Biopharmaceutical investigations of doxorubicin formulations used in liver cancer treatment

Studies in healthy pigs and liver cancer patients, combined with pharmacokinetic and biopharmaceutical modelling

ILSE R DUBBELBOER
Abstract

There are currently two types of drug formulation in clinical use in the locoregional treatment of intermediate hepatocellular carcinoma (HCC). In the emulsion LIPDOX, the cytostatic agent doxorubicin (DOX) is dissolved in the aqueous phase, which is emulsified with the oily contrast agent Lipiodol® (LIP). In the microparticular system DEBDOX, DOX is loaded into the drug-eluting entity DC Bead™.

The overall aim of the thesis was to improve pharmaceutical understanding of the LIPDOX and DEBDOX formulations, in order to facilitate the future development of novel drug delivery systems. In vivo release of DOX from the formulations and the disposition of DOX and its active metabolite doxorubicinol (DOXol) were assessed in an advanced multisampling-site acute healthy pig model and in patients with HCC. The release of DOX and disposition of DOX and DOXol where further analysed using physiologically based pharmacokinetic (PBPK) and biopharmaceutical (PBBP) modelling. The combination of in vivo investigations and in silico modelling could provide unique insight into the mechanisms behind drug release and disposition.

The in vivo release of DOX from LIPDOX is not extended and controlled, as it is from DEBDOX. With both formulations, DOX is released as a burst during the early phase of administration. The in vivo release of DOX from LIPDOX was faster than from DEBDOX in both pigs and patients. The release from DEBDOX was slow and possibly incomplete. The in vivo release of DOX from LIPDOX and DEBDOX could be described by using the PBBP model in combination with in vitro release profiles.

The disposition of DOX and DOXol was modelled using a semi-PBPK model containing intracellular binding sites. The contrast agent Lipiodol® did not affect the hepatobiliary disposition of DOX in the pig model. The control substance used in this study, cyclosporine A, inhibited the biliary excretion of DOX and DOXol but did not alter metabolism in healthy pigs. The disposition of DOX is similar in healthy pigs and humans, which was shown by the ease of translation of the semi-PBPK pig model to the human PBBP model.

Keywords: drug delivery system, in vivo release, PBPK modelling, hepatocellular carcinoma, doxorubicin, transarterial chemoembolization, drug disposition

Ilse R Dubbelboer, Department of Pharmacy, Box 580, Uppsala University, SE-75123 Uppsala, Sweden.

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“Wat zou het leven zijn als we niet wat durfden aanpakken?”
“What would life be if we had no courage to attempt anything?”

- Vincent van Gogh
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV **Dubbelboer, I.R.**, Sjögren, E., Lennernäs, H. Porcine and human *in vivo* predictions for doxorubicin-containing formulations used in locoregional HCC treatment. *In manuscript*

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On projects leading to Papers I and II, I was extensively involved in the planning and execution of experiments, data analysis of results and writing of the manuscripts. I was also extensively involved in all aspects of the projects leading to Papers III and IV.

*The authors contributed equally to the execution of the study and writing of the article.*
Additional papers not included in this thesis:


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Abbreviations

A  amount of compound  
(A)AFE  (absolute) average fold error  
AKR  aldo-keto reductase  
aMSA  advanced multi-sampling site acute  
AUC  area under the plasma concentration-time curve  
B:P  blood:plasma ratio of the drug  
C  concentration  
CBR  carbonyl reductase  
Cl  clearance  
CP  Child-Pugh  
CsA  cyclosporine A (ciclosporin)  
(c)TACE  (conventional) transarterial chemoembolization  
DCB  DC bead™  
DEB/DEE  drug-eluting beads/entities  
DEBDOX  drug-eluting beads (here DCB) loaded with DOX  
DOX(ol)  doxorubicin(ol)  
E_H  apparent hepatic extraction ratio  
F_e  fraction excreted  
F_rel  fraction released  
F_u,p  the fraction of unbound drug in plasma  
F_vol, tr/un  Volume fraction of treated or untreated liver section in models  
GFR  glomerular filtration rate  
GI  gastro-intestinal  
HCC  hepatocellular carcinoma  
IBS  intracellular binding site  
k  rate constant  
K_p,T  tissue-to-plasma concentration partitioning coefficient  
LIP  Lipiodol®  
LIPDOX  an aqueous doxorubicin solution emulsified with Lipiodol®  
NC1 or 2  non-clinical study 1 or 2  
NCA  non-compartmental analysis  
O/W  oil-in-water  
PBBP  physiologically based biopharmaceutical  
PBPK  physiologically based pharmacokinetic  
PK  pharmacokinetic(s)
PS  ECOG performance status
Q   blood flow
RD    DDS release of drug from formulation or drug delivery system
t  time
t_{1/2}  half-life
V  volume
W/O  water-in-oil
Background

Liver anatomy and function

Anatomy

The liver is composed of left and right lobes. The lobes are divided into a total of 8 segments (Figure 1a). The left lobe comprises segments 1–4 and the right lobe comprises segments 5–8. The liver has a dual blood supply from the portal vein (75–80%) and the hepatic artery (20–25%). Each segment has its own blood supply and biliary drainage.1

On a microscopic scale, the liver can be divided into lobules.2 The classic hepatic lobule forms a hexagonal structure (Figure 1b).2 The lobule comprises all hepatocytes that are drained into one central vein, and is bound by two or more portal triads.2 A triad consists of the terminal branches of a hepatic artery and a portal vein and a bile duct.2 The terminal branches of the portal

Figure 1. (a) The liver is divided into eight segments; segments 1–4 form the left lobe and segments 5–8 form the right lobe. To demonstrate the blood supply to a tumour by a branch of the hepatic artery, segment 3 in the diagram contains a hepatocellular carcinoma (orange/yellow). (b) The microarchitecture of the classic hepatic lobule. The classic lobule is hexagonal, with a central vein in the middle. Blood from the hepatic arterioles and portal venules mixes in the sinusoids (square) and empties into the central vein. Bile is collected in the bile canaliculi which empty into the bile ducts on the outside of the lobule. The hepatic arteriole, portal venule and bile duct comprise the portal triad. (c) The sinusoids are lined with hepatocytes. Endothelial cells line the vascular walls, and the space of Disse is situated between the endothelial cells and the hepatocytes. Kupffer and stellate cells are also situated in the sinusoids. The figure was adapted from Bismuth2 and Siriwardena et al.2, 3
vein and the hepatic artery converge into sinusoids, which drain into the central vein.\(^2\) The diameter of a portal venule or hepatic arteriole is in the range of 15–35 µm.\(^4\) The sinusoids are lined with hepatocytes, and are about 7-15 µm in diameter.\(^2,4\) Each hepatocyte secretes bile into the bile canaliculi, which end in bile ductules and finally in the bile duct.\(^2\)

The liver contains endothelial cells (2.8% of volume), Kupffer cells (2.1%), stellate cells (1.4%) and hepatocytes (~80%) (Figure 1c).\(^2\) Endothelial cells line the sinusoids, and the bile ductules and ducts.\(^2\) Endothelial cells in the sinusoids are fenestrated, i.e. have pores which are 0.05–0.15 µm in diameter.\(^5\) The fenestrations increase the flow of plasma solutes, but not blood cells, to the space of Disse.\(^2\) Kupffer cells are fixed macrophages that remove particulate matter, such as lipids, from the blood in the sinusoids.\(^2,6\) Stellate cells are found in the space of Disse; these play a central role in fibrogenesis after liver injury, which can lead to cirrhosis.\(^2\) Hepatocytes perform most functions attributed to the liver.

**Function**

The liver metabolizes, detoxifies and inactivates exogenous and endogenous compounds from the blood.\(^2\) Compounds that are degraded or biotransformed by the liver can be excreted back to the circulation or into the bile. Through the Kupffer cells, the liver filters particulate matter, such as bacteria, endotoxins, parasites and aging red blood cells, from the blood.

The liver stores and synthesizes important substances.\(^2\) Carbohydrates, peptides, vitamins, and some lipids from food can be stored by hepatocytes, and released when needed. Hepatocytes can synthesize plasma proteins (e.g., albumin), as well as substances that are important for the metabolic demands of the body (such as glucose, cholesterol, and phospholipids).

**Hepatocellular carcinoma**

**Pathology**

Hepatocellular carcinoma (HCC) is a solid tumour in the liver (Figure 1a).\(^7\) The majority of HCCs (70-90%) occur in cirrhotic livers.\(^7\) Either single or multiple tumours can be found and the tumours have different patterns of growth. Multinodular HCC (multiple tumours scattered throughout the liver) and diffuse HCC (multiple small nodules which mimic cirrhotic nodules) have been associated with a cirrhotic liver. Massive HCCs (where a solid tumour occupies much of the liver, possibly with some small satellite tumours) are found in noncirrhotic livers. The tumour itself can be expansive (distinct border) or spreading/infiltrative (no distinct border). Dependent on the time of diagnosis, an HCC can range from less than 1 to over 30 cm in
diameter. HCCs are usually highly vascular, with a blood supply from the hepatic artery.

Incidence, mortality and risk factors

About 80-95% of primary liver cancers are classified as HCC, and the terms are thus often used interchangeably.8 Around 782,000 people globally are affected by primary liver cancer each year and the incidence is rising.9 This makes primary liver cancer the sixth most common cancer form globally. The incidence ratio for women:men is 1:2.4, which means more men are diagnosed each year with primary liver cancer.9 The global incidence is 4.4-to 5-fold higher in less developed regions compared to more developed regions. For example, around 50% of the incidence occurs in China alone.

Nearly 745,000 people die yearly from primary liver cancer, making it the second most common cause of cancer death after lung cancer (1.6 million deaths).9 It has a poor prognosis, since the incidence:mortality ratio is around 1. There are no bigger differences in this ratio between sexes and regions.

The main causes of liver cirrhosis are the hepatitis B and C viruses and alcoholic liver disease. Hepatitis B and C increase the risk of HCC by 5- to 15-fold and 17-fold, respectively. It can take 2–3 decades for cirrhosis to develop in patients infected with hepatitis B or C. Heavy alcohol intake (>50–70 g/day) over prolonged time periods increases the incidence ratio for HCC 6-fold. Diabetes and obesity are risk factors as they increase the development and progression of hepatic fibrosis. Other risk factors for HCC are consumption of aflatoxin-contaminated foods, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, and tobacco smoking.7

Treatment

Clinical guidelines for the treatment of HCC have been published by the European Association for the Study of the Liver and the European Organisation for Research and Treatment of Cancer.10 In these guidelines, treatment strategies are based upon the staging, or severity, of the disease. The BCLC (Barcelona Clinic Liver Cancer) classification divides HCC into 5 stages, from very early to advanced. The BCLC classification is based upon tumour size and nodularity, the Eastern Cooperative Oncology Group performance status (PS), and the Child Pugh (CP) score. The PS is defined as the patient’s level of functioning on a scale of 0–5, i.e., the impact of the disease on the patient’s daily living abilities.11 The CP score reflects the prognosis of chronic liver disease and cirrhosis.12 A CP score of A to C is given, based on five clinical features (bilirubin and albumin levels, prothrombin time, degree of ascites and grade of hepatic encephalopathy).

The treatment of HCC depends on the stage of the liver cancer.10 At a very early stage (single nodule, <2cm; PS0; CPA) the tumour is resected. At
an early stage (single or up to 3 nodules, <3cm; PS0; CPA-B) the patient can receive a liver transplant or local ablation of the tumour by radiofrequency ablation or ethanol injection. At an intermediate stage (multinodular/multifocal tumour; PS0; CPA-B) the patient can receive image-guided transarterial tumour chemoembolization therapy. Patients with advanced stage HCC (multinodular/multifocal tumour with portal invasion; PS1–2; CPA-B) receive systemic treatment with the cytostatic agent sorafenib. Terminal stage patients (PS > 2; CPC) receive the best supportive care.

