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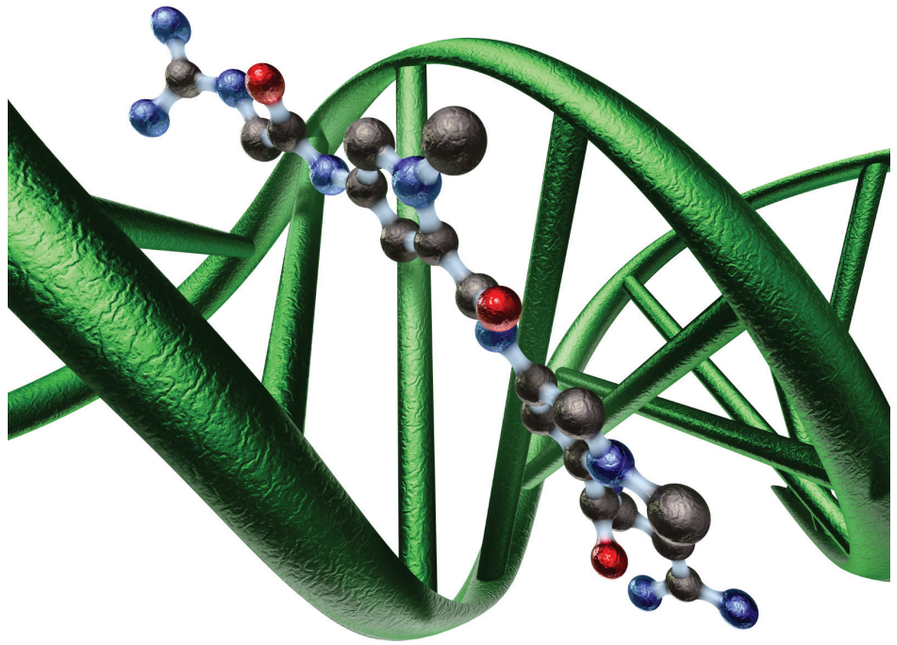

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# ABSTRACT

Cytochrome P450 2W1 (CYP2W1), is a monooxygenase enzyme endogenously expressed in fetal colon and normally silenced after birth. In adult life, the expression of *CYP2W1* occurs exclusively in tumor cells yielding high amounts of the enzyme in 30% of human colon cancers. Remaining the third most commonly diagnosed malignancy in the world, the colon cancer requires new chemotherapeutic strategies that would provide higher selectivity towards transformed cells. The unique confinement of CYP2W1 to tumor tissue opens the possibility for its targeting in colon cancer therapy. The studies in this thesis have focused on characterization of the CYP2W1 enzyme and the development of novel anticancer prodrugs designed for CYP2W1 - specific activation.

In cancer tissues and transfected cells CYP2W1 gives multiple immunoreactive bands, suggesting that the protein might be subjected to posttranslational modifications. Our *in vitro* and *in vivo* studies reveal that CYP2W1 undergoes glycosylation at Asn177, which is enabled by the unique inverted topology of the protein in the ER membrane. Regardless of the reversed emplacement, CYP2W1 retains its catalytic function in cell systems and is capable of converting inactive substrates to potent cytotoxic species, demonstrating the potential for prodrug activation.

With the goal to develop antitumor prodrugs for specific activation by CYP2W1, we have employed a library of novel duocarmycin-based compounds and identified ICT2705 and ICT2706 as the first molecules whose cytotoxic activity is dependent on CYP2W1 enzyme. Our studies reveal the results of the first attempt of CYP2W1 targeting in cancer, both *in vitro* and *in vivo*. We show that the colon cancer cell lines expressing CYP2W1 suffer substantial DNA damage and rapid loss of viability following incubation with ICT2705 and ICT2706. Moreover, we demonstrate that the CYP2W1-positive human xenografts undergo complete growth inhibition in mice dosed with ICT2706, with no apparent deleterious effects detected in any of the treated animals. In addition, we present *in vitro* evidence that the potent cytotoxic effect is most likely propagated by a bystander killing mechanism.

Polymorphisms in *CYP2W1* gene yield two alleles of significant frequencies, designated *CYP2W1*2* and *CYP2W1*6*. The resulting variant enzymes carry missense mutations that might affect the therapeutic outcome of prodrug targeting as well as the course of the metastatic disease. A recent study has associated *CYP2W1*2* with a decreased risk for colon cancer, suggesting the altered catalytic activity of this enzyme variant. Using the novel duocarmycin substrates, we were able to evaluate the catalytical properties of the variant enzymes for the first time. In contrast to previous findings, our studies show comparable catalytic capacity for all CYP2W1 enzymes, and no association between *CYP2W1*2* genotype and colon cancer risk based on 10-fold larger patient cohorts.

In conclusion, we have found that CYP2W1 is a glycosylated enzyme with reversed ER topology, whose catalytic activity is sustained in an intact cell system and unaffected by the most frequent polymorphic changes. We have developed first prodrugs for CYP2W1 targeting in cancer and demonstrated their antitumor efficacy in a preclinical setting. The work presented herein provides the basis for a novel therapeutic approach in colon cancer chemotherapy.

# LIST OF PUBLICATIONS

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

I. Alvin Gomez, Jana Nekvindova, **Sandra Travica**, Mi-young Lee, Inger Johansson, David Edler, Souren Mkrtchian and Magnus Ingelman-Sundberg (2009). Colorectal cancer-specific cytochrome P450 2W1: intracellular localization, glycosylation, and catalytic activity.
*Mol Pharmacol*, 78(6):1004-11

II. Helen M. Sheldrake*, **Sandra Travica***, Inger Johansson, Paul M. Loadman, Mark Sutherland, Lina Elsalem, Nicola Illingworth, Alexander J. Cresswell, Tristan Reuillon, Steven D. Shnyder, Souren Mkrtchian, Mark Searcey, Magnus Ingelman-Sundberg, Laurence H. Patterson, Klaus Pors (2013). Re-engineering of the duocarmycin structural architecture enables bioprecursor development targeting CYP1A1 and CYP2W1 for biological activity.
*J Med Chem, 56(15):6273–6277*

III. **Sandra Travica***, Klaus Pors*, Paul M. Loadman, Steven D. Shnyder, Inger Johansson, Mohammed N. Alandas, Helen M. Sheldrake, Souren Mkrtchian, Laurence H. Patterson, Magnus Ingelman-Sundberg (2013). Colon cancer-specific cytochrome P450 2W1 converts duocarmycin analogues into potent tumor cytotoxins.
*Clin Cancer Res*, 19(11):2952-2961

IV. Kristina Stenstedt*, **Sandra Travica***, Jia Guo, Isabel Barragan, Klaus Pors, Laurence Patterson, David Edler, Souren Mkrtchian, Inger Johansson, Magnus Ingelman-Sundberg (2013). Cytochrome P450 2W1 polymorphism: functional aspects and relation to risk for colorectal cancer.
*Pharmacogenomics, accepted for publication July 15$^{th}$ 2013.*

