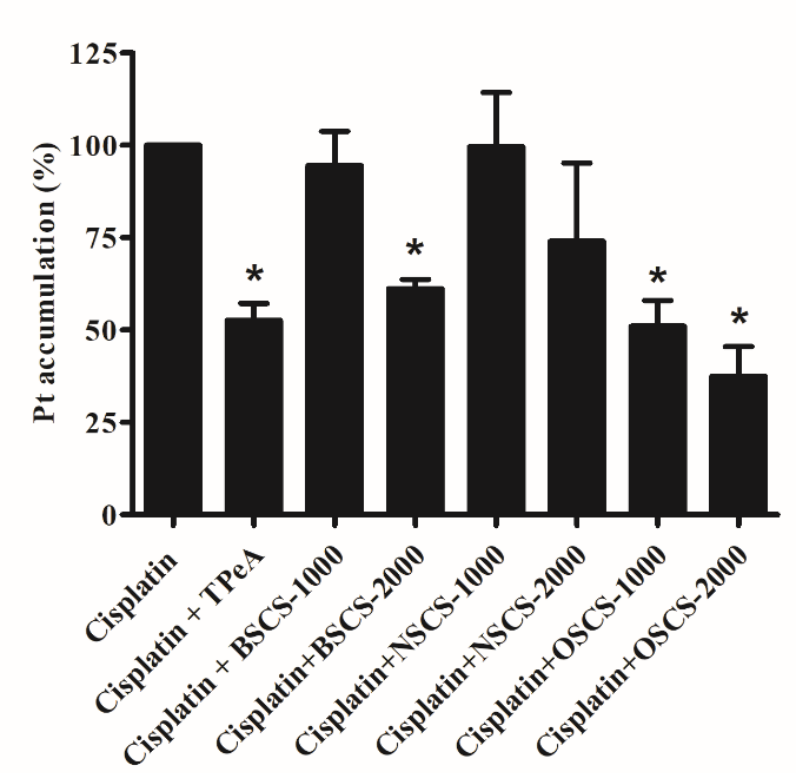


Figure 4.18 Effect of chitosan derivatives on cisplatin transport in human renal proximal tubular cells. RPTEC/TERT1 cells were incubated with 100 µM cisplatin alone or cisplatin plus the chitosan derivatives (1 mg/ml) or 1 mM TPeA for 4 h., then platinum (Pt) accumulation was measured. The Pt accumulations are expressed as % of cisplatin treatment (mean±S.E.). * Significant difference from control (*P<0.05)

4.3 Chitosan derivatives for prevention of cisplatin-induced nephrotoxicity and anticancer activity of cisplatin.

4.3.1 Cisplatin-induced nephrotoxicity in renal proximal tubular cells

Nephrotoxicity is the major side effect of cisplatin and its nephrotoxicity is mediated by. OCT2 [22, 34, 35, 110]. This study then investigated whether OSCS which produced more effect on cisplatin accumulation reduced the renal toxicity induced by cisplatin. Firstly, the effect of cisplatin on cell viability of RPTEC/TERT1 cells was determined. Cells were incubated with cisplatin in various concentration (0-500 µM) for 72 h, then the cell viability was determined by MTT assay. As shown in Fig. 4.19, cisplatin reduced cell viability of RPTEC/TERT1 cell in concentration dependent manner with the IC_{50} of 86 µM. Next, the concentration of cisplatin at 50 µM was selected for subsequent experiments.

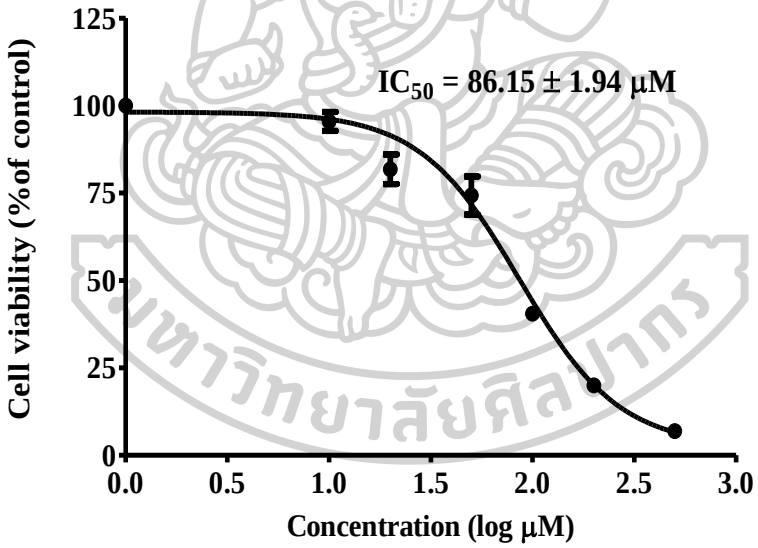


Figure 4.19 Effect of cisplatin on cell viability of RPTEC/TERT1 cells. The cells were incubated with cisplatin at 0-500 µM for 72 h. The data are expressed as mean±S.E. of percentage of control (n=3).

Next, the protective effect of the chitosan derivatives including BSCS, NSCS, and OSCS on cisplatin-induced cytotoxicity was determined. In addition, the effect of chitosan derivatives in the form of micelle on cisplatin toxicity was also evaluated. To validate the protective effect of the chitosan derivatives, the different methods for measurement of cytotoxicity including MTT assay, viable cell counting, and apoptotic cell using flow cytometry were selected to measure cytotoxicity. Since, the chitosan derivatives can form micelle at high concentration, the effect of micelle of the individual chitosan derivative on cisplatin-induced cytotoxicity was also included. RPTEC/TERT1 cells were incubated for 48-72h under four conditions: (1) vehicle; (2) medium containing 50 µM cisplatin; (3) medium containing 1 mg/ml chitosan derivative, and (4) medium containing chitosan derivative for 2 h before changing the medium to one containing 50 µM cisplatin plus chitosan derivative of chitosan derivative. At the end of incubation, cytotoxicity was determined using MTT assay, and apoptotic cells were stained by FITC-Annexin V and PI and were measured by flow cytometry.

4.3.2 Protective effects determination by MTT assay

As shown in Fig. 4.20, the RPTEC/TERT1 cells treated with 50 µM cisplatin for 72 h reduced cell viability. The reduction of cell viability induced by cisplatin was not attenuated by treatment with BSCS and BSCS-micelles. Exposure the cells to NSCS up to 3,000 µg/ml could not prevent cisplatin toxicity whereas its micelle form at 2,000 and 3,000 µg/ml attenuated cisplatin toxicity. In addition, both OSCS and OSCS-micelle (1,000 µg/ml) attenuated cisplatin toxicity. However, high concentration beyond 1,000 µg/ml induced toxicity. These results indicated that NSCS-micelles and OSCS/OSCS-micelle could attenuate the cisplatin-induced toxicity in RPTEC/TERT1 cells.

4.3.3 Effect of the OSCS on cisplatin-induced apoptosis in renal proximal tubular cells

Since OSCS showed more pronounce compared others, the protective effect of OSCS-polymer and OSCS-micelle on cisplatin-induced cytotoxicity was further confirmed by flow cytometry determination of cell apoptosis using FITC-Annexin V/PI staining. As shown in Fig. 4.21, following 48 h incubation, the viability cell of RPTEC/TERT1 cells cultured under normal conditions (control) was about 90% of total counted cell. In addition, RPTEC/TERT1 cells treated with OSCS-polymer or OSCS-micelles alone did not change cell viability compared with control. Cisplatin treatment significantly increased apoptotic cells compared with control (50% vs 10%). Co-treated with cisplatin and OSCS-polymer or OSCS-micelles showed a significantly reduced the apoptotic cells, indicating OSCS-polymer or OSCS-micelles were able to protect the cells against cisplatin-induced cytotoxicity.

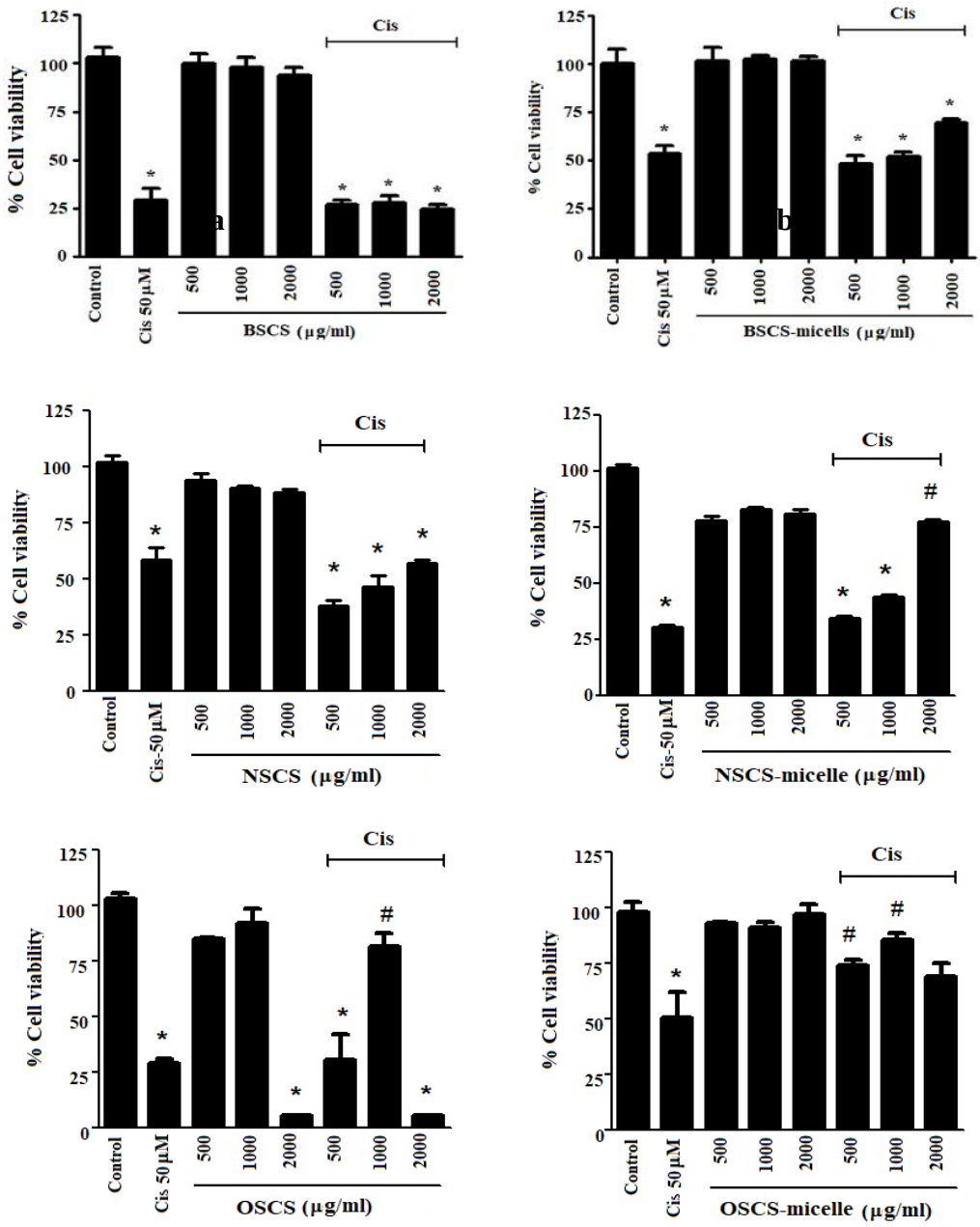


Figure 4.20 Effect of the chitosan derivatives in the form of (a) polymer and (b) micelles on cisplatin-induced toxicity using MTT assay. RPTEC/TERT1 cells were incubated with indicated for 72 h followed by measurement of cell viability using MTT assay. Data are expressed as mean±S.E. of % control (n=3). **P*<0.05 compared with control whereas $^{\#}P$<0.05 compared with cisplatin-treated cells.