**Image-guided transarterial chemoembolization tumour therapy / TACE**

Image-guided transarterial tumour therapy is described as “the intravascular delivery of therapeutic agents via selective catheter placement with imaging guidance for the treatment of malignancy”.[13] A schematic overview of this locoregional treatment option is given in Figure 2. Three treatment methods can be used, namely embolization, chemoembolization and radioembolization. Chemoembolization is most commonly used in the treatment of intermediate HCC. It involves the local infusion of a cytostatic agent combined with embolization of the blood vessel. Historically, image-guided tumour therapy with chemoembolization has been called transarterial chemoembolization (TACE).

The location of administration and the used drug formulations make the recommended image-guided transarterial tumour therapy with chemoembolization interesting from a pharmaceutical perspective. The locoregional administration route could be seen as passive targeting of the tumour.

Two types of drug formulation have been used with TACE. The first is a cytostatic Lipiodol® emulsion; this formulation combined with the image-guided treatment procedure is usually called conventional TACE (cTACE). Historically, cTACE has described the administration of an aqueous solution with one or more cytostatic agents emulsified with Lipiodol® followed by additional embolization. Administration of this cytostatic Lipiodol® emulsion without embolization has been called TOCE (transarterial oily chemoembolization) or TAI (transarterial infusion). However, recent guidelines

**Figure 2.** A schematic overview of image-guided transarterial tumour therapy. A catheter is placed through the femoral artery into the hepatic artery by the interventional oncologist or radiologist. The drug formulation is then administered through the catheter and deposited close to the hepatocellular carcinoma (HCC).
suggest the use of cTACE combined with a description of the procedure, independently of whether additional embolization was used.\textsuperscript{13}

The second type of drug formulation is a drug-eluting entity (DEE) which, when combined with the image-guided treatment procedure, is usually called DEE- or drug-eluting bead (DEB)-TACE. Both treatment options are used globally in the clinic to treat patients with HCC. The median survival of patients with untreated intermediate HCC is 16 months.\textsuperscript{10} Chemoembolization treatment improves median survival to 19–20 months.\textsuperscript{10, 14} Meta-analyses of studies comparing cTACE and DEE-TACE show varying results. Generally, a trend for better disease control and overall survival with DEE-TACE has been observed.\textsuperscript{15-18}

**Drug formulations used in TACE**

**Cytostatic Lipiodol\textsuperscript{®} emulsions**

A wide variety of cytostatic Lipiodol\textsuperscript{®} emulsions are used clinically worldwide. Both single-drug and multiple-drug emulsions are used, where doxorubicin (DOX) is the most commonly used additional drug.\textsuperscript{14, 19} Of 49 relevant studies published since 2016, 18 used DOX, either as a single or multiple drug emulsion.\textsuperscript{20-37}

This thesis focuses specifically on LIPDOX. LIPDOX describes any emulsion of an aqueous solution containing DOX mixed with Lipiodol\textsuperscript{®} (LIP).

**Lipiodol\textsuperscript{®}**

Lipiodol\textsuperscript{®} (Guerbet, France) is a poppy seed oil which is iodinated (37\% w/w, or 480 mg/mL iodine), making it suitable as a contrast agent.\textsuperscript{38} It has in fact been used in radiological applications since the 1920s. Since the 1980s, Lipiodol\textsuperscript{®} has been used in the treatment of HCC. It is registered as an approved drug product by the US Food and Drug Administration, and has the status of orphan drug designated for the management of known HCC since 2013.

When administered through the hepatic artery of rats, Lipiodol\textsuperscript{®} appears in the portal venules, and passes through to the sinusoids.\textsuperscript{39} This causes a temporary and partial stasis of the blood flow. In HCC tumours, Lipiodol\textsuperscript{®} is visible for up to 90 days after administration. Several hypotheses have been suggested for the uptake and retainment of Lipiodol\textsuperscript{®} by HCCs: (i) high vascularity and large microvessels improve Lipiodol\textsuperscript{®} accessibility to the tumour, (ii) lack of Kupffer cells and lymphatic system in the tumour microenvironment reduces Lipiodol\textsuperscript{®} elimination, and (iii) direct capture of Lipiodol\textsuperscript{®} by tumour and endothelial cells.\textsuperscript{38}
Composition of LIPDOX

A variety of different LIPDOX emulsions have been used clinically, although the exact composition of the LIPDOX formulation has not always been described in published studies. The aqueous phase in the emulsions can contain water or a combination of water and contrast agent. As Lipiodol® has a high iodine content, its density is 1.28, which is higher than most aqueous liquids. Equalizing the density of the aqueous phase to the Lipiodol® density increases the emulsion stability. Clinically, this can be done by adding a contrast agent to the aqueous phase. Of the previously mentioned 18 LIPDOX articles published since 2016, three stated that a contrast agent was used to dissolve the drug. The dissolution medium was not specified in the remaining 15 articles. No mention of an emulsifier to further stabilize the emulsion was made in any of these 18 articles.

The ratio of the aqueous to Lipiodol® phases ranges from 4:1 to 1:3.33 in the clinic, but is mostly reported as 1:1. Only five of the 18 LIPDOX articles published since 2016 mentioned the aqueous:Lipiodol® ratio, which ranged from 1:1–3.3. The ratio of the phases can affect the emulsion stability; lower Lipiodol® ratios make the emulsion less stable. The aqueous:Lipiodol® phase ratio can affect the type of emulsion obtained after mixing. Water-in-oil (W/O) emulsions with small droplets (2–3 µm in diameter) are formed when the aqueous:Lipiodol® ratio is 1:2–4. Oil-in-water (O/W) or more complex emulsions with bigger droplets (6–11 µm in diameter) are formed with aqueous:Lipiodol® ratios of 2–4:1.

Clinical preparation of the emulsion

LIPDOX is often prepared extemporaneously using a pumping technique. That is, the syringe containing the aqueous phase with DOX and the syringe containing Lipiodol® are connected with, for example, a three-way stopcock. The liquids are then mixed by pumping them back and forth between the two syringes, as shown in Figure 3.

This preparation procedure is not standardized, which affects the characteristics of the formed emulsion. For example, while using the pumping technique, droplet size ranges of 2–3 µm and 30–120 µm have been observed. The smaller size range was obtained by pumping the solutions back and forth for 5 minutes, while the larger size range was obtained by 20 pushes and pulls through the syringes.
The DOX dose is not standardized in LIPDOX. From the 18 reviewed LIPDOX articles published since 2016, the DOX dose ranged from 20 to 100 mg and 25 to 75 mg/m² per treatment procedure.20-37

Administration
LIPDOX can be administered lobularly, segmentally, or subsegmentally, and all administration sites are used clinically. LIPDOX administration can be followed by administration of embolic particles.38 These embolic particles can be biodegradable (e.g. gelatine sponge) or permanent (e.g. polyvinyl alcohol microparticles). From the 18 reviewed LIPDOX articles published since 2016, anywhere from 2-20 mL LIPDOX has been administered during a treatment procedure.20-37 The Lipiodol® dose is dependent on the tumour size, tumour physiology, and condition of the liver.40

Drug-eluting entities
Several types of DEEs can be used in image-guided TACE; for example, DC Bead™ (DCB), HepaSphere™, LifePearl® and Tandem™.47 Of these, DCB and HepaSphere™ are used globally48, with more publications related to DCB. In this thesis, the abbreviation DEBDOX is used to describe DOX-loaded DEEs (DCB was the specific DEB used) of any size and with any DOX loading.

DC Bead™
DCB (Biocompatibles Ltd, UK) is a polyvinyl alcohol-based hydrogel.40 The hydrogel contains negatively charged sulfonate groups, to which sodium ions are bound prior to loading. These hydrogels are biocompatible and non-biodegradable. DCB has been available for DEE-TACE since 2006. There are several sizes of DCB hydrogels currently on the market: 70–150 µm (M1), 100–300 µm, 300–500 µm, and 500–700 µm.49 The size ranges 700-900 µm and 900-1200 µm were previously available.

Loading of drug
Positively charged substances, such as DOX and irinotecan, can be loaded into DCB. Loading of DOX is achieved by an ion-exchange mechanism and self-aggregation of DOX.50 51 Upon removal of the sodium solution and addition of DOX solution, up to 40 mg DOX can be loaded per mL DCB.52 The loading time depends on the concentration of the loading solution, the amount to be loaded and the bead size, and can range from 30 min (70–150 µm, 25 mg/ml DOX solution) to 24h (500-700 µm, 2 mg/ml DOX solution). Clinically, DCB is usually loaded with 37.5 mg DOX per mL DCB, a total of 75 mg DOX is thus loaded into each vial of DCB.
Administration
The administration of DEBDOX is rather standardized, and has been described in both the literature and the package insert.\textsuperscript{49, 53} DEBDOX should be administered segmentally or subsegmentally (superselectively) whenever possible, to avoid embolization of vessels not leading to the tumour. It is recommended that the smallest size of DCB is administered first during treatment, as smaller sizes will penetrate deeper into the tumour. However, the hydrogel size should be chosen according to the pathology. Before administration, DEBDOX is mixed with a non-ionic contrast agent. During infusion, the administration rate should be slow and the syringe should be agitated to avoid sedimentation of DEBDOX in the syringe. A maximum dose of 150 mg DOX can be administered during one treatment.

The cytostatic drug doxorubicin
DOX, formulated in emulsion with Lipiodol\textsuperscript{®} or loaded into DEEs such as DCB, is the most commonly used cytostatic drug for treatment of HCC. It is a bright red powder and forms an orange-red solution in water. Structurally, DOX comprises an aglycone with a daunosamine group (sugar component) attached by a glycosidic bond.\textsuperscript{54} The physicochemical properties and molecular structure of DOX and its active metabolite doxorubicinol (DOXol) are presented in Table 1.

<table>
<thead>
<tr>
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<th>Doxorubicin</th>
<th>Doxorubicinol</th>
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<td>C\textsubscript{27}H\textsubscript{31}NO\textsubscript{11}</td>
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<td>Polar surface area (Å\textsuperscript{2})</td>
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<tr>
<td>Hydrogen bond donors</td>
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<td>7</td>
</tr>
<tr>
<td>Hydrogen bond acceptors</td>
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<tr>
<td>Solubility (mg/ml, PBS)</td>
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<tr>
<td>LogP\textsuperscript{58-61}</td>
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<tr>
<td>LogD\textsubscript{7.4}\textsuperscript{62}</td>
<td>2.42±0.08</td>
<td>1.19±0.06</td>
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</table>

Pharmacology
DOX is an antineoplastic agent, classified as a cytotoxic anthracycline antibiotic (ATC code: L01DB01). It is used in the treatment of multiple forms of
cancer such as soft-tissue sarcomas, non-Hodgkin's lymphomas, and ovary, breast, and stomach cancers. There are at least three anti-tumour mechanisms mediating its effects: (i) reversible binding to topoisomerase I and II, (ii) intercalation to DNA base pairs, and (iii) free-radical generation, which causes DNA damage (Figure 4). The concentration of DOX required for 50% growth inhibition (IC\textsubscript{50}) in vitro is reciprocal with time and cell-line-dependent. Unpublished IC\textsubscript{50} values for DOX in three HCC cell lines (HepG2, Huh7 and SNU449) were 3–120 µM at 24 h and 0.1–70 µM at 72 h.

**Pharmacokinetics**

When DOX is administered via the intravenous route, the plasma pharmacokinetics (PK) have been described by both two- and three-compartment models. The first (distribution) phase is rapid, and the terminal (elimination) phase is slow. The half-life ranges from 3 to 47 min (distribution) and 14 to 50 h (terminal). In humans, DOX PK are not affected by the dose in the range 20–70 mg/m\textsuperscript{2}. The oral administration route is not used for DOX because of its low bioavailability (<3%), poor and variable intestinal permeability (around 0.1*10\textsuperscript{-6} cm/s) and low fraction absorbed from the intestine (>20%) in rats.