*Equal contribution

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-FU | 5-flourouracil |
| ADEPT | Antibody-directed enzyme prodrug therapy |
| AEC | Antibody-enzyme conjugate |
| AhR | Aryl hydrocarbon receptor |
| APC | Adenomatous polyposis coli |
| Asn | Asparagine |
| CD20 | B-lymphocyte antigen CD20 |
| CPA | Cyclophosphamide |
| CRC | Colorectal cancer |
| CSC | Cancer stem cell |
| CYP | Cytochrome P450 (also P450) |
| EGFR | Epidermal growth factor receptor |
| ENDO H | Endoglycosidase H |
| ER | Endoplasmic reticulum |
| GDEPT | Gene-directed enzyme prodrug therapy |
| H2AX | H2A histone family member X |
| Her-2/NEU | Human Epidermal Growth Factor Receptor 2 |
| IFA | Ifosfamide |
| MGMT | O-6-methylguanine-DNA methyltransferase |
| MLH1 | MutL homolog 1 |
| MSH2 | MutS homolog 2 |
| MSH6 | MutS homolog 6 |
| MYH | MUTYH (mutY Homolog) |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| P450 | Cytochrome P450 (also CYP) |
| PAH | Polycyclic aromatic hydrocarbons |
| PMT | Prodrug monotherapy |
| PNGase | Peptide -N-Glycosidase |
| POR | Cytochrome P450 reductase |
| SNP | Single nucleotide polymorphisms |
| SRS | Substrate recognition site |
| TP53 | Tumor protein 53 |
| VDEPT | Virus-directed enzyme prodrug therapy |
| VEGF-A | Vascular endothelial growth factor A |

# 1 INTRODUCTION

## 1.1 CANCER

42 years have passed since the *War on Cancer* was officially launched by the U.S. President Richard Nixon. The ensuing investment in the study of cancer has been a major engine driving the ongoing revolution in cell and molecular biology. Yet, the clinical rewards have been less than remarkable. Each year, worldwide, nearly 13 million people are diagnosed with cancer and the disease continues to prevail as the leading cause of death in the developed world (Jemal et al., 2011).

Today, the term *cancer* encompasses more than 100 distinct diseases with diverse risk factors and epidemiology, which originate from most of the cell types in the human body and are characterized by unrestrained proliferation of cells that can invade distant organs (Stratton et al., 2009). All cancers nevertheless, arise as a result of changes in the DNA sequence of the crucial regulatory genes that tightly control cell growth, cell division, and the programmed cell death. The precise regulation of these processes is a requisite to the proper functioning of the organism. Occasionally, a single cell acquires a set of sufficiently advantageous mutations causing inactivation of regulatory tumor-suppressor genes or activation of proto-oncogenes, which confer autonomy of growth on a cell. Such opportune DNA changes are either inherited through the germline, or develop *de novo* within the neoplastic lineage though environmental exposures, diet, and aging. No single mutation however is sufficient to cause cancer; rather cancer results from multiple genetic and epigenetic alterations that eventually confer cells with an ability to completely escape normal regulation. Most, if not all, malignant tumors are equipped with the same set of functional capabilities commonly referred to as the hallmarks of cancer, which include: (1) self-sufficiency in growth signals; (2) insensitivity to antigrowth signals; (3) evasion of apoptosis; (4) limitless replicative potential; (5) sustained angiogenesis; (6) tissue invasion and metastasis, as well as additional hallmarks and enabling characteristics (Hanahan and Weinberg, 2011). Once endowed with these properties, a neoplastic cell rapidly gains the proliferative advantage and eventually outcompetes its surroundings in order to thrive and grow.

However general the hallmarks of malignancy may be, the specific routes and molecular mechanisms that drive the progression of a distinct tumor are extremely variable and complex. Such an extensive range of origins and features among cancer types poses the major challenge for the development of optimal anti-tumor drugs and therapies. The standard approaches for cancer management, i.e. surgery, systemic chemotherapy, and radiotherapy, are facing limitations either due to the unfeasibility of intervention, or induction of unspecific toxicity. Recent advancements in the understanding of molecular mechanisms of cancer development, progression, and

evolution, have revealed new biomarkers and potential molecular targets. These efforts have shifted from older, unspecific cytotoxic therapeutic options towards chemical and biological therapies that are precisely designed to target a critical gene or protein, opening avenues for new and improved cancer treatment.

## Colon Cancer

Colon cancer (colorectal cancer, CRC) is the third most frequently diagnosed malignancy in the world. The annual incidence is estimated to be around one million, while more than 500,000 patients die from the disease each year. Colon cancer is predominantly diagnosed in Western societies, putting emphasis on the etiological role of dietary and lifestyle factors (Kamangar et al., 2006). Concordantly, in 75% of cases the disease develops sporadically, whereas the remaining patients have a family history of CRC that suggests a hereditary contribution or common exposures among family members. Up to date, genetic mutations identified as the initiators of colon neoplasia account for only 6% of the cases overall, therefore it is likely that various undiscovered genes and background genetic factors significantly contribute to the heritable disease. The risk for developing the sporadic colon cancer is linked with old age, consumption of red meat, low-fiber diet, obesity, and smoking, and is more prevalent in men.

The complete spectrum of deleterious molecular alterations contributing to the pathogenesis of CRC is very extensive and not fully elucidated (Figure 1). The sporadic transition from normal epithelium to adenocarcinoma is associated with chromosomal instability, which causes aneuploidy and deleterious changes in the chromosomal structure. Residing on the lost portions of chromosomes 5q, 18q, and 17p, are important tumor suppressor genes, such as *APC*, *TP53*, and *SMAD4* (Lengauer et al., 1997). Chromosomal instability is therefore an efficient mechanism for causing the physical loss of their wild-type copies, whose normal activities oppose the malignant phenotype. In the remaining minor group of CRC patients, the mismatch-repair genes *MLH1*, *MSH2*, and *MSH6* are inactivated, triggering microsatellite instability. Their inactivation can be either inherited, as in the patients with Lynch syndrome, or acquired through silencing by methylation (Cunningham et al., 2010). An alternative route to CRC involves germ-line inactivation of *APC* gene or DNA-repair gene *MYH*, which manifest as a Familial Adenomatous Polyposis syndrome and confer a nearly 100% risk for colon carcinoma. Once developed, the colon cancer progression is promoted by mutations in several key oncogenes, namely *RAS* and *BRAF*, which drive the uncontrolled growth further through the MAPK signaling cascade (Markowitz and Bertagnolli, 2009).

Even though the sequence of transformation from adenoma to colon carcinoma involves pro-malignant mutations which are progressively acquired, the distinct mutations that dictate specific tumor characteristics, such as regional or distant metastases, have not been found. The full genome sequencing from primary colorectal cancers and their distant metastases unexpectedly shows that all the mutations in the

metastases are already present in the primary tumors, implying that the metastatic seeding requires no new triggering events and it happens rapidly (Jones et al., 2008).

It is likely however, that not all the cells within the tumor mass are equally capable of initiating metastatic dissemination. In recent years, the cancer stem cell (CSC) model has gained momentum postulating that a small subpopulation of self-renewing tumor cells has an enhanced potency to initiate distant metastases which not only resemble the primary tumor, but are more resistant to therapy (Vermeulen et al., 2008).

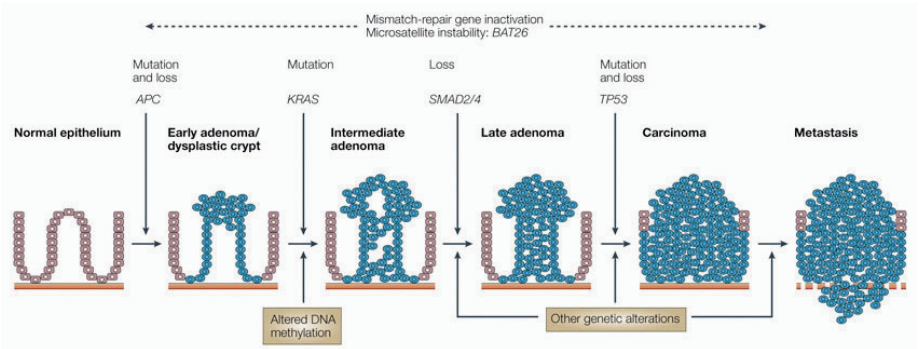


**Figure 1.** Progression from normal epithelium through adenoma to carcinoma is characterized by accumulated abnormalities of particular genes. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Cancer*, Davies et al., copyright (2005).