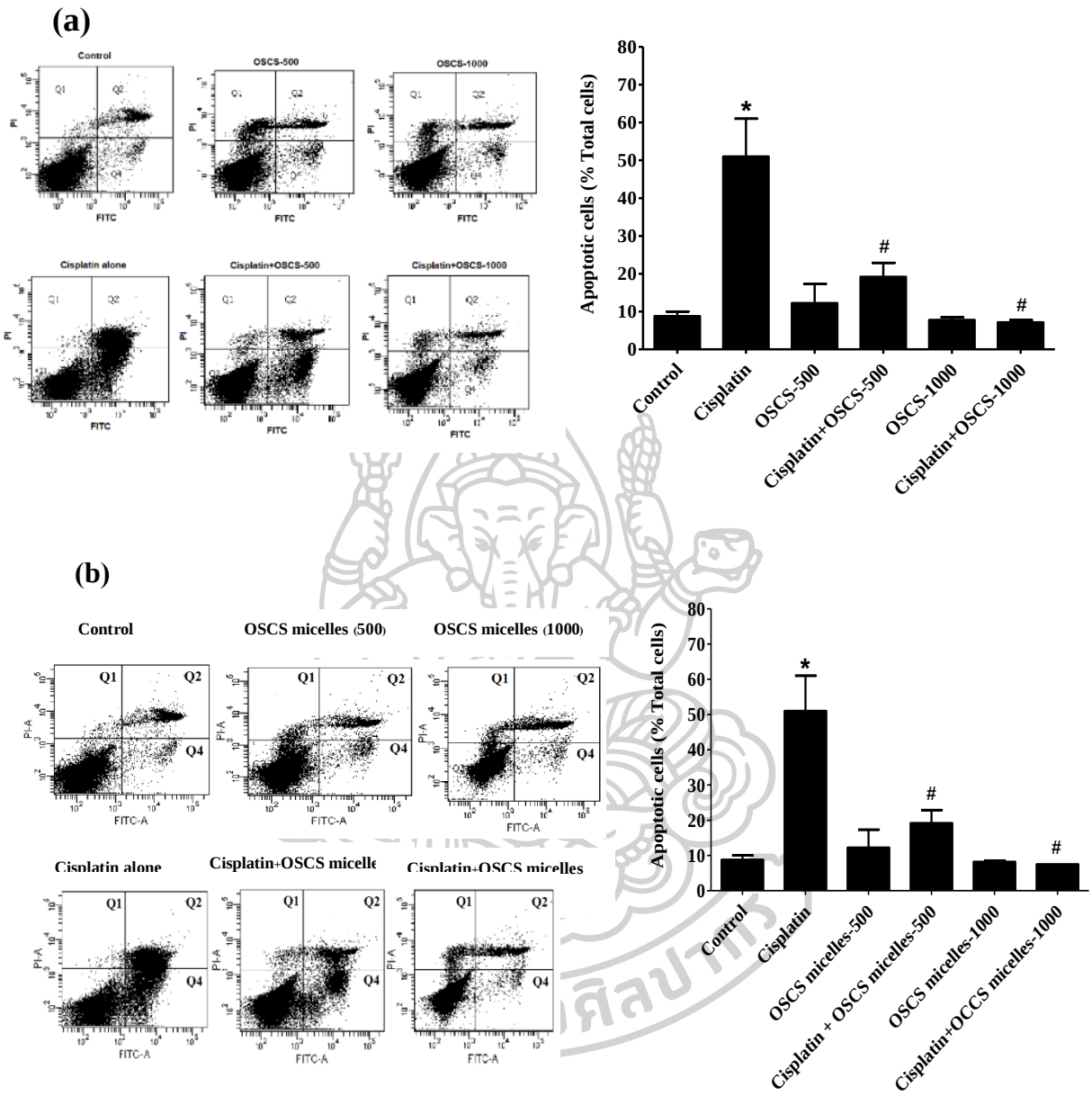


Figure 4.21 Effect of OSCS on cisplatin-induced cytotoxicity of renal proximal tubular cells. RPTEC/TERT1 cells were incubated with vehicle, 50 μM cisplatin, OSCS-polymer (**a**) or OSCS-micelle (**b**), cisplatin plus OSCS-polymer or micelle for 48 h. as indicated followed by determination of cell death using FITC-Annexin V and PI fluorescence measurement. Values are mean±S.E. for n=3. **P*< 0.05 compared with the vehicle-treated cells whereas $^{\#}P$<0.05 compared with cisplatin-treated cells as determined by one-way ANOVA.

4.3.4 Effect of OSCS on cisplatin-induced nephrotoxicity in mice

The present data revealed that OSCS attenuated cisplatin-induced cell apoptosis of renal proximal tubular cells. These data indicated that the inhibitory effect of OSCS on OCT2-mediated cisplatin uptake might be an anti-nephrotoxicity agent for cisplatin treatment. Therefore, the protective effect of OSCS on cisplatin-induced nephrotoxicity was determined in *in vivo* study. C57/BLJ6 mice were divided into 4 groups: 1) vehicle; 2) cisplatin (15 mg/kgBW i.p.); 3) OSCS (5 mg/kgBW i.v.); 4) cisplatin plus OSCS. After treatment protocols for 72 h, nephrotoxicity markers were determined. As shown in Fig. 4.22, cisplatin induced nephrotoxicity as evidences showing that cisplatin increased Ngal expression (an early nephrotoxic marker) and serum creatinine. The toxicity was correlated well with increase in accumulation of cisplatin in the kidney tissue. Interestingly, co-treatment with OSCS with cisplatin reduced cisplatin accumulation, Ngal expression, and serum creatinine. These results indicated that OSCS-polymer could attenuate the nephrotoxicity of cisplatin.

To determine whether OSCS-micelle revealed the preventing nephrotoxicity induced cisplatin as found in the OSCS-polymer, the effect OSCS-micelle on cisplatin's nephrotoxicity was determined using the same protocols as OSCS-polymer. Renal expression of Ngal in cisplatin plus OSCS-micelle treated mice was significantly lower than that of cisplatin-treated mice. This result indicated that OSCS-micelle could attenuate the nephrotoxic marker as same as OSCS-polymer. The data obtained from serum creatinine revealed that OSCS-micelle tended to decrease in serum creatinine induced by cisplatin (P value = 0.0871). Interestingly, co-treatment the animal with cisplatin and OSCS-micelle could not reduce cisplatin accumulation. The explanation for this notion is uncertain. It is possible that the remaining of cisplatin in blood supplying kidney affected the total content of cisplatin because of blood supplying the kidney did not be removed before tissue collection. Although, the similar effect of OSCS-polymer and OSCS-micelle was expected, the different effect on cisplatin accumulation of both forms could not be ruled out.

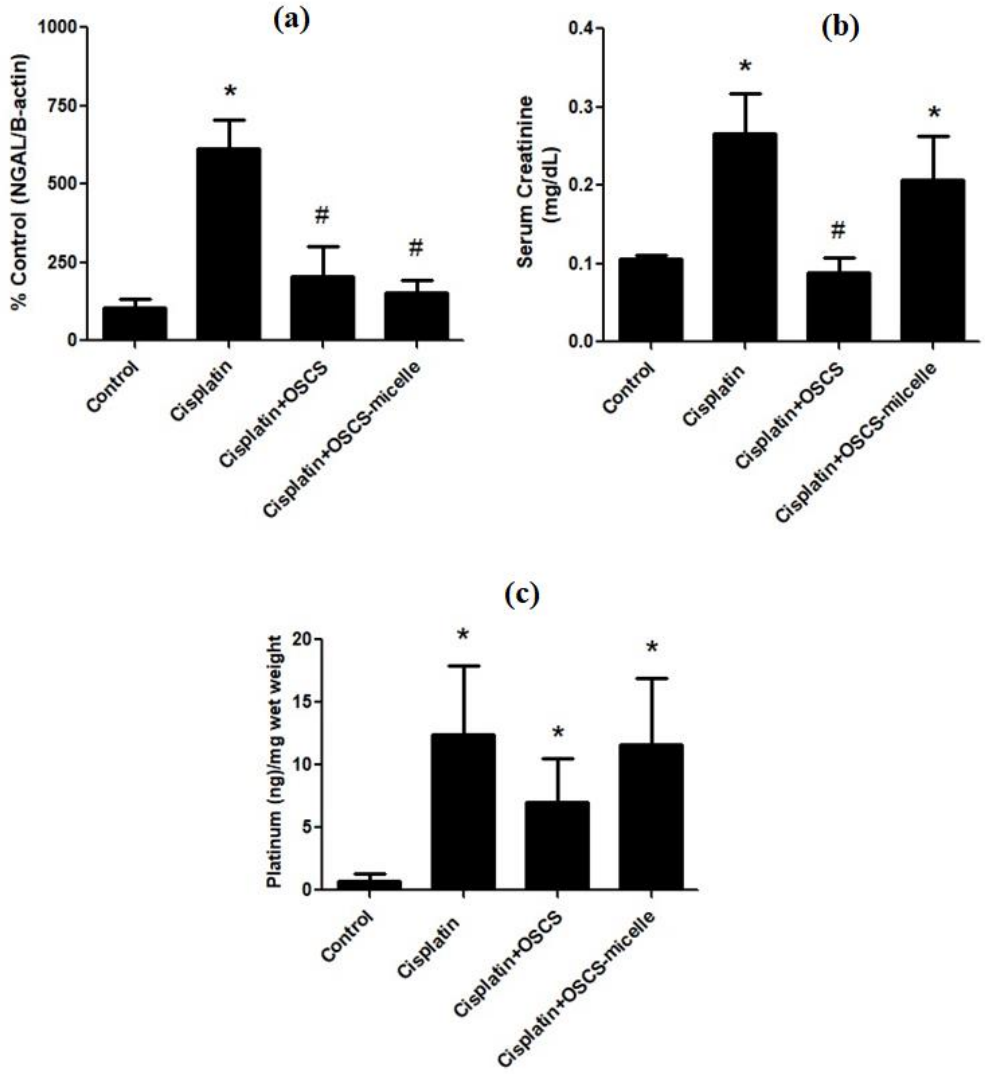


Figure 4.22 Effect of OSCS and OSCS-micelle on cisplatin-induced nephrotoxicity. Mice were received vehicle, cisplatin (15 mg/kgBW), and cisplatin plus OSCS-polymer (5mg/kgBW) for 72 h followed by determination of (a) NGAL protein expression in kidney, (b) serum creatinine, and (c) Pt accumulation in kidney. Data were obtained from 5-7 animals and expressed as mean±S.E. *P<0.05 compared with control and $^{\#}P$<0.05 compared with cisplatin-treated mice.

Cisplatin nephropathy in rodents is histologically characterized by degenerative changes in the proximal tubules that consist of tubular necrosis, luminal casts formation, Mononuclear cell infiltration, and tubular dilation. The present study has reported that cisplatin induced nephrotoxic marker protein and this event was attenuated by OSCS. Therefore, the effects of OSCS in form of both polymer and micelle on morphological changes in the kidney were revealed. As shown in Fig. 4.23, mice treated with either cisplatin (15 mg/kg) or cisplatin plus OSCS, and sacrificed at 72 h, displayed almost normal renal histology. There was no obvious tubular injury characteristics observed such as dilation of tubules, vacuole formation, and necrosis. This notion was supported by the semiquantitative scoring of tubular injury. This result indicated that change in nephrotoxic protein marker and kidney function were sensitive than that of renal histological changes. Although the data obtained from histology were not uncertain, the benefit effects of OSCS on nephrotoxic protein marker and kidney function data were revealed. The dose of cisplatin at 15 mg/kg might be the lower limit of nephrotoxic dose in the C57/bl/6 mice. To develop cisplatin-induced histological change of renal tissue, increase in dose of cisplatin (>15 mg/kg) in this murine strained is required.

