Biopharmaceutical Evaluation of Intra-arterial Drug-Delivery Systems for Liver Cancer

Investigations in healthy pigs and liver cancer patients

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Abstract


There are currently two types of intra-arterial drug-delivery system (DDS) in clinical use in the palliative treatment of primary liver cancer. The chemotherapeutic drug doxorubicin (DOX) can be formulated into a drug-in-lipiodol emulsion (LIPDOX) or a microparticulate drug-eluting bead system (DEBDOX). To facilitate development of future DDSs, we need to understand the release and local distribution of drug from these DDSs into the complex, in vivo, pathological environment.

The overall aim of this project was to assess and improve understanding of the in vivo release of DOX from LIPDOX and DEBDOX and its local disposition in the liver. These processes were investigated in detail in a multisampling-site, healthy pig model and in human patients with liver cancer. The mechanisms involved in DOX disposition were studied by examining potential interactions between DOX and lipiodol and/or cyclosporine A (CsA) in pigs.

In this project, the main elimination pathway for DOX and its primary metabolite doxorubicinol (DOXol) was via bile; their extensive canalicular carrier-mediated transport (e.g. ATP-binding cassette transporters ABCB1, ABCC1, ABCC2 and ABCG2) was inhibited by CsA. CsA had no effect on the carbonyl and aldo-keto reductases responsible for the metabolism of DOX into DOXol. LIPDOX released DOX more rapidly and to a greater extent into the circulation than DEBDOX, which had only released 15% of the dose in patients after 24 hrs. The systemic exposure to DOX was lower for DEBDOX than for LIPDOX. Greater fractions of DOXol were formed in blood and bile with LIPDOX than with DEBDOX. This may have been because DOX was more widely distributed into regions with increased metabolic capacity or because of increased intracellular uptake when DOX was delivered in LIPDOX. The excipient lipiodol in the LIPDOX formulation did not interact with transporters, enzymes or membranes that would explain the increased cellular uptake of DOX.

In conclusion, the release of DOX from DEBDOX is more controlled in vivo than that from LIPDOX, indicating that DEBDOX is a more robust pharmaceutical product. The formulations for future optimized DDSs should therefore be more similar to DEBDOX than to LIPDOX.

Keywords: in vivo release, drug delivery systems, local delivery, drug disposition, doxorubicin, hepatocellular carcinoma, transarterial chemoembolization, transarterial chemotherapy infusion

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List of Papers

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


III **Lilienberg E.**, Dubbelboer I.R., Sjögren E., Lennernäs H. Lipiodol Does Not Affect the Tissue Distribution of Intravenous Doxorubicin Infusion in Pigs. Submitted to *Molecular Pharmaceutics*.


Reprints were made with permission from the American Chemical Society.

*These authors contributed equally to the execution of the study and writing the article.

*My contribution to the papers included in this thesis was as follows:*  
I was highly involved in the project design, planning, project progress management and writing of the manuscripts for all Papers (I–IV). I was involved in the initiation of collaboration with the other researchers (Paper I and IV) and in the experimental work for all Papers. I performed the bioanalysis in Paper III and the data analysis for Paper I, III and IV.
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Abbreviations

A
Amount of drug or metabolite

ABC
ATP-binding cassette transporter

AKR
Aldo-keto reductase

AUC
Area under the concentration–time curve

BCLC
Barcelona clinic liver cancer

BCRP
Breast cancer resistance protein

C
Concentration of drug or metabolite

CBR
Carbonyl reductase

CL
Clearance

C_last
Last observed concentration

C_max
Maximum concentration

CsA
Cyclosporin A

cTACE
Conventional transarterial chemoembolization

CYP
Cytochrome P450

DDS
Drug-delivery system

DEBDOX
DOX-eluting microparticle bead DDS

DOX
Doxorubicin

DOXol
Doxorubicinol

E_H
Hepatic extraction

F
Bioavailability

f_e
Fraction of the dose excreted (to urine or bile)

F_{i,app}
Apparent intracellular availability

HCC
Hepatocellular carcinoma

IC_{50}
Concentration needed to cause 50% of the maximal inhibition

i.v.
Intravenous

k
Rate constant

K_p
Tissue-to-plasma concentration partitioning coefficient

LIPDOX
Lipiodol-based emulsion containing DOX

MRP
Multidrug resistance-associated protein

Pgp
P-glycoprotein

RD
Remaining fraction of the dose in liver and/or DDS

S.E.
Standard error of mean

SLC
Solute carrier
<table>
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<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>TACE</td>
<td>Transarterial chemoembolization</td>
</tr>
<tr>
<td>TAI</td>
<td>Transarterial infusion chemotherapy</td>
</tr>
<tr>
<td>$t_{\text{max}}$</td>
<td>Time to reach the maximum concentration ($C_{\text{max}}$)</td>
</tr>
<tr>
<td>UPLC-MS</td>
<td>Ultra-high performance liquid chromatography</td>
</tr>
<tr>
<td></td>
<td>coupled to mass spectrometry</td>
</tr>
<tr>
<td>UPLC-MS/MS</td>
<td>Ultra-high performance liquid chromatography</td>
</tr>
<tr>
<td></td>
<td>coupled to tandem mass spectrometry</td>
</tr>
<tr>
<td>V</td>
<td>Volume</td>
</tr>
<tr>
<td>VC/VH</td>
<td>Vena cava orifice from the hepatic veins</td>
</tr>
<tr>
<td>VF</td>
<td>Femoral vein</td>
</tr>
<tr>
<td>VH</td>
<td>Hepatic vein</td>
</tr>
<tr>
<td>VP</td>
<td>Portal vein</td>
</tr>
<tr>
<td>$V_{\text{ss}}$</td>
<td>Volume of distribution at steady state</td>
</tr>
<tr>
<td>$\lambda_z$</td>
<td>Terminal elimination rate constant</td>
</tr>
</tbody>
</table>
Introduction

There have recently been significant efforts to develop new parenteral drug-delivery systems (DDSs) with the aim of improving delivery of chemotherapeutic drugs in the treatment of patients with cancer. The therapeutic goal is to increase intracellular tumor exposure to chemotherapeutic drugs and consequently to improve the anti-tumor efficacy. However, it has proved a difficult challenge to find a drug or DDS that is specific to tumor cells, especially since tumor cells are derived from normal cells and thus share many of their cellular properties. Many relatively sophisticated DDSs have been developed through the years to improve drug delivery to tumors; these include, for example, nanoparticles (such as liposomes), microparticles and lipid emulsions. However, only a few of these have been successful enough to reach the clinic.

The primary objective of this thesis was to increase understanding of two clinically available DDSs that are currently in use in the palliative treatment of intermediate-stage hepatocellular carcinoma (HCC; primary liver cancer). The two DDSs investigated in the thesis, the lipiodol-based emulsion (LIPDOX) and microparticle drug-eluting beads (DEBDOX), are used to deliver the anti-cancer drug doxorubicin (DOX) to the tumor. LIPDOX is an emulsion of the contrast agent lipiodol with an aqueous solution of DOX. DEBDOX is a DOX-eluting bead system (DC Bead®). These DDSs are administered locally to the liver via intra-arterial delivery. Although LIPDOX and DEBDOX are in clinical use, little is known about their targeting and release mechanisms in vivo. Hence, to facilitate the development of future delivery strategies, this thesis specifically focused on increasing understanding of the in vivo release of DOX from these DDSs and its hepatic distribution, and the mechanisms specifically involved with these processes.

This thesis is the first part in the Target Exact Project – a liver cancer treatment optimization project that was initiated at the Department of Pharmacy, Uppsala University, Sweden.
Parenteral drug delivery and drug distribution

Parenteral administration of drugs includes all delivery methods that are not directly administered to the gastrointestinal tract. Mostly, these take the form of injection into the vascular system, subcutaneous injection, or injection into the muscles.¹ Parenteral delivery is often used to achieve a local and/or rapid effect. When a drug is administered directly into the vascular system, i.e. into veins or arteries, it reaches the circulatory system directly, and will thus be rapidly distributed in the body.¹ The distribution of a drug to an intracellular site of action is driven by vascular, transvascular, and interstitial transport and then by cellular uptake.² These processes are summarized in Figure 1.

![Figure 1](image)

**Figure 1.** Overview of the processes that govern the transport of a drug from the blood circulation into the cells. The figure shows tumor cells in unspecified tumor tissue, but the same transport processes apply to healthy tissue. These processes can, however, differ between different tissues and stages of disease as the biophysical properties of the vessel walls, cells and interstitium change. The figure is a modification of an illustration from Chauhan et al., Annu Rev Chem Biomol Eng, 2011.²
Vascular transport, i.e. the movement of a drug or a DDS within the blood, controls distribution of the drug into the organs and is dependent on the convective blood flow and the vascular morphology (the number, length, diameter and geometrical arrangement of the blood vessels). Transvascular transport, i.e. the passage of a drug or DDS across a blood vessel wall, is driven by a combination of convection and diffusion (pressure and concentration gradient) between the vessel and the organ interstitium. Transvascular transport is also dependent on the biophysical properties of the vessel wall and the physicochemical properties of the drug and/or DDS. Once in the interstitium, the drug moves towards the cells by interstitial transport. Interstitial transport is driven by a combination of convection and diffusion processes, and is dependent on the interstitial properties and the physicochemical properties of the drug or DDS. Finally, cellular uptake is mediated by diffusion, carrier-mediated transport of the free drug, and/or endocytosis of the DDS. The rate of drug release from the DDS affects both the efficacy and the adverse events. Once the drug is within the different cell types it can exert both the desired on-target effects and/or unwanted off-target effects (i.e. adverse reactions). For DDSs intended for parenteral drug delivery, the design and administration of the DDS, as well as biological pathophysiological factors also are important for the local drug deposition and distribution.

Elimination of drugs

The elimination of most drugs from the body occurs through metabolism in the liver and excretion into the bile and urine. Drug-metabolizing enzymes are available to various extents throughout the body, but the liver and the kidney have the highest activity. The liver and kidney are also the most important organs for excretion of drugs.

During metabolism, hydrophilic properties are added to detoxify and/or increase the possibility of eliminating the compound. The biotransformation of a hydrophobic compound into a more water-soluble form can be described by phase I and phase II metabolism stages. Phase I metabolism includes non-synthetic reactions such as oxidation, reduction or hydrolysis, where a functional group is modified or added to the compound. Enzymes that catalyze phase I metabolic processes include cytochrome P450 proteins (CYPs), carbonyl reductases and aldo-keto reductases. Phase II metabolism involves synthetic reactions where an endogenous substance such as glucuronide, sulfate or glutathione is conjugated to the compound. Phase II metabolites are formed by transferase enzymes. These metabolites are generally more polar than phase I metabolites, and therefore more likely to be excreted into urine. Metabolites formed in the liver may be further metabolized, excreted into the bile or transported back into the blood and then excreted into the urine.
Biliary and urinary excretion are processes that may involve transport proteins to various extents. Transport proteins involved in drug influx and efflux can be divided into two superfamilies: solute carriers (SLCs) and ATP-binding cassette transporters (ABCs).\(^6,\)\(^7\) The ABCs are directly driven by energy from ATP hydrolysis and therefore mediate primary active transport, mostly against a concentration gradient.\(^8\) Some SLCs are passive (facilitative) transporters that do not require any energy, and some SLCs are secondary active transporters that utilize energy from the surroundings, such as ion gradients, co-transport or counter transport. SLCs may be responsible for both influx and efflux while the ABCs that are important for drugs are mainly efflux proteins.\(^6\) Both ABCs and SLCs are highly expressed in the intestine, liver and kidney and are therefore important for the absorption, distribution and elimination of drugs. Compounds that have been transported to the bile are diverted to the intestine where they may be reabsorbed (enterohepatic cycle) or eliminated with the feces.\(^9\)

**The human liver**

The liver has a dual blood supply. Most of the blood (~75–80%) is delivered as nutrient-rich blood from the gastrointestinal tract and spleen via the portal vein (VP).\(^10\) The remainder is delivered as oxygen-rich blood directly from the aorta via the hepatic artery.\(^10\) The strategic location of the liver is ideal for elimination of exogenous compounds since compounds from the gastrointestinal tract have to pass through it before they reach the systemic circulation.