*Figure 4.* The toxicity and disposition of doxorubicin (DOX). DOX inhibits the binding of topoisomerase (TOP2A) to DNA, intercalates to DNA, and enzymatic reduction forms reactive oxygen species (ROS). These three mechanisms lead to cell death. DOX is distributed and eliminated by passive diffusion (arrows) and carrier-mediated transport (arrows with orange circle). DOX is metabolized to doxorubicinol (DOXol) by carbonyl reductases (CBR1, CBR3) and aldo-keto reductases (AKR1A1, AKR1C3). Information in the figure is based on published data.
Once infused into the blood circulation, DOX is distributed quickly to the tissues. Elevated DOX tissue concentration-time profiles have been observed in several species.\textsuperscript{77-79} Consequently, the tissue-to-plasma concentration partitioning coefficient \((K_{p,T})\) is high (ranging between 50 and 1300 in pig, rat, guinea pig and rabbit).\textsuperscript{80, 81} DOX is known to bind to DNA and the acidic phospholipid cardiolipin, which are found intracellularly in the nucleus and the mitochondria.\textsuperscript{82-86} Accordingly, intracellular distribution favours the nucleus (50-fold higher than intracellular), which is in good agreement with the reported anti-tumour mechanisms.\textsuperscript{54}

DOX passes through the cellular membrane by passive diffusion and carrier-mediated transport (Figure 4). Absorptive transport in Caco-2 cells has been speculated to occur mainly via the paracellular route.\textsuperscript{71} A multitude of transporters have been identified to facilitate DOX transport (Figure 4):\textsuperscript{37-40, 46}

- Solute carrier organic anion transporter family member 1A2 (OATP1A2, \textit{SLCO1A2}, uptake),
- Solute carrier family 22 member 16 (OCT6, \textit{SLC22A16}, uptake),
- Canalicular multispecific organic anion transporter 2 (MRP3, \textit{ABCC3}, efflux),
- Multidrug resistance protein 1 (MDR1, P-gp, \textit{ABCB1}, efflux),
- Breast cancer resistance protein (BCRP1, \textit{ABCG2}, efflux),
- Bile salt export pump (BSEP, \textit{ABCB11}, efflux),
- Canalicular multispecific organic anion transporter 1 (MRP2, \textit{ABCC2}, efflux),
- Multidrug resistance-associated protein 1 (MRP1, \textit{ABCC1}),
- Multidrug resistance-associated protein 6 (MRP6, \textit{ABCC6}),
- Multidrug resistance-associated protein 7 (MRP7, \textit{ABCC10}),
- ATP-binding cassette sub-family B member 8, mitochondrial (MABC1, \textit{ABCB8}),
- RalA-binding protein 1 (RLIP1, \textit{RALBP1})

DOX is eliminated by metabolism and excretion to bile and urine. It is metabolised to DOXol by aldo-keto and carbonyl reductases (AKR1A1, AKR1C3, CBR1, CBR3). These reductases are available in the cytosol of cells. The highest DOX-to-DOXol biotransformation rates have been found in the liver and kidneys, although intestine, heart, muscle and lung also have metabolic capacities.\textsuperscript{87} Other inactive, deglycosidated metabolites have been identified as well.\textsuperscript{88}

Within 6 to 7 days after administration of DOX, about 50% of the dose has been excreted to the bile and 12% to urine.\textsuperscript{89} The portion eliminated in the bile consists of unchanged DOX (about 50%), DOXol (23%), and other metabolites (27%). The portion eliminated in the urine is mainly DOX (about 66%), with metabolites making up the remainder.
Doxorubicinol
DOXol is about 75-fold less therapeutically active than DOX, but has been speculated to be the main cause of the cardio-related toxicity of DOX.\textsuperscript{90, 91} The half-life of DOXol in humans is similar to that of DOX, around 30h.\textsuperscript{66} The elimination half-life of DOXol is most likely formation rate-limited. The half-life after DOXol injection to dogs was 3.7 h, while it was up to 30h after administration of DOX.\textsuperscript{92}

Pharmacokinetic modelling
Mathematical or \textit{in silico} modelling is frequently used in the field of PK to describe or elucidate the mechanisms behind the concentration-time profiles from plasma, tissue or the gastro-intestinal (GI) tract.\textsuperscript{93} Different types of modelling strategies can be adopted for this purpose, where the most common one is by compartments connected to each other by mass-transport.\textsuperscript{93}

Compartmental models
Compartmental models usually contain a certain number of compartments needed to be able to describe the different phases of the plasma concentration-time curves: absorption, distribution and elimination.\textsuperscript{93, 94} One-compartment models describe the plasma concentration-time curve after intravenous administration of a substance with first-order elimination kinetics. The mass-transport to and from the compartment is commonly described by rate constants (k), or clearance (Cl) and volume (V) as in the following equation (Eq 1).

\[
\frac{dc}{dt} = -k \times C = -\frac{Cl}{V} \times C
\]  \hspace{1cm} (1)

Multi-compartment models are used when there is a multi-phase decline in the plasma concentration-time curve.\textsuperscript{94} Here the compartments describe a central compartment and one (or several) peripheral compartment(s). The central compartment usually describes the plasma, while the other compartments are used to represent drug distribution to other parts of the body, e.g., peripheral tissues. The mass transport between two compartments is described by Eq 2.

\[
V_1 \frac{dc_1}{dt} = -Cl \times C_1 + Cl \times C_2
\]  \hspace{1cm} (2)

When a compartmental model is fitted to the observed data, the values of the micro-constants (k, Cl, V) are estimated. From these values, PK parameters such as half-life (t\textsubscript{1/2}) and area under the plasma concentration-time curve (AUC) can be derived. Multi-compartmental models are usually used in a descriptive manner.
Physiologically based pharmacokinetic models

Physiologically based pharmacokinetic (PBPK) models are multi-compartmental models. Each compartment represents a specific physiologically entity, e.g. plasma or tissue, described with representative values such as tissue weight or volume. The tissue compartments are connected by flow rates which represent the blood flows to and from the tissue. PBPK models commonly include “lumped” tissue compartments. Lumped compartments describe a collection of tissues for which the specific concentrations are irrelevant to the purpose of the specific PBPK model, but which have the same properties, e.g. rapid or slow blood flow. PBPK models with lumped tissue compartments are usually described as semi-PBPK models.

Distribution of the drug to and from the tissue compartment is commonly regarded to be either perfusion or permeability limited. When the distribution is perfusion limited, Eqs. 3 and 4 describe the distribution of the drug from the concentration on entering the arterial blood ($C_{ab}$), to that on exiting the venous blood ($C_{vb,T}$) and that in the compartment tissue ($C_T$):

\[
V_T \frac{dC_T}{dt} = Q_T (C_{ab} - C_{vb,T}) \tag{3}
\]

\[
C_{vb,T} = \frac{C_{T+B:P} + F_{up}}{K_{p,T}} \tag{4}
\]

where $V_T$ describes the tissue volume, $Q_T$ the blood flow to and from the tissue, B:P the blood-plasma ratio of the drug and $F_{up}$ the fraction of unbound drug in plasma. However, if the tissue distribution is permeability limited, Eqs. 3 and 4 cannot be used. The tissue compartment will then probably be divided into two or more subcompartments describing the place of the rate-limiting step, such as the cell membrane. The distribution could be described by the rate of clearance over the permeability barrier.

Because of the physiological relevance of PBPK models, bottom-up approaches can be applied. This means that specific biological processes can be scaled up from in vitro experiments. For example, the intrinsic hepatic clearance could be scaled up to the whole liver from in vitro experiments on isolated hepatocytes.

PBPK modelling has been made easier in recent years by the availability of open source and commercial software, such as PK-sim, GastroPlus and Simcyp Population-Based Simulator.

Physiologically based biopharmaceutical (PBBP) models

PBPK models which emphasize the release of the drug from the formulation or drug delivery system are also known as PBBP models. This term was introduced in 2014 for a model that described the release and PK of 2-hydroxyflutamide from a modified-release formulation to prostate tissue and plasma.
Aims of the thesis

This thesis seeks to improve understanding of drug formulations used in the treatment of intermediate HCC. The long-term goal of this project is to develop novel drug delivery systems with improved clinical outcomes. In order to optimize any process, one first needs to understand how the basics function. In this case, the LIPDOX and DEBDOX formulations were studied from a biopharmaceutical perspective.

- The aim of **Paper I** was to evaluate the effect of the excipient Lipiodol® and cyclosporine A (ciclosporin; CsA) on the hepatobiliary disposition of DOX and DOXol in an advanced multisampling-site acute pig model. CsA was used as a positive control in this study, since it has an inhibitory effect on the biliary excretion of DOX. Multi-compartment PK modelling was used to evaluate the effect of CsA on the disposition of DOX and DOXol.
- The aim of **Paper II** was to evaluate the *in vivo* release of DOX from LIPDOX and DEBDOX in human patients with HCC. In addition, urinary excretion of DOX from these formulations, and their efficacy and safety in human patients with HCC, was assessed.
- The aim of **Paper III** was to describe the PK of DOX and DOXol in healthy pigs using PBPK modelling, in order to describe the pronounced intracellular binding of these compounds.
- The aim of **Paper IV** was to predict the *in vivo* release of DOX from LIPDOX and DEBDOX in healthy pigs and human patients with HCC using biopharmaceutical modelling and simulation.
Methods

In vivo work

Two in vivo studies were carried out (these are described in Papers I and II). Paper I describes the effect of Lipiodol® and/or cyclosporine A (CsA) on the disposition of DOX and DOXol in the advanced multi-sampling site acute (aMSA) pig model. This pig study is also referred to as non-clinical study 2 (NC2) throughout this thesis. Drug concentrations in the tissues sampled in this study were published in Paper ii.80 Paper II describes the in vivo release of DOX and DOXol from LIPDOX and DEBDOX in a clinical study of patients with HCC, and the subsequent plasma PK.

Our group has previously published another DOX study in the aMSA pig model, by Lilienberg et al. (2014).98 This study is referred to in this thesis as non-clinical study 1 (NC1). NC1 comprised three treatment groups: those receiving a 50-minute intravenous infusion of DOX into an ear vein (IV group), those receiving LIPDOX via the hepatic artery (LIPDOX group), and those receiving DEBDOX via the hepatic artery (DEBDOX group). Plasma concentration-time and biliary excretion profiles were collected. Although the NC1 study was not performed as part of this thesis nor by the thesis author, the study is mentioned here as the historical data were used in the developed in silico models used in Papers III and IV.

Study design

Effects of Lipiodol® and CsA on DOX and DOXol disposition (NC2)

Twelve male, mixed-breed pigs were divided into four treatment groups (TI-TIV; Figure 5). Each pig received two consecutive intravenous infusions of 57.8 µmol DOX (at 0-5 and 200-205 min) into the right ear vein. Before the second dose of DOX was administered, each treatment group received additional treatment administered into the portal vein. Thus, at 165–185 min, groups TI and TII received an infusion of saline, while groups TIII and TIV received an infusion of 250 mg CsA. Then, at 190–195 min, groups TI and TIII received Lipiodol® emulsion (6 mL Lipiodol®, 1.8 mL H₂O).

Blood was sampled from the hepatic vein and the hepatic artery (before and after the liver, local samples) and the femoral artery (systemic, peripheral samples) during the whole study period (Figure 5). Bile was sampled at 20
Figure 5. The NC2 study design for the advanced multi-sampling site pig model. Doxorubicin (DOX, 57.8 µmol, black box) was administered into the right ear vein at 0-5 and 200-205 minutes. Lipiodol® (LIP, 6 mL in 1.8 mL sterile water, grey box) was administered via the portal vein to groups II and IV at 190-195 minutes. Cyclosporine A (CsA, 250 mg, grey box) was administered to groups III and IV at 165-185 min. When no Lipiodol® or CsA was administered, saline solution was administered (white box). Plasma ( | ) and bile (—X—) were sampled throughout the study period. Urine was collected continuously and sampled at the end of the study period (—*). Tissue samples from kidney, liver, lung and intestine were collected at the end of the study period (∆).

minute intervals. Urine was collected throughout the study period. At the end of the study period (360 min), the urine was sampled. The pigs were euthanized and tissue samples from liver, kidney, heart and intestine were collected (Paper ii).