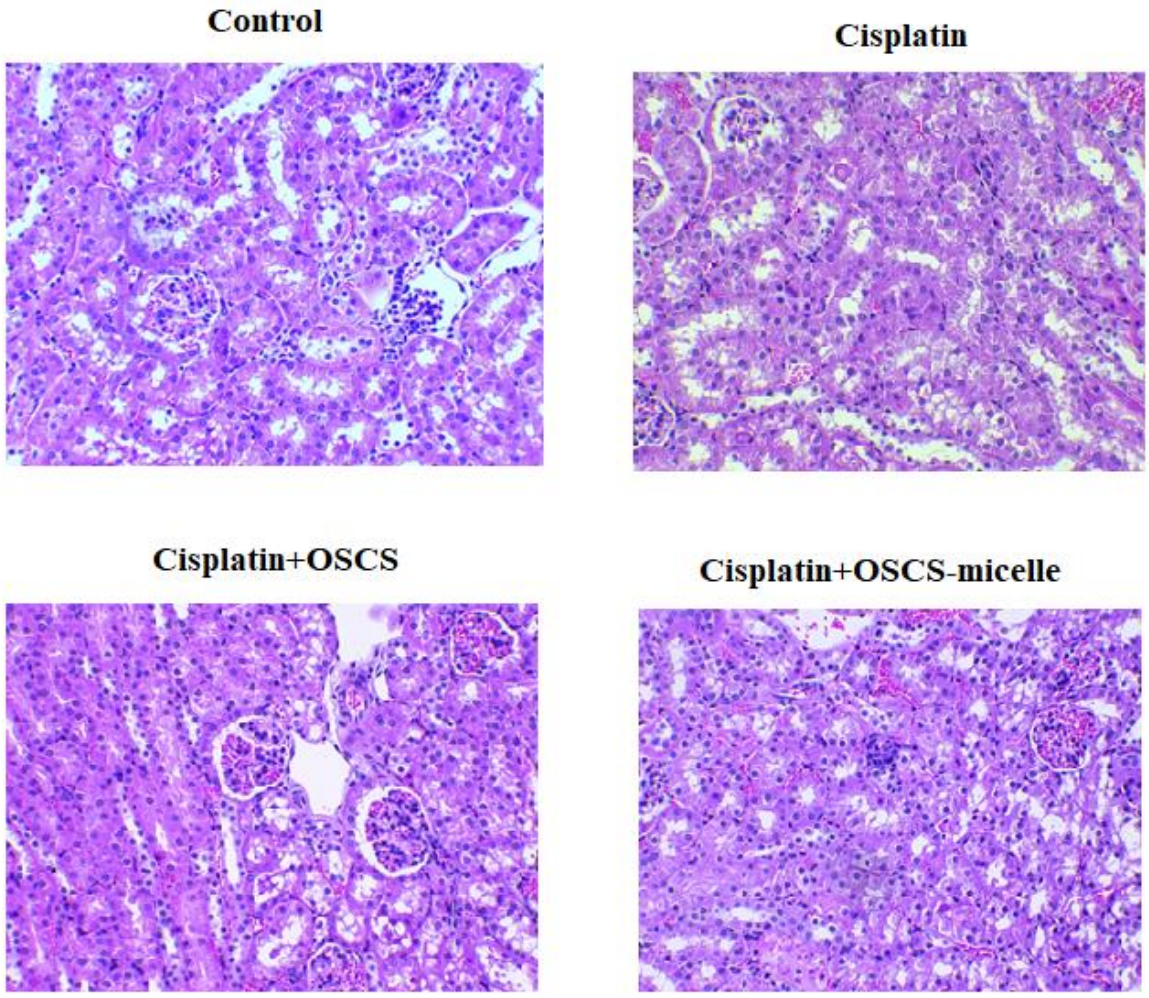


Figure 4.23 Effect of OSCS on cisplatin-induced kidney damage in mice. Mice were treated with (A) PBS, (B) cisplatin (15 mg/kg i.p.), (C) cisplatin (15 mg/kg i.p.) plus OSCS polymer (5 mg/kg i.v.), and (D) cisplatin (15 mg/kg i.p.) plus OSCS micelle (5 mg/kg i.v.) for 72 h. The representative kidney slides were stained with H&E.

4.3.5 Effect OSCS on anticancer activity of cisplatin in cancer cell lines

Since OSCS showed protective effect of cisplatin-induced nephrotoxicity, it is interesting whether OSCS altered the anticancer activity of cisplatin in cancers. To address this concern, the effect of OSCS on anticancer activity of cisplatin was determined in cancer cells. Firstly, the effect of OCCS on cell viability of cancer cell lines including HN22 cell (head & neck cancer), HegG2 cell (hepatocarcinoma), and MCF-7 cell (breast cancer) was detrmined. The cells were incubated with OSCS at 250-1,000 µg/ml 48 h followed by MTT assay. As shown in Fig. 4.24, the results showed that OSCS at 250 µg/ml significantly reduced cell viability of HN22, HepG2 and MCF-7 cells compared with vehicle-treated cells. Increasing the concentration of OSCS led to further reduction of cell viability. Interestingly, the concentration of OSCS that produced an effect on cell viability of theses cancer cell lines did not cause cytotoxicity on normal kidney cells. These data indicated that OSCS has a selective cytotoxic effect.

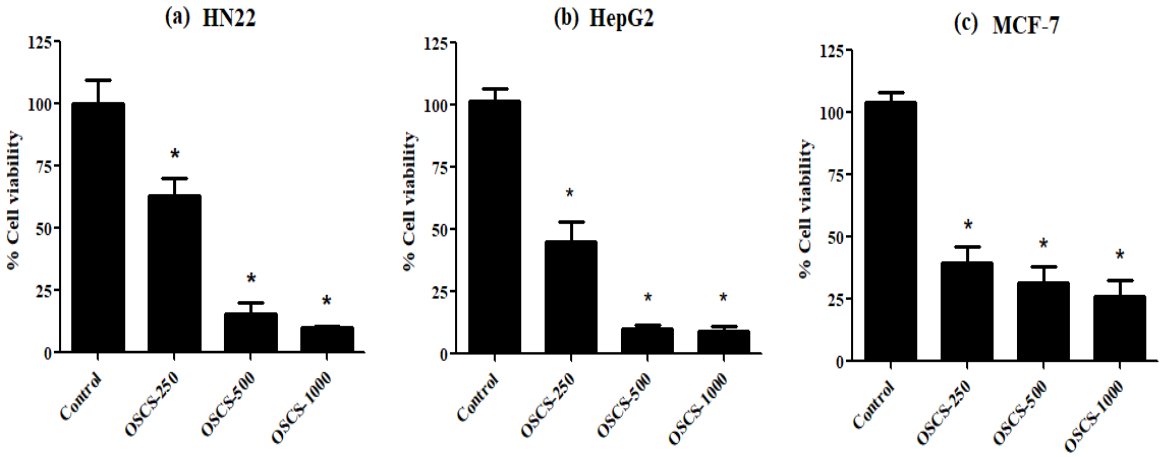


Figure 4.24 Effect of OSCS on cell viability of (a) HN22, (b) HepG2, and (c) MCF-7 cells. The cells were incubated with OSCS at 250-1000 µg/ml for 48 h. Data are expressed as mean±S.E. from 3 experiments. *P<0.05 compared with control

Next, the effect of OSCS on cisplatin's effect in HN22, HepG2, and MCF-7 cells was evaluated. The cells were incubated with vehicle, cisplatin (20 µM), OSCS (250 µg/ml), and cisplatin plus OSCS for 48 h. As shown in Fig.4.25, cisplatin decreased cell viability of HN22 and HepG2 cells to 50% control. Exposure the cells to OSCS did not alter the effect of cisplatin on cell viability of HN22 and HepG2 cells. Increasing concentration of OSCS to 500 µg/ml led to decrease in cells viability of these cell lines. These data indicated that OSCS at low concentration produced no effect on anti-cancer activity of cisplatin whereas OSCS at high concentration (500 µg/ml) produced an additive effect of cisplatin. Cisplatin showed a marked effect on cell viability of MCF-7 cells (about 20% control). Interestingly, cell viability of MCF-7 following exposure the cells with cisplatin and OSCS (250 µg/ml) was higher than that of cisplatin alone. These data indicated that OSCS at 250 µg/ml attenuated anti-cancer activity of cisplatin. The effect of OSCS at 500 µg/ml on cisplatin's anti-cancer activity in MCF-7 cells was further determined. The results showed that OSCS at 500 µg/ml had no effect on anti-cancer activity of cisplatin in MCF-7 cells. Taken together, OSCS at concentration (500 µg/ml) that had no effect on cell viability of normal kidney cells might not attenuate the anti-cancer activity of cisplatin.

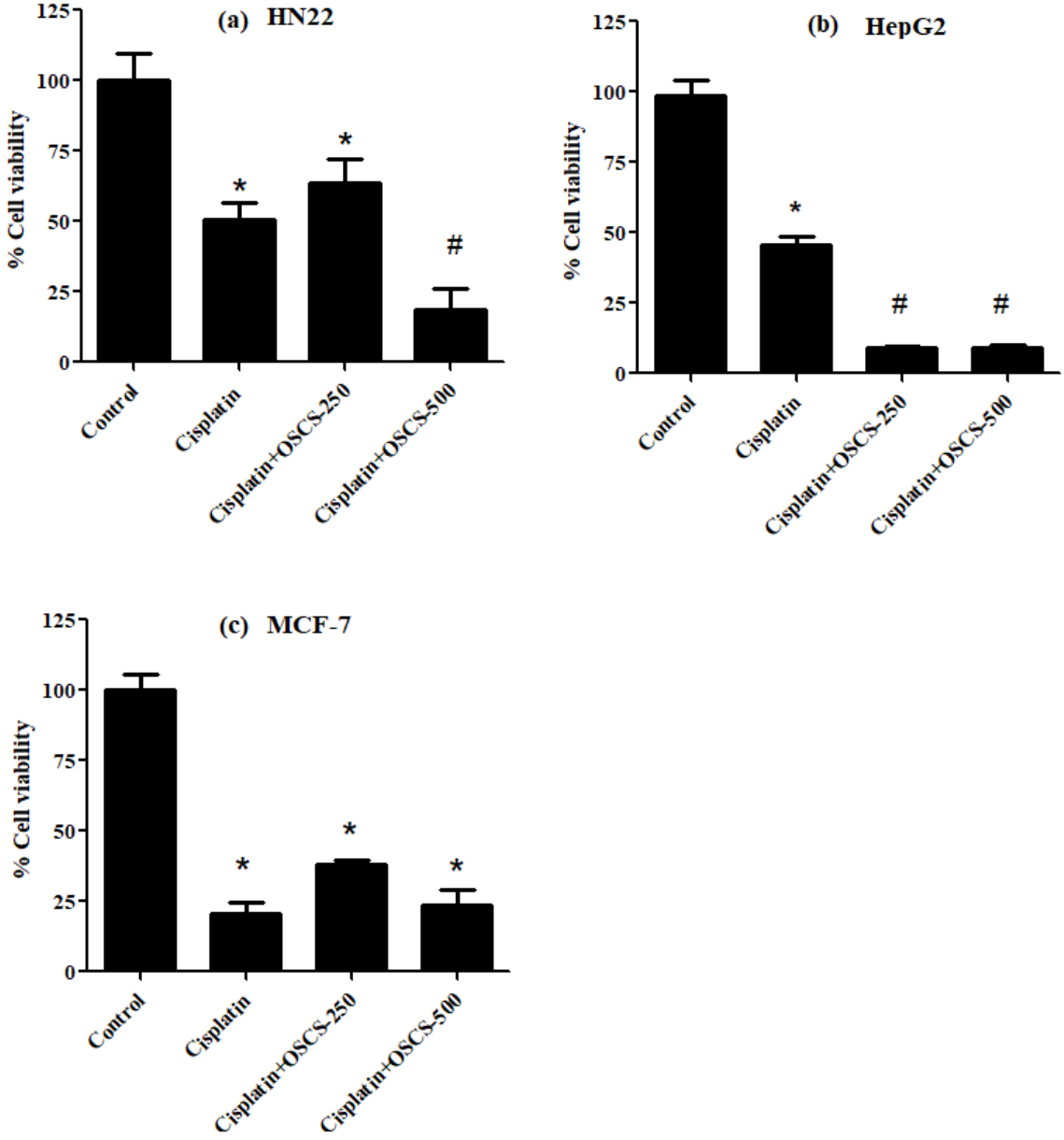


Figure 4.25 Effect of OSCS (250 and 500 µg/ml) on anti-cancer activity of cisplatin in cancer cells (a) HN22, (b) HepG2, and (c) MCF-7 cells. Data are expressed in mean±S.E. of 3 experiments. **P*<0.05 compared with control whereas $^{\#}P<$ 0.05 compared with cisplatin-treated cells.

4.4 Cisplatin-OSCS complex for prevention of cisplatin-induced nephrotoxicity and anticancer activity of cisplatin

4.4.1 Preparation of cisplatin-OSCS complex

First, blank micelles of chitosan derivatives were prepared. BSCS, NSCS, and OSCS blank polymeric micelles were successfully prepared by dialysis method. They are amphiphilic chitosan which can from polymeric micelles by self-aggregation via attractive force among hydrophobic segments. The characteristics of micelles ares shown in Table 4.1. All blank polymeric micelles showed nanosize and negative charges. Due to the succinic groups on the chitosan backbone, polymeric micelles were highly negatively charged. The particle size of blank polymeric micelles prepared by BSCS, NSCS, and OSCS which are containing different hydrophobic moieties were not different.

Next, the cisplatin-OSCS complex was prepared and characterized. In this study, the OSCS blank polymeric micelles was chosen for platinum-OSCS complex. The octhyl group in OSCS backbone is a hydrophobic part that can form polymeric self-assembled structure. In addition, the carboxylic moieties available on the OSCS backbone were employed to form coordinate bonds with cisplatin 5 (Trummer et al, 2018). The pH values of the blank micelles were fixed at 8.5. This is because huge improvements in cisplatin loading into these micellar systems were observed at pH 8 – 8.5 (Trummer et al, 2018). The OSCS polymeric micelles were successfully complexed with cisplatin which the %EE of cisplatin-OSCS was 64.50% and the LC was 0.28. The particle sizes, surface charges, pH, and osmolarity of cisplatin-OSCS complex are shown in Table 4.1.