## 1.2 CANCER THERAPY

In theory, non-hematological cancers can be cured if entirely removed by surgery. However, such precise intervention is not always possible. Even small localized tumors are being increasingly recognized as possessing metastatic potential, as even a single lingering cancer cell invisible to the naked eye can rapidly regrow into a new tumor. Once the tumor has spread beyond the lymph nodes and metastasized to distant sites of the body, the complete surgical excision becomes usually impossible and adjuvant therapies must be introduced.

The treatment of cancer over the past decades has relied primarily on the use of various forms of cytotoxic chemotherapy and radiation therapy. Emitted against localized tissue or administered systemically, these regiments take advantage of the increasing sensitivity of dividing cancer cells to DNA or microtubule damage. Even though such interventions have had profound positive results on many hematologic and a few solid tumors, the most prevalent of malignancies still resist these approaches. In all cases, the effectiveness of cytotoxic treatments has been limited by the side effects on normal cells which are affected alongside cancerous tissue almost indiscriminately.

Targeted anticancer strategies aimed to bypass the high price of toxicity are mostly focused on inhibiting proximal events in the signal transduction cascades, rather than affecting the downstream DNA replication and microtubule assembly. However, the inhibition of these upstream targets (i.e. Bcr:abl, CD20, Her-2/NEU, EGFR, *etc.)* is overpowered by pathway redundancies that can create resistance to the targeted therapy effects (Hait and Hambley, 2009).

### Colon cancer therapy

The treatment of colon cancer depends on how advanced the disease has become. If diagnosed early, CRC is highly treatable through surgery. Regrettably, due to the late manifestation of symptoms, most of the patients are diagnosed at the late stage of the disease, once the metastatic lesions in liver or even lung have already formed. Therefore, for the vast majority of patients the surgical intervention is insufficient or unfeasible, and the only treatment option is chemotherapy directed at the disseminated metastatic colonies.

Since 1957, 5-fluorouracil (5-FU) has played an important role in the treatment of colon cancer. This chemotherapeutic belongs to the family of cytotoxic agents called antimetabolites, which include folate antagonists and purine or pyrimidine analogues capable of subverting pathways of DNA synthesis. These agents act by inhibiting nucleotide metabolism and induce apoptosis by depleting cells of dNTPs. Moreover, by mimicking the building blocks of nucleic acids these drugs can impair replication by becoming incorporated into the DNA (Helleday et al., 2008).
5-FU acts by inhibiting the action of thymidylate synthase and therefore blocks the synthesis of the pyrimidine thymidine. Due to the scarcity in dTMP, rapidly dividing

cancerous cells undergo thymineless death (Longley et al., 2003). Even though 5-FU's effects fall most heavily upon rapidly dividing cells, the cytotoxic burden is felt system wide. With the goal to reduce the normal tissue exposure, a 5-FU prodrug capecitabine was designed to be activated preferentially in tumor tissue. This precursor requires a cascade of three enzymatic steps and two intermediary metabolites to form 5-fluorouracil as the terminal toxin (Rautio et al., 2008). Although infrequent, the side-effects of capecitabine involve severe cardiotoxicity, particularly if given in connection to other drugs (Molteni et al., 2010; Schneiders et al., 2011). The 5-FU prodrug is today a part of the standard combination treatment regimens for colon cancer, together with folinic acid and cytotoxic drugs irinotecan and oxaliplatin (Soreide et al., 2011).

Irinotecan is a semisynthetic analogue of the natural alkaloid camptothecin derived from Chinese tree *Camptotheca acuminata* Decne. Metabolism of irinotecan includes its hydrolysis into a 1000-fold more potent metabolite SN-38 capable of inhibiting the function of topoisomerase I and thereby generating DNA strand breaks that harm the integrity of the genome. Such topoisomerase poisons kill cells not by simply inhibiting the enzyme catalysis but by increasing the rate of DNA cleavage or decreasing the rate of DNA religation by the topoisomerase (Lavelle et al., 1996). Analogously to 5-FU, the effects of irinotecan are nonspecific and directed against the cell cycle. The most significant side effects (e.g. immunosuppression) are even more profound in a subgroup of patients genetically predisposed for impaired inactivation of SN-38 in the liver. Irinotecan is thus one of the first widely used chemotherapeutics that is dosed according to the recipient's genotype (O'Dwyer and Catalano, 2006).

The first platinum-based antineoplastic agent to be approved for the treatment of colorectal cancer is oxaliplatin. This planar water soluble coordination complex is non-enzymatically converted into active derivatives that can react with guanine in DNA, resulting in the formation of intra- and interstrand Pt-DNA crosslinks. The detrimental DNA crosslinking impedes the replication fork progression causing non-targeted cytotoxicity (Graham et al., 2004; Krause-Heuer et al., 2009).

As a consequence of improved understanding of the molecular pathology of cancer, several targeted agents have been developed and demonstrated improved outcome in metastatic CRC patients.

Bevacizumab is a recombinant humanized monoclonal antibody that specifically targets vascular endothelial growth factor A (VEGF-A) to prevent its interaction with appropriate receptors in vascular endothelial cells. As a result, cell signaling pathways that enhance angiogenesis, and thus the blood supply for tumors, are diminished. In addition, by acting through specific targeting of endothelial cells, this drug may avoid potential tumor cell resistance. Used in combination with standard chemotherapeutic agents (e.g. 5-FU), bevacizumab improves the overall survival of metastatic CRC patients by approximately 5 months. Nonetheless, by targeting a general phenomenon such as blood vessel formation, bevacizumab can induce toxicities of multiple organs including hypertension, bleeding, and proteinuria.

Another target, EGFR, is a transmembrane tyrosine kinase receptor known to be overexpressed in tumors of epithelial origin, including colon cancer. EGFR activation

by peptide growth factors initiates both the RAS/RAF/MAPK and the PI3K signaling pathways directly affecting cell proliferation and survival, and therefore contributing to metastatic progression. Anti-EGFR therapies for colon cancer include monoclonal antibodies and tyrosine kinase inhibitors, whose side effects are commonly manifested as skin toxicities (Hagan et al., 2013).

## Prodrug strategies

Important approach toward improving the specificity of chemotherapy is the use of less toxic prodrug forms that can be selectively activated in cancer tissue. Among the several mechanisms for selective activation, the exploitation of a property unique to the tumor represents the most direct route. In prodrug monotherapy (PMT), inherent physiological or metabolic aberrations within the tumor tissue are used to trigger the conversion of innocuous compounds into tumor-confined cytotoxins. In order to expand the range of tumors susceptible to prodrug therapy, activating enzymes can be delivered to tumor cells by using antibodies or genes. Such binary approaches, e.g. antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT) are modern strategies for augmenting and restricting toxicity solely to cancer cells.