Clinical study of DOX release from LIPDOX and DEBDOX and PK comparison
Patients with HCC admitted to treatment centres at Uppsala University Hospital and Karolinska University Hospital were divided into two study arms (according to the hospital they attended) for this open, prospective, non-randomized, multicentre study. All patients were scheduled for 4 visits (Table 2). During Visit I, patients were screened for inclusion and exclusion criteria, their blood was sampled and their tumours were imaged. During Visit II, patients received the image-guided transarterial tumour therapy standard with chemoembolization at each clinic. Local (orifice hepatic vein in vena cava) and systemic (femoral vein) blood samples for PK analysis were collected up to 6 hours after the end of treatment. Systemic blood samples for PK analysis and safety sampling were collected at 24h. Urine was collected for 24h and then sampled. During Visit III, blood samples were collected for PK analysis and safety. During Visit IV, patients received follow-up tumour imaging.
Table 2. Study design for the clinical trial

<table>
<thead>
<tr>
<th>Time period</th>
<th>Visit I</th>
<th>Visit II</th>
<th>Visit III</th>
<th>Visit IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening</td>
<td>Pre-treatment</td>
<td>0–24h</td>
<td>5–7 days</td>
<td>4–6 weeks</td>
</tr>
<tr>
<td>Blood sample: safety</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Blood sample: PK</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Tumour imaging</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

**Uppsala University hospital**

- Included patients: 15
- Tumour imaging technique: MR
- Catheter placement: Lobular
- Drug delivery system: LIPDOX (no embolization)

**Karolinska University Hospital**

- Included patients: 15
- Tumour imaging technique: CT
- Catheter placement: Subsegmental
- Drug delivery system: DEBDOX

CT = computed tomography; DEBDOX = doxorubicin-loaded DC Bead™; LIPDOX = doxorubicin-in-Lipiodol® emulsion; MR = magnetic resonance; PK = pharmacokinetics.

Patients at Uppsala University Hospital received LIPDOX without additional embolization via a catheter with lobular placement. The LIPDOX emulsion contained an aqueous solution of 2.56 mL iohexol, 0.44 mL sterile water and 50 mg DOX mixed with 10 mL Lipiodol®. LIPDOX was constituted extemporaneously immediately before administration by pumping the liquids back and forth between two syringes.

Patients at Karolinska University Hospital received DEBDOX via a catheter placed superselectively (subsegmentally). DEBDOX consisted of DOX-loaded DCB (37.5 mg DOX/mL DCB). All available bead sizes were used. Unloaded DCB hydrogels were administered if blood flow stasis was not obtained after the administered dose (150 mg DOX).

**Sampling, sample work-up and analysis of biological matrices**

Blood samples were collected in EDTA vacutainers and stored on ice. Blood samples were centrifuged within 30 min of collection (Papers I and II). Urine was collected continuously until the end of the blood sampling period (Papers I and II). Bile was sampled on ice at 20 min intervals during the whole study period (Paper I). All biological matrices were aliquoted to dark polypropylene vials to protect DOX from light and were stored at −20 °C pending analysis.
All further sample work-up and analysis of the biological matrices was performed by employees at SVA (Statens Veterinärmedicinska Anstalt/National Veterinary Institute). Sample work-up and analysis with ultraperformance liquid chromatography-tandem mass spectrometry is described in Papers I and II, as well as in Lilienberg et al.98

**In silico models**

Different types of *in silico* models were used to describe and explore the disposition of DOX and DOXol in pigs and humans. A multi-compartment model was used in Paper I to elucidate the effect of CsA on DOX and DOXol disposition. In Paper III, semi-PBPK models were developed to describe the disposition of DOX and DOXol after intravenous administration to healthy pigs. PBBD models were used to evaluate the *in vivo* release of DOX from LIPDOX or DEBDOX in pigs and HCC patients (Paper IV).

**Multi-compartment model**

**Model development**

The mechanisms behind the effects of Lipiodol® or CsA on DOX and DOXol disposition in the NC2 study were investigated with a multi-compartment PK model (Figure 6). A three-compartment physiological model structure, representing plasma (central), tissue and liver, was adopted with first-order reactions describing the disposition processes (distribution/elimination). Eq. 5 describes the first-order mass transport in the model:

$$\frac{dA}{dt} = C \times k \times V$$

where A is the amount of compound and t the time.

![Figure 6. Multi-compartmental model describing the disposition of doxorubicin (DOX) and its metabolite doxorubicinol (DOXol). Ovals represent the distribution compartments for DOX and DOXol. Distribution to and from tissue, plasma, liver, bile and urine is shown with solid arrows, while metabolism of DOX and DOXol is shown with dashed arrows.](attachment://Figure%206.png)
Study design
The multi-compartmental model was fitted to the reference phase (0-160 min, P1) of all the observed NC2 data (n=12) to estimate 12 model parameters. Thereafter the model and the estimated parameters were used to simulate the effect of Lipiodol® or CsA on the disposition of DOX and DOXol. Inhibition of metabolic and biliary excretion parameters was simulated by reducing parameter values to 1% of the estimated values in various combinations. The simulated inhibition started at 200 min and was constant until the end of the simulation (360 min). These simulations were compared with the observed porcine data.

Semi-PBPK models
Two semi-PBPK models were developed in Paper III to describe the observed DOX and DOXol PK profiles. These PK profiles consisted of data from the NC198 (IV group) and NC2 (Paper I and Paper II) studies.

Model development
The two semi-PBPK models, one generic and one binding-specific, followed the same basic model structure (Figure 7). The models comprised six tissue compartments, two blood compartments (arterial and venous) and two excretion compartments (bile and urine). The liver and kidney compartments were divided into three sub-compartments: vascular, extracellular and cellular. Lung, GI/spleen, and slowly and rapidly perfused tissues were each described as one compartment. The binding-specific semi-PBPK model also included an intracellular binding site (IBS) in each tissue compartment or tissue cellular subcompartment (liver and kidney). This model structure was adapted to describe the disposition of DOX and DOXol. The mass transfer between blood and tissue compartments was described by differential Eq.s 3 and 4 (see section in Background headed “Physiologically based pharmacokinetic models”, page 22). Distribution between the vascular and extracellular sub-compartments in the liver and kidney was described by the capillary wall diffusion clearance.

Intracellular binding sites (IBSs) were available in the binding-specific model, and distribution to and from the IBSs occurred as described in Eq. 6. Here the association clearance (Cl_on) is set to its estimated value; as the intracellular binding site concentration (C_t,IBS) reaches its maximum binding, Cl_on was set to 0. The dissociation clearance (Cl_off) remained equal to its estimated value.

\[ V_{t,IBS} \frac{dC_{t,IBS}}{dt} = Cl_{on} * C_t - Cl_{off} * C_{t,IBS} \]  (6)
The model structure for the generic and binding-specific semi-PBPK models. Note that the intracellular binding site is only included in the binding-specific model.

Metabolism in the liver and kidney was described by Eq.s 7 and 8, where $V_{\text{max}}$ and $K_m$ are constants of the Michaelis Menten equation, $C_{\text{cel}}$ is the concentration in the cellular subcompartment, $SF_{\text{met}}$ is the scaling factor correcting for interspecies and organ differences, $MTPMH$ is the total protein content of hepatocytes, and $HPGL$ is the hepatocellularity.

$$\text{Cl}_{\text{met}} = \frac{J_{\text{max}} C_{\text{cel}}}{K_m + C_{\text{cel}}} \times SF_{\text{met}}$$ (7)

$$J_{\text{max}} = V_{\text{max}} \times MTPMH \times HPGL \times V_t$$ (8)

Hepatic and renal excretion ($Cl_{\text{excr}}$) to bile and urine was described by unidirectional linear kinetics. Biliary excretion was modelled to take place from the hepatocellular subcompartment, while urinary excretion was specified from the vascular space via glomerular filtration (glomerular filtration rate, GFR) and from the kidney cellular space via renal excretion.

**Study design**

The generic and binding-specific semi-PBPK models were fitted to NC2 data from pigs not receiving CsA (groups TI and TII; Paper I and Paper ii\textsuperscript{80}). The parameters were estimated during the fitting of the model to the plasma concentration-time profiles, biliary and urinary excretion profiles and liver and kidney concentrations, simultaneously. The model that fitted the observed data best was chosen to continue the study.
The performance of the best model (binding-specific) was then evaluated by simulating the observed data from the NC1 data\textsuperscript{98} (subset: IV group). Here, 4 pigs received a 50-minute infusion of DOX into an ear vein. Data for plasma concentrations and biliary excretion were available.

Because of differences observed between simulated and observed data from NC1, the model was fitted to the IV group of the NC1 data set. A reduced number of parameters was estimated, as urinary excretion and tissue concentrations were not available in this study.

Finally, a sensitivity analysis was performed. The estimated parameters were increased and reduced 2-fold from the original value. Each parameter was evaluated separately from other parameter changes.

PBBP models

Porcine and human HCC PBBP models were developed from the binding-specific PBPK model. The aim of the PBBP models was to describe DOX PK profiles after administration of LIPDOX or DEBDOX to pigs and HCC patients.

Model development

The following alterations were made for the porcine and human HCC PBBP models:

- The liver compartment was split into two sections: untreated and treated. Each liver section contained the vascular, extracellular and cellular sub-compartments. Distribution parameters and blood flow were adjusted to the chosen size of the liver section.
  The treated and untreated liver sections were supplied with blood from the portal vein (80%) and the hepatic artery (20%) in the pig PBBP model. In the human PBBP model, the treated section was supplied with blood from the hepatic artery (100%). Blood flow to the untreated section was adjusted accordingly.
- The administration site was changed to the vascular compartment of the treated liver section.

The following alterations were also included in the human HCC PBBP model:

- Species-specific parameters were changed from porcine to human values.
- The option to simulate different degrees of liver cirrhosis in the untreated liver section was included, classified using the CP classes. Simulated liver cirrhosis altered the cardiac output, organ blood flow, GFR, fraction of unbound drug, blood:plasma ratio and the functional liver mass.\textsuperscript{99, 100}
DOX release from LIPDOX and DEBDOX

In the PBPK models, the input of DOX into the system was modelled as a constant infusion, i.e., a constant inflow of DOX per time unit during the administration period. When administering LIPDOX or DEBDOX, the input of DOX into the system is equal to the release rate of DOX from each of the formulations. The release rate of DOX from these formulations is not constant over time. To model the input of DOX into the pig and human HCC PBBP models, 53 release data sets were obtained from published reports or via personal communication. A modified version of the Weibull equation (Eq. 9) was fitted to the release data sets to mathematically describe the release profiles and obtain release parameter estimates.

\[ A_{rel} = \text{dose} \times F_{rel,\text{max}} \times (1 - e^{-At^B}) \]  

where \( A_{rel} \) is the cumulative amount/percentage/fraction released, \( \text{dose} \) is the dose loaded into the formulation, \( F_{rel,\text{max}} \) is the maximum fraction of the dose that could be released from the formulation, \( t \) is time (min), and \( A \) and \( B \) are constants affecting the shape of the release curve.