Table 4.1 Physicochemical characteristics of blank polymeric micelles and cisplatin-OSCS complex (mean ± SD, n=3)

Formulation	Size (nm)	PDI	ZP (mV)	pH	Osmolarity (mOsm/kg H_2O)	EE (%)	LC
BSCS blank micelles	225.80 ± 5.34	0.15	-34.90 ± 1.40	4.84	N/A	N/A	N/A
NSCS blank micelles	162.07 ± 7.16	0.53	-28.80 ± 0.37	4.76	N/A	N/A	N/A
OSCS blank micelles	276.23 ± 6.46	0.39	-29.17 ± 1.68	5.00	530	N/A	N/A
Cisplatin-OSCS complex	317.67 ± 5.86	0.55	-19.23 ± 0.31	8.00	0	64.50 ± 7.63	0.28 ± 0.03

The sizes of BSCS, NSCS, and OSCS blank micelles were 225.80, 162.07, 276.23 nm, respectively. Cisplatin-OSCS complex was 317 nm in particle size. The zeta potential of blank micelles was negative in the range of -28.80 to -34.90 mV. For cisplatin-OSCS complex, it showed the increasing in zeta potential (-19.23 mV) compared with OSCS blank micelles. These results were similar to the previous study of Trummer et al. (2018). These suggested that the deprotonated carboxylic acid groups of the succinic acid moieties bound to cisplatin by co-ordinate bonds resulted in the reduction of the negative charge surface.

4.4.2 Anti-cancer activity of cisplatin-OSCS complex in HN22 cells

One of the strategies to reduce the cisplatin-induced nephrotoxicity is to develop platinum-based polymeric drug delivery for the treatment of cancers. The advantages of platinum-based nanocarriers are such as enhancing drug efficacy, prevention of side effects, and increasing in cellular accumulation mediated drug resistance. The carboxylic moieties on the polymer backbone could be employed to form coordinate bonds with cisplatin. The platinum-based polymeric complex was synthesized and characterized by using OSCS as a polymer backbone. In addition, the anti-cancer activity and side effect on renal proximal tubular cells of the synthesized platinum-based polymeric complex were determined.

The anti-cancer activity of cisplatin-OSCS complex and the free drug (cisplatin) was investigated in HN22 cells. Cells were continuously exposed for 4 days (96 h) to various concentrations of complex or free drug corresponding to cisplatin concentrations in a range of 50 to 500 μM. At the end of incubation, the cell viability was assessed by using MTT assay. The percentage of cell viability are shown in Fig. 4.26.

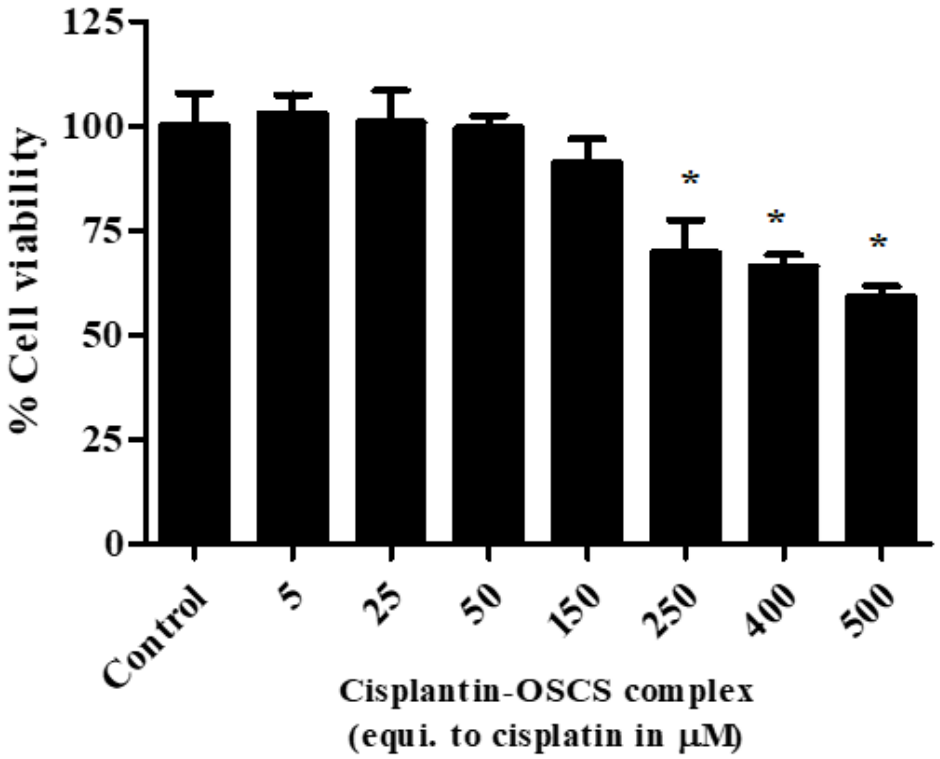


Figure 4.26 Cytotoxic effect of cisplatin-OSCS complex in HN22 cells. The data are mean±S.E. of n=3. *P<0.05 compared with control.

4.4.3 Effect of cisplatin-OSCS complex in renal proximal tubular cells

4.4.3.1 Cytotoxity of cisplatin-OSCS complex in renal proximal tubular cells

This experiment was setup to determine whether the cisplatin-OSCS complex showed the cytotoxic effect as found in free drug. RPTEC/TERT1 cells were incubated with cisplatin-OSCS complex that contains platinum at 9.75, 19.5, 97.5 µg/ml for 5 days. These concentrations were equivalent to cisplatin at 50, 100, and 500 µM µM, respectively. At the end of incubation period, cell viability was determined by MTT assay. As shown in Fig. 4.27, cisplatin-OSCS complex that contains platinum at 9.75 and 19.5 µg/ml did not affect cell viability of RPTEC/TERT1 cells. Interestingly, exposure the cells to cisplatin at 50 and 100 µM that were equivalent to the cisplatin-OSCS complex showed a marked reduction of cell viability. These data indicated that cisplatin-OSCS complex could prevent cytotoxic effect of cisplatin in renal proximal tubular cells that are the side effect of cisplatin treatment. Increasing the concentration of cisplatin-OSCS complex to 97.5 and 130 µg/ml of platinum significantly could reduce cell viability. The cytotoxic effect of cisplatin-OSCS complex at 97.5 µg/ml of platinum was similar as found in cisplatin treatments.

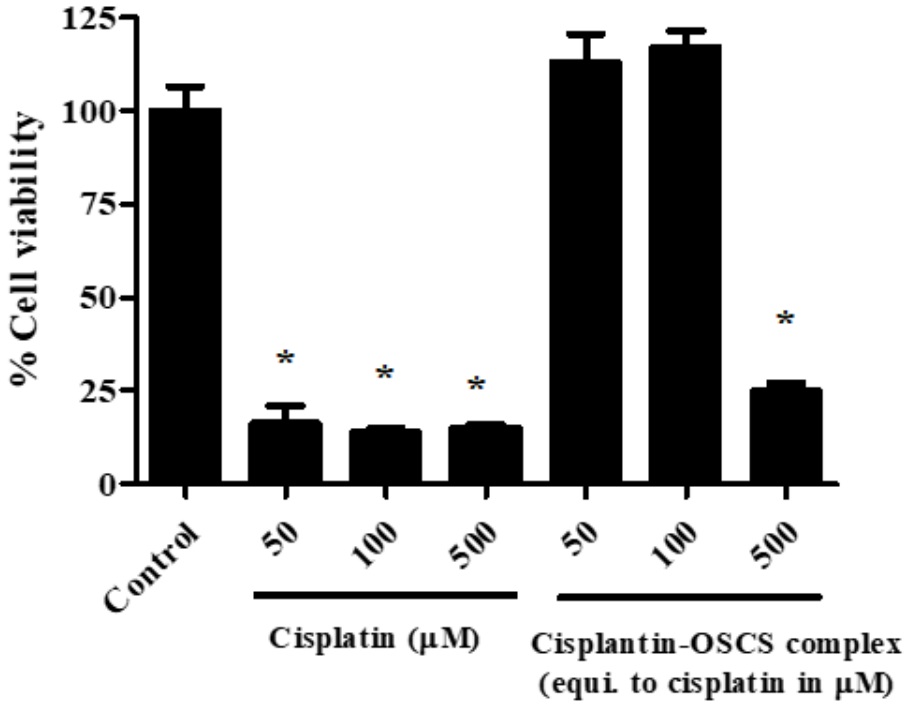


Figure 4.27 Cytotoxic effects of cisplatin-OSCS complex and cisplatin in RPTEC/TERT1 cells. The data are mean+S.E. of n=3. *P<0.05 compared with control.

4.4.3.2 Cisplatin-OSCS complex induced apoptosis in renal proximal tubular cells

The protective effect of cisplatin-OSCS complex on renal proximal tubular cells was confirmed by determining drug-induced apoptosis. The RPTEC/TERT1 cells were incubated with vehicle, cisplatin at 50 and 100 µM, and cisplatin-OSCS complex equivalent to cisplatin 50 and 100 µM for 48 h followed by measurement of apoptotic cells using Annexin V-FITC and PI staining.

The results demonstrated that cisplatin at 50 and 100 µM significantly increased % apoptotic cells by 60% and 80%, respectively. As expected, cisplatin-OSCS complex did not increase % apoptotic cells (Fig. 4.28). These results have proved that cisplatin-OSCS complex could prevent cisplatin-induced cytotoxicity of renal proximal tubular cells. To be certain that the cisplatin-OSCS complex could be an agent that is used to treat cancer and reduce nephrotoxicity, the data obtained from cancer-xenograft animal model are future required.

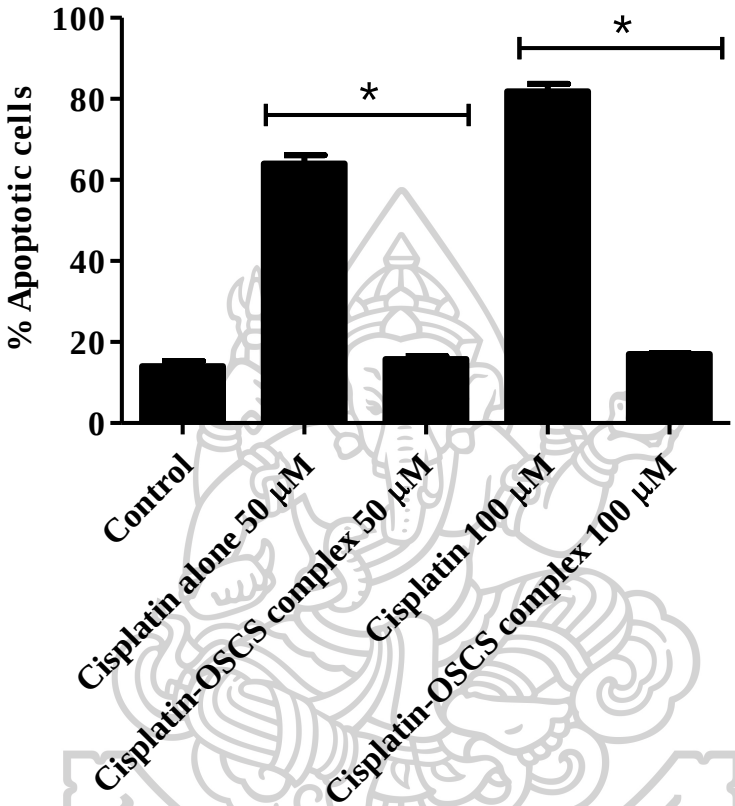


Figure 4.28 Effect of cisplatin and cisplatin-OSCS complex on cell apoptosis of renal proximal tubular cells. Data were obtained from 3 experiments and expressed as mean±S.E. *P<0.05

4.4.3.3 Protective effect of cisplatin-OSCS complex in mice

The concentration of platinum in murine kidney tissue was determined by ICP-MS. As shown in Fig. 4.29, the platinum concentration in kidney tissue results demonstrated that cisplatin-OSCS complex showed slightly lower concentration of the drug than that of free cisplatin (free drug). These results implied that the cisplatin-OSCS complex might be less uptake into the tissue of kidney than the free drug. the larger structure might interfere the binding between cisplatin and OCT2 subsequently reduce in tissue accumulation.