### ADEPT

ADEPT is a two-step therapy that employs antibody-enzyme conjugates (AEC) to activate prodrugs selectively in the vicinity of cancer cells. Administered AEC binds to a tumor-specific antigen on the malignant cell membrane. Subsequently, the inactive prodrug is given systemically and activated by the enzyme of the targeted AEC, thereby generating a highly cytotoxic drug at the tumor site. Over the last two decades, a number of prodrugs derived from cytotoxic agents such as nitrogen mustards, antitumor antibiotics (discussed below), and 5-FU have been proposed for application in ADEPT (Denny, 2004). Despite the considerable efforts, the clinical application of ADEPT-suitable prodrugs is still pending due to a rather narrow therapeutic window accompanied by comparably low cytotoxicity of the formed drug.

### GDEPT

In GDEPT, also named suicide-gene therapy, targeting vectors are utilized to transduce genes into the genome of cancer cells, where they cause or enrich the expression of a specific enzyme. Upon subsequent systemic administration of the nontoxic prodrug, intracellular enzymatic conversion yields high levels of cytotoxin in tumor tissue (Figure 2). Regarding the required vectors, various systems have been proposed for gene delivery, addressing safe administration and their selective incorporation into tumor cells. If recombinant viruses are used, the approach is referred to as virus-directed enzyme prodrug therapy (VDEPT), whereas non-viral

transfer systems include bacteria or DNA complexed with cationic lipids, peptides, liposomes, or nanoparticles. Prominent established GDEPT systems include e.g. *herpes simplex* virus thymidine kinase/gancyclovir, cytosine deaminase/5-fluorocytosine, and cytochrome P450/cyclophosphamide, whereas pioneering GDEPT systems designed to treat glioma and prostate cancer have completed or are now entering late-stage clinical trials, respectively (Both, 2009; Tietze and Schmuck, 2011). The challenges of the GDEPT strategy, notably the specific delivery of the therapeutic gene followed by its sufficient expression and high catalytic activity of the enzyme product, are the subject of continuous optimization attempts.

Whatever the mechanism of delivery, only a small proportion of any tumor cell population is likely to become competent to activate a prodrug. Therefore, the success of both GDEPT and ADEPT relies not only on the cell-specific activity by the delivered enzyme, but moreover on a strong local bystander effect. The activated drug can diffuse to surrounding cancer tissue, killing even the untransformed or antigen-negative cells.

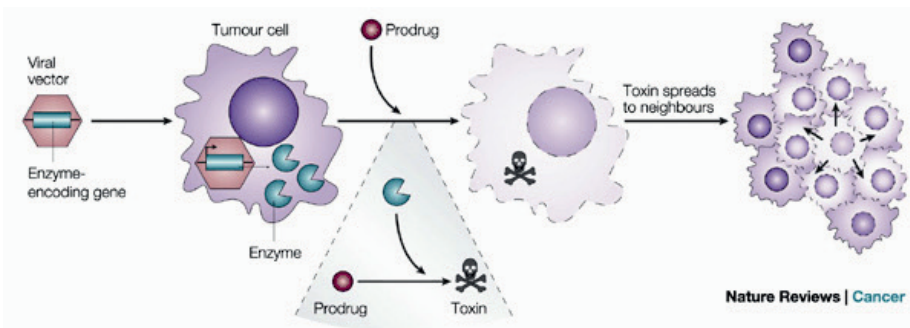


**Figure 2.** Mechanism of Gene-Directed Enzyme Prodrug Therapy (GDEPT). The vector delivers a gene encoding a prodrug-converting enzyme to tumor cells. The subsequently delivered prodrug is converted to the active, cytotoxic metabolite in the tumor cells. The toxin diffusion to neighboring tissue confers a potent bystander effect. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Cance*r, McCormick, copyright (2001).

## PMT

Targets for prodrug monotherapy are key differences between healthy and cancerous tissues, such as membrane-associated receptors, pH value, hypoxia, or elevated concentrations of special enzymes (de Groot et al., 2001; Denny, 2004). Advantageously and in contrast to binary strategies, there is no need for tumor-directed carriers such as antibodies or vectors, rendering PMT far less complex, safer, and more cost-effective approach that may also be augmented by bystander effect. Over the history of anticancer drug design, the targeting of endogenous tumor-specific

enzymes has remained a very attractive strategy, however the ideal cancer proteins that are entirely absent from normal tissues are still pending discovery. Majority of today's promising enzyme targets, such as beta-glucuronidase, are differentially represented in transformed vs. normal cells to a level sufficient to justify therapeutic actions, however their success still lies in a narrow gap between the insufficient therapeutic outcome and the unwanted toxicity (Chen et al., 2013).
Among the potential prodrug activators, the specific CYP enzymes, discussed in detail beneath, are receiving growing attention as novel antitumor targets due to their significant overexpression in cancer tissues.

## Alkylating agents in anticancer therapy

Most cancer cells proliferate more rapidly than their normal counterparts, rendering cell cycle the common target of anticancer drugs. Alkylating agents constitute a major class of frontline chemotherapeutics that inflict cytotoxic DNA damage as their main mode of action. These reactive compounds can generate a variety of covalent DNA adducts ranging from simple methyl groups to complex alkyl additions which impede the replication process. Alkylating agents act by forming carbonium ion that carries only six electrons in its shell and can therefore react instantaneously with an electron donor such as amine or hydroxyl group. Being strongly nucleophilic, the nitrogen atom at position 7 (N7) of guanine molecule is the main molecular target for alkylation in DNA, although N1 and N3 of adenine and N3 of cytosine may also be affected (Hurley, 2002). The pattern of DNA lesions generated by an alkylating agent depends on its particular chemistry ($SN_1$ vs. $SN_2$-type nucleophilic substitution) and the number of reactive sites within the compound (Fu et al., 2012). Most of the alkylating drugs are bifunctional i.e. containing two reactive groups that can bind to separate DNA bases and thereby cause intra- or interchain crosslinking. This interferes severely with both transcription and replication of DNA. Other effects of alkylation are excision of the guanine base or aberrant pairing of the alkylated guanine with thymine. Their main impact is seen during S phase when unpaired regions of DNA are accessible for alkylation, resulting in cell cycle block and subsequent apoptosis. Main types of classical alkylating agents include nitrogen mustards such as cyclophosphamide and ifosfamide, alkyl sulfonates, and lipid-soluble nitrosoureas capable of crossing the blood-brain barrier. Among novel experimental alkylators, the derivatives of CC-1065 and duocarmycin (bizelesin), as well as glutathione S-transferase-activated prodrugs (TLK286) are evaluated in early clinical trials (Teicher and Chari, 2011; Tew, 2005).

## Antitumor antibiotics

Cytotoxic antibiotics represent a highly potent group of drugs that produce their effects mainly through direct actions on DNA. Anthracyclines (doxorubicin), dactinomycin, and bleomycin are fermentation products capable of evoking a range of nuclear damages, from disruption of nucleic acid synthesis to topoisomerase inhibition and DNA fragmentation; whereas antibiotics such as duocarmycins are activated to give alkylating metabolites (Yang and Wang, 1999). In contrast to non-specific alkylating agents, antitumor antibiotics seem to have better selectivity for cancer cells than normal cells, as well as significantly greater DNA sequence selectivity than the classical alkylators (Hurley, 2002).

### Duocarmycins

The duocarmycins, isolated from a culture broth of *Streptomyces* species, are parent members of a family of antitumor antibiotics that also includes CC-1065 and yatakemycin (Figure 3). These natural compounds represent a class of extremely potent cytotoxic agents which derive their biological activity from an ability to bind and sequence-selectively alkylate DNA at the N3 of adenine in the minor groove, initiating a cascade of events that terminates in apoptotic cell death (Ghosh et al., 2009).