The release of DOX into the porcine PBBP model was described using a derivative of the Weibull equation (Eq. 10) and the estimated release parameters from the release data sets. It was assumed that the formulation was administered as a bolus dose.

\[ R_{DDS} = \text{dose} \times F_{rel,\text{max}} \times A \times B \times t^{B-1} \times e^{-At^B} \]  

where \( R_{DDS} \) is the rate of release of DOX from the formulation. In the human HCC PBBP model, the following equations (11 and 12) were used to describe the release of DOX from the administered formulation after the first minute, assuming a constant administration rate.

\[ R_{DDS,t=i} = \frac{\text{dose} \times F_{rel,\text{max}} \times A \times B \times (t_i - (i))^{B-1} \times e^{-A(t_i-(i))^B}}{t_{inf}} \]  

\[ R_{DDS,\text{total}} = \sum_{i}^{t_{inf}} R_{DDS,t=i} \]

Embolication of treated vessels

In the porcine and human HCC PBBP models, it was assumed that LIPDOX and DEBDOX caused embolization of the blood supplying arteries (hepatic artery and portal vein in pig, and hepatic artery in human HCC). The embolization caused by LIPDOX is described by Eq.s 13 and 14. Blood flow (Q) in the treated area (\( F_{vol,tr} \)) is gradually recovered after administration of LIPDOX (after 3 days in rats).

\[ Q_{tr} = Q_{HA or VP} \times F_{vol,tr} \times (1 - e^{-k \times t}) \]  

\[ k = \frac{\ln(2)}{\text{time to recovery}/7} \]
DEBDOX is administered until complete and irreversible embolization in the treated hepatic artery is achieved. However, the treated section is often supplied by two or more hepatic arteries and DEBDOX will only embolize the specific vessel used for its administration. In line with this, a permanent embolization of 50% from the first minute of administration was applied during simulations with DEBDOX.

**Study design**

The PBBP models were used to predict the DOX plasma concentration-time profiles of HCC patients treated locoregionally with LIPDOX or DEBDOX.

In order to predict these concentrations, 53 release data sets were collected and values for release parameters were estimated. The release parameters from each data set (separate) were used in the porcine PBBP model to simulate in vivo plasma concentration-time profiles and biliary excretion. The simulations were compared with the observed PK porcine data from NC1 (groups LIPDOX and DEBDOX). The DOX release data sets with the best predictive properties were identified.

The identified release data sets were thereafter used in the human HCC PBBP model. The resulting simulated plasma concentration-time curves were compared with observed plasma concentration-time curves for human HCC patients (*Paper II*).

**Software and software parameters**

The multi-compartmental model was developed using Berkeley Madonna (Version 8.3.18, University of California at Berkeley). All parameter estimations were performed using Phoenix 64 WinNonlin 6.3 (Certara USA, Inc., USA). Standard settings used in model options were: 1/ŷ² weighting scheme for plasma PK profiles, and uniform weighting scheme for biliary and urinary excretion.

**Data analysis**

**PK data analysis**

**Non-compartmental analysis**

DOX and DOXol plasma concentration-time curves, and biliary and urinary excretion were analysed using non-compartmental analysis (NCA) using Phoenix WinNonLin 6.3 (*Papers I, II, IV*). All NCAs were performed using the pre-set settings with some adjustments. The linear and logarithmic trapezoidal computational method was used for the AUC calculations. A 1/ŷ² weighting scheme was used for plasma PK profiles, and a uniform weighting scheme was used for biliary and urinary excretion. The following PK param-
eters were reported from the NCA: maximum concentration ($C_{\text{max}}$), time to $C_{\text{max}}$ ($t_{\text{max}}$), AUC, $t_{1/2}$, Cl, volume of distribution at steady state ($V_{\text{ss}}$), fraction excreted ($f_e$). Other PK parameters were calculated from these PK parameters: apparent hepatic extraction ratio ($E_H$), apparent biliary clearance ($C_{\text{app,bile}}$), apparent urinary clearance ($C_{\text{app,urine}}$).

**Compartmental analysis**

The plasma concentration-time profiles of the reference study phase (P1, 0-160 min) for the NC2 data set (Paper I) were evaluated using compartmental analysis. The plasma concentration–time curves for DOX from each sampling site (portal, hepatic and femoral vein) were analysed using one-, two- and three-compartment models, adopting a $1/\hat{y}^2$ weighting scheme.

**Deconvolution: in vivo release from LIPDOX and DEBDOX**

In Paper II, the in vivo release rate of DOX from LIPDOX and DEBDOX was estimated by deconvolution of the HCC patient plasma concentration-time curve data using WinNonLin and a three-compartment model. As no individual reference intravenous plasma concentration-time curves were available, observed intravenous DOX plasma concentration-time curves for HCC patients were obtained from the literature. Frel from LIPDOX or DEBDOX into the systemic (femoral vein) and local (orifice hepatic vein/vena cava) sampling sites was determined at 6h and 24 h (systemic only).

**Analysis of additional output**

The results from the models were compared during model development, curve fitting and parameter estimation, and model discrimination. Different methods were used to compare output.

The Akaike Information Criterion (AIC) and Schwarz Bayesian Criterion (SBC) relate the weighted residual sum of squares to the number of parameters used in the curve fitting. The lower the number, the better the result. These values were used to evaluate whether a one-, two-, or three-compartment model best fitted the observed data (Paper I), to evaluate the best fitting developed multi-compartment model (Paper I), and to evaluate the best fitting semi-PBPK model (Paper III).

The results were also visually evaluated; fitted or simulated curves that best followed the observed PK profiles were deemed the best. This was applied in Papers I, III and IV.

Finally, the results of model development (Papers III and IV), and agreement between observed and simulated or fitted data sets, or agreement between two simulated data sets, were evaluated with the absolute average fold error (AAFE, eq. 15) and the average fold error (AFE, eq 16).
The AAFE can only be 1 or greater than 1 and the accuracy of the simulation gets better the closer to 1 AAFE comes. An AAFE value of one indicates a perfect agreement between data sets while a value of two indicates an average 2-fold difference between evaluated data sets. The AAFE does not indicate how the data sets relate to each other, it only indicates a difference. AFE shows the direction of the difference between the data sets, and was used as such.

\[
AAFE = 10 \frac{\sum |\log(\text{new/old})|}{N} \quad (15)
\]

\[
AFE = 10 \frac{\sum \log(\text{new/old})}{N} \quad (16)
\]
Results and discussion

*In vivo* release of DOX from LIPDOX and DEBDOX

An *in vivo* input rate of DOX from LIPDOX and DEBDOX in human HCC patients was obtained in Paper II, by deconvolution of plasma concentration-time profiles. The *in vivo* input rate of DOX from LIPDOX and DEBDOX in pigs was previously studied in NC1.

Importance of site of measurement or sampling sites

The site of measurement, which here is equal to the sampling site, is important when determining the *in vivo* release rate, or rather the *in vivo* input rate. As the name suggests, the input rate describes the rate of DOX coming in to the sampling site, while the release rate describes the rate of DOX released from the formulation. For both LIPDOX and DEBDOX, the assumption was that the formulation would stay close to the administration site, i.e. passive targeting of the liver tumour.

The input rate and cumulative input of DOX could be lower than the release rate or cumulative release of DOX from the formulations depending on the distance from the site of administration (e.g. pre-tumour) to the site of measurement (e.g. femoral vein), because of distribution of DOX to the tissues. This theory was confirmed by the observed decrease ($P = 0.0039$) in cumulative DOX input from LIPDOX from the local to systemic sampling sites in the pig (Table 3). No significant decrease was observed for cumulative input from DEBDOX or in human HCC patients. However, it should be

<table>
<thead>
<tr>
<th>Sampling site/ site of measurement</th>
<th>Species</th>
<th>LIPDOX</th>
<th>DEBDOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vena hepatica</td>
<td>Pig (NC1)</td>
<td>100±44% (n=4)</td>
<td>30±11% (n=4)</td>
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<tr>
<td>Orifice vena hepatica/vena cava</td>
<td>HCC patient</td>
<td>60±21% (n=13)</td>
<td>14±5.6% (n=11)</td>
</tr>
<tr>
<td>Systemic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral vein</td>
<td>Pig (NC1)</td>
<td>48±29% (n=4)</td>
<td>16±6.5% (n=4)</td>
</tr>
<tr>
<td>Femoral vein</td>
<td>HCC patient</td>
<td>51±17% (n=13)</td>
<td>12±4.6% (n=11)</td>
</tr>
</tbody>
</table>
noted that the local sampling sites in pig and human were slightly different. The local sampling site (the hepatic vein) was closer to the administration site for the pig than the sampling site (the orifice of the hepatic vein in the vena cava) for the HCC patients. This means that the human blood sample from the hepatic vein could have been diluted with blood from the vena cava. Accordingly, the local cumulative input from LIPDOX was higher ($P = 0.0075$) for pigs than for human HCC patients (Table 3). The systemic site of measurement was the femoral vein for both species, and no significant differences in cumulative input were found between these.

The importance of the sampling site was also demonstrated with the porcine and HCC patient PBBP models (Paper IV). Predictions of in vivo PK profiles were improved when the local rather than systemic cumulative input from pigs was used to simulate release of DOX from LIPDOX and DEB-DOX. However, the peak plasma concentration was still 2-fold underpredicted when the in vivo local cumulative input data sets were used, suggesting an underprediction of the actual release rate.

These results strongly suggest that the cumulative input into the site of measurement is not similar to the in vivo cumulative release from a formulation. The input into the site of measurement will be affected by release from the formulation and disposition of the drug en route to the sampling site. Consequently, the in vivo release from the formulation can be higher, but not lower, than the input to the measuring site.

**DOX release from LIPDOX**

Preferentially, parenteral emulsions are O/W emulsions with an oil-soluble drug dissolved in the oil phase, and have a droplet size under 3 µm to prevent oil embolisms. The objective with a LIPDOX emulsion is the opposite: temporary embolization is wanted as an effect in addition to the assumed drug delivering properties of the emulsion.

**Formulation specifics affecting release**

The release of DOX from LIPDOX should be controlled and extended, and multiple studies have identified factors which influence this release (in vitro). One important aspect in in vitro studies is the emulsion type, since W/O emulsions result in the extended release of DOX from LIPDOX. Preliminary solubility studies showed that DOX is practically insoluble in Lipiodol® (0.006 mg/ml) and slightly soluble in pure water (8 mg/ml) and the contrast agent iohexol (4 mg/ml). When formulated as a LIPDOX emulsion, (preliminary) results showed that only 0.5–6% of the DOX content had partitioned to Lipiodol® within one hour of preparation, which suggests slow partitioning into Lipiodol®. Thus, when formulated in a W/O emulsion, DOX first needs to traverse the continuous outer Lipiodol® phase, which is the rate-limiting step. When formulated in an O/W emulsion, the DOX is
dissolved in the outer phase, and can mix instantaneously with the release media in \textit{in vitro} release studies.

Generally, the volumes of the two liquids determine the emulsion formed; the liquid with the bigger volume forms the outer or continuous phase.\footnote{113} This has been observed for LIPDOX emulsions as well. With an aqueous phase consisting of water or contrast agent, prepared with the pumping technique, and without addition of emulsifiers or densifiers, W/O emulsions are formed with aqueous:Lipiodol\textsuperscript{®} ratios of 1:2–4.\footnote{4524, 86, 88}

Another factor affecting extended release from LIPDOX is the stability of the emulsion. \textit{In vitro}, LIPDOX emulsions have been stabilized with emulsifiers (e.g. hydrogenated castor oil) and densifiers (e.g. contrast agent).\footnote{41, 106, 108} Emulsion stability was also affected by the speed of incorporation of DOX solution into Lipiodol\textsuperscript{®}. A slower incorporation created more stable emulsions.\footnote{44}

\textbf{In vivo release}

The \textit{in vivo} release rate from a LIPDOX emulsion was studied in Paper II. The LIPDOX formulation was composed of 3 mL aqueous phase (0.44 mL sterile water, 2.56 mL iohexol, 50 mg DOX) and 10 mL Lipiodol\textsuperscript{®}, and it was emulsified with the pumping technique. This should, in theory, form a stabilized W/O emulsion.