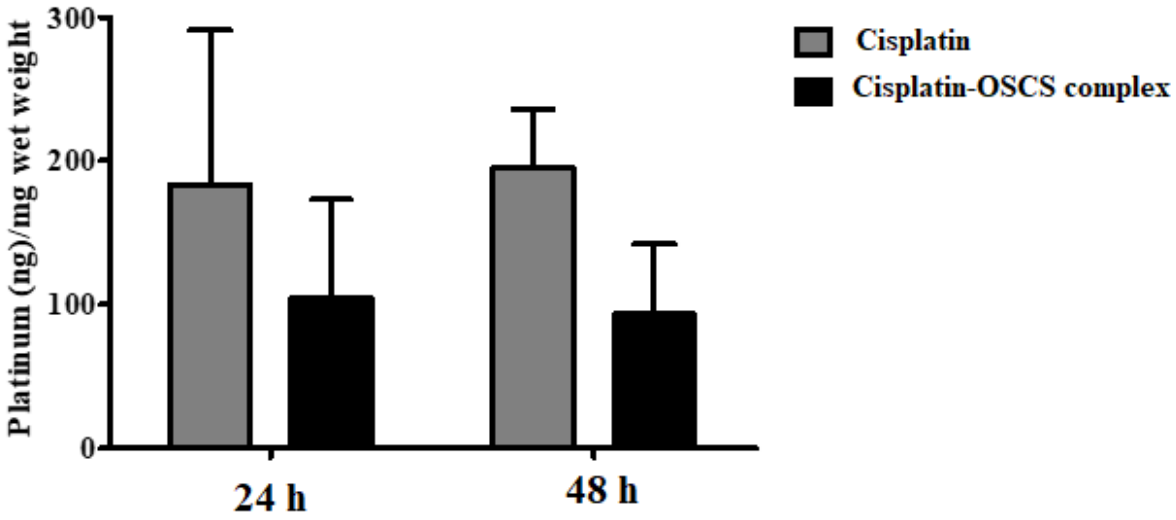


Figure 4.29 Platinum accumulation in the kidney tissue following 24 h, and 48 h of treatment with cisplatin (free drug) or cisplatin-OSCS complex. Data are mean±S.E. from 5 animals.

CHAPTER 5 CONCLUSION

The increasing evidences showing pharmaceutical excipients have directly or indirectly alter the activity of drug transporters. These excipients may alter the pharmacokinetic of an incorporated drug thereby affecting its intended therapeutic efficacy and/or enhancing adverse side effects. This study, the effects of the pharmaceutical excipients including conventional excipients and chitosan derivatives on transport function of OCT1 and OCT2 was investigated. Moreover, the potential therapeutic application of the excipients on reducing nephrotoxicity of anticancer drug (cisplatin) was revealed.

The present demonstrates the inhibitory effect of frequently used pharmaceutical excipients, especially Tween20 Tween60, and Tween80, on the transport function of OCT1 and OCT2 which play crucial roles for the pharmacokinetics, drug-drug interactions and tissue deposition of many cationic drugs. In addition, the effect of novel excipient chitosan and its derivative on OCT1 and OCT2 was revealed. Chitosan derivatives including MDM, BSCS, NSCS and OSCS, inhibited transport function of OCT1 and OCT2. Co-administration of cisplatin, an OCT2 substrate, plus OSCS significantly reduced cisplatin accumulation compared with receiving cisplatin alone. This result was accompanied by decreasing nephrotoxicity without reversing activity of cisplatin in cancer cells. This study also demonstrated that cisplatin-OSCS complex induced cytotoxicity of cancer cells whereas produced less effect on human renal proximal tubular cells that might be accompanied with less cellular accumulation of cisplatin in renal proximal tubular cells compared with cisplatin. Taken together, the present study demonstrates the pharmaceutical excipients including Tweens and chitosan derivatives inhibit the transport function of OCT1 and OCT2 that might affect pharmacokinetic of drug and/or their efficacy and toxicity.

APPENDIX

APPENDIX A

Table A.1 Uptake study of Tweens in rb-OCT1-CHO-k1 cells (expressed as percentage of control)

Sample	Concentration (µg/ml)	Concentration (log µg/ml)	Uptake (%control) n1	n2	n3	Average	SD
Tween 20	1	0.00	102.88	92.12	94.17	96.39	5.71
	10	1.00	88.45	91.65	95.8	91.97	3.69
	100	2.00	52.19	55.44	51.51	53.05	2.10
	200	2.30	36.5	34.98	33.25	34.91	1.63
	1000	3.00	17.52	21.03	16.21	18.25	2.49
	2000	3.30	13.06	17.78	15.37	15.40	2.36
Tween 60	1	0.00	103.7	98.44	94.59	98.91	4.57
	10	1.00	84.02	80.78	77.73	80.84	3.15
	100	2.00	43.27	45.43	54.58	47.76	6.00
	200	2.30	45.62	38.95	42.28	42.28	3.34
	1000	3.00	17.52	24.68	32.99	25.06	7.74
	2000	3.30	25.76	25.1	17.13	22.66	4.80
Tween 80	1	0.00	101.44	99.76	97.88	99.69	1.78
	10	1.00	83.21	81.25	78.44	80.97	2.40
	100	2.00	60.48	59.39	65.31	61.73	3.15
	200	2.30	44.68	39.21	42.38	42.09	2.75
	1000	3.00	25.91	30.22	27.56	27.90	2.17
	2000	3.30	20.53	17.45	19.84	19.27	1.62

Table A.2 Uptake study of Chitosan derivatives in rb-OCT1-CHO-k1 cells (expressed as percentage of control)

Sample	Concentration (µg/ml)	Concentration (log µg/ml)	Uptake (%control) n1	n2	n3	Average	SD
MDM	1	0.00	97.39	99.32	103.3	100.00	3.01
	10	1.00	89.32	92.65	95.39	92.45	3.04
	100	2.00	34.78	30.64	31.13	32.18	2.26
	200	2.30	23.98	23.38	24.03	23.80	0.36
	1000	3.00	16.61	16.07	16.93	16.54	0.43
	2000	3.30	14.3	15.05	12.36	13.90	1.39
BSCS	1	0.00	99.00	103.00	97.00	99.67	3.06
	10	1.00	94.00	99.00	96.00	96.33	2.52
	100	2.00	90.00	102.00	104.00	98.67	7.57
	200	2.30	72.00	82.00	76.00	76.67	5.03
	400	2.60	73.00	81.00	79.00	77.67	4.16
	600	2.78	59.00	53.00	53.00	55.00	3.46
	1000	3.00	42.00	33.00	33.00	36.00	5.20
	2000	3.30	18.00	25.00	27.00	23.33	4.73
	3000	3.48	18.00	20.00	20.00	19.33	1.15
NSCS	1	0.00	112.00	83.00	105.00	100.00	15.13
	10	1.00	77.00	89.00	90.00	85.33	7.23
	100	2.00	87.00	87.00	72.00	82.00	8.66
	200	2.30	67.00	90.00	61.00	72.67	15.31
	400	2.60	78.00	81.00	69.00	76.00	6.24
	600	2.78	64.00	67.00	83.00	71.33	10.21
	1000	3.00	39.00	41.00	48.00	42.67	4.73
	2000	3.30	25.00	24.00	32.00	27.00	4.36
	3000	3.48	23.00	20.00	25.00	22.67	2.52
OSCS	1	0.00	89.00	101.00	105.00	98.33	8.33
	10	1.00	101.00	112.00	76.00	96.33	18.45
	100	2.00	64.00	68.00	77.00	69.67	6.66
	200	2.30	60.00	65.00	61.00	62.00	2.65
	400	2.60	62.00	52.00	61.00	58.33	5.51
	600	2.78	43.00	47.00	39.00	43.00	4.00
	1000	3.00	28.00	31.00	32.00	30.33	2.08
	2000	3.30	14.00	11.00	12.00	12.33	1.53
	3000	3.48	10.00	12.00	10.00	10.67	1.15

Table A.3 Uptake study of Tweens in rb-OCT2-CHO-k1 cells (expressed as percentage of control)

Sample	Concentration (µg/ml)	Concentration (log µg/ml)	Uptake (%control)				
			n1	n2	n3	Average	SD
Tween 20	1	0.00	98.81	102.64	108.54	103.33	4.90
	100	2.00	99.00	99.44	92.48	96.97	3.90
	200	2.30	79.86	81.49	67.54	76.30	7.63
	1000	3.00	40.33	45.41	46.10	43.95	3.15
	2000	3.30	45.67	44.72	42.28	44.22	1.75
Tween 60	1	0.00	94.68	93.83	95.42	94.64	0.80
	10	1.00	84.94	81.48	86.86	84.43	2.73
	100	2.00	52.76	52.76	50.04	51.85	1.57
	200	2.30	40.10	40.68	39.03	39.94	0.84
	1000	3.00	36.11	36.37	35.79	36.09	0.29
	2000	3.30	30.31	31.21	31.37	30.96	0.57
Tween 80	1	0.00	98.81	102.64	108.54	103.33	4.90
	10	1.00	80.17	85.19	82.44	82.60	2.51
	100	2.00	70.15	72.17	68.99	70.44	1.61
	200	2.30	40.33	45.41	46.10	43.95	3.15
	1000	3.00	20.15	18.36	19.10	19.20	0.90
	2000	3.30	10.00	9.61	7.21	8.94	1.51

Table A.4 Uptake study of chitosan derivatives in rb-OCT2-CHO-k1 cells (expressed as percentage of control)

			Uptake (%control)				
Sample	Concentration (µg/ml)	Concentration (log µg/ml)	n1	n2	n3	Average	SD
MDM	1	0.00	109.16	93.94	88.38	97.16	10.76
	10	1.00	83.83	90.33	93.29	89.15	4.84
	100	2.00	35.51	38.39	39.78	37.89	2.18
	200	2.30	23.18	31.8	27.26	27.41	4.31
	1000	3.00	22.07	19.75	17.06	19.63	2.51
	2000	3.30	20.36	22.81	20.21	21.13	1.46
BSCS	1	0	107.99	96.72	103.07	102.59	5.65
	100	2	87.17	102.39	102.1	97.22	8.70
	200	2.3	85.53	85.53	91.72	87.59	3.57
	500	2.7	86.28	71.28	86.28	81.28	8.66
	1000	3	65.6	74.34	76.72	72.22	5.86
	2000	3.3	33.74	33.74	39.63	35.70	3.40
	3000	3.48	18.07	19.78	23.59	20.48	2.83
	5000	3.7	10.75	8.59	9.71	9.68	1.08
NSCS	1	0.00	113.37	99.58	95.72	102.89	9.28
	100	2.00	103.80	94.75	92.73	97.09	5.90
	200	2.30	102.39	99.23	101.16	100.93	1.59
	500	2.70	103.97	96.25	99.14	99.79	3.90
	1000	3.00	79.21	79.74	86.59	81.85	4.12
	1500	3.18	56.73	58.93	62.79	59.48	3.07
	2000	3.30	34.52	36.45	37.15	36.04	1.36
	3000	3.48	17.83	16.96	18.19	17.66	0.63
	5000	3.70	17.00	18.00	12.00	15.67	3.21
OSCS	1	0.00	103.00	112.00	84.00	99.67	14.29
	100	2.00	65.00	98.00	106.00	89.67	21.73
	200	2.30	92.00	89.00	93.00	91.33	2.08
	500	2.70	104.00	86.00	92.00	94.00	9.17
	1000	3.00	76.00	85.00	75.00	78.67	5.51
	1500	3.18	41.00	32.00	43.00	38.67	5.86
	2000	3.30	19.00	19.00	23.00	20.33	2.31
	3000	3.48	5.00	5.00	6.00	5.33	0.58

Table A.5 Uptake study of Tweens in HepG2 cells (expressed as percentage of control)

Sample	Concentration (µg/ml)	Concentration (log µg/ml)	Uptake (%control)				
			n1	n2	n3	Average	SD
Tween 20	1	0.00	82.12	112.35	106.04	100.17	15.95
	10	1.00	94.02	84.98	84.62	87.87	5.33
	100	2.00	109.02	98.42	91.52	99.65	8.81
	200	2.30	100.57	96.16	86.88	94.54	6.99
	500	2.70	74.15	70.34	57.37	67.29	8.80
	1000	3.00	58.68	45.47	43.57	49.24	8.23
	2000	3.30	38.09	34.64	35.00	35.91	1.90
Tween 60	1	0.00	101.78	110.37	87.85	100.00	11.37
	10	1.00	96.43	93.59	68.71	86.24	15.25
	50	1.70	86.08	86.62	83.58	85.43	1.62
	100	2.00	94.62	86.28	78.96	86.62	7.84
	500	2.70	69.30	75.77	71.70	72.26	3.27
	1000	3.00	62.18	59.87	59.43	60.49	1.48
	5000	3.70	42.90	44.22	43.73	43.62	0.67
Tween 80	1	0.00	96.28	101.62	102.10	100.00	3.23
	10	1.00	81.07	80.00	80.00	80.36	0.62
	50	1.70	97.03	103.72	100.00	100.25	3.35
	1000	3.00	94.82	78.70	80.00	84.51	8.96
	5000	3.70	47.25	43.85	53.19	48.10	4.73