The liver can be divided anatomically into eight functionally independent segments, according to Couinaud’s scheme (Figure 2). Basically, the main VP is separated into left and right branches, dividing the liver into left and right lobes that are separated by the middle hepatic vein (VH).\(^11,\)\(^12\) Separate blood supply (from the VP and hepatic artery), vascular outflow and biliary drainage systems further divide the lobes into eight segments. These blood vessels and bile ducts subdivide repeatedly within the liver and the terminal branches lead into the functional units of the liver, the hepatic lobules. The human liver contains about one million of these lobules.\(^13\)
The microstructure of the liver lobule

The liver lobule has a hexagonal shape and is composed of two major cell types: parenchymal and non-parenchymal cells. The parenchymal cells, i.e. hepatocytes, represent 60% of the cellular population in the liver and occupy almost 80% of the liver volume. It is the hepatocytes that are responsible for the liver metabolism and excretion of endogenous compounds and xenobiotics such as drugs. The hepatocytes have many other important functions as well, such as the storage of proteins, the transformation of carbohydrates and the synthesis of proteins, cholesterol, bile salts and phospholipids. The non-parenchymal cells include sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells (macrophages of the liver) and cholangiocytes (biliary epithelial cells).

The organization of the cells in the liver lobule is shown in Figure 3. The central vein is located in the center of the lobule, from whence the hepatocytes extend radially in linear chords to the lobule periphery. These chords are usually one cell-layer thick. The portal and arterial vascular branches and the bile duct form the “portal triad”, which is located in 4–8 places in the periphery of the lobule. Portal and arterial blood is mixed in the sinusoids.
located between the hepatic chords. From the sinusoids, the blood continues through the lobule into the central vein, from which it moves into the VHs and eventually leaves the liver to flow into the vena cava. Most hepatocytes are exposed to the sinusoidal vasculature on two sides to optimize exchange. The unique physiology of liver sinusoidal endothelial cells facilitates molecular exchange with the blood; they are highly fenestrated and lack a basement membrane. The endothelial cells are separated from the hepatocytes by the space of Disse, in which the hepatic stellate cells reside. The stellate cells are important for the storage of fat and vitamin A and for regulating the turnover of extracellular fluid in the space of Disse. The Kupffer cells mainly reside within the sinusoids.

Figure 3. The microstructure of the hepatic lobule. Blood enters the lobule via branches of the portal vein and hepatic artery, mixes in the sinusoids and leaves the structure through the central vein. The sinusoids surround the chords of hepatocytes that run from the central vein. The inset shows the bile canaliculi between adjacent hepatocytes. These canaliculi form a network that transfers bile in the opposite direction to the blood flow, towards the bile duct in the periphery of the lobule. Liver sinusoidal endothelial cells line the sinusoidal vessel wall. The hepatic stellate cells are situated in the space of Disse, and the Kupffer cells are mainly located in the sinusoids. The figure was reprinted with kind permission from A. Vildhede.
Bile canaliculi are formed in the membrane between two adjacent hepatocytes. Tight junctions separate the canalicular membrane from the basolateral membrane. These canaliculi form an interconnected network that transfers bile in the opposite direction to that of the sinusoidal blood, i.e. into the bile duct in the periphery of the lobule. The bile ducts are lined by cholangiocytes, which have an important function that involves secretion of bicarbonates and water. While the hepatocytes produce the primary bile, the cholangiocytes modify the volume and composition of the bile during its transport through the network (the biliary tree). The bile components travel through the bile ducts via the common hepatic duct to the gallbladder, from which they are released into the small intestine.

The hepatocyte

Hepatocytes are cubic, polarized cells with two distinct membranes: the basolateral membrane that faces the sinusoids and adjacent cells, and the canalicular (apical) membrane that forms the bile canaliculi. Both the basolateral and canalicular membranes are richly covered with microvilli to increase the surface area for molecular exchange. About 68% of the hepatocyte surface area is in contact with other hepatocytes, 22% with sinusoids and 10% with bile canaliculi. Transporter proteins are expressed to a high degree by both the sinusoidal and canalicular membranes of the hepatocyte, thus facilitating cellular influx and efflux of endogenous compounds and xenobiotics.

Hallmarks of cancer

The development of cancer is a multistep process where normal cells accumulate several genomic mutations over time. The many different cell types in the body enable many possibilities for different mutations and, hence, cancer is a highly heterogeneous disease. This heterogeneity is one of the reasons why finding a cure for cancer has not been successful, at least in the search for a “magic bullet” that would treat all forms of cancer. There are several common hallmarks of cancer, although the underlying mechanisms may differ between tumors. These include self-sufficiency in growth signals and insensitivity to anti-growth signals, resistance to apoptosis, induced angiogenesis, limitless replicative potential, and a tendency for tissue invasion and metastasis. Most cancer mutations damage somatic cells, and it is only when they damage the DNA in germ cells that the mutation will be inherited by the next generation. Tumor cells have increased mitosis and apoptosis activity compared to healthy cells.
Cell cycle control and DNA damage

The mutations associated with tumor cells and cancer are likely to be the result of DNA damage in combination with defects in DNA repair and the regulation of the cell cycle.\textsuperscript{24} The cell cycle consists of a series of highly regulated phases and irreversible events that are mandatory for proliferation.\textsuperscript{25} Each phase has a specific checkpoint that must be passed before the cell can move into the next phase. In adults, most cells are dormant, outside of the cell cycle, and are thus not subject to cell division. These cells are in the G\textsubscript{0}-phase. Growth factors can stimulate these cells to move from G\textsubscript{0} to the first gap phase, G\textsubscript{1}, in which they prepare for DNA replication. Once the cell has passed the G\textsubscript{1} restriction point there is no return, and the cell will complete the cell cycle. After G\textsubscript{1}, the cell enters the synthesis (S) phase of DNA replication, followed by the G\textsubscript{2} phase, in which the cell is controlled and damaged DNA is repaired. After DNA repair, the cell continues into mitosis, the M phase. In the M phase, the cell is divided and the duplicated genetic material is equally separated into two daughter cells.\textsuperscript{26} These daughter cells can now either re-enter the cell cycle or become dormant in the G\textsubscript{0} phase.

To avoid the accumulation of unwanted cells, such as those with damaged DNA, there is a well regulated system for cell death. The two main modes of cell death are apoptosis and necrosis.\textsuperscript{27} Apoptosis is ATP-dependent and is considered to be a “clean and tidy” process for killing cells. The mechanism includes special proteins (caspases) that inactivate important proteins in the cell. Inactivated cells or cell parts are typically engulfed and destroyed by phagocytes (specialized immune cells).\textsuperscript{28} Necrosis is ATP-independent and was for a long time considered to be unordered. However, there is now evidence that necrosis can also occur in a regulated fashion.\textsuperscript{29} The process of necrosis is typically described as a swelling of the cell and rupturing of the cell membrane, which causes leakage of the intracellular components, commonly followed by inflammation.\textsuperscript{27} Programmed cell death is important as a protective mechanism against cancer. Impaired or inactivated apoptotic processes can promote cancer, since cells with mutations are not cleared from the body.

Tumor microenvironment

Tumor cells are surrounded by stromal cells, endothelial cells, and macrophages. These cells are in close contact with each other and together they create a unique environment in which the tumor can proliferate.\textsuperscript{30} Common pathophysiological changes give tumors special characteristics that contribute to membrane and interstitial transport barriers that drugs or DDSs have to cross.\textsuperscript{3}
1. **Growth-induced solid stress.** Tumor growth occurs within a restricted area. Solid stress is the result of tissue expansion to the extent that it comes into contact with the surrounding normal cells. This solid stress accumulates in the tumor as a result of continuous increases in cell number and overproduction of the interstitial matrix. Eventually, this leads to compression of blood and lymphatic vessels, which results in reduced blood flow, increased vascular resistance and reduced fluid drainage by the lymphatic system.

2. **Abnormal vascular network.** Tumor cells produce a variety of pro-angiogenic factors that induce new blood vessels to grow from the surrounding normal vessels; these new vessels are required for the tumor to be able to grow beyond 2 mm in size. The new vessels typically lack order in branch hierarchy and they are heterogeneous and hyper-permeable to fluids and macromolecules (>40 kDa). This is because of the presence of fenestrae in the vessel walls, transendothelial channels, and large pores throughout the vasculature. The loss of fluid from the blood vessels leads to increased viscosity of the remaining blood, which reduces the blood flow.

3. **High interstitial fluid pressure.** The impaired lymphatic system, the hyperpermeable blood vessels, and the accumulation of fluid and macromolecules in tumors all lead to high interstitial fluid pressure. These factors cause a substantial reduction in transvascular and interstitial fluid pressure gradients. Without the pressure gradient, the convective flow is reduced and the tumor distribution is mainly driven by diffusion.

4. **Dense interstitial structure.** For the reasons mentioned above (1–3), tumors are highly cellular and accumulate macromolecules. They also have a high proportion of stromal cells. This increases the density of the tumor structure and, consequently, also causes steric hindrances to drug transport. Since many drugs are highly bound to the matrix and cellular components, the interstitial transport rate is reduced even further.

These intratumor transport barriers reduce the penetration of the drug into the tumor, resulting in poor, heterogeneous drug distribution. The impaired spatial distribution from the blood vessels has been demonstrated for DOX, mitoxantrone, and topotecan. However, the accumulation of macromolecules that is characteristic of the tumor microenvironment enables passive targeting of macromolecular DDSs into this area. This indirect accumulation of drugs in the tumor is called the enhanced permeability and retention effect and was first described by Maeda in the middle of the 1980s.
Hepatocellular carcinoma – HCC

Liver cancer was the sixth most common cancer form globally in 2012 (782,000 cases), and the second most lethal cancer form (745,000 deaths).\textsuperscript{34} Survival rates and prognosis are poor, with a reported yearly mortality-to-incidence ratio of 0.95.\textsuperscript{34} The highest incidence of liver cancer is observed in Asia and Africa, while the incidence in Southern Europe and Northern America is intermediate (Figure 4).\textsuperscript{34} However, it has been estimated that the incidence of liver cancer will increase globally and especially in developed countries over the next ten years.\textsuperscript{35} HCC represents 70–85% of all primary liver cancers.\textsuperscript{36}

\hspace{1cm}

Figure 4. This world incidence map illustrates the age-standardized incidence of liver cancer in both sexes in 2012 per 100,000 individuals. Data from ref.\textsuperscript{35} Available from: http://globocan.iarc.fr, accessed on 21 October 2015.

The development of HCC is usually highly correlated with advanced chronic liver disease, i.e. liver cirrhosis. The liver cirrhosis is often associated with hepatitis C virus, hepatitis B virus, or over-consumption of alcohol. Ingestion of aflatoxins has also been associated with a risk of developing HCC.\textsuperscript{37} Recent studies have identified obesity and diabetes type 2 as additional risk factors.\textsuperscript{38}
Pathophysiology

HCC is a primary liver cancer that originates from mutations in hepatocytes. The microstructure of a HCC tumor is typically characterized by distorted chords of hepatocytes that are 2–10 cell layers thick, as opposed to the single-layer, ordered chords of hepatocytes in the healthy liver. These cell layers typically get thicker as the HCC progresses. The tumor cells of HCC have distinct cell membranes and are associated with atypical, often enlarged, nuclei. Some cells may appear transparent due to accumulation of lipids or glycogen. Kupffer cells are typically quantitatively reduced in HCCs, and bile pigment is present in approximately 20% of all HCCs. The hepatic stellate cells undergo transformation into cells that produce tumor growth factors and pro-angiogenic factors and secrete extracellular matrix proteins to a higher degree.