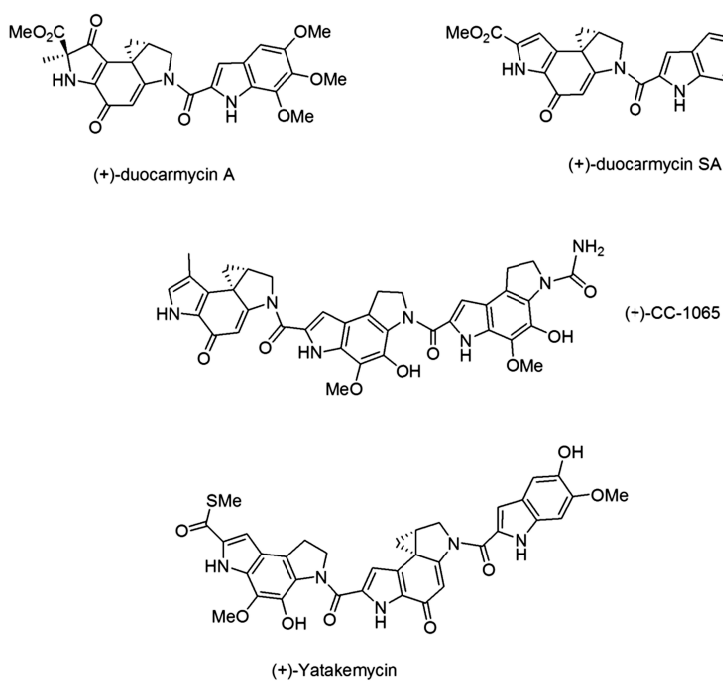


**Figure 3**. Structures of duocarmycin family of compounds.

Duocarmycins are planar, curved molecules whose structures are characterized by carboxamide-linked heterocycles where indole moiety enables binding and the alkylating activity is conferred to cyclopropane ring (Figure 4). The DNA recognition subunit of the molecule binds preferentially to the narrow, deep, AT-rich minor groove, where adenine is correctly positioned to interact with the reactive cyclopropane. These compounds however, are extremely stable until bound to DNA, and are therefore not substrates for any other biological nucleophile (Searcey, 2002). In essence, they have the ability to "wait around" for the transient dissociation of the DNA-histone complexes that allows them to recognize their specific target sequence. The DNA binding induces conformational change in the duocarmycin molecule that twists and destabilizes the linking amide and activates the cyclopropane ring for nucleophilic attack. Once trapped and alkylated, the DNA of a proliferating cell cannot longer replicate.

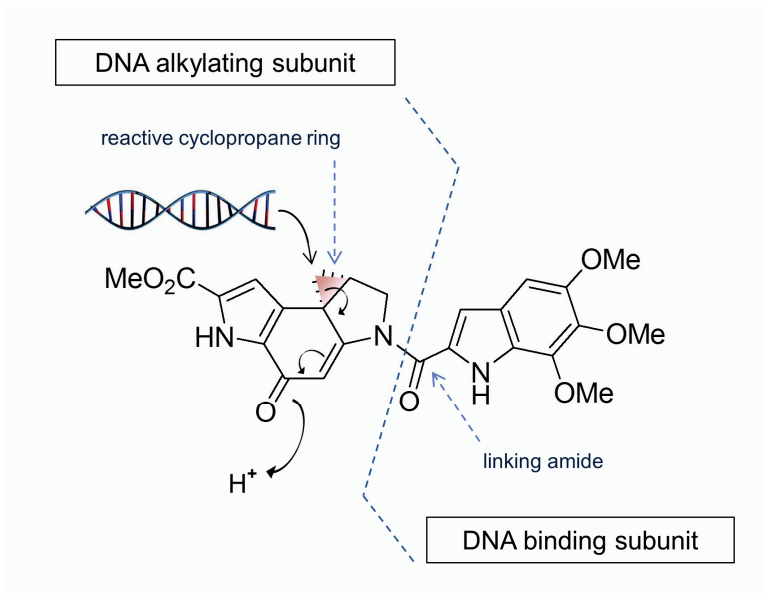


**Figure 4**. The mechanism of DNA alkylation by duocarmycin SA.

Duocarmycins are the most potent tumor antibiotics discovered to date, however their exceptional potency has proven to pose a hinder for clinical utilization. CC-1065 and duocarmycin SA (Figure 3), which have shown significant antitumor activity in *in vivo* models, cannot be used systemically in humans due to delayed death profile which results from catastrophic toxicity in experimental animals (Ghosh et al., 2009). The failure of clinical progression of these natural products has motivated extensive efforts to design duocarmycin analogues that could retain the potency and antitumor activity, but bypass the adverse toxicity. Crucial consideration in such design is the sensitive balance between stability and biological activity, which should result in a compound stable enough to enter the cell, yet sufficiently reactive to alkylate DNA.

In this regard, various duocarmycin alkylation subunits have been extensively studied and redesigned and their analogues combined to create dimers and hybrid molecules with novel properties. Such creative efforts have yielded four compounds, analogues of CC-1065 (adozelesin, carzelesin, and bizelesin) and duocarmycin B2 (KW-2189), with properties sufficiently improved to warrant clinical trials. Screened against several advanced cancer types, these drugs have unfortunately exhibited only marginal antitumor efficacy due to dose-limiting toxicities to crucial normal tissues, and will therefore unlikely progress towards phase III (Ghosh et al., 2009). Presently, new and improved highly potent analogues of CC-1065 and the duocarmycins are emerging and are being investigated as candidate prodrugs for targeted anticancer therapy.

## Duocarmycin prodrugs

Phenol-containing *seco*-analogues of duocarmycin have the great potential for prodrug design and have so far been explored in both mono- and binary antitumor strategies. Within these structures, the reactive cyclopropane ring is *opened* (*"seco"*) and must therefore be restored in order to trigger the DNA alkylation. The cyclopropane formation involves spirocyclization of the chloromethylindoline fragment, which is a mechanism dependent on the free phenolic hydroxyl group (Figure 5). The possibility of halting this spirocyclization event by blocking the hydroxyl group is an attractive approach for inactivating the duocarmycin before it reaches the tumor tissue. Up to date, several *seco*-compounds in which the blocked OH- group is subsequently released by enzymatic cleavage or by bioreduction in hypoxic tumor environment have shown great potential as anticancer agents (Jin et al., 2007; Tietze et al., 2008) however, in analogy to similar molecules (e.g. carzelesin and KW-2189) the clinical potential of such prodrugs remains unclear.

An alternative strategy for the design of cancer-specific *seco*-duocarmycin prodrug is a complete removal of the OH- group, instead of its masking. Within the cancer cells, this lost group can be regenerated in the form of new, active hydroxyl metabolites that are formed through prodrug oxidation by unique, tumor-specific cytochrome P450 enzymes (Pors et al., 2011).