Table A.6 Uptake study of chitosan in HepG2 cells (expressed as percentage of control)

Sample	Concentration (µg/ml)	Concentration (log µg/ml)	Uptake (%control)				
			n1	n2	n3	Average	SD
MDM	5	0.70	98.68	105.49	102.05	102.07	3.41
	10	1.00	70.77	57.82	59.32	62.64	7.08
	100	2.00	114.77	97.63	106.16	106.19	8.57
	500	2.70	102.64	85.96	112.3	100.30	13.32
	1000	3.00	132.42	128.91	124.04	128.46	4.21
	2000	3.30	81.77	77.88	114.62	91.42	20.18
BSCS	1	0.00	109.82	102.43	87.34	99.86	11.46
	100	2.00	93.92	90.39	79.00	87.77	7.80
	501	2.70	76.11	72.41	61.82	70.11	7.42
	1000	3.00	77.55	59.73	60.70	65.99	10.02
	2000	3.30	55.24	54.11	62.14	57.16	4.35
	5000	3.70	20.73	22.65	25.86	23.08	2.59
NSCS	1	0.00	83.92	113.79	102.67	23.08	2.59
	100	2.00	98.53	99.17	91.54	96.41	4.23
	200	2.30	81.06	79.79	86.78	82.54	3.72
	500	2.70	74.39	72.48	70.00	72.29	2.20
	1000	3.00	49.29	46.43	49.60	48.44	1.75
	1500	3.18	40.71	43.57	34.67	39.65	4.54
	2000	3.30	27.68	31.17	24.18	27.68	3.50
	4000	3.60	17.51	16.88	16.56	16.98	0.48
OSCS	1	0.00	101.42	104.44	93.56	99.81	5.62
	100	2.00	95.62	90.54	79.54	88.57	8.22
	500	2.70	84.98	78.09	67.46	76.84	8.83
	1000	3.00	55.85	65.40	63.71	61.65	5.10
	2000	3.30	28.18	28.66	31.68	29.51	1.90
	4000	3.60	18.51	20.32	17.66	18.83	1.36
	5000	3.70	18.26	20.08	19.59	19.31	0.94

Table A.7 Uptake study of Tweens in RPTEC/TERT1 cells (expressed as percentage of control)

			Uptake (%control)				
Sample	Concentration (µg/ml)	Concentration (log µg/ml)	n1	n2	n3	Average	SD
Tween 20	1	0.00	102.61	113.84	104.59	107.01	5.99
	10	1.00	88.74	94.47	98.87	94.03	5.08
	100	2.00	53.30	64.75	58.36	58.80	5.74
	200	2.30	45.60	44.71	42.07	44.13	1.84
	300	2.48	38.33	39.87	35.47	37.89	2.23
	1000	3.00	28.86	34.59	32.83	32.09	2.94
Tween 60	1	0.00	98.00	89.00	100.00	95.67	5.86
	10	1.00	67.78	79.50	71.69	72.99	5.97
	100	2.00	34.65	34.48	32.96	34.03	0.93
	200	2.30	27.35	28.71	34.14	30.07	3.59
	300	2.48	20.39	14.95	15.63	16.99	2.96
	1000	3.00	18.35	20.89	16.31	18.52	2.29
	5000	3.70	12.91	9.34	15.12	12.46	2.92
Tween 80	1	0.00	100.00	100.00	100.00	100.00	0.00
	10	1.00	93.72	91.13	98.52	94.46	3.75
	50	1.70	68.76	74.86	74.31	72.64	3.37
	100	2.00	57.12	68.76	53.23	59.70	8.08
	500	2.70	28.84	25.32	41.59	31.92	8.56
	1000	3.00	20.70	18.48	24.21	21.13	2.89
	5000	3.70	12.94	12.20	13.12	12.75	0.49

Table A.8 Uptake study of chitosan in RPTEC/TERT1 cells (expressed as percentage of control)

Sample	Concentration (µg/ml)	Concentration (log µg/ml)	Uptake (%control)				
			n1	n2	n3	Average	SD
MDM	1	0.00	86.78	104.72	102.97	98.16	9.89
	10	1.00	98.66	107.53	99.53	101.91	4.89
	20	1.30	88.59	84.28	90.47	87.78	3.17
	50	1.70	58.02	61.71	60.15	59.96	1.85
	100	2.00	63.71	59.77	63.65	62.38	2.26
	200	2.30	59.77	55.58	59.15	58.17	2.26
	1000	3.00	44.02	50.96	53.40	49.46	4.87
	2000	3.30	46.64	43.14	47.39	45.72	2.27
BSCS	1	0.00	101.74	97.74	99.59	99.69	2.00
	100	2.00	89.41	94.96	85.72	90.03	4.65
	200	2.30	82.33	72.16	78.01	77.50	5.10
	500	2.70	80.78	84.17	88.18	84.38	3.70
	1000	3.00	57.67	52.74	55.20	55.20	2.47
	1500	3.18	41.03	39.79	40.00	40.27	0.66
	2000	3.30	31.47	30.86	28.39	30.24	1.63
	4000	3.60	19.45	20.38	35.17	25.00	8.82
NSCS	1	0.00	93.86	107.77	99.58	100.40	6.99
	100	2.00	97.67	97.13	96.31	97.04	0.68
	500	2.70	67.95	81.58	73.13	74.22	6.88
	1000	3.00	55.40	65.22	60.00	60.21	4.91
	1500	3.18	44.76	43.13	38.76	42.22	3.10
	2000	3.30	37.94	35.22	28.12	33.76	5.07
	4000	3.60	27.03	34.12	30.85	30.67	3.55
OSCS	1	0.00	103.00	112.00	84.00	99.67	14.29
	100	2.00	100.00	98.00	106.00	101.33	4.16
	200	2.30	92.00	89.00	93.00	91.33	2.08
	500	2.70	104.00	86.00	92.00	94.00	9.17
	1000	3.00	76.00	85.00	75.00	78.67	5.51
	1500	3.18	41.00	32.00	43.00	38.67	5.86
	2000	3.30	19.00	19.00	23.00	20.33	2.31
	3000	3.48	5.00	5.00	6.00	5.33	0.58

APPENDIX B

Cytotoxicity of cisplatin and protective effect of chitosan derivatives on cisplatin-induced cytotoxicity in RPTEC/TERT1 cells

Table B.1 Cytotoxicity of cisplatin in RPTEC/TERT1 cells

			% Cell viability				
Sample	Concentration (µg/ml)	Concentration (log µg/ml)	n1	n2	n3	Average	SD
Cisplatin	1	0.00	109.99	98.35	108.12	105.49	6.25
	10	1.00	91.33	97.59	90.78	93.23	3.78
	20	1.30	69.26	86.50	87.38	81.05	10.22
	50	1.70	38.42	58.62	66.19	54.41	14.36
	100	2.00	35.68	39.30	42.70	39.23	3.51
	200	2.30	18.11	27.77	23.93	23.27	4.86
	500	2.70	6.59	6.92	6.92	6.81	0.19

Table B.2 Effect of the BSCS in the form of polymer and micelles on cisplatin-induced toxicity using MTT assay

		% Cell viability				
Sample	Concentration (µg/ml)	n1	n2	n3	Average	SD
Control		105.51	110.40	101.72	105.88	4.35
Cisplatin 50 µM		32.24	39.08	35.41	35.58	3.42
BSCS	500	95.00	105.00	99.00	99.67	5.03
	1000	91.96	101.97	99.89	97.94	5.29
	2000	90.12	97.08	95.50	94.23	3.65
Cisplatin 50 µM+BSCS	500	25.00	27.00	29.00	27.00	2.00
	1000	30.77	29.06	23.20	27.68	3.97
	2000	24.06	22.10	26.74	24.30	2.33
Control		89.88	104.65	99.05	97.86	7.46
Cisplatin 50 µM		47.27	54.58	52.71	51.52	3.80
BSCS micelles	500	103.25	98.12	93.92	98.43	4.67
	1000	103.72	103.87	99.68	102.42	2.38
	2000	104.65	100.30	101.54	102.16	2.24
Cisplatin 50 µM+BSCS micelles	500	44.01	44.16	50.69	46.29	3.82
	1000	53.18	49.14	53.80	52.04	2.53
	2000	69.04	67.64	71.53	69.41	1.97

Table B.3 Effect of the NSCS in the form of polymer and micelles on cisplatin-induced toxicity using MTT assay

Sample	Concentration (µg/ml)	% Cell viability				
		n1	n2	n3	Average	SD
Control		106.83	115.11	116.27	112.74	5.15
Cisplatin 50 µM		67.25	55.33	46.48	56.87	14.69
NSCS	500	105.02	92.38	85.84	34.57	5.06
	1000	92.16	86.13	90.35	40.38	8.25
	2000	95.79	84.61	82.72	56.24	3.18
Cisplatin 50 µM+NSCS	500	28.76	36.97	37.98	98.67	4.56
	1000	32.32	40.02	48.80	30.91	2.07
	2000	52.58	57.74	58.39	77.94	4.02
Control		93.98	103.10	98.94	98.67	4.56
Cisplatin 50 µM		29.38	33.27	30.09	30.91	2.07
NSCS micelles	500	82.57	75.93	75.31	77.94	4.02
	1000	81.95	82.92	85.04	83.30	1.58
	2000	77.52	82.48	83.54	81.18	3.21
Cisplatin 50 µM+NSCS micelles	500	37.08	31.59	34.16	34.28	2.75
	1000	42.65	43.54	43.01	43.07	0.45
	2000	78.23	79.03	74.96	77.40	2.16

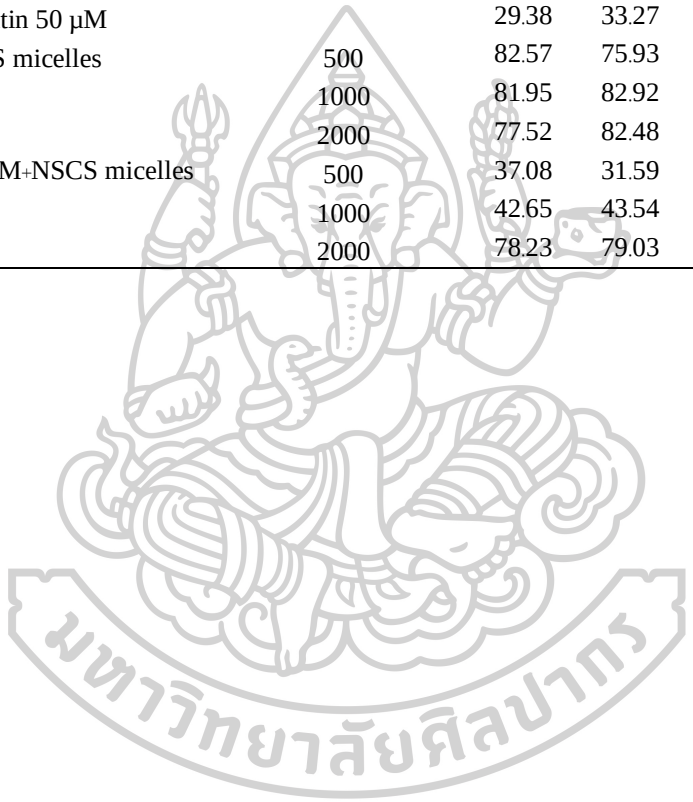


Table B.4 Effect of the OSCS in the form of polymer and micelles on cisplatin-induced toxicity using MTT assay

Sample	Concentration (µg/ml)	% Cell viability				
		n1	n2	n3	Average	SD
Control		105.51	98.31	101.72	101.85	3.60
Cisplatin 50 µM		32.24	39.08	35.41	35.58	3.42
OSCS	500	83.90	86.83	85.48	30.81	19.46
	1000	103.19	102.70	80.96	81.37	10.83
	2000	5.74	5.74	5.37	5.54	0.14
Cisplatin 50 µM+OSCS	500	14.04	26.26	52.14	100.23	5.65
	1000	87.31	87.93	68.87	54.41	14.36
	2000	5.62	5.37	5.62	92.83	0.54
Control		99.07	106.37	95.25	100.23	5.65
Cisplatin 50 µM		38.42	58.62	66.19	54.41	14.36
OSCS micelles	500	93.08	92.21	93.19	92.83	0.54
	1000	91.66	86.83	91.55	90.01	2.76
	2000	96.16	92.76	95.83	94.91	1.88
Cisplatin 50 µM+OSCS micelles	500	75.41	76.40	72.78	74.86	1.87
	1000	85.00	88.69	83.42	85.71	2.70
	2000	61.14	68.06	70.03	66.41	4.67