The majority of HCCs arise in cirrhotic livers. Cirrhosis is an advanced stage of fibrosis that is associated with scar tissue in the space of Disse, lost fenestrations in the sinusoidal endothelial cells, distorted hepatic vasculature, and regenerative nodules on the hepatocytes. All of these characteristics lead to impaired hepatocyte functionality and a gradual increase in intrahepatic resistance.

HCC manifests as a highly vascularized tumor; in contrast to normal liver tissue, which has a dual blood supply, the blood in HCC tumors mainly (>90%) comes from the hepatic artery. The changes to the blood supply during hepatocarcinogenesis start during the early phases. As the portal venous flow diminishes, hypoxia develops in the tumor, resulting in angiogenesis from pre-existing blood vessels or development of new tumor vessels. Eventually, well or moderately differentiated HCCs are predominantly supplied with arterial blood. In poorly differentiated HCCs, the tumor shifts into an anaerobic metabolism and the arterial blood supply decreases. It is common to find a focal area in the tumor with hemorrhage or necrosis. HCCs can be single or multiple and usually range in size from less than 1 cm to 30 cm in diameter.

Treatments

The Barcelona Clinic Liver Cancer (BCLC) classification system is widely used to classify HCC into five stages: very early stage HCC (BCLC 0), early stage HCC (BCLC A), intermediate stage HCC (BCLC B), advanced stage HCC (BCLC C) and terminal stage HCC (BCLC D). The classification depends on prognostic predictions (tumor status such as size, number, vascular invasion, and patient characteristics such as liver function and health status) and parameters known to affect the therapeutic outcome (e.g. bilirubin and portal hypertension).
There are curative treatment options for patients with early HCC (BCLC 0 and A). The other BCLC stages (B–D) are considered unresectable. Patients diagnosed with BCLC stages B–C are usually offered palliative treatment options. Treatment guidelines from the European association for the study of the liver and the European organisation for research and treatment of cancer can be viewed in Table 1.42

Table 1. Treatment recommendations for patients with hepatocellular carcinoma (HCC) according to guidelines from the European association for the study of the liver and the European organisation for research and treatment of cancer.42

<table>
<thead>
<tr>
<th>BCLC classification</th>
<th>Recommended treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCLC 0 and A (early HCC)</td>
<td>Resection, liver transplantation, local ablation with radiofrequency or percutaneous ethanol injection</td>
</tr>
<tr>
<td>BCLC B (intermediate stage)</td>
<td>Transarterial chemoembolization (TACE)</td>
</tr>
<tr>
<td>BCLC C (advanced stage)</td>
<td>Sorafenib (anti-angiogenic drug, multi-tyrosine kinase inhibitor)</td>
</tr>
<tr>
<td>BCLC D (terminal stage)</td>
<td>Best supportive care</td>
</tr>
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Transarterial chemoembolization

This thesis focuses on improving understanding of drug delivery from LIPDOX and DEBDOX, two of the DDSs used in transarterial chemoembolization (TACE) in intermediate-stage HCC. The enhanced arterial blood supply of HCC, compared to normal liver tissue, enables a locoregional delivery approach that, in theory, will increase local drug concentrations close to the tumor, while normal liver tissue is protected. In TACE, the DDS is delivered via a catheter through the hepatic artery close to the tumor, with lobar, segmental (selective) or superselective placement. The DDS is usually loaded with a single chemotherapeutic agent or a drug cocktail that is infused together with a contrast agent and embolic agents. The embolic agents (such as polyvinyl alcohol particles, microparticles, gel foam or gel sponge) are used to reduce the tumor blood flow as much as possible so as to deprive the tumor of oxygen and, at least in theory, increase the time that the drug resides in the tumor.

Generally, TACE techniques can be subdivided as follows: transarterial infusion chemotherapy (TAI), also called transarterial oily chemoembolization or lipiodolization, where the DDS consists of an emulsion based on the oily contrast agent lipiodol mixed with an aqueous drug solution;42, 43
conventional TACE (cTACE), where administration of the lipiodol-based emulsion is followed by administration of embolic agents; and drug-eluting bead TACE, where the DDS consists of beads loaded with drug and functions as an embolic agent while simultaneously delivering the drug. TACE treatment may be repeated on several occasions, depending on the individual tumor response and the occurrence of adverse events.

The survival benefits of intra-arterial therapies have been the subject of many clinical trials, and show contradictory results. \(^{44-47}\) cTACE is recommended as the standard of care for HCC patients (BCLC B) on the basis of a meta-analysis that proved a survival benefit. \(^{46}\) This meta-analysis showed that the expected overall median survival (i.e length of time that the patient survives after start of treatment) was increased to about 20 months with cTACE compared to 16 months with symptomatic treatment. \(^{46}\) In 2007, a meta-analysis comparing cTACE, embolization only, and TAI concluded that embolization and cTACE increase the time of survival whereas no benefit was found for TAI. \(^{43}\) Many of the studies in this analysis included several chemotherapeutic drugs, and possible differences in potency of those drugs were neglected. In another randomized clinical trial where cTACE and TAI with the same drug was compared, it was concluded that embolization (cTACE) does not increase survival compared to TAI. \(^{48}\) The methodology in this TAI vs cTACE study was however subsequently questioned. \(^{49}\)

The lack of strong evidence to indicate the most beneficial method for transarterial treatments (i.e. TAI, cTACE or drug-eluting bead-TACE) has resulted in a field with tremendous variations in treatment among clinical centers and interventional radiologists. These variations commonly involve the choice and dosage of the chemotherapeutics, the choice of embolic agents (if used), the selectivity of the transarterial catheter, and the frequency of repeated treatments.

**Doxorubicin – DOX**

DOX is one of the most commonly used cytotoxic drugs in TACE. \(^{43}\) DOX was investigated in this thesis because it is loadable into the two DDSs in question. DOX is produced by the bacterium *Streptomyces peucetius*, and is a potent cytotoxic antitumor drug that has been classified as an anthracycline antibiotic. \(^{50}\) It is chemically described as a hydrophobic planar tetracyclic ring system connected to a more polar deoxy(amo)nosugar by a glycosidic bond, see Figure 5. It is an ampholytic molecule with two pKa values: 8.2 and 10.2. \(^{51}\) DOX is often used as the starting material in formulations as the hydrochloride salt. DOX hydrochloride has a water solubility of 10 mg/ml at unspecified pH and has a log \(D_{7.5}\) of 2.42. \(^{52}\) It forms an orange-red solution in water.
For over 30 years, DOX has been used as a chemotherapeutic agent against multiple types of cancer, e.g. bone sarcoma, Hodgkin’s disease, and ovary, breast, lung, and liver cancer.\textsuperscript{53} DOX exerts its cytotoxic effect intracellularly by intercalation to the DNA double strand, inhibition of topoisomerase II (an enzyme that separates the DNA strands from each other before transcription and replication), and induction of reactive oxygen species.\textsuperscript{53,54} These mechanisms all cause DNA damage, which leads to apoptosis or cell cycle arrest.\textsuperscript{55-57} DOX is most effective in mitotic cells but can also be effective in other phases of the cell cycle.\textsuperscript{53} Since DOX does not specifically target tumor cells, its use is associated with several adverse effects such as nausea, vomiting, bone marrow depression, hair loss, local tissue necrosis, kidney failure, and cardiotoxicity.\textsuperscript{53} Since it causes cumulative, irreversible toxicity, and mainly because of the risk of cardiotoxicity, the cumulative intravenous (i.v.) dose of DOX is limited to 500 mg/m\textsuperscript{2} body surface area.\textsuperscript{57,58} Because of this broad, dose-limiting toxicity and the poor permeability of the intestinal wall to DOX (0.1 × 10\textsuperscript{-6} cm\textsuperscript{2}/s), its delivery is restricted to the parenteral route.\textsuperscript{59,60} Following an i.v. dose, the plasma concentration–time curve for DOX is best described by a three-compartment model; it has a reported terminal half-life of 35 h and a large volume of distribution (22 L/kg), which reflects extensive binding to tissue, mainly liver, kidneys, heart, and lungs.\textsuperscript{61,62}

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{doxorubicin_structure.png}
\caption{The molecular structure of doxorubicin. The functional group R is a ketone. In the primary metabolite doxorubicinol, the only difference is the transformation of the ketone into an alcohol.}
\end{figure}
Hepatic disposition of DOX

The major metabolizing and eliminating organ for DOX is the liver. Cellular uptake is mediated by both passive diffusion and carrier-mediated transport, followed by metabolism. The parent drug and metabolites are primarily eliminated through the bile. After i.v. administration of DOX, 40–50% of the dose is eliminated in the bile within a week (50% as the parent drug and 50% as metabolites, mainly doxorubicinol, DOXol). Renal excretion of unchanged DOX is much less, with only 12% excreted in the six days after an i.v. dose. Transporters and enzymes important for the hepatic disposition of DOX are summarized in Figure 6.

Figure 6. Doxorubicin (DOX) enters the cell mainly by passive diffusion, but is also a substrate for the solute carriers SLC01A2 and SLC22A16. In the cell, DOX may be metabolized into its active metabolite doxorubicinol (DOXol) or other inactive metabolites by carbonyl reductases (CBR1 and CBR3) and/or aldo-keto reductases (AKR1A1 and AKR1C3). DOX and DOXol exert their effects by causing DNA damage in the nucleus. DOX and DOXol may diffuse back into the blood or be transported into bile by transport proteins such as ABCB1 (P-glycoprotein), ABCC1 and ABCC2 (multidrug resistance-associated protein 1 and 2), ABCG2 (breast cancer resistance protein) and Ral binding protein1 (RALBP1). The figure is based on information from Thorn et al.65
Although passive diffusion is thought to be the main uptake mechanism for DOX, the drug is a substrate for at least two transporters on the basolateral membrane of the hepatocyte: SLC22A16 and SLC01A2. Following cellular uptake of DOX, it may be metabolized into its primary, active metabolite DOXol, which is 75-fold less potent than DOX. This biotransformation is catalyzed by cytosolic carbonyl reductases (CBR1, CBR3) and aldo-keto reductases (AKR1A1 and AKR1C3) which reduce the ketone group in DOX into an alcohol, see figure 5. CBRs and AKRs are available in many cells, but are most active in the liver and kidney. DOX and DOXol may be further metabolized into inactive aglycones. In order to be eliminated, the aglycones need to be glucuronidized. However, these inactive metabolites are present in negligible amounts in human biological fluids compared to DOX and DOXol. DOX and DOXol are transported to the bile via transporters in the canalicular membranes of the hepatocytes. Mainly by energy-dependent ABCs such as ABCB1 (P-glycoprotein, Pgp); ABCC1 and ABCC2 (multidrug resistance-associated proteins MRP1 and MRP2); and ABCG2 (breast cancer resistance protein, BCRP) and by Ral binding proteins such as RALBP1.

Local drug delivery systems

As mentioned, the two local DDSs investigated in this thesis are LIPDOX and DEBDOX (Figure 7). A recent meta-analysis of randomized clinical trials where DOX was delivered with LIPDOX (given as cTACE) or DEB-DOX showed a similar overall survival rate, although DEBDOX was associated with a higher objective tumor response rate. There are reports of a higher incidence of DOX-related adverse events and serious liver toxicity when using LIPDOX delivery for DOX compared to DEBDOX. It has, however, been demonstrated that DEBDOX is associated with a 6.6-times higher risk of developing liver/biliary events such as a dilated bile duct, VP narrowing, portal venous thrombosis or biloma/liver infarct than LIPDOX give as cTACE. The incidence of post-embolization syndrome (i.e. abdominal pain, nausea, vomiting and fever) is reported to be similar between the two DDSs.