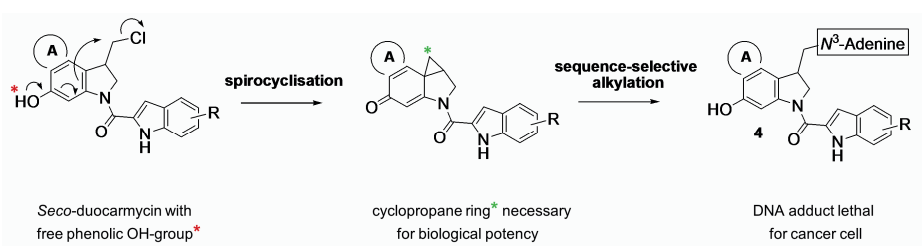


**Figure 5.** The mechanism of action of *seco*-duocarmycins.

## 1.3 THE CYTOCHROME P450 ENZYMES

Cytochromes P450 (CYP) comprise a large superfamily of heme-containing enzymes which represent major catalysts involved in the oxidation of a wide variety of endogenous and exogenous substrates. Over their long history of more than 3.5 billion years, the CYPs have occupied almost every realm of life from Bacteria and Archaea to Eukaryotes, evolving into pivotal chemical defense system against environmental and endogenous toxins (Danielson, 2002). These ubiquitous enzymes play an essential role in the metabolism of more than 70% of medical drugs, chemical carcinogens, and other xenobiotics, leading to both activation and inactivation of compounds, while several are also involved in the biosynthesis of physiologically relevant molecules such as steroids, eicosanoids, and fatty acids. Typically, CYPs employ molecular oxygen and an enzymatic reductase component to catalyze reactions whereby lipophilic drugs or metabolites, that would otherwise reach toxic levels in cell membranes, are rendered suitably water-soluble to be excreted in the urine. In this monooxygenase reaction, one of the oxygen atoms is inserted into a substrate, while the second oxygen is reduced to a water molecule, utilizing two electrons provided by NAD(P)H via cytochrome P450 reductase (POR) (Porter, 2002). The basic stoichiometry of this process is represented by the equation:

$$RH + O_2 + 2e^- + 2H^+ \rightarrow ROH + H_2O$$

Aside from monooxygenation, certain CYPs can catalyze other chemical reactions such as deaminations, epoxidations, N-, S- and O- dealkylations, *etc*. (Meunier et al., 2004).

Cytochromes P450 got their name from their unusual spectral properties, displaying a characteristic absorption peak at 450nm when the reduced protein is complexed with carbon monoxide. Human genome carries 57 CYP genes and 58 CYP pseudogenes, grouped on the basis of their amino acid sequence similarity. Enzymes belonging to the same CYP family hold 40% sequence identity (CYP1, CYP2, *etc.*), while those grouped within a subfamily (CYP2A, CYP2B, *etc.*) are 55% identical (Nelson, 2006). Even though the amino-acid sequences of CYP proteins are extremely diverse, with levels of identity as low as 16% in some cases, their topography has remained highly conserved throughout evolution (Werck-Reichhart and Feyereisen, 2000). All eukaryotic CYPs are tethered to the membrane of endoplasmic reticulum or inner membrane of mitochondria through a hydrophobic transmembrane helix at the N-terminus of the protein, which also serves as a targeting sequence. Bound to the organelle, the bulk of the CYP protein is exposed on the cytosolic side, available for interactions with substrates and reductase partner.

In order to become a functionally active enzyme, the nascent CYP protein must be complexed with a heme iron cofactor. This amendment is only one of several co- and posttranslational modifications CYPs submit to, including phosphorylation, ubiquitination, nitration, and glycosylation. Protein glycosylation, a site-specific supplementation of sugar moieties to a peptide, occurs through stepwise actions of several ER- and Golgi-confined glycosyltransferases. Most common types of glycosylation are *N*-linked and *O*-linked glycosylation which differ in their occurrence

and complexity. In *N*-glycosylation, which yields 90% of glycoproteins, the oligosaccharides become attached to asparagine (Asn or N) residue of the nascent protein as it is being translated and transported into the ER. This type of linkage can only occur in the context of canonical peptide sequence Asn-X-Ser/Thr, where X represents any amino acid. Glycosylation of human CYP enzymes is a rarely described event. Placental CYP19A1, which is the only well explored N-glycosylated human P450, displayed no significant relationship between glycosylation and enzyme activity (Aguiar et al., 2005). Other studies on this modification have mostly focused on its relevance in P450 localization, leaving the role of glycosylation in CYP enzymes largely unexplored (Loeper et al., 1998; Szczesna-Skorupa and Kemper, 1993). The first paper included in this thesis reveals a unique example of a CYP protein glycosylation and its implication in enzyme localization and topology.

In mammals, CYPs are predominantly expressed in the liver corresponding to the metabolic function of the organ, whereas the extrahepatic expression is usually much lower and mainly linked to endogenous functions. Some P450s however significantly or predominantly reside outside the liver, especially in the gastrointestinal and respiratory tissues which are the portals of entry for foreign compounds. In the biotransformation of xenobiotics, the major players are polymorphic CYPs from families 1-3, which have received wide attention due to their role in the metabolism of pharmaceuticals. This relevant group harbors not only liver enzymes but also most of the extrahepatic CYPs, including those overexpressed in tumor cells (Ding and Kaminsky, 2003). Although liver P450s play the predominant role in the drug metabolism, the extrahepatic CYPs may determine the tissue sensitivity to a given xenobiotic, affecting the outcome of drug treatment or conferring a targeted tissue effect. This possibility of locoregional drug activation is particularly important in the domain of cancer therapy, bringing attention to tumor-confined enzymes such as CYP1B1, CYP2W1, and CYP1A1.

## CYP2W1

CYP2W1 is the youngest member of the P450 family of enzymes. A part of the CYP2W1 sequence was first found in the year 2000 in a cDNA library from the human hepatoma cell line HepG2. The CYP2W1 gene is located on chromosome 7 and shows the typical family 2 structure with nine exons. Translation results in 490 amino acids that assemble an evolutionary conserved protein, embedded in the membranes of endoplasmic reticulum and mitochondria (Karlgren et al., 2005).

Over the years, multiple expression analyses have been conducted on a broad panel of fetal and adult tissues and showed that CYP2W1 is significantly expressed only during fetal development of the colon, with traces of mRNA present in fetal kidney, liver, and lung. No significant levels of mRNA or the enzyme have been detected in healthy adult tissues, including complete absence of CYP2W1 from the ER of hepatic cells (Karlgren et al., 2006). The initial discovery of this enzyme in hepatoma cells however, prompted the search for its expression in multiple cancer cell lines and

human tumor tissues. High levels of both CYP2W1 mRNA and protein were found in more than 30% of human colon cancers, whereas the expression in surrounding normal tissue was absent or negligible. In addition, the moderate amounts of gene transcript were present in adrenal gland and lung tumors. Between colon cancer samples, CYP2W1 has displayed not only varying expression levels, but furthermore an unexplained difference in molecular weight, indicating that the protein might be a substrate for posttranslational modifications. Analogously, CYP2W1 gave several immunoreactive bands in transfected cells. Aside from HepG2, the presence of CYP2W1 protein was confirmed in a human colon cancer cell line CaCo-2TC7 (Gomez et al., 2007; Karlgren et al., 2006; Karlgren and Ingelman-Sundberg, 2007).

Clinical analyses performed on several hundreds of CRC patients have revealed that CYP2W1 has a value as a biomarker and a prognostic factor (Edler et al., 2009; Stenstedt et al., 2012). These studies have shown that the level of CYP2W1 expression correlates with the degree of tumor malignancy, and that the increased levels of the enzyme predict decreased 10-year survival in colon cancer patients. However significant the associations between CYP2W1 and tumor phenotype may be, the endogenous function of this protein in neoplasia is still unknown. Furthermore, its plausible role in developing intestine remains a mystery.

