Prediction of transporter-mediated drug-drug interactions in the liver: Based on in vitro inhibition study and clearance concept

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The liver mainly regulates the ADME (absorption, distribution, metabolism and excretion) properties of numerous endogenous and exogenous compounds with metabolic enzymes such as cytochrome P450 (CYP) and membrane transporters such as organic anion transporting polypeptides (OATPs) and organic cation transporters (OCTs). Recently, clinical studies investigating drug-drug interactions (DDIs) or pharmacogenetics (PGx) have revealed that, in addition to drug metabolizing enzymes, an alteration in the membrane transport activity can also affect the pharmacokinetics and subsequent pharmacodynamic/toxicological effect of their substrates (1). Thus, pharmaceutical companies are now trying to avoid or to predict these events in the early phase of drug development.

However, the accurate prediction of such DDIs remains challenging, mainly for two difficulties. One difficulty is the estimation of the inhibitor protein unbound concentration at the interaction site. The other one is that hepatic clearance consists of multiple intrinsic elimination processes such as uptake into hepatocytes, basolateral efflux from hepatocytes into blood, metabolism, and biliary efflux. Thus, it is difficult to estimate the effects of the change in each elimination process on the overall hepatic elimination.

By considering these difficulties, we tried to predict the degree of transporter-mediated DDIs (TP-DDIs), particularly involving OATP substrates, using the inhibition constants obtained with in vitro inhibition experiments and several assumptions on inhibitor concentration or the effect of each elimination pathway to the hepatic clearance as explained (2). As a result, the AUCRs of OATP substrates were predicted within a 3-fold difference of observed values in 49 out of 54 cases. The number of false-negative predictions was decreased by these assumptions: (i) theoretical maximum concentration of coadministered drugs at the inlet to the liver was used (Ito K et al., Pharmacol Rev, 50: 387–412 (1998)); (ii) the product of maximum inhibitory effects of
both hepatic uptake and efflux/metabolic processes was considered; and (iii) FaFg was set to 1 when the inhibition of intestinal CYP3A4/P-gp/BCRP was thought to occur.

In conclusion, based on the in vitro inhibitory potencies and several assumptions, AUCRs were predicted well and avoidance of false-negative predictions was maximized. This method will be helpful for preventing unnecessary clinical DDI studies involving drug transporters, particularly at the early phase of drug development.

<references>

(1) Yoshida, K. et al., Prediction of the altered pharmacokinetics of drugs by hepatic and intestinal transporter-mediated drug-drug interactions and pharmacogenetics from in vitro experimental results. Annu Rev Pharmacol Toxicol. Accepted.

Preparation of tiny liposomes using pressure microfluidizer for gene delivery to posterior segment of eye

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**Purpose:** Blood-retinal barrier (BRB) remains as a challenging obstacle in drug delivery to retinal pigment epithelium (RPE) and neurosensory retina. Epithelial tight junctions limit greatly the access of molecules and particles. It is understood that smaller particles generally penetrate the biological tissues more easily than large particles. The choroidal capillaries are fenestrated with small pores of about 80 nm in diameter. In this study we prepared different types of liposomes by using microfluidizer, and investigated the effects of manufacturing parameters on physical properties of the liposomes. We also made preliminary *in vivo* studies on delivery of these liposomes to the retina of rats.

**Materials & methods:** Neutral, cationic and anionic phospholipids were used to produce the liposomes. Plasmid DNA (pDNA) was used as a model drug to investigate the release from the liposomes. Liposomes were prepared by two different principal methods. In one protocol, the liposomes were first manufactured by film hydration method and then processed with the microfluidizer (Microfluidics M-110P) in aqueous solution. In second protocol, the liposomes are prepared directly in microfluidizer (Microfluidics M-110P or LV-1) using a novel double emulsion method, which is done by mixing immiscible aqueous and organic phases. Phospholipids form layers at the phase border and the liposomes are eventually formed by evaporating the organic phase from the fine double emulsion. Size of the liposomes was determined by dynamic light scattering, zeta potential was measured by electrophoretic light scattering, and release of pDNA from liposomes was determined by fluorescence detection method. Retinal delivery was investigated by confocal microscope.