Lipiodol-based emulsion – LIPDOX

Lipiodol was introduced for chemoembolization in HCC in the 1980s. It accumulates in tumor tissue, where it resides for several months after administration. This is believed to be a consequence of the enhanced permeability and retention effect in the tumor microenvironment. The rationale behind using lipiodol as an intra-arterial DDS is based on the assumption
that lipiodol releases the drug where the emulsion is accumulated. In a rabbit tumor model, LIPDOX increased the intratumoral concentrations of DOX compared to intra-arterial infusion of a DOX solution. However, it has been demonstrated that the lipiodol accumulation is a poor quantitative predictor of local concentrations of DOX, which raises concerns about the potential drug-targeting advantage of lipiodol. In the following sections, the characteristics of lipiodol and the lipiodol–based emulsion are summarized briefly.

**Lipiodol**

Lipiodol is marketed as Lipiodol Ultra Fluid® and Lipiodol® by Guerbet and is available in 47 countries. Although widely used globally for HCC, it was not until recently (2013-2014) that lipiodol was approved for TACE with the indication HCC in France, USA and Japan. In Sweden it is prescribed by the physician as a compassionate-use DDS.

Lipiodol consists of di-iodinated ethyl esters of poppy seed oil, which is a heterogeneous mixture of linoleate (~60%), oleate, palmitate, stearate and linolenate. The iodine content is 37–39% (i.e. 480 mg/ml) and this is the reason why lipiodol is visible in radiological techniques. Lipiodol is a heavy oil with a higher density than water (1.280 g/cm³ at 15°C) and has a high viscosity (25 mPa/s at 37°C, 50 mPa/S at 25°C). Intra-arterial infusion of lipiodol generates temporary, plastic embolization (i.e. adapted to vessel size) in the microvasculature. This sinusoidal embolization causes the sinusoids to atrophy in healthy animals. In animal models, lipiodol that passes the sinusoids is shunted forward to the portal venules where it accumulates and slows the sinusoidal blood flow. However, the microvascular changes that occur during HCC development, i.e. development of leaky, irregular vasculature, may impair the transport of lipiodol into the portal areas.

As lipiodol consists of lipids, many of the mechanisms involved in its distribution and elimination are the same as for other lipids. In vitro studies suggest that lipiodol is incorporated into the cell membrane and transported into the cell by pinocytosis (liquid endocytosis). Many active transporters (such as ABCA1, ABCB4, ABCB11, ABCD1, ABCG1, and ABCG5/8) are associated with lipid transport. Lipiodol is partly cleared from the blood as a result of phagocytosis by Kupffer cells. A study of the distribution of [¹⁴C]-lipiodol found that it is mainly eliminated to bile, while only trace amounts are found in the urine.

**Use of lipiodol-based emulsions as a drug-delivery system**

There is wide heterogeneity in the reported emulsion properties of LIPDOX. The properties can vary with respect to the aqueous solution:lipiodol volume ratio, the use of densifiers, and the mixing procedure. Several authors have highlighted the importance of a stable emul-
sion in improving the in vivo release and pharmacokinetic profile of the drug. In vitro and in vivo studies have indicated that the most stable LIPDOX emulsion is a water-in-oil emulsion with a volume ratio of one part drug (aqueous solution) and 2–4 parts lipiodol, where the specific gravity of the aqueous phase has been adjusted to that of lipiodol. However, the stability of the most stable LIPDOX emulsion is still poor. Therefore, the final formulation is traditionally prepared by the clinician minutes prior to administration by mixing with a syringe-pumping technique 10–15 times, see Figure 7A.

Figure 7. A) Preparation of the lipiodol-based emulsion. The physician receives two syringes from the hospital pharmacy, one containing lipiodol and the other containing an aqueous solution of DOX. These two syringes are connected via a connector and the emulsion is prepared by pumping the solutions back and forth about 10–15 times. The figure was used with kind permission from the creator I.R. Dubbelboer. B) A syringe loaded with DC bead® mixed with a non-ionic contrast agent for visualization during the infusion.

Drug-eluting beads – DEBDOX

The drug-eluting bead system DC Bead® was approved in 2007 as a novel DDS for the transarterial treatment of HCC. DC Bead® (Biocompatibles, BTG, UK) is indicated for treatment of hypervascular tumors and approved for loading with DOX. It is CE-marked (i.e. the product complies with European product directives) and, hence, is approved as a medical device instead of as a drug. Currently, it is approved in most of Europe, Russia, Canada, Australia, parts of Asia, Africa and Latin America; however, it is not yet approved in the US.
DC Bead® is a nondegradable, microparticulate DDS that consists of a polyvinyl alcohol-based hydrogel with associated chains of 2-acrylamido-2-methylpropane sulfonic acid sodium salt. The sulfonate sodium salt groups on these chains allow positively charged DOX molecules to replace the sodium ions during the loading process. The DOX-loaded DC Bead® (DEBDOX; Figure 7 B) releases the drug via a similar mechanism, by diffusion of ions into the bead, ion-exchange with DOX, and then diffusion of DOX out of the bead. After superselective administration of DEBDOX, the beads cause permanent embolization of the targeted vessels at the same time as DOX is released slowly. The beads are available in several different size ranges: 70–150 µm, 100–300 µm, 300–500 µm, and 500–700 µm. One and three months after administration of DEBDOX, the local tissue concentrations have been reported to be in or above the cytotoxic range for DOX (IC50 0.024–6.0 µM) for a range of 0.6 mm from the edges of the beads.

In vitro and in vivo studies have shown that the release rate of DOX is higher for smaller than for larger beads. The increase in release rate is probably related to the shorter diffusion path in the bead and the larger total surface area. In addition, smaller beads are associated with improved toxicity and efficacy, which may be the result of better penetration and more distal embolization in tumor tissue.
Aims of the thesis

The overall aim of this thesis was to assess the in vivo release of DOX from the currently used clinical formulations for palliative treatment of HCC (LIPDOX and DEBDOX) and its disposition in the surrounding tissue. A better understanding of the release mechanisms and the hepatic disposition of DOX from these DDSs in vivo is essential in order to evaluate which formulation achieves the best risk–benefit ratio, and to form the basis for future formulations and treatment strategies for this disease.

Specific aims

- To examine the effects of lipiodol on the disposition of DOX in plasma, urine and bile (Paper II) and in tissue (Paper III) in a multisampling-site, healthy pig model. Also, to investigate the mechanisms involved in increased tissue uptake of DOX.

- To study the in vivo release of DOX from the formulations LIPDOX and DEBDOX and the hepatic disposition of DOX after intra-arterial administration in a multisampling-site, healthy pig model (Paper I).

- To compare the in vivo release and hepatic disposition of DOX from the formulations LIPDOX and DEBDOX after intra-arterial administration to patients diagnosed with HCC. In addition, to evaluate the efficacy, safety and urinary excretion of these formulations in the patients (Paper IV).
Methods

Ethics and surgical/interventional procedures

The in vivo multisampling-site pig model

In Papers I, II and III, an advanced multisampling-site acute pig model was applied. This pig model was developed and validated by Petri et al. in 2006. The multiple sampling sites enable sampling prior to the blood reaching the liver (VP), after the liver (VH) and systemically (femoral vein; VF) as well as quantitative collection of bile and urine up to six hours after administration of a drug. These sampling sites and the large number of samples that can be collected provide a model suitable for mechanistic in vivo investigations in a healthy animal. The pig model was expected to be suitable for studies of transarterial treatment, since the pig liver is similar to that of a human in segmental anatomy, vascularization and size.

The in vivo studies were approved by the local ethics committee for the use of laboratory animals in Uppsala, Sweden (C40/11). The pigs included in the studies were 10–12 weeks old, of mixed breed (Yorkshire and Swedish Landrace) and weighed on average 26.0 ± 1.3 kg in Paper I and 26.7 (range 24.2–30.3) kg in Papers II/III. The anesthesia and surgical procedures are explained in detail in Papers I and II. In brief, the pigs were sedated, anesthetized, and connected to a respirator. They were continuously monitored for heart rate, blood gases, arterial and central venous pressure, and body temperature and electrocardiograms were run continuously, to maintain normal physiological values throughout the experiment. A midline incision of the abdominal cavity gave access to position the catheters in the VP, VH, VF, and bile duct. A tube was inserted into the bladder to collect urine. At the end of the experiment each animal was euthanized with an i.v. bolus dose of 20–30 nmol potassium chloride while still under anesthesia.
The clinical trial in patients with liver cancer

The clinical trial described in Paper IV was performed in patients diagnosed with intermediate-stage HCC (BCLC stage B). These patients were treated with either LIPDOX as TAI or DEBDOX as TACE. The study was a multi-center study carried out at two hospitals in Sweden. The study protocol was approved by the regional ethical review board in Uppsala (Dnr 2013/227) and the study was registered in the European Clinical Trials Database (Eudra CTnr 2013-001244-56). All enrolled patients provided written informed consent. The inclusion and exclusion criteria are reported in Paper IV.

The intra-arterial procedure was equivalent at both hospitals, as described in Paper IV. Briefly, two catheters were placed intravascularly, as shown in Figure 8. As in the normal clinical procedure, a catheter was inserted into the hepatic artery via the right femoral artery for administration of the DDS. This catheter was placed lobarly for LIPDOX, in order to treat any eventual tumor satellites, and close to the tumor in the tumor-feeding arteries (super-selective) for DEBDOX. In this study, a blood sampling catheter was also placed in the vena cava orifice from the hepatic veins (VC/VH catheter) in order to obtain local measurements. This placement was accessed via the left VF through an introducer. Peripheral samples were taken directly from the introducer.

![Figure 8. Catheters inserted intra-arterially during TAI or TACE treatment. One was used for drug administration and the other was used to collect local blood samples in the vena cava/hepatic vein orifice.](image-url)
Study designs

Drug–excipient and drug–drug interaction studies in healthy pigs

In Papers II and III, the mechanisms behind a potential LIPDOX-induced increased cellular uptake of DOX were investigated. This was done using a drug–excipient and drug–drug interaction approach between DOX and lipiodol and/or cyclosporin A (CsA).

This study was designed with four parallel treatment groups (TI–TIV, Figure 9). There were two treatment phases: the reference phase (phase 1, 0−160 min) and the test phase (phase 2, 200−360 min). At times 0 and 200 min, all pigs received an i.v. infusion of DOX into an ear vein for 5 min. These two i.v. doses were given so that the pigs could serve as their own controls. Just prior to the second dose of DOX, i.e. phase 2, the treatment groups TII–TIV received an additional 5 min infusion of drug-free lipiodol emulsion (TII), a 20 min CsA infusion (TIII) or both (TIV) into the VP. If no other infusion was scheduled before the test phase, the volume was compensated for with physiological saline into the VP. CsA was used because it is a known inhibitor of efflux transporters important for DOX elimination and of several biotransformation enzymes important for drug metabolism. Its main role was thus to investigate whether lipiodol interacts with transporters or enzymes important for DOX disposition. The experiment lasted for six hours, during which time samples were collected at predetermined time points from the VP, VH, and VF. Bile and urine were collected quantitatively (data presented in Paper II). At the end of the experiment, the pigs received a lethal dose of potassium chloride and pre-selected tissue samples (i.e. heart, liver, kidney and intestine) were collected (data presented in Paper III).