Our \textit{in vivo} results suggested, however, that release of DOX from this LIPDOX emulsion was not controlled. The input rate of DOX from LIPDOX steadily decreased over time and did not settle at a plateau (Figure 8), as would have been expected with a controlled-release product. The maximum input rate of DOX from LIPDOX was reached during the administration period. The period of release was slightly extended, as the cumulative

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Input rate (mg/min) and cumulative input (% of dose) of doxorubicin from LIPDOX into local and systemic sites of measurement. Data were obtained from deconvoluted hepatocellular carcinoma patients (human, n=11, Paper II) and porcine (pig, n=4, NC1\textsuperscript{98}) plasma data. Data are shown as means.}
\end{figure}
input reached a plateau within 1 h for 11 of 13 patients. After 6 h, the input rate was negligible. This suggests that the input rate is steered by the administration rate and also slightly by extended release from the formulation. Similar results were observed in the NC1 pig study (Figure 8), although a more gradual decline in input rate was observed.98 Simulations with the PBBP model (Paper IV) confirmed that this LIPDOX emulsion had no or only slightly extended release, as tested data sets of LIPDOX emulsions with extended in vitro release overpredicted the time to reach peak plasma concentrations. These simulations further suggested that >90% of the loaded DOX dose was released within 0.8 to 2.1 hours after administration. The simulations also suggested that in vitro release methods using a diffusion barrier, such as a dialysis membrane, do not produce in vivo-like release data sets.

The effect of different emulsion specifics on the plasma PK of DOX has been studied in vivo. In rabbits with VX2 carcinomas, lower peak plasma concentrations and plasma exposure were observed for up to 1 day for LIPDOX_W/O compared to LIPDOX_O/W or a DOX solution, and for a 1:4 (aq:LIP) emulsion compared to a 1:1 emulsion.43, 107, 116 In healthy dogs, a stabilized LIPDOX emulsion had a longer terminal half-life than a non-stabilized emulsion, causing lower peak plasma concentrations but higher 48h plasma exposure.108 In the NC1 pig study, where the same LIPDOX emulsion was used as in the HCC patient study (Paper II), there were no differences in the terminal half-life of DOX from LIPDOX or intravenous infusion.98 Again, this could suggest that the release of DOX from LIPDOX is not or is only slightly extended, as this would have altered the terminal half-life.

One possible reason for the lack of profoundly extended in vivo release of DOX from LIPDOX could be that the emulsion breaks upon contact with the blood stream. In fact, during administration of LIPDOX emulsions to healthy rats, the emulsion appeared as an oil drop containing water drops in the blood stream45 and then, in the sinusoids and upon contact with blood flow, the water droplets were washed away from the oil droplets. In general, the emulsions were distributed and circulated as if Lipiodol® were administered alone. Furthermore, the stability of a LIPDOX formulation is seldom tested in in vivo-like conditions, e.g. administered into high flows resembling blood flow. Breaking of a W/O emulsion would cause the inner aqueous phase to mix with the blood instantaneously, and no extended release from the aqueous phase would be possible. Only if DOX were dissolved in the Lipiodol® phase would extended release be possible. However, as mentioned above, the amount of DOX diffusing into the Lipiodol® phase is negligible.

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DOX release from DEBDOX

Formulation and method specifics affecting release

The *in vitro* release of DOX from DEBDOX has been extensively investigated. The size of the DCB hydrogels in DEBDOX affects the DOX release: as the diffusion pathway decreases with decreasing bead sizes, the *in vitro* release rate increases. A higher loading of DOX increased the release rate from DCB hydrogels, while the release rate was seemingly independent of DOX loading for DCB hydrogels. DCB hydrogels shrink during loading and swell during DOX release, and the minimum and maximum diameters of larger hydrogels change more. This difference in the change of diffusion pathway length could account for the impact of loading on release rate for the larger bead sizes.

The *in vitro* release method specifics can also influence the release rate. Increasing the ionic strength or the temperature, but not the pH, of the release medium increased the DOX release rate from DEBDOX. Increasing the flow rate of or the rotation speed in the release medium increased the release rate, which indicates an effect of hydrodynamics on release. Finally, complete DOX release from DEBDOX was only achieved when the release medium was repeatedly replaced, or when the release medium consisted of 9% NaCl or a 30:70 water:ethanol mixture containing 20% w/v potassium chloride.

In vivo release

The *in vivo* release rate of DOX from DEBDOX was studied in Paper II. DEBDOX was composed of DCB loaded with 37.5 mg DOX per mL DCB. All available bead sizes were used in the study: 70–150 µm (M1, 5 patients), 100–300 µm (10 patients), 300–500 µm (4 patients), and 500–700 µm (2 patients) as loaded DEBDOX, and 300–500 µm (2 patients), 500–700 µm (2 patients), and 700–900 µm (1 patient) as unloaded DEBDOX, to achieve complete embolization. Multiple bead sizes were used in most patients. Our *in vivo* results showed a burst release of DOX from DEBDOX in the deconvoluted human HCC plasma data. This is shown in Figure 9, where the input rate declined after a sharp increase for DEBDOX. A burst release, or the release of an initial bolus of drug before the release rate reaches a stable profile, is not uncommon for controlled-release formulations. A burst release from DEBDOX has been observed *in vitro* from hydrogels loaded with 5 mg, 10 mg or >45 mg DOX/mL DCB, although the percentage was not mentioned. Others have not mentioned an *in vitro* burst release from DCB. In the clinical study, a portion of DEBDOX was first mixed with a contrast agent, and then diluted with saline solution prior to administration. Upon mixing with saline solution, ions necessary for the release of DOX become available. It could thus be speculated that a small amount of
DOX had already been released from DEBDOX before its intra-arterial administration. However, ions needed for release are also available immediately after administration to blood.

Like the in vitro observations, the in vivo results suggested that the release of DOX from DEBDOX is extended and controlled. The input rate of DOX from DEBDOX reached a plateau after 1.5–2 hours, which was maintained for at least 14 hours (Figure 9). Cumulative input did not reach a plateau at the systemic sampling site; it increased from 12% at 6h to 15% at 24h. These results were again a good match with the NC1 pig data. The peak input rate occurred later in the clinical study than in the porcine study, which could simply be explained by the longer administration times in humans (mean 13 min) compared to pigs (~2 min). The simulations with the PBBP model (Paper IV) confirmed the incomplete release of DOX from DEBDOX over 6h (NC1 pig study) and 24h (human study). The simulations suggested release of ~32% of the loaded DOX after 6h.

The in vivo cumulative input and the simulations are in good agreement with the incomplete release of DOX from DEBDOX, which has been observed over up to 90 days in porcine and human tissue samples. One reason for this incomplete release could be the formation of DEBDOX aggregates, which have been observed in the arteries. These aggregates or clusters containing more hydrogels result in an increased percentage of the loaded dose (~42%) being left in the formulation after 80 days. It was speculated that this happens because of a saturated drug environment. Another explanation might be the longer diffusion pathway in larger aggregates for both DOX and the counter ion needed for drug release. A third explanation could be that fibrotic tissue forms around DEBDOX. This phe-
nomenon, which resembles a fibrotic encapsulation or “foreign body reaction”, has been observed in humans\textsuperscript{122} and can occur within three weeks\textsuperscript{123} possibly severely reducing the release of DOX to the surrounding tissues.

**Effect of Lipiodol\textsuperscript{®} on DOX and DOXol disposition**

In NC2 (\textit{Paper I}), the administration of Lipiodol\textsuperscript{®} to pigs via the portal vein did not affect the disposition of DOX administered via the ear vein. DOX and DOXol concentration-time curves and biliary and urinary excretion were not altered by the administration of Lipiodol\textsuperscript{®}. Tissue concentrations of DOX and DOXol were not altered by administration of Lipiodol\textsuperscript{®} (\textit{Paper ii}).\textsuperscript{80} For visual confirmation, see the section headed “\textit{Variability in PK of DOX in pig and human}”, Figure 13. These results suggest that Lipiodol\textsuperscript{®} does not alter the uptake, metabolism or excretion of DOX or DOXol in healthy pigs when it is administered separately from DOX.

In the NC1 pig study\textsuperscript{98}, Lipiodol\textsuperscript{®} and DOX were administered as LIPDOX. The administration of LIPDOX did not significantly alter the biliary excretion, plasma clearance and terminal half-life compared to an intravenous infusion of DOX alone. These results, combined with the results from \textit{Paper I}, suggest that Lipiodol\textsuperscript{®} does not alter the disposition of DOX or DOXol in healthy pigs. Both studies were acute studies, where samples were collected over 6h. The half-life of DOX has a reported half-life of 14–50 h\textsuperscript{66}, and later effects of Lipiodol\textsuperscript{®} on DOX or DOXol disposition could thus not be observed.

Interestingly, \textit{in vitro} experiments and clinical studies have shown that Lipiodol\textsuperscript{®} can affect DOX disposition. Uptake of DOX into liver cancer cells was enhanced in the presence of Lipiodol\textsuperscript{®}.\textsuperscript{124, 125} It has been suggested that DOX is taken up together with Lipiodol\textsuperscript{®} by pinocytosis into the cell, increasing the intracellular DOX concentrations. Intra-arterial administration of LIPDOX (without additional embolization) compared to DOX alone lowered the DOX peak plasma concentration and 1h exposure, but not the total exposure or terminal half-life.\textsuperscript{41, 112, 126} DOX tumour concentrations were increased compared with the surrounding liver tissue after intra-hepatic LIPDOX administration to VX2 rabbits and patients with HCC.\textsuperscript{41, 126, 127} However, the intra-tumour distribution of DOX does not correlate with the intra-tumour distribution of Lipiodol\textsuperscript{®}.\textsuperscript{128, 129}

In all, it appears that Lipiodol\textsuperscript{®} alone does not affect any of the disposition processes of DOX or DOXol in healthy pigs. When DOX is emulsified with Lipiodol\textsuperscript{®}, however, it is possible that DOX is taken up with Lipiodol\textsuperscript{®} into the cells, increasing the intra-tumoral concentrations.\textsuperscript{41, 126, 127}
Effect of CsA on DOX and DOXol disposition

The effect of CsA on the disposition of DOX and DOXol was examined as a control for the possible effect of Lipiodol® in Paper I (NC2). As was discussed in the previous section, Lipiodol® did not have an effect on the PK profiles of DOX and DOXol. Therefore, treatment groups TI and TII were combined into one group (–CsA, n=6) as were groups TIII and TIV (+CsA, n=6).

DOX plasma concentrations were not significantly altered by administration of CsA (Figure 10). However, biliary and urinary excretion of DOX were significantly decreased after treatment with CsA (Figure 11). We conclude that CsA inhibits the canalicular located efflux transporters of DOX (P-gp, BCRP, BSEP, and/or MRP2),58-61 which is in agreement with previous studies.130-133 The decrease in urinary excretion of DOX after addition of CsA may be explained by the inhibition of renal tubular efflux mediated by P-gp and/or MRP2.74 CsA is also an inhibitor of the OATP1A2 transporter, and has the potential to affect DOX plasma concentrations by decreasing hepatic uptake. This was not observed in Paper I, probably because of the short sampling time. Several studies with longer sampling periods (>24h) have shown increased DOX plasma concentrations when CsA is co-admini-

Figure 10. Porcine plasma concentration-time curves for DOX and DOXol during the 6h study. All pigs received DOX at 0–5 min (reference phase; 0-160 min) and 200–205 min (test phase; 200-360 min). Of these, 6 pigs also received cyclosporine A (+ CsA) at 160-180 minutes. Peak plasma concentrations (Cmax) and exposure (AUC) during the test phase are shown in the table. The grey area and the asterisk (*) in the graph depict the time points where the plasma concentration significantly differed (P < 0.05) between the two treatment groups.
stered,\textsuperscript{130, 134} while studies with short sampling times (<4h) did not observe an effect on plasma concentration.\textsuperscript{130-133}

For DOXol, both peak plasma concentrations and plasma exposure were significantly increased when CsA was administered (Figure 10). Biliary excretion of DOXol was significantly decreased after treatment with CsA (Figure 11). Our results support previous suggestions that DOXol is transported by P-gp, as its biliary excretion is inhibited by CsA.\textsuperscript{130-132} We further suggest that DOXol may be transported by the same carrier-mediated uptake and efflux mechanisms as DOX, as CsA inhibited the biliary excretion of both DOX and DOXol to the same extent. The inhibition of biliary excretion of DOX would probably lead to higher intracellular availability of DOX for metabolism to DOXol. The combination of increased DOXol formation and decreased excretion to bile could cause a redistribution of DOXol to plasma. This theory is supported by the observed increased DOXol plasma concentrations.