**Results:** Neutral liposomes with different sizes ranging from less than 30 nm to over 300 nm in diameter were produced. Also cationic and anionic liposomes, with diameters as small as about 55 nm and 65 nm respectively, were produced. The liposomes were physically stable for about two months in aqueous solutions and stored in a refrigerator. The pDNA amounts were less than 1% in solutions, and more than 70% inside the liposomes. Microscope images indicated that the liposomes can reach the target area in the eye.
Mechanism study of Diclofenac induced toxicity

in the intestine ex vivo

Xiaoyu Niu
(Groningen University, the Netherlands)

Now I am working in Chiba University as an exchange PhD student for 3 months. It is my pleasure to join the Global Education Seminar in Japan, and introduce you my work in the field of toxicology.

P1-P3: A short introduction of myself.
2002-2009 Bachelor and master in Beijing University of traditional Chinese medicine, Beijing, China.
2009 Exchange master in Medical University of Graz, Graz, Austria.
2009-2013 PhD student in Groningen University, Groningen, The Netherlands.
2012/8-2012/10 Exchange PhD in Chiba University, Chiba, Japan.

P4: I will present part of my PhD project which is entitled the mechanism of diclofenac induced toxicity in the intestine ex vivo.

P6-P9 Background
P6: Diclofenac (DCF) is a nonsteroidal anti-inflammatory drug (NSAID): relieve pain in conditions such as arthritis or acute injury. Though wildly used, DCF is associated with a high prevalence of gastrointestinal side effects: the symptoms are abdominal pain, constipation, diarrhea, intestinal ulcers. The underlying mechanisms of diclofenac induced enteropathy are not fully understood, one major reason is there is no good in vitro or ex vivo model.

P7: In vivo studies show that DCF is mainly metabolized in liver by two pathways: DCF could be oxidized by Cyp enzymes to hydroxyl DCF, further conjugated with glucuronic acid or sulfuric acid. Besides, though quinone imine intermediate to form GSH adducts. The second path way is glucuronidation, DCF could be glucuronidated by UGTs to form DAG which is the main biliary metabolites.

P8: DCF undergoes almost complete biotransformation in man and lower species. Theoretically, the hydroxyl DCF can induce oxidative stress due to the reactive intermeate quinone imine. DAG contains electrophilic carboxyl carbon which can be attacked by protein, forming protein-drug adducts, induce protein modification or immune response.

P9: Precision cut intestinal slices (PCIS) represents an organ mini-model with all cell types present. Pictures show the way to prepare the PCIS.

P10 Objectives of the project
**P11-P26 Results**

P12: Viability of colon and small intestinal slices was assessed by ATP, gene expression, morphology as well as metabolic rate. 5h incubation time was selected for further study.

P13: Toxicity was evaluated by ATP, LDH leakage, Morphology. Dose-dependent toxicity of DCF was shown by PCIS.

P14: Diclofenac metabolism in the intestine was measured by HPLC and Mass spectrometry. Main metabolites 4’-OH DCF, 5-OH DCF and DAG were detected.

P17: Human tissue resources.

P18: Species differences in toxicity (rat vs human). Human intestine showed more resistance to DCF toxicity.

P19: Species differences in metabolism. Human tissue produced more metabolites with high variation among different individuals.

P21: Immunohistochemistry staining using anti-diclofenac antibody detected the drug-protein binding by 5-OH DCF and DAG.

P22: Co-incubation (liver+intestine)did not further increase the intestinal toxicity.

P24: Decreased metabolites formation by the addition of inhibitors Borneol and Cimetidine.

P25: Enhanced toxicity in the presence of inhibitors.

P26: Decreased drug-protein adducts formation by the inhibition.