![Figure 9](image-url). The study design for papers II and III.
The *in vivo* release studies in healthy pigs and HCC patients

**Healthy pigs (Paper I)**

In this study, the primary aim was to investigate the effects of the intra-arterial DDSs on the pharmacokinetics of DOX in pigs. The study had a randomized, parallel design and four treatment groups, TI–TIV (Table 2). The pigs in TI were the control group and received a 55-minute i.v. infusion of DOX solution in an ear vein. The pigs in TII and TIII received a five-minute intra-arterial infusion of either LIPDOX as TAI (TII) or of DEBDOX (TIII) into the hepatic artery. In TIV, LIPDOX was administered through the hepatic artery and the bile duct simultaneously, in order to increase the local availability of the drug. The intended dose in all groups was 2 mg/kg. During the six-hour experiment, samples were collected at predetermined time-points from the VP, VH, and VF and continuously from the bile.

**Table 2. The study design for Paper I**

<table>
<thead>
<tr>
<th>Group</th>
<th>Formulation</th>
<th>Dose(^a) (mg)</th>
<th>Administration-route</th>
<th>Sampling sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>TI (n=4)</td>
<td>Solution</td>
<td>47 ± 0.3</td>
<td>Intravenous(^c)</td>
<td>Yes</td>
</tr>
<tr>
<td>TII (n=4)</td>
<td>Lipiodol emulsion</td>
<td>3.1 ± 0.8</td>
<td>Intra-arterial</td>
<td>Yes</td>
</tr>
<tr>
<td>TIII (n=4)</td>
<td>Drug-eluting beads</td>
<td>50 ± 0.0</td>
<td>Intra-arterial</td>
<td>Yes</td>
</tr>
<tr>
<td>TIV (n=2)</td>
<td>Lipiodol emulsion</td>
<td>22 ± 3.5</td>
<td>Intra-arterial and via bile duct</td>
<td>Yes</td>
</tr>
</tbody>
</table>

\(^a\)Mean ± S.E., \(^b\)samples were collected from the portal, hepatic and femoral veins, \(^c\)constant infusion for 55 min given through the ear vein

**HCC patients (Paper IV)**

Data from the 14 patients who were included in the ongoing study during the period from January 2014 to March 2015 were assessed in the interim study analysis presented in Paper IV. The study was an open, prospective, multi-center study performed at the Uppsala University Hospital and the Karolinska University Hospital, both in Sweden. The patients were divided into two study arms based on the hospital to which they were admitted, as the hospitals had different standard treatments. Six patients were enrolled at Uppsala University Hospital where LIPDOX was given as TAI, and eight
patients were enrolled at Karolinska University Hospital where DEBDOX was given. The inclusion and exclusion criteria are reported in Paper IV.

The investigation schedule for each patient, presented in Table 3, can be summarized as follows: during and after TAI or TACE treatment, blood samples were collected at predetermined time-points from the VC/VH catheter to measure local concentrations (up to 6 h) and from a peripheral vein to measure the systemic concentrations (up to 7 days). After six hours, the VC/VH catheter was removed. Peripheral samples collected at 24 hours and 5–7 days were accessed through a needle in the arm. Urine was quantitatively collected from the start of treatment up to 24 hours. Safety parameters in the blood were measured before, 24 hours after and 5–7 days after treatment. Specifically, these parameters were: aspartate amino transferase, alanine amino transferase, alkaline phosphatase, prothrombin-international normalized ratio, bilirubin, creatinine, urea, hemoglobin, thrombocytes, leukocytes, albumin, sodium, potassium and C-reactive protein. In addition, the necrotic efficacy was evaluated by computed tomography or magnetic resonance imaging before and 4–6 weeks after treatment.

Table 3. Study flowchart for each patient. The pharmacokinetic blood samples were taken at visit 2 at pre-defined time points: before the infusion, and at 0, 5, 15, 30, and 60 min and 2, 6, and 24 h after the end of the infusion. Urine was collected during visit 2. One additional blood sample was taken at visit 3. The tumor response was assessed with imaging techniques such as computed tomography and magnetic resonance imaging.

<table>
<thead>
<tr>
<th>STUDY PERIOD</th>
<th>Visit 1 Baseline assessment</th>
<th>Visit 2 TAI/TACE treatment Day 1 (0–24 h)</th>
<th>Visit 3 Return to hospital 5–7 days</th>
<th>Visit 4 Return to hospital 4–6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmacokinetic samples</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Safety measurements*</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Imaging</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

*Safety measurements: aspartate amino transferase, alanine amino transferase, alkaline phosphatase, prothrombin-international normalized ratio, bilirubin, creatinine, urea, hemoglobin, thrombocytes, leukocytes, albumin, sodium, potassium and C-reactive protein.
Bioanalytical methods

Two methods were used to quantify DOX and DOXol concentrations in various biological matrices: ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS), and ultra-high performance liquid chromatography coupled with mass spectrometry (UPLC-MS). Internal standards ([13C,2H3]-DOX and [13C,2H3]-DOXol) were used in all methods.

The quantification of DOX and DOXol in plasma, bile and urine was carried out at the National Veterinary Institute (SVA), Uppsala, Sweden. These analyses were performed by UPLC-MS/MS as described in detail in Papers I, II and IV. Quantification in tissue homogenates was carried out at Uppsala University using a UPLC-MS system. Before injection onto the UPLC-MS system, the tissue homogenate was prepared by protein precipitation. The specific method is described in detail in Paper III.

Pharmacokinetic data analysis

The pharmacokinetic parameters for DOX and DOXol in Papers I, II and IV were calculated using non-compartmental methods with WinNonLin 5.2 and 6.3 software (Pharsight, Mountain View, CA). In Papers I and II, compartmental models were also applied. The maximum plasma concentration (C_max) and the time to reach C_max (t_max) were directly derived from the plasma concentration–time curve. The area under the concentration–time curve (AUC) for plasma and bile represents the drug exposure over time and was calculated with the linear and logarithmic trapezoidal method for the ascending and descending parts to the last data point (C_last), respectively. AUC_0–t was calculated from time zero to time t. AUC_0–∞ was calculated by adding the residual area, which was estimated based on the slope (λ₂) of the two to three last measurable observations after C_max. The terminal half-lives in the studies were calculated by ln2/λ₂.

Following an i.v. dose of DOX in Papers I and II, the plasma clearance (CL) of DOX was calculated as follows:

\[ CL = \frac{Dose_{i.v.}}{AUC_{0–∞,VF}} \]  
(eq.1)

where \( AUC_{0–∞,VF} \) is the AUC extrapolated to infinity based on the measured plasma concentrations from the VF (i.e. systemic concentrations).
The capacity of the liver to remove DOX from the circulation, defined as hepatic extraction ($E_H$), was calculated based on data from the available sampling sites in the pigs before (VP) and after (VH) the liver:

$$E_H = \frac{(AUC_{VP} - AUC_{VH})}{AUC_{VP}} \quad \text{(eq.2)}$$

where all AUCs were the observed AUCs up to six hours.

Further, the fraction of the DOX or DOXol dose excreted ($f_e$) to bile and urine during the study period was calculated according to the following equation:

$$f_e = \sum(C \cdot V)/Dose \quad \text{(eq.3)}$$

where $C$ is the concentration in the collected volume ($V$) of either bile or urine. The biliary excretion was assessed only in the pig studies while renal excretion was assessed in both Papers II and IV.

**Tissue distribution of DOX and DOXol**

In Paper III, the tissue distribution of DOX and DOXol was investigated. The tissue distribution was defined as the tissue-to-plasma partitioning coefficient ($K_p$) as in equation 4.

$$K_p = \frac{C_{tissue}}{C_{plasma}} \quad \text{(eq.4)}$$

where $C_{tissue}$ is the tissue concentration corrected for its blood content after six hours, as described in Paper III, and $C_{plasma}$ is the VF plasma concentration measured at six hours in the same pig.

**Mechanistic interaction between cyclosporin and DOX**

In Paper II, a mathematical multicompartment model was built in WinNonLin (v 6.3) to increase the mechanistic understanding of CsA-induced alterations to DOX and DOXol pharmacokinetics (Figure 10). The model was derived from a three-compartment model with a central plasma compartment and two tissue compartments: liver and peripheral tissue. The mass transport in the model was calculated as a first order reaction according to equation 5;

$$\frac{dA}{dt} = C \cdot k \cdot V \quad \text{(eq.5)}$$

where $A$ is the amount of compound, $C$ is the concentration of compound in the compartment, $k$ is the rate constant, and $V$ is the volume of the compartment. For more details about the model, see Paper II.
Figure 10. Schematic overview of the multicompartment model describing the disposition of doxorubicin (DOX) and doxorubicinol (DOXol). Grey compartments with solid lines represent compartments that describe observed data (i.e. from plasma, urine and bile). Dashed arrows represent metabolism from DOX to DOXol or from DOXol to other metabolites.

The model was fitted to the observed data after administration of the first dose of DOX, i.e. in the reference phase, and 11 model parameters were estimated. Thereafter, a sensitivity analysis was done to simulate what would happen if the estimated rate constants for metabolism and/or elimination to bile were systematically reduced by 99% for DOX and DOXol. These simulations were compared to the observed data, and were used to elucidate the mechanism responsible for the effects on DOX and DOXol disposition by lipiodol and/or CsA.

Bioavailability and in vivo release of DOX

The bioavailability (F) is an approximation of the fraction of the dose that reaches the systemic circulation, which in this case is reached after intra-arterial hepatic drug administration. F may therefore be used to approximate the fraction released from parenteral DDSs in vivo. In Paper I, the bioavailability was calculated by the following equation;

\[ F = \frac{CL \cdot AUC_{0-6,DDS}}{Dose_{DDS}} \]  

(eq.6)
Another approach to approximate in vivo drug release is by deconvolution using WinNonLin software. Briefly, the plasma concentrations in the groups receiving the DDS were set as the response function, \( r(t) \), which was weighted against data obtained after an i.v. dose of DOX, the weighting function, \( w(t) \), which was derived from compartmental modeling of the i.v. dose. In Paper I, a two-compartment model best described the pig data collected over six hours after an i.v. dose. In Paper IV, human i.v. data were taken from literature and were best described by a three-compartment model.\(^{115}\) Deconvolution was then executed and the input function, \( i(t) \) was obtained; \( i(t) = r(t)/w(t) \). This function represents the apparent in vivo release of drug from the DDSs into the local and systemic sampling sites.

**Apparent intracellular availability of DOX**

Since metabolism of DOX to DOXol mainly occurs in the hepatocytes, the exposure of bile to DOX and DOXol was used to approximate the apparent intracellular availability of DOX, \( F_{i;app} \), in Papers I and II (Equation 7). The following assumptions were made: i) that the metabolism followed linear distribution, ii) that reabsorption from the bile duct was limited, and iii) that there was no saturation of the efflux transporters and/or enzymes responsible for the biotransformation.

\[
F_{i;app} = \frac{AUC_{bile,DOXol}}{(AUC_{bile,DOX} + AUC_{bile,DOXol})} \quad (eq.7)
\]

**Statistical analysis**

In the pig studies, non-parametric tests (i.e. Kruskal-Wallis or Mann-Whitney) were used to test for significance between the treatments. In the clinical trial, Student’s t-tests were used to compare the treatments. Differences were considered to be statistically significant when \( p \) was <0.05. Post hoc analyses for multiple comparisons were performed when applicable. For details about the statistical analyses, see the relevant Paper.
Results and Discussion

In vivo pharmacokinetics of DOX following an i.v. dose in pigs

DOX was rapidly and extensively distributed to tissues following five- and 55-minute i.v. infusions (ear vein; Papers II and I, respectively; Figure 11). The longer infusion time reduced the $C_{\text{max}}$ of DOX about 8-fold and the fraction of the formed metabolite DOXol (DOXol-to-DOX AUC$_{0-3h}$ ratio) about 9-fold (Figure 11, Table 4). The infusion rate-dependent metabolism of DOX into DOXol might be explained by saturation of the DOXol metabolism and/or excretion while plasma and tissue concentrations of DOX were high. The plasma concentration–time curves for DOXol declined in parallel with those for DOX, indicating that the pharmacokinetics of DOXol were limited by its rate of formation (Figure 11).