The observed plasma and biliary PK profiles were replicated with the multi-compartment model (\textbf{Paper I}) by inhibiting biliary excretion of DOX and DOXol by 99\%, thus confirming the above suggestions. The effect of CsA on DOX distribution was also evaluated with the binding-specific semi-PBPK model (\textbf{Paper III}). DOX and DOXol PK profiles were adequately predicted, with a 90\% decrease in biliary excretion (AAFE < 2). This further supported the above conclusion that CsA inhibits the biliary excretion of these substances.

\textbf{Figure 11.} The fraction of doxorubicin (DOX) and doxorubicinol (DOXol) excreted to bile (0–3 and 3–6 h) and urine (0–6 h) in pigs is shown with box-and-whisker plots on a logarithmic scale, showing min-max and median. All pigs received DOX at 0–5 and 200–205 min. Of these, 6 pigs also received cyclosporine A (CsA: +) at 160–180 minutes. Closed circles show individuals not receiving Lipiodol\textsuperscript{®} treatment, and open circles show individuals receiving additional Lipiodol\textsuperscript{®} treatment at 190–195 minutes. The asterisk (*) indicates a significant difference (\textit{P} < 0.05) between the two treatment groups.
Description of DOX tissue binding in PBPK modelling

In Paper III we concluded that the disposition of DOX and DOXol could only be described by a PBPK model when there was a possibility of tissue retention.

Effect of $K_{p,T}$ on DOX disposition

In the generic semi-PBPK model, tissue distribution to non-eliminating, non-metabolizing tissues was described using the traditional $K_{p,T}$. A $K_{p,T} > 1$ indicates distribution of the compound to the investigated tissue. The proper definition of $K_{p,T}$ is the total tissue-plasma concentration ratio at equilibrium. However, in PBPK models, $K_{p,T}$ is used to describe the distribution ratio both at equilibrium and when equilibrium is not present. Several methods of calculating $K_{p,T}$ for a substance have been devised. $K_{p,T}$ prediction methods have included using the octanol:water partitioning coefficient; the vegetable oil:water partitioning coefficient; the solubility of the substance in the lipid, phospholipid and water content of tissues; and the in vivo-based volume of distribution and in vivo partitioning coefficients.

The possibility of using the predicted $K_{p,T}$ for non-eliminating, non-metabolizing tissue was evaluated during the early development of the generic semi-PBPK model in Paper III. In vivo and in vitro partitioning coefficients (either taken directly from or calculated using plasma/tissue data) from multiple species at multiple time points were collected, and were used as input in the generic semi-PBPK model. All the $K_{p,T}$ values underpredicted the peak plasma concentration 4- to 100-fold. This indicated that the used $K_{p,T}$ overpredicted the tissue distribution during the distribution phase. $K_{p,T}$ calculated using lipid and water content in tissues also poorly predicted the observed DOX plasma concentrations in NC2. Interestingly, the authors of this in silico prediction method concluded that the predicted $K_{p,T}$ values for DOX under-predicted the observed $K_{p,T}$ values. Even when the $K_{p,T}$ values were estimated by curve-fitting the generic model to the observed data, the fit of DOX plasma concentration-time profiles was poor.

The metabolizing, eliminating tissues (kidney, liver) were not described as perfusion-limited compartments, but as permeability-limited tissues. Instead of using $K_{p,T}$ to describe the disposition to these tissues, clearance over vascular and cellular membranes and the fraction of unbound drug were used to increase the tissue concentration. Decreasing the fraction of unbound drug from 0.1 to 0.01 improved the simulated concentrations in these tissues greatly. This, however, worsened the curve fittings to the observed DOX and DOXol plasma concentration-time profiles and urinary excretion.
Effect of intracellular binding sites on DOX disposition

To improve the description of the DOX and DOXol disposition by the PBPK model, an intracellular binding site was included in each tissue compartment (Figure 7). The drug distribution to the intracellular binding sites was described by association and dissociation binding clearances, which were identical in all tissues. \( K_{p,T} \) was accordingly set to 1 for all non-metabolizing and non-eliminating tissues. There is no indication that facilitated cell membrane translocation of DOX and DOXol is important in these tissues.

The addition of the intracellular binding sites improved the overall curve fitting. Descriptions of DOX plasma concentration-time profiles and biliary excretion and liver and kidney concentrations were improved, as were the DOX biliary excretion and kidney concentrations. The intracellular binding sites represented all intracellular binding for DOX and DOXol, e.g. DNA and cardiolipin. These results agree well with results from a previously published PBPK model incorporating specific binding of DOX to DNA and cardiolipin. This model was able to predict concentration-time profiles in several tissues and plasma from mouse, dog and human.

The estimated association clearance was larger than the dissociation clearance for both DOX and DOXol. This indicates prolonged tissue binding, and thus prolonged residence time in the tissue, which is in good agreement with studies showing high tissue concentrations or high \( K_{p,T} \) values. The prolonged residence time could well be explained by the binding of DOX to DNA, which is in good agreement with the reported antitumour mechanisms.

Similarity in human and porcine doxorubicin disposition

Throughout this thesis, the pig has been used as a model for the human HCC patient. The healthy porcine liver is macroscopically different from the healthy human liver, in that it has three lobes instead of two. However, and more importantly, the segmental anatomy, vascularity, and biliary tree of the livers are similar. Most porcine and human transporters and metabolizing enzymes are closely related and located similarly. For example, activity and expression levels of CYP isoforms are similar in humans and mini pigs. OATP1A2 is closely related in pigs and humans and is located in the liver in both species. P-gp and MRP2 are highly expressed in the livers of humans and pigs. Both CBR1 and AKR1C3 have been detected in humans and pigs. All this indicates that the pig is an appropriate model for investigations of DOX and DOXol disposition after administration of LIPDOX and DEBDOX. However, one important difference is the liver status of the two species: human HCC patients have impaired liver function while pigs
have healthy livers. Another difference is age: the studied pigs were young adults (around 12 weeks), while human HCC patients were more senior (>50 years).

The dose- and weight-corrected plasma concentration-time data from humans and pigs treated with LIPDOX and DEBDOX are shown in Figure 12. The data show that the DOX plasma concentrations decreased faster in humans than in pigs. The DOXol plasma concentrations increased more slowly in humans than in pigs. The formation of DOXol still exceeded its elimination after 6 h in humans, but had already turned after 2 h in pigs.

In **Paper IV**, the binding-specific semi-PBPK model was translated from the healthy pig to the healthy human. When simulating intravenous DOX infusions to humans with healthy livers, the observed DOX plasma curves were well described by the model (AAFE < 1.6). This suggests that the pig is a good model for PK studies of DOX, as the only alteration to the model was to change physiological parameters from porcine to human where appropriate. However, the DOXol plasma concentrations were underpredicted in

![Figure 12. Dose- and weight-corrected (1 mg, 1 kg) plasma concentration-time profiles after administration of LIPDOX (a, b) or DEBDOX (c, d) to healthy pigs (NC1) and human HCC patients (**Paper II**). (a, c) Doxorubicin PK (left-hand graphs) and (b, d) doxorubicinol PK (right-hand graphs).](image-url)
HCC patients. It is likely that there were uncertainties in the estimated parameter values for DOXol. The observed PK data set (Paper I and Paper ii) was less comprehensive for DOXol than for DOX; DOXol concentrations were only measurable in kidney tissue, not in liver, heart and intestine. Therefore, association and dissociation binding clearances for DOXol were more variable than those for DOX.

**Effect of liver cirrhosis on DOX disposition**

The binding-specific semi-PBPK model was further developed from a pig PBPK model to a human PBPK, a human cirrhosis PBPK and finally a human HCC PBPK model in Paper IV. The effects of chronic liver disease/cirrhosis on DOX disposition could thus be examined with the model.

Simulations from this PBBP model suggested that the PK of DOX does not change with changed liver status. Liver extraction of DOX has been reported as 0.05 to 0.5 during intrahepatic infusion and 0.03 to 0.105 after intravenous injection. DOX is thus classified as a low to intermediate hepatic extraction drug. In a well-stirred model, total clearance of DOX would largely be determined by plasma protein binding and intrinsic clearance via the main elimination route. In our PBBP model, intrinsic clearance was described intracellularly by metabolism and excretion into bile. To adhere to the reported changes in liver function in a cirrhotic liver, metabolic and excretory clearances were decreased. As plasma albumin and α1-acid glycoprotein decrease with increasing levels of cirrhosis, the fraction of unbound DOX was calculated to increase. Interestingly, the model simulations suggested that the effects of decreased intrinsic clearance and increased fraction of unbound DOX with increasing liver impairment/cirrhosis cancelled each other out for the total clearance of DOX. This explains the lack of effect of cirrhosis on DOX plasma concentration-time profiles.

Literature reviews have found contradictory results regarding the effect of chronic liver disease/cirrhosis/primary hepatic malignancies on the PK of DOX. In short, multiple studies have found increased DOX plasma exposure and an increased half-life in patients with impaired liver function, e.g. hyperbilirubinaemia, while others have not been able to confirm these results. It has been suggested that liver cirrhosis or impaired liver function might alter DOX PK, but that primary liver tumours will not affect DOX PK. Unfortunately, none of these studies classify liver impairment with CP classes, making a more specific comparison with our results difficult.
Variability in DOX PK in pig and human

Variabilities in the disposition of DOX and DOXol in the pig were noticed when the binding-specific PBPK model was used to simulate the observed intravenous data from NC1\(^9\) using parameter estimates from NC2 (Paper III). When simulating NC1 data, prediction of DOX plasma concentrations was acceptable. DOX 3h plasma exposure and clearance were similar in the studies: mean AUC 0.44 (NC1) vs 0.6 µM*min (NC2), and mean clearance 59 (NC1) vs 48 ml/min/kg (NC2) (Figure 13a).\(^{160}\) However, DOXol plasma concentrations from NC1 were 4.5- to 6.8-fold overpredicted when NC2 parameter values were used and a 10-fold lower DOXol plasma exposure was observed in NC1 compared to NC2 data.\(^{160}\) The fraction excreted to bile was two-fold higher for DOX and DOXol in NC1 than in NC2. From Figure 13 it is clear that the pigs in the two studies had different DOX and DOXol dispositions. In three pigs in NC1 the disposition of DOX and DOXol differed from that in the pigs in NC2, while one behaved more like the NC2 group of pigs.