**P27 Summary and conclusion**

**P28 Acknowledgment**
Development of organic acid-crosslinked gelatin as a coating material for drug-eluting stent

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Drug-eluting stents (DESs) are important medical devices in coronary intervention therapy. DESs support vessels against elastic recoil, which helps to prevent smooth muscle cell growth during the release of drugs. DESs have non-biodegradable or slow-biodegradable polymeric matrices, which act as drug reservoirs and release drugs over a period of several weeks or months. DESs successfully suppress the restenosis rate to below 10% in some cases. However, recent studies suggest that there are some complications associated with DESs use, such as stent thrombosis, an exaggerated inflammatory response, prevention of endothelialization, and poor attachment of stents to the arterial wall at the implant site. In-stent thrombosis, particularly late angiographic stent thrombosis (LAST), was the most common complication. When bare metal stents (BMS) were used, only a few cases of LAST were observed. Therefore, many kinds of biometal coating materials have been developed. However, these coatings are not ideal biocompatible materials, because they are not cytocompatible. Therefore it is essential to develop coatings that are highly biocompatible, antithrombogenic and capable of promoting endothelialization, for use in vascular devices.

Here, we report that biocompatible alkali-treated gelatin (AlGelatin) matrix with endothelialization and antithrombogenic properties. Trisuccinimidyl citrate (TSC)-crosslinked AlGelatin (AlGelatin-TSC) was prepared with various crosslinking densities, and its biochemical and physicochemical properties, including gelation time, water content, concentration of residual functional groups in AlGelatin-TSC, and endothelialization and antithrombogenic properties, were investigated. Quantitative analysis of inflammation reaction was performed using transgenic mice expressing nuclear factor-kappa B (NF-κB) receptor to compare the biocompatibility of the AlGelatin-TSC with that of glutaraldehyde crosslinked AlGelatin (AlGelatin-GA).

Thrombus formation on the surface of AlGelatin-TSC was less than that of AlGelatin-GA. The number of endothelial cells cultured on the surface of the AlGelatin-TSC increased as the concentration of TSC was increased to 20 mM, and then decreased with further increases in TSC concentration. On the AlGelatin-GA, no HUVECs were detected. The inflammation reaction of the AlGelatin-TSC was lower than that of AlGelatin-GA by bioluminescence imaging. Physicochemical analysis of AlGelatin-TSC with different TSC concentrations showed that the high concentration of the cell adhesion sequence, arginine–glycine–aspartic acid, contributed to the promotion of endothelial cell adhesion and subsequent endothelial cell growth. Analysis of the carboxyl groups in the AlGelatin-TSC showed that the antithrombogenic activity was due to
the increased negative charge derived from the hydrolyzed active ester groups of TSC. The biocompatibility of the matrix was evaluated by bioluminescence imaging, indicated that the inflammation reaction of the AlGelatin-TSC was lower than that of the AlGelatin-GA. These findings show that AlGelatin-TSC has the potential for use a coating material for drug-eluting stent.

**SELECTED PUBLICATIONS:**

**Lubrication properties of potential alternative lubricants, glycerin fatty acid esters and chitosan-laurate, to magnesium stearate**

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**Purpose:** Magnesium stearate (Mg-St) is widely used as a lubricant in solid pharmaceutical formulations, however also is widely known that, as the concentration in a formulation increases, it will cause manufacturing problems such as reduction in tablet hardness and prolonged disintegration time. Precaution for potential biological contamination is important in the use of excipients of animal origin that includes some of Mg-St brands. In addition, to assure the uniform quality of pharmaceutical products, it is necessary to discard initial tablets after starting compression until the uniform lubrication effect takes place. As for alternative lubricants to Mg-St, we firstly focused on 2 types of glycerin fatty acid esters. To study the usefulness of glycerin fatty acid esters as lubricants, pressure transmission ratio, ejection force, tensile strength and disintegration time were measured at different concentrations for granules and tablets.