![Figure 11. Dose-normalized plasma concentration–time curves for doxorubicin (DOX) and doxorubicinol (DOXol) following an i.v. infusion of DOX. The data were obtained from Paper I, in which four pigs received 55-min i.v. infusions of DOX, and Paper II, in which 12 pigs received 5-min i.v. infusions of DOX. The striped line represents the last datapoint measured during the 55-min infusion.](image-url)
Table 4. Summary of pharmacokinetic parameters for doxorubicin (DOX) and doxorubicinol (DOXol) in plasma, bile and urine following an i.v. dose of DOX into an ear vein in healthy pigs.

<table>
<thead>
<tr>
<th></th>
<th>Pigs (Paper I) a</th>
<th>Pigs (Paper II) b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DOX</td>
<td>DOXol</td>
</tr>
<tr>
<td>No. of pigs</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Infusion time (min)</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>Dose (µmol)</td>
<td>85.7 (84.6–86.8)</td>
<td>-</td>
</tr>
<tr>
<td>C_{max} (nM)</td>
<td>7.7 (6.5–10.3)</td>
<td>0.06 (0.045–0.24)</td>
</tr>
<tr>
<td>AUC_{0–3h} (µM·min)</td>
<td>0.44 (0.37–0.55)</td>
<td>0.006 (0.005–0.027)</td>
</tr>
<tr>
<td>DOXol-to-DOX AUC ratio (%)</td>
<td>1.4 (1.1–6.3)</td>
<td>-</td>
</tr>
<tr>
<td>CL (mL/min/kg)</td>
<td>59 (53–74)</td>
<td>-</td>
</tr>
<tr>
<td>E_H (%)</td>
<td>41 (22–57)</td>
<td>-</td>
</tr>
<tr>
<td>f_{biliary} (%)</td>
<td>20 (6.6–23)</td>
<td>4.2 (2.6–5.5)</td>
</tr>
<tr>
<td>f_{urine} (%)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Data were from the femoral vein samples and are presented as medians (ranges); b data were from the portal vein samples and are presented as means ± standard deviations; c data were normalized after the administered dose; d measured for three pigs, six hours after two consecutive i.v. doses of DOX (dosing interval three hours), data presented as medians (ranges). AUC = area under the plasma concentration–time curve; C_{max} = maximum concentration; CL = clearance; E_H = hepatic extraction; f_e = fraction excreted.

**Plasma clearance and hepatic extraction of DOX**

The average plasma CL of DOX was about 48–59 mL/min/kg in pigs (Table 4). This CL was higher than in humans (13 ± 4.2 mL/min/kg) studied over seven days. The higher CL in pigs may have been the result of under-estimation of the residual area in these short studies (3 h and 6 h for the 5- and 55-min infusion studies, respectively). The plasma concentration–time curves in the pig studies were best described by a two-compartment model, although DOX has previously been best described by tri-exponential equations with a long terminal half-life (35 h).

The simultaneous blood sampling from the VP and VH enabled direct quantification of the E_H of DOX. The E_H was dependent on the infusion time; it was higher after the 55-minute infusion than after the five-minute infusion (Table 4). In Paper II (55-min infusion), it was also found that E_H
varied with time; and that it rapidly decreased once the infusion stopped. A time- and route-dependent phenomenon for $E_H$ has previously been observed for other drugs with this model (e.g. finasteride, verapamil and raloxifene). An intermediate $E_H$ of about 40% was observed for DOX during the longer infusion, which is also consistent with other data from domestic pigs and humans. The intermediate $E_H$ value indicates that the hepatic clearance of DOX depends on the hepatic blood flow, the intrinsic metabolizing capacity of the liver, and the fraction of free drug in blood.

**Biliary and urinary transport of DOX**

The main elimination route for DOX was via bile, with about 4.5–23% of the dose excreted as unchanged drug during the first six hours after the infusions in pigs (Table 4). The dose-fraction of DOXol excreted was about five-fold less. The dose-fraction excreted to urine was lower than that excreted to bile: 5.8% and 2% for DOX and DOXol, respectively.

The exposure of bile to DOX and DOXol was extensive compared to that of plasma. For example, in Paper I, the bile-to-plasma $AUC_{0-6h}$ ratios for DOX and DOXol were 640 (410–670) and 5000 (1500–6200), respectively, given as median (range). The ten-fold higher bile-to-plasma $AUC_{0-6h}$ ratios for DOXol than for DOX may be explained by the higher initial concentrations of DOX in plasma directly after administration. Consequently this would result in a lower bile-to-plasma AUC ratio for DOX since DOXol is formed intracellularly. Within the cell, DOX (and probably also DOXol) is a substrate for several canalicular transporters (i.e. Pgp, BCRP, MRP1 and MRP2) which direct transport out of the cell to the bile rather than to the plasma. In addition, the passive diffusion rate across lipophilic membranes is slower for DOXol than for DOX as a result of its more hydrophilic properties.

**Tissue distribution of DOX**

DOX was extensively distributed in all the investigated tissues ($K_p >140$) after six hours, with the following rank order: kidney > liver > heart > intestine (Paper III). This is in agreement with the high volume of distribution ($V_{ss}= 130 \pm 98$ L) in the same pigs (Paper II). Explanations for this extensive tissue distribution include the amphiphilic properties of DOX, and also its high affinity for DNA, which would result in intracellular accumulation. Indeed, it has been reported that DOX concentrations in the cell nucleus are 50-fold higher than those in the cytoplasmic compartment, while intracellular concentrations are generally 10- to 500-fold higher than in the extracellular space.
Drug–excipient and drug–drug interaction studies in healthy pigs

The effects of lipiodol on the pharmacokinetics of DOX

Lipiodol did not affect the disposition of DOX and DOXol after an i.v. infusion of DOX into an ear vein in healthy pigs. Plasma, bile and tissue concentrations were no different from those in control pigs (Figure 12). The $f_{uc}$ into urine and the individual DOXol-to-DOX AUC ratio were not altered either. These results indicate a lack of direct interaction between lipiodol and DOX. Hence, there is no evidence of an interaction with transporters, enzymes and/or biological membranes important for the hepatobiliary disposition of DOX and DOXol.

**Figure 12.** The effect of lipiodol (LIP) and cyclosporin A (CsA) on doxorubicin (DOX; left columns) and doxorubicinol (DOXol; right columns) plasma concentrations, amounts accumulated in bile, and tissue concentrations in healthy pigs. DOX was given as an i.v. dose at 0 and 200 min. LIP and/or CsA were administered before the second dose of DOX (indicated by the red striped line). *p <0.05, **p <0.01.
We suggest that the *in vivo* increase in tissue uptake of DOX (mainly by the tumor) when DOX is emulsified in lipiodol is related more to the encapsulation of DOX in the formulation than to excipient–drug interactions on a membrane, enzyme or transport level. The complexity of the biological systems makes it difficult to study the detailed effects of formulation properties on drug distribution with *in vitro* models. Therefore, more systematic *in vivo* approaches are needed to elucidate the mechanisms behind the increased uptake of DOX by tumor tissue.

The effects of cyclosporin on the pharmacokinetics of DOX

When CsA was administered with DOX to pigs, the plasma exposure to DOX was unaffected but that to DOXol was increased (Figure 12, Table 5). In addition, there was a clear reduction in the biliary excretion of both DOX and DOXol. CsA treatment (TIII and TIV) caused an increase in liver, kidney and intestine concentrations of DOX compared to control (TI and TII, p <0.05). These effects of CsA on DOX and DOXol disposition in plasma and bile are in accordance with those in other short (<4 h) *in vivo* studies.

<table>
<thead>
<tr>
<th>Table 5. Pharmacokinetic parameter changes after infusion of cyclosporin (CsA) to pigs (groups TIII and TIV) in combination with doxorubicin administration, compared to control groups without CsA (TI and TII). No change (±30%) is indicated by ↔, a 30–60% change is indicated by ↑ or ↓, a 60–80% decrease is indicated by ↓↓ and a 80–100% decrease is indicated by ↓↓↓.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
</tr>
<tr>
<td>AUC</td>
</tr>
<tr>
<td>C_{\text{max}}</td>
</tr>
<tr>
<td><strong>Bile</strong></td>
</tr>
<tr>
<td>f_e, bile</td>
</tr>
<tr>
<td>AUC_{\text{bile}}/AUC_{\text{plasma}}</td>
</tr>
<tr>
<td>F_{\text{i;app}}</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
</tr>
<tr>
<td>f_e, urine</td>
</tr>
<tr>
<td><strong>Tissues</strong></td>
</tr>
<tr>
<td>Liver conc.</td>
</tr>
<tr>
<td>Kidney conc.</td>
</tr>
<tr>
<td>Heart conc.</td>
</tr>
<tr>
<td>Intestine conc.</td>
</tr>
</tbody>
</table>

AUC = area under the concentration–time curve; conc. = concentration; C_{\text{max}} = maximum concentration; f_e = fraction excreted; F_{\text{i;app}} = apparent intracellular availability; N.A. = not applicable; N.D. = not detected.
We suggest that DOX and DOXol use the same canalicular transport proteins, since the inhibition of biliary transport by CsA was similar for the parent drug and the metabolite.

CsA is a known inhibitor of the efflux transporters Pgp, MRP2 and BCRP, and of influx transporters (e.g. OATPs) and metabolic enzymes (e.g. CYPs). The unchanged plasma exposure to DOX following CsA treatment suggests that the interaction with DOX was not caused by inhibition of basolateral influx transporters. For other drugs where CsA is known to interact with basolateral uptake transporters, CsA treatment increased plasma exposure to and decreased hepatic clearance of the parent drug. This theory is further supported by simulations made during sensitivity analysis of the multi-compartment model; it was necessary for the metabolism of DOX to DOXol to be unaffected in the model in order to fit the observed increase in plasma concentrations of DOXol. In analogy with the lack of reports, it suggests that CsA does not inhibit AKRs and CBRs.

Pgp, MRP2 and BCRP are also expressed in tissues other than the liver, which may explain the observed increase in DOX concentrations in kidney and intestine, and the decrease in urine. Reduced efflux of DOX to bile, urine and the intestinal lumen will increase the concentrations in liver, kidney and intestine. Higher intracellular concentrations of DOX increase the availability of substrate for metabolism into DOXol. However, the DOXol concentrations in kidney and the renal excretion of DOXol were unchanged after inhibition by CsA. This observation might be explained by varying expression and activity of cytosolic CBRs and AKRs in the different tissues. The high intracellular concentrations of DOX caused by CsA inhibition may have saturated these enzymes in the kidney but not in the liver or intestine, and/or they may have induced other metabolic pathways leaving DOXol concentrations in the kidney unchanged.

**The in vivo release studies in healthy pigs and HCC patients**

This section discusses the *in vivo* release and local distribution of DOX after administration with LIPDOX and DEBDOX. There is an important difference in the administration approach between these two DDSs. LIPDOX is administered selectively to one liver lobe without injecting additional embolic agents, resulting in transient embolization. DEBDOX is administered superselectively to the tumor-feeding arteries close to the tumor, where it provides complete embolization and slowly releases DOX.
Dosage and tumor characteristics

The tumor characteristics and dosage of DOX in the DDSs for the preclinical and clinical study subjects are summarized in Table 6.