APPENDIX C

Cytotoxicity of cisplatin and chitosan derivatives in HN22, HepG2, and MCF-7 cells

Table C.1 Cytotoxicity of cisplatin and OSCS in HN22 cells

Sample	Concentration (µg/ml)	% Cell viability				
		n1	n2	n3	Average	SD
Control		112.91	107.31	89.34	103.19	12.31
Cisplatin 20 µM		51.43	59.67	52.09	54.40	4.58
Cisplatin 20 µM+OSCS	250	60.00	64.45	60.82	61.76	2.37
Cisplatin 20 µM+OSCS	500	26.51	28.29	18.19	24.33	5.39
OSCS	250	60.00	69.40	52.91	60.77	8.27
	500	10.23	10.89	20.03	13.72	5.48
	100	10.30	9.68	9.87	9.95	0.32

Table C.2 Cytotoxicity of cisplatin and OSCS in HepG2 cells

Sample	Concentration (µg/ml)	% Cell viability				
		n1	n2	n3	Average	SD
Control		96.07	104.54	97.00	99.21	4.65
Cisplatin 20 µM		56.15	51.48	50.48	52.70	3.03
Cisplatin 20 µM+OSCS	250	57.08	48.18	42.00	49.09	7.58
Cisplatin 20 µM+OSCS	500	9.07	9.70	7.77	8.85	0.98
OSCS	250	36.40	52.63	46.10	45.04	8.16
	500	9.13	9.47	11.57	10.06	1.32
	100	11.34	8.69	7.61	9.21	1.92

Table C.3 Cytotoxicity of cisplatin and OSCS in MCF-7 cells

Sample	Concentration (µg/ml)	% Cell viability				
		n1	n2	n3	Average	SD
Control		81.29	81.55	104.49	89.11	13.32
Cisplatin 20 µM		17.50	14.93	18.90	17.11	2.02
Cisplatin 20 µM+OSCS	250	35.22	40.63	38.77	38.21	2.75
Cisplatin 20 µM+OSCS	500	34.17	16.30	20.51	23.66	9.34
OSCS	250	90.35	87.77	83.24	87.12	3.60
	500	34.35	20.03	13.58	22.65	10.63
	100	19.57	14.74	15.83	16.71	2.53

APPENDIX D

Phosphate buffer saline (PBS)

For 1 liter of PBS, add these chemicals into approximately 900 ml of cell culture water while stirring;

Component	Final concentration (mM)	Amount (g/L)
NaCl	137	8.0
KCL	2.68	0.2
Na_2HPO_4	10.14	1.44
KH_2PO_4	1.47	0.24

The pH of buffer was adjusted to 7.4 with HCl or NaOH. Then, make the volume of buffer to 1 liter with cell culture grade water. The buffer was sterilized by autoclaving for 20 minutes at 121^{o}C under the pressure of 15 1b/sq inch on liquid cycle and kept at room temperature.

APPENDIX E

Waymouth Buffer (WB)

Component	Final concentration (mM)	Amount (g/L)
NaCl	135	7.88
KCL	5	0.38
HEPES	13	3.10
$CaCl_2$. H_2O	2.5	0.36
$MgCl_2$	1.2	0.24
$MgSO_4.7H_2O$	0.8	0.20
D-glucose	28	5.04

The buffer's pH was adjusted to 7.4 with HCl or NaOH.

REFERENCES

1. Elder, D.P., M. Kuentz, and R. Holm, *Pharmaceutical excipients - quality, regulatory and biopharmaceutical considerations.* Eur J Pharm Sci, 2016. **87**: p. 88-99.
2. Engel, A., et al., *Pharmaceutical excipients influence the function of human uptake transporting proteins.* Mol Pharm, 2012. **9**(9): p. 2577-81.
3. Paus, R., A. Prudic, and Y. Ji, *Influence of excipients on solubility and dissolution of pharmaceuticals.* Int J Pharm, 2015. **485**(1-2): p. 277-87.
4. Strickley, R.G., *Solubilizing excipients in oral and injectable formulations.* Pharm Res, 2004. **21**(2): p. 201-30.
5. Goole, J., et al., *The effects of excipients on transporter mediated absorption.* Int J Pharm, 2010. **393**(1-2): p. 17-31.
6. Sajomsang, W., et al., *Synthesis and anticervical cancer activity of novel pH responsive micelles for oral curcumin delivery.* Int J Pharm, 2014. **477**(1-2): p. 261-72.
7. Sajomsang, W., et al., *Quaternization of N-aryl chitosan derivatives: synthesis, characterization, and antibacterial activity.* Carbohydr Res, 2009. **344**(18): p. 2502-11.
8. Sajomsang, W., et al., *Synthesis and characterization of N-aryl chitosan derivatives.* Int J Biol Macromol, 2008. **43**(2): p. 79-87.
9. Yamagata, T., et al., *Effect of excipients on breast cancer resistance protein substrate uptake activity.* J Control Release, 2007. **124**(1-2): p. 1-5.
10. Yamagata, T., et al., *Characterization of the inhibition of breast cancer resistance protein-mediated efflux of mitoxantrone by pharmaceutical excipients.* Int J Pharm, 2009. **370**(1-2): p. 216-9.
11. Li, L., T. Yi, and C.W. Lam, *Interactions between human multidrug resistance related protein (MRP2; ABCC2) and excipients commonly used in self-emulsifying drug delivery systems (SEDDS).* Int J Pharm, 2013. **447**(1-2): p. 192-8.
12. Jin, X., et al., *Paclitaxel-loaded N-octyl-O-sulfate chitosan micelles for superior cancer therapeutic efficacy and overcoming drug resistance.* Mol Pharm, 2014. **11**(1): p. 145-57.
13. Opanasopit, P., et al., *Methylated N-(4-pyridinylmethyl) chitosan as a novel effective safe gene carrier.* Int J Pharm, 2008. **364**(1): p. 127-34.
14. Opanasopit, P., et al., *Nuclear localization signal peptides enhance transfection efficiency of chitosan/DNA complexes.* Int J Pharm, 2009. **382**(1-2): p. 291-5.
15. Woraphatphadung, T., et al., *Synthesis and characterization of pH-responsive N-naphthyl-N,O-succinyl chitosan micelles for oral meloxicam delivery.* Carbohydr Polym, 2015. **121**: p. 99-106.
16. Woraphatphadung, T., et al., *pH-Responsive polymeric micelles based on amphiphilic chitosan derivatives: Effect of hydrophobic cores on oral meloxicam delivery.* Int J Pharm, 2016. **497**(1-2): p. 150-60.
17. Jonker, J.W. and A.H. Schinkel, *Pharmacological and physiological functions of the polyspecific organic cation transporters: OCT1, 2, and 3 (SLC22A1-3).* J Pharmacol Exp Ther, 2004. **308**(1): p. 2-9.
18. Okuda, M., et al., *Molecular mechanisms of organic cation transport in OCT2-*

expressing Xenopus oocytes. Biochim Biophys Acta, 1999. **1417**(2): p. 224-31.
19. Otsuka, M., et al., *A human transporter protein that mediates the final excretion step for toxic organic cations.* Proc Natl Acad Sci U S A, 2005. **102**(50): p. 17923-8.
20. Urakami, Y., et al., *Creatinine transport by basolateral organic cation transporter hOCT2 in the human kidney.* Pharm Res, 2004. **21**(6): p. 976-81.
21. Wright, S.H. and W.H. Dantzler, *Molecular and cellular physiology of renal organic cation and anion transport.* Physiol Rev, 2004. **84**(3): p. 987-1049.
22. Ciarimboli, G., et al., *Cisplatin nephrotoxicity is critically mediated via the human organic cation transporter 2.* Am J Pathol, 2005. **167**(6): p. 1477-84.
23. Koepsell, H., K. Lips, and C. Volk, *Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications.* Pharm Res, 2007. **24**(7): p. 1227-51.
24. Wright, S.H., *Role of organic cation transporters in the renal handling of therapeutic agents and xenobiotics.* Toxicol Appl Pharmacol, 2005. **204**(3): p. 309-19.
25. Hosoyamada, M., et al., *Molecular cloning and functional expression of a multispecific organic anion transporter from human kidney.* Am J Physiol, 1999. **276**(1 Pt 2): p. F122-8.
26. Schomig, E., et al., *Molecular cloning and characterization of two novel transport proteins from rat kidney.* FEBS Lett, 1998. **425**(1): p. 79-86.
27. International Transporter, C., et al., *Membrane transporters in drug development.* Nat Rev Drug Discov, 2010. **9**(3): p. 215-36.
28. Shu, Y., et al., *Functional characteristics and steroid hormone-mediated regulation of an organic cation transporter in Madin-Darby canine kidney cells.* J Pharmacol Exp Ther, 2001. **299**(1): p. 392-8.
29. Somogyi, A., et al., *Reduction of metformin renal tubular secretion by cimetidine in man.* Br J Clin Pharmacol, 1987. **23**(5): p. 545-51.
30. Yokoo, S., et al., *Differential contribution of organic cation transporters, OCT2 and MATE1, in platinum agent-induced nephrotoxicity.* Biochem Pharmacol, 2007. **74**(3): p. 477-87.
31. Ozols, R.F., et al., *Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study.* J Clin Oncol, 2003. **21**(17): p. 3194-200.
32. Posner, M.R., et al., *Cisplatin and fluorouracil alone or with docetaxel in head and neck cancer.* N Engl J Med, 2007. **357**(17): p. 1705-15.
33. Williams, S., et al., *Adjuvant therapy of ovarian germ cell tumors with cisplatin, etoposide, and bleomycin: a trial of the Gynecologic Oncology Group.* J Clin Oncol, 1994. **12**(4): p. 701-6.
34. Pabla, N. and Z. Dong, *Cisplatin nephrotoxicity: mechanisms and renoprotective strategies.* Kidney Int, 2008. **73**(9): p. 994-1007.
35. Filipski, K.K., et al., *Contribution of organic cation transporter 2 (OCT2) to cisplatin-induced nephrotoxicity.* Clin Pharmacol Ther, 2009. **86**(4): p. 396-402.
36. Miller, R.P., et al., *Mechanisms of Cisplatin nephrotoxicity.* Toxins (Basel), 2010. **2**(11): p. 2490-518.
37. Tanihara, Y., et al., *Protective effect of concomitant administration of imatinib*

on cisplatin-induced nephrotoxicity focusing on renal organic cation transporter OCT2. Biochem Pharmacol, 2009. **78**(9): p. 1263-71.

38. Socaciu, C., R. Jessel, and H.A. Diehl, *Competitive carotenoid and cholesterol incorporation into liposomes: effects on membrane phase transition, fluidity, polarity and anisotropy.* Chem Phys Lipids, 2000. **106**(1): p. 79-88.
39. Porter, C.J., N.L. Trevaskis, and W.N. Charman, *Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs.* Nat Rev Drug Discov, 2007. **6**(3): p. 231-48.
40. Minato, S., et al., *Application of polyethyleneglycol (PEG)-modified liposomes for oral vaccine: effect of lipid dose on systemic and mucosal immunity.* J Control Release, 2003. **89**(2): p. 189-97.
41. Oku, N. and Y. Namba, *Glucuronate-modified, long-circulating liposomes for the delivery of anticancer agents.* Methods Enzymol, 2005. **391**: p. 145-62.
42. Hugger, E.D., et al., *A comparison of commonly used polyethoxylated pharmaceutical excipients on their ability to inhibit P-glycoprotein activity in vitro.* J Pharm Sci, 2002. **91**(9): p. 1991-2002.
43. Uekama, K., F. Hirayama, and T. Irie, *Cyclodextrin Drug Carrier Systems.* Chem Rev, 1998. **98**(5): p. 2045-2076.
44. Casadidio, C., et al., *Chitin and Chitosans: Characteristics, Eco-Friendly Processes, and Applications in Cosmetic Science.* Mar Drugs, 2019. **17**(6).
45. Ngo, D.H. and S.K. Kim, *Antioxidant effects of chitin, chitosan, and their derivatives.* Adv Food Nutr Res, 2014. **73**: p. 15-31.
46. Fathi, M., et al., *Chitosan-based multifunctional nanomedicines and theranostics for targeted therapy of cancer.* Med Res Rev, 2018.
47. Naskar, S., K. Koutsu, and S. Sharma, *Chitosan-based nanoparticles as drug delivery systems: A review on two decades of research.* J Drug Target, 2018: p. 1-41.
48. Qu, G., et al., *N-octyl-O-sulfate chitosan-modified liposomes for delivery of docetaxel: preparation, characterization, and pharmacokinetics.* Biomed Pharmacother, 2012. **66**(1): p. 46-51.
49. Qu, G., et al., *PEG conjugated N-octyl-O-sulfate chitosan micelles for delivery of paclitaxel: in vitro characterization and in vivo evaluation.* Eur J Pharm Sci, 2009. **37**(2): p. 98-105.
50. Tonglairoum, P., et al., *Development and evaluation of N-naphthyl-N,O-succinyl chitosan micelles containing clotrimazole for oral candidiasis treatment.* Pharm Dev Technol, 2017. **22**(2): p. 184-190.
51. Rojanarata, T., et al., *Methylated N-(4-N,N-dimethylaminobenzyl) chitosan for novel effective gene carriers.* Eur J Pharm Biopharm, 2008. **70**(1): p. 207-14.
52. Mo, R., et al., *The mechanism of enhancement on oral absorption of paclitaxel by N-octyl-O-sulfate chitosan micelles.* Biomaterials, 2011. **32**(20): p. 4609-20.
53. Zhang, C., et al., *Biological evaluation of N-octyl-O-sulfate chitosan as a new nano-carrier of intravenous drugs.* Eur J Pharm Sci, 2008. **33**(4-5): p. 415-23.
54. Koepsell, H., *Polyspecific organic cation transporters and their biomedical relevance in kidney.* Curr Opin Nephrol Hypertens, 2013. **22**(5): p. 533-8.
55. Matsuzaki, T., et al., *Altered pharmacokinetics of cationic drugs caused by down-regulation of renal rat organic cation transporter 2 (Slc22a2) and rat multidrug and toxin extrusion 1 (Slc47a1) in ischemia/reperfusion-induced acute*