**Methods:** Two types of glycerin fatty acid esters (Poem TR-FB® (TR-FB) and Poem TR-HB® (TR-HB)) were examined. Mg-St, TR-FB or TR-HB was added to the granules, which were composed of lactose monohydrate, corn starch, and hydroxypropyl cellulose and was mixed for 5 min, using a V-shaped mixer. Each lubricant was added at concentration from 0.1 to 3.0%. The pressure transmission ratio or the ejection force was calculated or measured as lubricant property. The tensile strength was measured and the disintegration tests were performed according to the JP15.

**Results:** When each lubricant was mixed at 0.1-3.0%, the increase in the pressure transmission ratio that was equal to or greater than that of Mg-St as well as the reduction in the ejection force was observed at a low concentration in both TR-FB and TR-HB, proving that they have excellent lubrication performance. The granules that were lubricated with TR-FB and TR-HB at even low concentration showed a more stable and sufficiently lower ejection force than with Mg-St from the first tablet after the start of compression. As for the tensile strength and the disintegration time, a decreased tensile strength and a prolonged disintegration time, which are disadvantages of Mg-St, were not observed in TR-FB and TR-HB.

**Conclusion:** Both TR-FB and TR-HB had excellent lubrication performance than Mg-St and did not cause the decrease of tablet hardness and prolonged disintegration time. Based on these results, it was concluded that TR-FB and TR-HB are useful as alternative lubricants to Mg-St. In the latter part of my talk, I will introduce new results of other novel lubricant, chitosan-laurate.
Nano-crystalline drug production and a novel HDL-like gold nanoparticles formulation with anti-inflammatory effect on vascular endothelial cells

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Nagoya City University

Nano-crystal of poorly water soluble drugs has been developed to enhance drug dissolution and absorption. Nano-crystalline drug production technologies recently published and a novel one are introduced involving the top-down and bottom-up methods.

Atherosclerosis plays an important role in the progression of vascular-related serious diseases including myocardial infarction and stroke. Although supportive care is effective, novel and fundamental therapeutic approaches have been developing. Atherosclerosis involves slow inflammatory process, and therefore, the inhibition of inflammation is effective for progress of the diseases. In the present study, we developed a novel high density lipoprotein (HDL) nanoparticles formulation. This formulation includes sphingosine-1-phosphate (S1P) and apolipoprotein AI (ApoAI). The S1P/ApoAI nanoparticles were characterized, and then, the combination effect of S1P with ApoAI on the anti-inflammation in vitro was also investigated.

Synthesis of HDL nanoparticles: Nano-sized gold particles were obtained by mixing Gold (III) chloride aqueous solution and 1-Octadecanethiol (protecting group, in chloroform) in reductive condition. The resulting 1-Octadecanethiol-coated gold nanoparticles were mixed with S1P solution (in methanol), and accordingly, S1P was inserted on the surface of the nanoparticles. ApoAI protein was then attached on the surface of nanoparticle by gentle mixing, and then, S1P/ApoAI nanoparticles were obtained. The formulation parameters (Diameter, Zeta potential, S1P content, ApoAI content) of nanoparticles were evaluated. Evaluation of anti-inflammatory effect in vitro: HUVECs were used as the model of vascular cells in vitro. Inflammation was induced by the treatment with LPS, and subsequently, S1P/ApoAI nanoparticles were added to the cells. The total RNA was isolated and the mRNA level of plasminogen activator inhibitor-1 (PAI-1) gene as the marker of inflammation was determined by real time RT-PCR. The diameter of gold particles was controllable by the addition of 1-Octadecanethiol (from 5 nm to submicron order). Estimated characteristics of nanoparticles were as follows: 50 molecules of S1P and 20 molecules of ApoAI/one nanoparticle (20 nm, PDI=0.3). The nanoparticles were stable at 4 degree (>1 month). The treatment of S1P/ApoAI nanoparticles significantly reduced PAI mRNA level (>70%) within a few hours compared with LPS alone. We succeeded in the synthesis of HDL-like nanoparticles with small size distribution. Our results suggest that the combination of S1P and ApoAI has anti-inflammatory effect in vitro, and consequently, our formulation is promising for the novel therapy of atherosclerosis.