In healthy pigs (Paper I), it was impossible to administer the intended dose of LIPDOX (50 mg of DOX) as an unexpected stasis occurred in the artery after the infusion. As a consequence, a lower dose was given. Pigs seem to be more sensitive to the highly viscous lipiodol than humans, maybe because of differences in blood vessel anatomy and/or physiological adaptations. It has, for example, been reported that younger pigs (aged three months) have a narrower VP with a thinner vessel wall than humans. The droplet sizes in relation to the blood vessel size may also be an important factor for the increased vessel resistance, since larger droplets have higher embolic capacities. Despite these difficulties in administering LIPDOX, the DDS released DOX sufficiently well to be able to quantify the concentrations in plasma. In liver cancer patients (Paper IV), the treated tumors were larger at screening in the LIPDOX group than in the DEBDOX group (p <0.05).

Table 6. Baseline characteristics and dosage of intra-arterial LIPDOX and DEBDOX treatment in the in vivo release studies with healthy pigs and human liver cancer patients.

<table>
<thead>
<tr>
<th></th>
<th>Pigs (Paper I)</th>
<th>Patients (Paper IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LIPDOX</td>
<td>DEBDOX</td>
</tr>
<tr>
<td>No of included subjects</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Baseline tumor size (mm)a</td>
<td>No tumor</td>
<td>No tumor</td>
</tr>
<tr>
<td>Child-Pugh (A/B/na)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dose of DOX (mg)b</td>
<td>2.3 (2.3–5.4)</td>
<td>50 (50–50)</td>
</tr>
<tr>
<td>No. of treated tumors</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

aMedian (range). DEBDOX = microparticle DOX-eluting beads; DOX = doxorubicin; LIPDOX = lipiodol-based emulsion containing DOX; na = not available

The pharmacokinetics of DOX after delivery with LIPDOX or DEBDOX

The dose-normalized plasma concentrations of DOX and DOXol in pigs and patients (Paper IV) following LIPDOX and DEBDOX delivery are displayed in Figure 13. The Cmax of DOX was 10–11 times higher after LIPDOX than after DEBDOX in both species. For both DDSs, the Cmax was followed by a rapid distribution phase. The plasma exposure to DOX (AUC0–6h in pig and AUC0–24h in patients) was 2–3 times higher after LIPDOX than after DEBDOX. The higher exposure to LIPDOX was observed both locally and
systemically. This relationship between the two DDSs is consistent with previously reported data in patients. In summary, LIPDOX released DOX to the systemic circulation to a greater extent than DEBDOX during the study.

It has been suggested that DEBDOX is associated with fewer DOX-related adverse events because of the lower systemic exposure. However, the recommended clinical dose for DEBDOX is three times higher than that for LIPDOX (150 mg vs 50 mg), which compensates for the difference in systemic exposure. For example, using the local bioavailability of DOX in patients, this corresponds to about 16 mg of DOX released from both DDSs after six hours. The C_{max} of DOX is, however, still lower for DEBDOX, which might affect the frequency of adverse events.

Figure 13. Dose-normalized plasma concentration–time curves after administration of doxorubicin (DOX) in a lipiodol-based emulsion (LIPDOX) or DOX-eluting microparticle beads (DEBDOX) to pigs [(A) DOX and (B) doxorubicinol (DOXol)] and to patients [(C) DOX and (D) DOXol]. Measurements were taken for six hours in pigs and for seven days in patients; the insets display the first six hours in patients.

45
Release from LIPDOX

In most of the pigs and patients, 50% of the dose was released from LIPDOX into the circulation within the first 30 min (Figure 14, Table 7).

The rapid release from LIPDOX suggests that the emulsion breaks down once it is infused into the circulation, just as it does when it comes into contact with elution media in vitro. Indeed, the sustained delivery of DOX is highly correlated with the physicochemical properties of the emulsion. In Papers I and IV, the physical stability of the emulsion was optimized with a densifier and the most optimal volume ratio between lipiodol and the aqueous DOX solution (1:3.3 aqueous DOX in lipiodol) was used. Despite these measures, the emulsion still starts to separate 10 min after preparation in vitro.

Figure 14. The cumulative fraction of the dose of doxorubicin (DOX) released (approximated by deconvolution) as a function of time at the systemic (A) and local (B) sampling sites over six hours following intra-arterial injection of a lipiodol-based emulsion containing DOX (LIPDOX) or DOX-eluting microparticle beads (DEBDOX) in pigs and liver cancer patients. The results for liver cancer patients over seven days are presented in graph (C). LIPDOX released DOX more rapidly than DEBDOX.
The release mechanism for DOX from the emulsion involves partitioning between the water and oil phases as well as diffusion through the oil phase.\textsuperscript{137} The fraction of DOX that partitions into the lipid phase of the emulsion has the potential to follow lipiodol into the tissues. Inter-individual differences in tumor size and vessel anatomy may be of greater importance for LIPDOX than for DEBDOX because of the unstable nature of the emulsion, since the fixed position of DEBDOX and the reduced blood flow will enhance the probability that DOX will reach the arteries adjacent to the administration site.\textsuperscript{2}

Table 7. The bioavailability (in this case, the release) and local distribution of doxorubicin (DOX) following intra-arterial delivery in a lipiodol-based emulsion (LIPDOX) or DOX-eluting microparticle beads (DEBDOX) to healthy pigs (Paper I) and liver cancer patients (Paper IV).

<table>
<thead>
<tr>
<th>Bioavailability/ release</th>
<th>Species</th>
<th>LIPDOX</th>
<th>DEBDOX</th>
<th>LIPDOX</th>
<th>DEBDOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>F (%)\textsuperscript{a,b}</td>
<td>Pig</td>
<td>29 (22–44)</td>
<td>14 (9.4–18)</td>
<td>85 (70–94)</td>
<td>24 (18–29)</td>
</tr>
<tr>
<td>F\textsubscript{deconv, 6h} (%)\textsuperscript{a,d}</td>
<td>Pig</td>
<td>38 (28–56)</td>
<td>14 (11–21)</td>
<td>110 (92–120)</td>
<td>32 (24–38)</td>
</tr>
<tr>
<td>F\textsubscript{deconv, 6h} (%)\textsuperscript{c,d}</td>
<td>Human</td>
<td>51 ± 7.6</td>
<td>11 ± 5.6</td>
<td>69 ± 25</td>
<td>13 ± 6.5</td>
</tr>
<tr>
<td>F\textsubscript{deconv, 24h} (%)\textsuperscript{c,d}</td>
<td>Human</td>
<td>51 ± 7.6</td>
<td>15 ± 8.5</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Local distribution</th>
<th>Species</th>
<th>Systemic</th>
<th>Local</th>
</tr>
</thead>
<tbody>
<tr>
<td>F\textsubscript{i;app}\textsuperscript{a}</td>
<td>Pig</td>
<td>32 (22–43)</td>
<td>18 (17–21)</td>
</tr>
<tr>
<td>AUC\textsubscript{last,Doxorubicin/DOX}\textsuperscript{c}</td>
<td>Human</td>
<td>1.2 ± 0.53</td>
<td>0.54 ± 0.21</td>
</tr>
<tr>
<td>RD,\textsubscript{6h} (%)\textsuperscript{c}</td>
<td>Human</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data are shown as medians (interquartile ranges); \textsuperscript{b}F was obtained using equation 6; \textsuperscript{c}data are shown as means ± standard deviations; \textsuperscript{d}data were derived from deconvolution. Deconvoluted values are only presented up to 24 h due to few observations at 5–7 days. AUC = area under the concentration–time curve; F = bioavailability; F\textsubscript{i;app} = apparent intracellular availability; NA = not applicable; RD = remaining dose in the liver, calculated by 100-F\textsubscript{local} for each patient.

Release from DEBDOX

Approximately 32% of the dose was released from DEBDOX into the local compartment in pigs after six hours. In patients, the corresponding fraction of the dose released was 13% locally after six hours and 15% systemically after 24 hours (Figure 14, Table 7). This difference in \textit{in vivo} release between pigs and patients (32% vs 13%) may be the result of species differences, as well as the absence of tumors or cirrhotic livers in the pigs. DOX could therefore have been distributed more freely in pigs, as they lacked the transport barriers associated with the tumor microenvironment.\textsuperscript{2} Comparison of the half-lives\textsubscript{5–7d} of DOX between the DDSs indicates that the release rate
from DEBDOX is lower than the elimination rate of DOX (approximately 3-fold). The mechanism for release of DOX from DEBDOX is based on ion exchange and is then driven by diffusion out of the beads followed by diffusion through a stagnant layer surrounding the beads to reach its target site.

The fixed position of DEBDOX in the artery after the administration may have implications for the release of DOX, especially as this DDS is supposed to deliver DOX over a significantly longer period than LIPDOX. Both in vitro and in vivo studies have shown a slow release rate from DEBDOX, which suggests that the interaction between DOX and the functional unit (sulfonate ions) of the DDS is strong. For example, it has been reported that 48% and 89% of the dose is released in healthy pigs after one and three months, respectively. In addition, a foreign body response has been observed around DEBDOX in vivo, leaving a fibrotic capsule around the beads. The static environment created around the beads may restrict the release of DOX, raising concerns that the loaded dose might not be completely released.

Local distribution of DOX delivered by LIPDOX and DEBDOX

For both DDSs, the fraction of the dose released at the local sampling site was higher than that at the systemic sampling site in both pigs and humans, with a 2- to 3-fold difference between the sites in pigs and a smaller difference in patients (1.1- to 1.3-fold; Table 6). The concentrations at the systemic sampling sites were lower because DOX mixes with the circulation and is distributed to other tissues.

It is important to know the local distribution, and hence concentrations, at the target site in order to predict the anti-tumor effects and adverse events. In Papers I and IV, we approximated the availability in the liver and the hepatocytes with different approaches.

Intracellular availability of DOX

In pigs, the biliary excretion of DOX and DOXol was used to approximate the apparent intracellular availability (F\text{\textsubscript{i,app}}) of DOX, which appeared to be higher for LIPDOX than for DEBDOX (Table 6). The biliary flow was not affected by either of the formulations (median flow of 14–18 µL/min/kg). In patients, the local plasma DOXol-to-DOX AUC ratio was 2.7-fold higher after LIPDOX delivery than after DEBDOX delivery (p <0.001; Table 6). The higher ratio for LIPDOX than for DEBDOX may be explained by differences in the local distribution of DOX and the subsequent metabolism. One hypothesis for explaining these two independent observations is that increased intracellular delivery of DOX from LIPDOX would lead to more substrate available for DOXol formation. This is consistent with observations made by Furuta et al. in 1988. In the liver cancer patients, who had cirrhotic livers to a greater or lesser extent, the increase in the DOXol-to-
DOX AUC ratio could have been the result of wider distribution of DOX after LIPDOX administration than after DEBDOX. Thus, DOX from LIPDOX could reach the healthier liver regions and/or other tissues with a greater metabolic capacity than the areas in close vicinity to the infused DEBDOX. The liver and kidney have the greatest capacity (highest intrinsic clearance) of the main sites in the body for conversion of DOX to DOXol.69, 71 The metabolic capacity of CBRs and AKRs in the cirrhotic liver and in patients with HCC is not yet fully understood but it is expected to be reduced since many CYP enzymes that are important for the metabolism of drugs other than DOX are down-regulated in the cirrhotic liver and HCC patients.142

**Dose of DOX remaining in the liver**

In patients, the fraction of the dose that was released to the local sampling site was used to evaluate how much of the dose still remained in the liver after six hours (RD; see equation 2 in Paper IV). The RD at six hours was 31 ± 25% for LIPDOX and 87 ± 6.4% for DEBDOX. This fraction might still be bound to the DDS and/or be distributed to the different compartments of the liver (i.e. extra- and intracellular compartments in tumor or nontumor tissue). The first option is less likely for LIPDOX with its fast release, although DOX may follow LIPDOX into the cells. For DEBDOX, on the other hand, the slow release and strong interaction between DOX and the beads suggest that most of the dose is still bound to the DDS after six hours.