kidney injury. Drug Metab Dispos, 2008. **36**(4): p. 649-54.
56. Zhang, C., et al., *Pharmacokinetics, biodistribution, efficacy and safety of N-octyl-O-sulfate chitosan micelles loaded with paclitaxel.* Biomaterials, 2008. **29**(9): p. 1233-41.
57. Wang, L. and D.H. Sweet, *Renal organic anion transporters (SLC22 family): expression, regulation, roles in toxicity, and impact on injury and disease.* Aaps J. **15**(1): p. 53-69.
58. Nies, A.T., et al., *Organic cation transporters (OCTs, MATEs), in vitro and in vivo evidence for the importance in drug therapy.* Handb Exp Pharmacol, 2011(201): p. 105-67.
59. Bacq, A., et al., *Organic cation transporter 2 controls brain norepinephrine and serotonin clearance and antidepressant response.* Mol Psychiatry, 2012. **17**(9): p. 926-39.
60. Barendt, W.M. and S.H. Wright, *The human organic cation transporter (hOCT2) recognizes the degree of substrate ionization.* J Biol Chem, 2002. **277**(25): p. 22491-6.
61. Ciarimboli, G., *Membrane transporters as mediators of Cisplatin effects and side effects.* Scientifica (Cairo), 2012. **2012**: p. 473829.
62. Koepsell, H., *The SLC22 family with transporters of organic cations, anions and zwitterions.* Mol Aspects Med, 2013. **34**(2-3): p. 413-35.
63. Grundemann, D., et al., *Drug excretion mediated by a new prototype of polyspecific transporter.* Nature, 1994. **372**(6506): p. 549-52.
64. Lips, K.S., et al., *Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium.* Am J Respir Cell Mol Biol, 2005. **33**(1): p. 79-88.
65. Muller, J., et al., *Drug specificity and intestinal membrane localization of human organic cation transporters (OCT).* Biochem Pharmacol, 2005. **70**(12): p. 1851-60.
66. Kimura, N., M. Okuda, and K. Inui, *Metformin transport by renal basolateral organic cation transporter hOCT2.* Pharm Res, 2005. **22**(2): p. 255-9.
67. Ciarimboli, G., et al., *Organic cation transporter 2 mediates cisplatin-induced oto- and nephrotoxicity and is a target for protective interventions.* Am J Pathol, 2010. **176**(3): p. 1169-80.
68. Rosenberg, B., L. Vancamp, and T. Krigas, *Inhibition of Cell Division in Escherichia Coli by Electrolysis Products from a Platinum Electrode.* Nature, 1965. **205**: p. 698-9.
69. Dasari, S. and P.B. Tchounwou, *Cisplatin in cancer therapy: molecular mechanisms of action.* Eur J Pharmacol, 2014. **740**: p. 364-78.
70. Ozkok, A. and C.L. Edelstein, *Pathophysiology of cisplatin-induced acute kidney injury.* Biomed Res Int, 2014. **2014**: p. 967826.
71. Hanigan, M.H. and P. Devarajan, *Cisplatin nephrotoxicity: molecular mechanisms.* Cancer Ther, 2003. **1**: p. 47-61.
72. Bellomo, R., J.A. Kellum, and C. Ronco, *Acute kidney injury.* Lancet, 2012. **380**(9843): p. 756-66.
73. Yao, X., et al., *Cisplatin nephrotoxicity: a review.* Am J Med Sci, 2007. **334**(2): p. 115-24.
74. Schetz, M., et al., *Drug-induced acute kidney injury.* Curr Opin Crit Care, 2005.

11(6): p. 555-65.
75. Pabla, N., et al., *The copper transporter Ctr1 contributes to cisplatin uptake by renal tubular cells during cisplatin nephrotoxicity.* American Journal of Physiology-Renal Physiology, 2009. **296**(3): p. F505-F511.
76. Siddik, Z.H., *Cisplatin: mode of cytotoxic action and molecular basis of resistance.* Oncogene, 2003. **22**(47): p. 7265-79.
77. Arany, I. and R.L. Safirstein, *Cisplatin nephrotoxicity.* Semin Nephrol, 2003. **23**(5): p. 460-4.
78. Townsend, D.M., et al., *Metabolism of Cisplatin to a nephrotoxin in proximal tubule cells.* J Am Soc Nephrol, 2003. **14**(1): p. 1-10.
79. Marullo, R., et al., *Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions.* PLoS One, 2013. **8**(11): p. e81162.
80. Pan, H., et al., *Cannabidiol attenuates cisplatin-induced nephrotoxicity by decreasing oxidative/nitrosative stress, inflammation, and cell death.* Journal of Pharmacology and Experimental Therapeutics, 2009. **328**(3): p. 708-714.
81. Li, S., et al., *Anti-inflammatory effect of fibrate protects from cisplatin-induced ARF.* Am J Physiol Renal Physiol, 2005. **289**(2): p. F469-80.
82. Arany, I., et al., *Cisplatin-induced cell death is EGFR/src/ERK signaling dependent in mouse proximal tubule cells.* Am J Physiol Renal Physiol, 2004. **287**(3): p. F543-9.
83. Kim, Y.K., et al., *Role of ERK activation in cisplatin-induced apoptosis in OK renal epithelial cells.* J Appl Toxicol, 2005. **25**(5): p. 374-82.
84. Jo, S.K., et al., *MEK inhibitor, U0126, attenuates cisplatin-induced renal injury by decreasing inflammation and apoptosis.* Kidney Int, 2005. **67**(2): p. 458-66.
85. Sprowl, J.A., et al., *Cisplatin-induced renal injury is independently mediated by OCT2 and p53.* Clin Cancer Res, 2014. **20**(15): p. 4026-35.
86. Kim, D.H., et al., *SIRT1 activation by resveratrol ameliorates cisplatin-induced renal injury through deacetylation of p53.* Am J Physiol Renal Physiol, 2011. **301**(2): p. F427-35.
87. Dong, G., et al., *Inhibitors of histone deacetylases suppress cisplatin-induced p53 activation and apoptosis in renal tubular cells.* American Journal of Physiology-Renal Physiology, 2010. **298**(2): p. F293-F300.
88. Molitoris, B.A., et al., *siRNA targeted to p53 attenuates ischemic and cisplatin-induced acute kidney injury.* J Am Soc Nephrol, 2009. **20**(8): p. 1754-64.
89. Wei, Q., et al., *Activation and involvement of p53 in cisplatin-induced nephrotoxicity.* Am J Physiol Renal Physiol, 2007. **293**(4): p. F1282-91.
90. Wei, Q., et al., *The pathological role of Bax in cisplatin nephrotoxicity.* Kidney Int, 2007. **72**(1): p. 53-62.
91. Dursun, B., et al., *Caspases and calpain are independent mediators of cisplatin-induced endothelial cell necrosis.* Am J Physiol Renal Physiol, 2006. **291**(3): p. F578-87.
92. Seth, R., et al., *p53-dependent caspase-2 activation in mitochondrial release of apoptosis-inducing factor and its role in renal tubular epithelial cell injury.* J Biol Chem, 2005. **280**(35): p. 31230-9.
93. Liu, H. and R. Baliga, *Endoplasmic reticulum stress-associated caspase 12 mediates cisplatin-induced LLC-PK1 cell apoptosis.* J Am Soc Nephrol, 2005.

16(7): p. 1985-92.
94. Atessahin, A., et al., *Effects of lycopene against cisplatin-induced nephrotoxicity and oxidative stress in rats.* Toxicology, 2005. **212**(2): p. 116-123.
95. Wang, S., et al., *ERK-mediated suppression of cilia in cisplatin-induced tubular cell apoptosis and acute kidney injury.* Biochim Biophys Acta, 2013. **1832**(10): p. 1582-90.
96. Clark, J.S., et al., *Cisplatin induces apoptosis through the ERK-p66shc pathway in renal proximal tubule cells.* Cancer Lett, 2010. **297**(2): p. 165-70.
97. Ouchi, A., et al., *Comparison of short and continuous hydration regimen in chemotherapy containing intermediate- to high-dose Cisplatin.* J Oncol, 2014. **2014**: p. 767652.
98. Launay-Vacher, V., et al., *Prevention of cisplatin nephrotoxicity: state of the art and recommendations from the European Society of Clinical Pharmacy Special Interest Group on Cancer Care.* Cancer chemotherapy and pharmacology, 2008. **61**(6): p. 903-909.
99. Muraki, K., et al., *Hydration with magnesium and mannitol without furosemide prevents the nephrotoxicity induced by cisplatin and pemetrexed in patients with advanced non-small cell lung cancer.* Journal of Thoracic Disease, 2012. **4**(6): p. 562-568.
100. Santoso, J.T., et al., *Saline, mannitol, and furosemide hydration in acute cisplatin nephrotoxicity: a randomized trial.* Cancer Chemother Pharmacol, 2003. **52**(1): p. 13-8.
101. Cornelison, T.L. and E. Reed, *Nephrotoxicity and hydration management for cisplatin, carboplatin, and ormaplatin.* Gynecol Oncol, 1993. **50**(2): p. 147-58.
102. Dumas, M., et al., *Influence of hydration on ultrafilterable platinum kinetics and kidney function in patients treated with cis-diamminedichloroplatinum(II).* Cancer Chemother Pharmacol, 1990. **26**(4): p. 278-82.
103. Daley-Yates, P.T. and D.C. McBrien, *A study of the protective effect of chloride salts on cisplatin nephrotoxicity.* Biochem Pharmacol, 1985. **34**(13): p. 2363-9.
104. Asavapanumas, N., et al., *Fenofibrate down-regulates renal OCT2-mediated organic cation transport via PPARalpha-independent pathways.* Drug Metab Pharmacokinet, 2012. **27**(5): p. 513-9.
105. Soodvilai, S., A. Chatsudthipong, and V. Chatsudthipong, *Role of MAPK and PKA in regulation of rbOCT2-mediated renal organic cation transport.* Am J Physiol Renal Physiol, 2007. **293**(1): p. F21-7.
106. Malo, C. and A. Berteloot, *Analysis of kinetic data in transport studies: new insights from kinetic studies of Na(+)-D-glucose cotransport in human intestinal brush-border membrane vesicles using a fast sampling, rapid filtration apparatus.* J Membr Biol, 1991. **122**(2): p. 127-41.
107. Hilgendorf, C., et al., *Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines.* Drug Metab Dispos, 2007. **35**(8): p. 1333-40.
108. Aschauer, L., et al., *Expression of xenobiotic transporters in the human renal proximal tubule cell line RPTEC/TERT1.* Toxicol In Vitro, 2015. **30**(1 Pt A): p. 95-105.
109. Wieser, M., et al., *hTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics.* Am J Physiol Renal

Physiol, 2008. **295**(5): p. F1365-75.
110. Harrach, S. and G. Ciarimboli, *Role of transporters in the distribution of platinum-based drugs.* Front Pharmacol, 2015. **6**: p. 85.

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AWARD RECEIVED	Royal Golden Jubilee Ph.D. Program (Grant No. PHD 0139/2557)