The tumor-specific expression of CYP2W1 in adult life is associated with the loss of epigenetic control (Gomez et al., 2007). The exon-1/intron-1 junction of CYP2W1 gene harbors a CpG island which is methylated in nontransformed cells, silencing the gene after birth. In cancer cells, this epigenetic mark is lost, constituting a requirement for aberrant overexpression. This finding however, does not illuminate the full complexity of CYP2W1 regulation, which is probably complemented by the actions of transcriptional regulators such as AhR (Tan et al., 2011).

Analogously to other members of CYP2 family, several single nucleotide polymorphisms (SNPs) have been reported for *CYP2W1* (Hanzawa et al., 2008). Three of these changes lie within the gene's exons, with one being silent and two coding for missense mutations. The two variant alleles, named *CYP2W1*2* (Ala181Thr) and *CYP2W1*6* (Pro488Leu), are defined by the changes that lie outside of the predicted substrate recognition site, but are nevertheless conserved within the CYP2 family. The frequencies for the Ala181Thr mutation are 0.8-8.3, 0.8, 7.1, and 0-4.2% respectively, in Caucasian, African, African-American, and Asian populations, whereas the Pro488Leu frequency remains to be reported (Karlgren and Ingelman-Sundberg, 2007). Regarding the alleles' relation to cancer, up to date the *CYP2W1*2* variant has been associated with decreased CRC risk (Gervasini et al., 2010). Thorough exploration of the relationships between CYP2W1 genotype, enzyme catalysis, and colon cancer risk, is presented in Paper IV of this thesis.

Despite the orphan status, the possibility of utilizing CYP2W1 as a target in colon cancer therapy has inspired efforts to elucidate its catalytic activity and substrate affinity. Expressed in bacteria or mammalian cells, CYP2W1 has demonstrated variable activity towards few candidate substrates (Tan et al., 2011; Wang and Guengerich, 2012; Wu et al., 2006; Xiao and Guengerich, 2012; Yoshioka et al., 2006), none of which have proven to be CYP2W1-specific.

## 1.4 CYPs AND CANCER

The CYP enzymes have three major roles in cancer:

- they activate or inactivate dietary and environmental components to ultimate carcinogens
- they activate or inactivate chemotherapeutic drugs
- they are specific targets for anticancer therapy

### Cancer predisposition and CYPs

A large number of procarcinogens, particularly polycyclic aromatic hydrocarbons (PAHs), can be activated by the CYP enzymes (Rodriguez-Antona and Ingelman-Sundberg, 2006). Due to the possible impact on cancer susceptibility, hundreds of studies have been carried out in the past with the aim to define CYP genetic variants related to certain types of tumor. Despite the extensive work, these studies have provided inconclusive results. This is to a great extent due to the complexity of cancer etiology as well as the difficulties in controlled study design which must take into account a plethora of confounding factors. Moreover, the major CYP enzymes triggering procarcinogen activation (the CYP1 family, CYP2E1, and the CYP3A family) are well conserved xenobiotic metabolizers which do not exhibit critical functional alterations (Rodriguez-Antona et al., 2010). In the absence of the relevant polymorphisms, it is very difficult to elucidate these enzymes' role in neoplasia. In addition, due to their overlapping substrate specificities, in most cases several enzymes contribute to the metabolism of a single procarcinogen.

Besides the carcinogen formation, the extrahepatic CYP2J2 was found to promote the neoplastic phenotype by stimulating cell proliferation and invasiveness through metabolism of arachidonic acid. The inhibition of CYP2J2-mediated biosynthesis was therefore suggested as a beneficial approach in anticancer therapy (Chen et al., 2011).

### Metabolism of chemotherapeutic drugs

Activation or inactivation of anticancer drugs takes place mainly in the liver. These aggressive cytotoxins have narrow therapeutic indexes and their effects are therefore highly dependent on the factors that can determine the final concentration of the active compound. Polymorphic P450 enzymes such as CYP2D6, CYP2C19, and CYP2B6, have important role in hepatic anticancer drug metabolism and their genetic variability may be responsible for the interindividual differences in therapeutic and toxic effects (Rodriguez-Antona et al., 2010). One of the best examples illustrating the impact of CYP genotype on the therapeutic outcome of anticancer drugs is tamoxifen. This estrogen receptor modulator, used worldwide in breast cancer prevention and treatment, is converted to active metabolites by CYP2D6. In Caucasians, 7% of the

population carries defective CYP2D6 alleles associated with diminished metabolic function, and consequently with the worsen breast cancer outcome in women undergoing tamoxifen therapy (Newman et al., 2008; Rodriguez-Antona et al., 2010).

A variety of chemotherapeutics are entirely ineffective before encountering a P450 activator in the liver. In addition to systemic activation of several antitumor prodrugs, such as oxazaphosphorines cyclophosphamide (CPA) and ifosfamide (IFA) (Giraud et al., 2010), hepatic P450s have been introduced to cancer cells with the aim to shift the metabolic balance and corresponding toxicity burden from liver to tumor tissue. Currently, among various projects in progress, attention is being drawn to a retroviral gene delivery vector encoding CYP2B6, called MetXia. In this GDEPT strategy, pioneered by Chen and Waxman in 1995, CPA is administered systemically after the CYP2B6 infection showing clinical benefit in patients with advanced breast cancer or melanoma (Braybrooke et al., 2005; Gunzburg and Salmons, 2005). In a recent GDEPT study, rat CYP2B1 was retrovirally introduced into human pancreatic tumor cells, rendering them more sensitive to IFA (Hlavaty et al., 2012).

At the simplest level, the inactivation function of the P450s involves clearance of cytotoxins from the body. Excessive CYP-mediated metabolism can therefore attenuate the availability of an anticancer drug. In addition, certain CYPs, such as extrahepatic CYP1B1 have been specifically implicated in deactivation of antitumor agents and thereby proposed to contribute to the development of resistance (Rochat, 2005). These findings however have not been verified by subsequent studies (Martinez et al., 2008).

## Targeting of cancer-specific CYP enzymes

In addition to the variable hepatic P450 profile, the presence of CYPs in cancer cells may affect their susceptibility to systemic chemotherapy. Due to the genetic and epigenetic DNA instability, tumor cells have the capacity to modulate P450 regulation and can either overexpress enzymes already present in normal cells (i.e. CYP3A, CYP2J2), or more importantly, produce unique CYP proteins.

Among several tumor-populating CYPs, only few enzymes are restricted to transformed cells sufficiently to constitute cancer-specific drug targets. CYP1B1 is the best studied example found in a wide range of malignant tumors and in metastatic disease (McFadyen et al., 2001). Even though the CYP1B1 mRNA has been found in healthy tissues (Rieder et al., 1998; Stoilov et al., 1998), the corresponding protein is generally not detected, opening avenues for CYP1B1 targeting (Gibson et al., 2003; McFadyen and Murray, 2005). Several cancer therapy approaches involving both CYP1B1 prodrug activation and CYP1B1 immunotherapy are under development. Aryl oximes, resveratrol, and Phortress, are some of the anticancer prodrugs investigated for activation by CYP1B1. An immunotherapy approach aimed to destroy cancer cells through induction of the T-cell response is the CYP1B1 vaccine ZYC300, which has warranted clinical evaluation (Rodriguez-Antona et al., 2010).