In general, there was wide interindividual variability in DOX and DOXol disposition in these studies. At any given time point, the plasma concentrations between pigs within each study could vary as much as 4.5-fold for DOX and up to 10-fold for DOXol. The fraction excreted to bile could vary 3.5-fold for both DOX and DOXol. Wide interindividual variation has also been observed in humans.\(^{69, 161}\) Plasma exposure and terminal rate constants varied at least 3-fold in cancer patients (no involvement of the liver).\(^{69}\) Another study reported a coefficient of variation (CV%) of over 38% for plasma exposure, elimination rate constant and clearance in cancer patients (no involvement of the liver).\(^{161}\) The pigs included in the NC1\(^9\) and NC2 studies were male and of a mixed race (Yorkshire and Swedish landrace). They were not genetically identical, as is usual for rats and mice used in research, for example. However, in this respect, the pigs resembled the human population. One reason behind the wide variability could be genetics. Different polymorphisms exist for the OATP1A2, MRP2, MRP3, P-gp and BCRP drug transporters.\(^{162}\) Polymorphisms of P-gp, but not BCRP, affect the PK of DOX.\(^{163}\) MRP1 and MRP2 polymorphisms have been associated with an increased risk of anthracycline-induced cardiotoxicity.\(^{164}\) It could be speculated that the polymorphisms of the other transporters could also affect the DOX PK. Polymorphisms for the aldo-keto reductases and carbonyl reductases have also been reported.\(^{165, 166}\) Polymorphisms of AKR1C3 and CBR1, but not CBR3, have been shown to have an effect on the PK of DOX.\(^{167, 168}\) Most of these polymorphisms were identified in humans. However, considering the similarities between the two species, one could assume that similar polymorphisms are present in pigs.
Figure 13. Dose (50 mg)- and weight (27 kg)-corrected plasma exposure (A, B), biliary excretion (C, D), fraction excreted to bile and urine after 6h (E), and tissue concentrations (F) for doxorubicin (DOX) (A, C, E, F) and doxorubicinol (DOXol) (B, D, E, F). Individual data are shown for non-clinical study 1 (NC1, n=4) and study 2 (NC2, n=6, Paper I and Paper ii). DOX was administered to the right ear vein in pigs over 50 min (NC1) or 5 min, twice (NC2, at 0 and 200 min). Individuals TII04, TII05, TII06 received 6 mL Lipiodol® at 190-195 min.
Consequently, the variations between the individual pigs in the NC1 study could be the result of variations in excretion caused by transporter or metabolizing enzyme polymorphisms. The binding-specific semi-PBPK model was curve-fitted to the NC1 data set by estimating hepatic excretion, the scaling factor for metabolism, and cell membrane clearance (Paper III). This resulted in a good fit to the observed data. The results suggested that the hepatic excretion rate of DOX was increased 2-fold in the three pigs that had lower DOXol plasma curves and higher DOX biliary excretion. Interestingly, the estimated metabolism did not differ between the different pigs. The variability between NC1 and NC2 in the hepatic excretion parameter estimations for both DOX and DOXol seems to suggest that the biliary variability in excretory transporters is more important than the variability in metabolizing enzymes.
Conclusions

In this thesis, the pharmacokinetic profiles of DOX and DOXol were investigated in vivo. The release of DOX from the clinically used formulations LIPDOX and DEBDOX and the effect of Lipiodol® and CsA on the disposition of DOX and DOXol were studied. PBPK and PBBP models were developed to provide a better understanding of these processes.

Specifically:
- The in vivo release of DOX from LIPDOX did not reach a steady release rate and was not or was only slightly extended (Papers II and IV).
- The in vivo release of DOX from DEBDOX reached a steady release rate after 2 h, which was continued for at least 14 h (Papers II and IV).
- DOX was released from LIPDOX and DEBDOX as a burst in the early phase of administration (Papers II and IV).
- The choice of sampling site/site of measurement is of importance when determining in vivo release rate (Papers II and IV).
- Lipiodol® by itself did not affect the hepatobiliary disposition of DOX or DOXol in healthy pigs (Paper I and Paper ii).
- CsA (a positive control for Lipiodol®) seemed not to affect the metabolism of DOX, but inhibited the excretion of DOX and DOXol to bile in the healthy pig (Papers I, III, Paper ii).
- The prolonged tissue binding of DOX was only established when intracellular binding sites were added to the semi-PBPK model describing the PK of DOX and DOXol (Paper III).
- There was good agreement between healthy porcine and human disposition data for DOX, as shown by the ease of translation of the semi-PBPK pig model to the human PBBP model (Paper IV).
- In vivo release of DOX from LIPDOX and DEBDOX was described using the human HCC PBBP model using in vitro release profiles as input (Paper IV).
Primär levercancer, eller hepatocellulärt carinom (HCC), är en elakartad tumör i levern. Globalt insjuknar årligen ca 780 000 människor i HCC och 745 000 avlider i sjukdomen. Det gör HCC till den näst dödligaste cancerformen globalt. I Norra Europa är det årliga insjukningstalen 6 500 och 6 300 avlider. Fler män än kvinnor drabbas och HCC är vanligare i utvecklingsländer. Den maligna levertumören uppstår oftast i en redan sjuk lever, till exempel cirrotiskt lever eller skrumplever. Sjukdomsförloppet kan delas in i fem stadijer; intermediär HCC är ett av dessa stadijer.


I avhandlingen har det undersöks vad som händer med läkemedlet doxorubicin i kroppen efter injicering av LIPDOX eller DEBDOX. Doxorubicin frisätts nämligen från formuleringarna och fördelas sig i kroppens organ och vävnader. Därefter kan doxorubicin utsöndras från kroppen med urinen och via galla till avföring. Doxorubicin kan även brytas ner i kroppen till sin nedbrytningsprodukt doxorubicinol. Doxorubicinol har en svagare effekt än doxorubicin. Hur mycket doxorubicin som frisätts från formuleringen och med vilken hastighet frisättningen sker är beroende av formulerings egenskaper och kroppens fysiologi. Ett av målen med båda formuleringarna är att långsamt frisätta doxorubicin nära tumören. Ett annat är att täppa till blodkärl nära tumören, vilket stoppar blodflödet och gör att doxorubicin inte kan spolas bort från tumören. I teorin gör den långsamma frisättningen tillsammans med det stoppade blodflödet att tumören blir utsatt
för höga halter av doxorubicin under en längre period. Det borde då öka doxorubicinets effekt på tumören.


Med all information vi samlat in kunde vi programmera så kallade farmakokinetiska datormodeller, som beskriver allt som händer med doxorubicin i kroppen. Dessa datormodeller kombinerades med information om hur doxorubicin frisätts från formuleringarna *in vitro* (d.v.s. i provröra) som gjorts av andra forskare. När *in vitro*-resultaten kombinerades med modellerna kunde vi således simulera vilka halter doxorubicin HCC-patienter hade i blodet. Simuleringsarna bekräftade resultaten som visades i studien med HCC-patienterna. Simulerings från våra farmakokinetiska modeller visar även att grisen är en bra försöksmodell för vad som händer i människor. Genom att ändra fysiologiska värden (som organvikt) från gris till människa i modellen kunde doxorubicinhalten i blodet hos människor med frisk lever förutses. Våra simuleringar visar även att skrumplever inte påverkar doxorubicinets fördelning och nedbrytning i kroppen.

Man vet att vissa substanser kan ha effekt på vävnadsfordelning, nedbrytning eller utsöndring av doxorubicin. I vår forskning undersökte vi huruvida oljan i LIPDOX, Lipiodol, har en sådan effekt i friska grisar. Det visades sig att Lipiodol inte ändrade någon av doxorubicinets tidigare nämnda processer i friska grisar. Vi testade även läkemedlet ciklosporin, där det har visats tidigare att den blockerar proteiner som har som roll att utsöndra främmande ämnen ur kroppen. Vi visade att ciklosporin minskade doxorubicinets utsöndring till galla och urin i friska grisar. Eftersom utsöndringen av doxorubicin minskade bildades mer DOXol, vilket ökade dess halt i blodet.

Sammanfattningsvis har avhandlingen bidragit till ökad kunskap om hur doxorubicin frisätts från två formuleringar efter att dessa har injicerats nära en levertumör. Kombinationen av försök i friska grisar och människor med HCC i kombination med farmakokinetiska matematiska modeller ger tyngd åt våra slutsatser.

Populairwetenschappelijke samenvatting

Primaire leverkanker wordt ook wel hepatocellulair carcinoom (HCC) genoemd en is een kwaadaardige tumor die in de lever ontstaat. Jaarlijks worden er wereldwijd ongeveer 782.000 mensen met deze ziekte gediagnostiseerd en overlijden er 745.000 mensen aan de ziekte. Dit maakt HCC de op een na dodelijkste kankervorm. In West Europa werden er in 2012 ongeveer 20.500 mensen gediagnostiseerd en overleden er 18.900 personen aan HCC. Deze cijfers zijn hoger in ontwikkelingslanden en voor mannen. Deze kwaadaardige tumor ontwikkelt zich voornamelijk in een chronisch ontstoken lever, waarin littekenvorming is ontstaan, dit wordt ook wel een cirrotische lever genoemd. Het verloop van HCC kan in verschillende stadia worden ingedeeld, waarvan intermediaire HCC er één is.


In dit promotietraject hebben we onderzocht wat er met het geneesmiddel doxorubicine gebeurd vanaf het moment dat LIPDOX of DEBDOX in de leverslagader geïnjecteerd zijn. Doxorubicine komt namelijk vrij uit het preparaat en verdeelt zich daarna over de tumor en de rest het lichaam. Doxorubicine kan daarna uitgescheiden worden via de gal, en vervolgens de ontlasting, of via de urine. Doxorubicine kan ook afgebroken worden in de metaboliet doxorubicinol. Doxorubicinol heeft een minder sterk effect in vergelijking met doxorubicine. De snelheid en de volledigheid waarmee
doxorubicine uit de preparaten vrijkomt wordt mede bepaald door eigenschappen van het preparaat, maar ook door de fysiologie van het lichaam. Het eerste doel van deze preparaten is om doxorubicine langzaam vrij te laten komen in en om de tumor. Het tweede doel is om een afsluiting van het bloedvat bij de tumor te veroorzaken, waardoor doxorubicine niet met het bloed weggespoeld kan worden. Deze twee doelen zouden er in theorie voor moeten zorgen dat de tumor gedurende een langere periode aan hoge concentraties doxorubicine blootgesteld wordt, wat de cytotoxische werking van doxorubicine zou kunnen verbeteren.

Onze resultaten in patiënten met HCC wijzen erop dat doxorubicine snel uit LIPDOX vrij komt: binnen 1-2 uur is alle doxorubicine vrijgekomen in het lichaam. Door de snelle verdeling van doxorubicine naar andere weefsels, en de eliminatie van doxorubicine via de gal en urine dalen de concentraties in het bloed snel en krijgt de tumor een lagere dosis doxorubicine. Met DEBDOX zien we een ander patroon: doxorubicine komt langzaam vrij uit dit preparaat. Doxorubicine er komt zelfs zo langzaam uit, dat het niet zeker is of alle doxorubicine ooit vrij zal komen. Na één dag is er namelijk nog maar 15% afgegeven. Met de verzamelde gegevens konden er zogenaamde farmacokinetische modellen op de computer gemaakt worden die de doxorubicine concentraties in het bloed konden simuleren. Het vrijkomen van doxorubicine uit deze preparaten is verder ook in vitro (in reageerbuis) door andere onderzoekers getest. Door de modellen met de in vitro resultaten te combineren, konden we de conclusies van de humane studies bevestigen. De simulaties met de farmacokinetische modellen duiden er verder op dat het gezonde varken een goed experimenteel model voor de mens is. Door fysiologische waarden (zoals orgaan gewicht) van varkens naar menselijke waardes te veranderen, konden doxorubicine concentraties in het bloed van mensen zonder leverkanker worden voorspeld.

Verschillende stoffen kunnen de verdeling in of eliminatie van doxorubicine uit het lichaam veranderen. We hebben onderzocht of Lipiodol een dergelijke werking heeft in gezonde varkens. Onze conclusie was dat Lipiodol niet de verdeling of eliminatie van doxorubicine uit het lichaam van gezonde varkens verandert. We testten ook de stof ciclosporine, waar het wetenschappelijk aangetoond is dat het de eliminatie naar de gal en urine van doxorubicine wordt verminderd. Onze resultaten bevestigden dat dit ook het geval is gezonde varkens. Door de verminderde eliminatie van doxorubicin, wordt er extra doxorubicine omgezet naar de metaboliet doxorubicinol. Hierdoor stegen de concentraties van doxorubicinol in het bloed.

In conclusie heeft dit proefschrift bijgedragen tot het vergroten van de kennis over hoe doxorubicine uit deze twee preparaten vrijkomt, nadat ze dicht bij de tumor geïnjecteerd zijn. De combinatie van testen in gezonde varkens, HCC patiënten en modellen sterken onze conclusies.
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*En toen kwam er een olifant
met een hele lange snuit
en die blies het hele verhaaltje uit*
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