**Safety and anti-tumor efficacy of the DDSs with respect to the pharmacokinetics of DOX**

In Paper IV, the efficacy and safety of the two loaded DDSs were not the primary objective. However, it was of interest to relate those parameters to the release and pharmacokinetics of DOX. The number of included patients was too low for adequate comparison between the DDSs regarding safety and efficacy. The responses and adverse events are presented in Table 8.

The short-term necrotic effect of the treatment tended to be better with DEBDOX, as expected.73, 74 This is because DEBDOX causes complete embolization from a fixed location, which induces ischemia, in contrast to LIPDOX which exerts transient embolization and is more widely spread in the liver tissue. Unloaded beads have approximately the same necrotic effect as DEBDOX, with a longer time to progression of the tumor when DOX is present.143 The stasis caused by DEBDOX and the subsequent blood clotting and necrosis may affect the release and pharmacokinetics of DOX. It has been shown in explants from DEBDOX-receiving patients that the DOX concentrations are higher in necrotic areas than in non-necrotic areas after one month.105 This may be related to the low cellularity in necrotic areas,
which will result in less restricted diffusion than in the tumor micro-
environment.\textsuperscript{144}

Table 8 indicates that the incidence of post-embolization syndrome (i.e. abdominal pain, nausea, vomiting and fever) was similar between LIPDOX and DEBDOX recipients, while liver failure only occurred in LIPDOX recipients. This observation might also be related to the degree of embolization and the wider spread of LIPDOX in the liver. However the observations are few and therefore one should be careful with interpretations before more patients are included in the study.

Table 8. Treatment responses according to mRECIST and adverse events reported during the study period in Paper IV. The mRECIST classification was assessed by computed tomography or magnetic resonance imaging prior to and 4–6 weeks after transarterial treatment of patients with hepatocellular carcinoma with the lipiodol-based emulsion containing doxorubicin (LIPDOX) or doxorubicin-eluting micro-
particle beads (DEBDOX).

<table>
<thead>
<tr>
<th>mRECIST\textsuperscript{a}</th>
<th>LIPDOX</th>
<th>DEBDOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive disease</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>2 (50%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Partial response</td>
<td>1 (25%)</td>
<td>5 (62.5%)</td>
</tr>
<tr>
<td>Complete response</td>
<td>1 (25%)</td>
<td>3 (37.5%)</td>
</tr>
</tbody>
</table>

\textbf{Adverse events and causality to treatment}\textsuperscript{b}

| Probable                  | Post-embolization syndrome\textsuperscript{c}: 3 | Post-embolization syndrome\textsuperscript{c}: 5 |
|                          | Liver failure: 2                                   | Infection in necrotic part of tumor: 1 |
| Possible                 | Death: 1                                         | Back pain: 1 |
| Unlikely                 | Rectal bleeding: 1                                | Urinary tract infection: 1 |
|                          | Confusion: 1                                      | |
|                          | Headache: 1                                       | |

\textsuperscript{a}The Modified Response Evaluation Criteria in Solid Tumors include tumor size and the extent of necrosis in the tumor in the evaluation.\textsuperscript{145} \textsuperscript{b}Reported in absolute numbers; the relationship with treatment is indicated as probable, possible or unlikely. \textsuperscript{c}Post-embolization syndrome is an expected adverse event following embolization and includes abdominal pain, nausea, vomiting and fever.
Conclusions

The main objective of this thesis was to evaluate two current clinical formulations (LIPDOX and DEBDOX) of the chemotherapeutic drug DOX from a biopharmaceutical perspective. Specifically, the focus was on assessing the effects of these DDSs on the disposition of DOX in vivo. Understanding the in vivo release and disposition of a drug from these DDSs is an important step in the development and optimization of new DDSs. With novel DDSs there are opportunities for improving the risk–benefit ratio for patients with HCC by prolonging their overall survival and/or improving their quality of life.

The main conclusions drawn from the investigations in this thesis were:

- DOX and DOXol were extensively distributed to tissue and bile in pigs. The hepatic extraction of DOX was time- and infusion-dependent. The intermediate E_H (~40%) following a 55-minute i.v. infusion suggests that the hepatic elimination of DOX is dependent on the hepatic blood flow, intrinsic metabolizing capacity, and fraction of free drug.

- The excipient lipiodol did not interact with transporters or enzymes important for DOX disposition in vivo. Nor did it alter the membrane fluidity to enhance the cellular uptake of DOX. Instead it is suggested that the increased tissue accumulation of the drug delivered in lipiodol could be related to the properties of the formulation (drug + excipients) rather than to a direct interaction between excipient and drug.

- The transport protein inhibitor CsA inhibited carrier-mediated excretion of DOX to bile and urine. The excretion of DOXol to bile was also inhibited. The site of inhibition of biliary transport was assumed to be at the canalicular membrane rather than at the basolateral membrane, since plasma concentrations of DOX were unchanged but those for DOXol were increased. An inhibition on the basolateral membrane would have affected the plasma concentrations of the parent drug.\textsuperscript{131, 132} Multi-compartment modeling and sensitivity analysis confirmed that the transport inhibition by CsA occurred at the canalicular membrane. In addition, it is suggested that CsA does not inhibit enzymes responsible for the metabolism of DOX to DOXol (i.e. AKRs and CBRs).
• DOX was released faster from LIPDOX than from DEBDOX *in vivo* and was distributed more widely in the body, which could be associated with a higher risk of DOX-related adverse events. However, the three-times-higher recommended dose of DOX with DEBDOX (150 mg vs 50 mg) may lead to a similar systemic plasma exposure to DOX as LIPDOX.

• DEBDOX only released 15% of the dose to the circulation during 24 hours, which confirms its relatively extended release and prolonged tumor exposure. Future *in vivo* studies need to investigate whether this release rate for DOX is too slow and whether the total dose might not be released.

• Local distribution of DOX from LIPDOX and DEBDOX differed in patients, with 31% and 87% of the dose remaining in the DDS or liver after six hours, respectively. DOX from LIPDOX is likely to have been distributed in the liver because of its fast, extensive release characteristics, while most of the DOX from DEBDOX is likely to still reside within the DDS after six hours.

• DOXol was formed to a greater extent after delivery by LIPDOX than after DEBDOX in both pigs and patients. This can be explained by i) more extensive intracellular delivery from LIPDOX, since DOX could be co-transported with the lipid part of the emulsion, and/or ii) wider distribution of DOX from LIPDOX into regions with higher metabolic capacity and activity.

• The plasma concentration–time curve for DOX was similar in both healthy pigs and liver cancer patients. This indicates the usefulness of the advanced multisampling-site pig model when assessing *in vivo* release and distribution of DOX from intra-arterial DDSs for treatment of HCC. However, the pig model was less suitable for the lipid-based emulsion because the formulation induced unexpected early stasis of the blood vessel on administration. This has to be taken into consideration if a high dose is required.
Future perspectives

Finding a treatment for patients with cancer is challenging. It is well known that formulation of an active drug into a DDS can facilitate the targeting of the tumor by the drug while reducing the likelihood of adverse events. It therefore makes sense to develop efficient delivery systems for chemotherapeutic drugs known to be potent against cancer. In this thesis, DDSs used clinically in palliative treatment for primary liver cancer were studied in detail in vivo to learn which approach delivers the drug most efficiently.

The studies conducted in this thesis concluded that future optimized novel drug delivery strategies should be more similar to DEBDOX than to LIPDOX. This is because DEBDOX is a more robust pharmaceutical product from which the drug does not immediately separate on contact with blood causing a high extrahepatic DOX exposure. With controlled release, as in DEBDOX, the inter-individual response and frequency of drug-related adverse events may be easier to predict. However, it is important to further investigate the impact of a number of pathophysiological factors on the slow release of DOX from DEBDOX. The local drug–tumor exposure relationship has not been established and further in-depth investigation is required.

This thesis is part of a liver cancer treatment-optimization project at the Department of Pharmacy, Uppsala University, Sweden. Apart from the work in this thesis, the project also includes studies of the in vitro release of DOX from LIPDOX and DEBDOX as well as physiologically-based biopharmaceutical models based on the in vitro and in vivo studies. These studies will create a knowledge base for the future development of novel DDSs with optimal in vivo drug release. The primary goal in the future is to design an injectable formulation that can be given repeatedly and is easily monitored using imaging techniques, and that can release a combination of drugs effectively in the vicinity of the tumor tissue.

Syftet med denna avhandling var att undersöka två läkemedelsbärare som idag används vid behandling av levercancer. Den ena läkemedelsbäraren består av en vatten-i-olja emulsion (ungefär som en salladsdressing) och den andra består av mikropartiklar. Båda bärarna är laddade med doxorubicin när man ger det till patient. Emulsionen baseras på oljan lipiodol som är ett kontrastmedel som man kan följa på röntgen. Där kan man se att oljan ansamlas i tumörer upp till flera månader efter behandlingen. Syftet med denna avhandling var främst att ta reda på hur bra dessa läkemedelsbärare släpper ifrån sig och fördelar doxorubicin lokalt i och runt tumören. Detta för att kunna avgöra vilken strategi som är bäst vid utveckling och optimering av nya bärarsystem som skulle kunna förbättra behandlingen mot levercancer så att patienten lever längre och får en bättre livskvalitet. Frisläppningen och fördelningen av doxorubicin från emulsionen och mikropartiklarna studerades både i friska grisar och i patienter med levercancer genom att ta blodprover vid flera tidpunkter från flera ställen (både lokalt och ute i blodomloppet) och genom att samla urin. I grisar samlades
också gälla. Detta för att bestämma läkemedelshalten av doxorubicin och dess nedbrytningsprodukt doxorubicinol i kroppen.

Den lipiodol-baserade emulsionen släppte doxorubicin mycket snabbare och mer omfattande ut i blodomloppet än vad mikropartiklarna gjorde både i grisor och i patienter. Emulsionen släppte ifrån sig ca 50% av den laddade dosen till blodomloppet inom 30 minuter medan mikropartiklarna enbart släppte ifrån sig 15% av den laddade dosen under de första 24 timmarna efter dosering i patienter. Det är tveksamt om mikropartiklarna kan släppa ifrån sig allt doxorubicin som de laddats med i kroppen och därför behövs ytterligare forskning och fördjupning på området för att förstå detta. En större andel doxorubicinol bildades när doxorubicin gavs i emulsionen än vad det gjorde när det gavs i mikropartiklarna. Två hypoteser kan förklara detta. Antingen sprider emulsionen doxorubicin till en större del av levern och/eller andra regioner i kroppen som har en annan förmåga att bryta ned doxorubicin, eller så ökar emulsionen upptaget av doxorubicin in till cellerna där nedbrytningen sker. Den tumörödande effekten var jämföbar mellan läkemedelsbärarna, man kan dock ana en liten fördel med mikropartiklarna.


Sammanfattningsvis har denna avhandling bidragit med ny kunskap om hur dessa två läkemedelsbärare, som är i klinisk användning, släpper ifrån sig och fördelar doxorubicin. Dessutom har möjliga mekanismer för ökat cellupptag av doxorubicin studerats. Framtida läkemedelsbärare ämnade för livsuppehållande behandling mot levercancer borde likna mikropartiklarna mer än emulsionen eftersom mikropartiklarna släpper ifrån sig doxorubicin mer kontrollerat. Detta gör att förutsättningarna är bättre för att kunna förutse hur bra effekt behandlingen kommer att ha och för att förutse hur många eventuella biverkningar läkemedeleet kommer att ge.
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