Since the discovery of its cancer-specific expression, CYP2W1 has been discussed in the context of tumor specific prodrug activation. This intriguing approach would allow for utilization of a particularly potent agent, whose concealed cytotoxic capacity would be released without jeopardizing the healthy cells. During the course of recent years, several compounds, including endogenous molecules (lysophospholipids), carcinogens (benzopyrene), and drugs (A4QN), have been proclaimed CYP2W1 substrates. Many of these however, are metabolized at a very low rate or, due to the experimental design are not clearly associated with CYP2W1 activity (Karlgren et al., 2006; Nishida et al., 2010; Wu et al., 2006; Xiao and Guengerich, 2012; Yoshioka et al., 2006). Recently, fluorobenzothiazoles GW 610 and 5F 203 (Phortress), have emerged as novel substrates for CYP2W1 (Wang and Guengerich, 2012). These anticancer molecules undergo multistep metabolism by extrahepatic CYPs to generate potent DNA alkylating species. The lead compound, 5F 203 is predominantly metabolized by CYP1A1, whereas GW 610 seems to be more CYP2W1 selective *in vitro*. Possible role of these agents in CYP2W1-mediated antitumor activity remains to be verified by *in vivo* studies. Our work presented in Papers II and III of this thesis reveals newly-designed duocarmycins as first CYP2W1-activated prodrugs whose potent antitumor effect is demonstrated against human colon cancer xenografts.

Results from the human genome project have placed CYP2W1 in the CYP2 family based on its amino acid sequence. However, a comparison of the predicted substrate recognition site (SRS) has shown that CYP2W1 shares highest identity with CYP1A1 (Karlgren and Ingelman-Sundberg, 2007). This active site homology can explain the overlapping specificity between the two enzymes for substrates such as GW 610 and 5F 203.

CYP1A1 is under normal conditions present at minor levels in lung, placenta, and fetal liver, while its marked overexpression is detected in respiratory and breast cancers. During smoking or environmental exposures, the levels of CYP1A1 escalate in both healthy and tumor tissues (Androutsopoulos et al., 2009). Regardless of its general inducibility, CYP1A1 is considered as a target for several candidate anticancer agents. Previously mentioned GW 610 and 5F 203, act not only as substrates but also as inducers of CYP1A1 (Tan et al., 2011; Wang and Guengerich, 2012). Recently, novel duocarmycin prodrugs were developed with the goal to target CYP1A1, and the selected truncated *seco*-analogue named ICT2700 has shown potent antitumor activity both *in vitro* and *in vivo* (Pors et al., 2011; Sutherland et al., 2013).

# 2 AIMS

The overall goal of this thesis was to explore if the unique tissue expression pattern of CYP2W1 enzyme can be employed for the development of a highly specific therapy for colon cancer.

More specifically, this thesis aimed to:

- characterize the membrane topology and posttranslational modification(s) of CYP2W1 protein

- develop specific anticancer prodrugs for CYP2W1-mediated activation in colon cancer and determine the potential of this novel approach in a preclinical setting

- evaluate the catalytic properties of CYP2W1 polymorphic variants as well the relationship between CYP2W1 genotype and colon cancer risk

# 3 RESULTS AND DISCUSSION

## 3.1 CYP2W1 GLYCOSYLATION, LOCALIZATION, AND CATALYTIC ACTIVITY

### Paper I

In transfected cells and colon cancer tissues, CYP2W1 manifests as two immunoreactive bands (52 and 54 kDa), suggesting possible involvement of posttranslational modifications in this phenomenon. By performing multiple *in silico* analyses of CYP2W1 sequence, we have identified the N-glycosylation at Asn177 to be the plausible posttranslational event.
In order to investigate if addition of sugar moieties does indeed generate a distinct pool of CYP2W1, microsomes from transfected HEK293 cells and from human colon tumor tissues were treated with deglycosylating enzymes PNGase and Endo H, resulting in a significant shift of the heavier immunoreactive band. The predicted site Asn177 was confirmed as the glycosylated residue in CYP2W1 by the means of site-directed mutagenesis and mass spectrometry analysis.

The scarcity of glycosylation in CYP enzymes is a consequence of their membrane topology. ER-resident CYPs are typically oriented towards the cytoplasm and are therefore inaccessible to glycosyltransferases which reside in the luminal space of the endoplasmic reticulum. Nevertheless, our findings show that CYP2W1 glycosylation occurs both *in vitro* and *in vivo,* suggesting atypical topology of this enzyme in the ER membrane. The unique inverse orientation of CYP2W1 was revealed using protease protection assay in multiple cell lines, which showed that the major portion of the protein molecule is indeed facing the ER lumen, thus co-localizing with the glycosyltransferases. Among the drug-metabolizing CYPs, a subfraction (2-3%) of the proteins can be inversely incorporated into the ER-membrane dependent on the specific features of the N-terminal anchoring sequence (Neve and Ingelman-Sundberg, 2000, 2010), however the predominant luminal orientation found in the case of CYP2W1 is unique.

The inverse topology of CYP2W1 undoubtedly questions the possibility of its interaction with the crucial members of the P450 electron transport chain, and consequently the biological activity *per se*. The lack of known endogenous substrates and the rather low catalytic activity towards numerous xenobiotic substrates screened in the past (Wu et al., 2006; Yoshioka et al., 2006), could be perceived as a reflection of a fairly silent CYP2W1 enzyme. However, we found that CYP2W1 displays a significant catalytic activity in the context of an intact cell system. Using transfected HEK293 cells, we showed that CYP2W1 is active in the metabolism of indoline substrates, generating unique derivatives that were not formed in control cells. Moreover,

CYP2W1 was able to metabolize aflatoxin B1 into cytotoxic products, causing dose-dependent loss of cell viability, which demonstrated the enzyme's potential for antitumor prodrug activation.

An important question to address is the role of glycosylation in CYP2W1. It was previously shown that this modification can facilitate selective transport of proteins to the cell surface (Gladysheva et al., 2008) and that some members of the CYP family localize to the plasma membrane (Neve and Ingelman-Sundberg, 2000, 2010). In order to explore these prospects with relation to CYP2W1, we have employed immunofluorescence microscopy and cell surface biotinylation assay which revealed that approximately 10% of the CYP2W1 travels to the plasma membrane. At this alternative location however, the extent of CYP2W1 glycosylation remains unchanged relative to the intracellular pool (4%), suggesting that glycosylation does not give any preferential advantage for plasma membrane targeting of CYP2W1. Even though the targeting mechanism remains to be explained, the amount of cell surface localized CYP2W1 is the highest amount of a microsomal P450 detected in this fraction, thereby raising the possibility for therapeutic targeting of colon tumor cells by using CYP2W1-specific antibodies.

## 3.2 CYP2W1 - MEDIATED ACTIVATION OF NOVEL DUOCARMYCIN PRODRUGS AS A NEW APPROACH IN COLON CANCER THERAPY

### Papers II and III

*Seco*-duocarmycins are highly reactive molecules that can spontaneously reshape their structure by spirocyclization to become ultrapotent DNA-alkylating agents (Figure 5). With the goal to modify these reactive compounds into prodrugs, the spirocyclization event can be halted by blocking the critical OH- group, a strategy that has been employed in tumor targeting approaches (Ghosh et al., 2009). Intended for PMT however, *seco*-duocarmycins can be inactivated in a way that allows for more cancer-specific activation of the prodrug. Rather than masking the crucial OH- group, our aim was to inactivate the duocarmycins by complete OH- removal, and to then harness the unique capability of tumor-specific CYPs to regenerate the removed hydroxyl moiety by oxidation (Figure 6). Once the OH- group is restored via CYP metabolism, the active drug rapidly forms and alkylates the DNA of cancer cell.

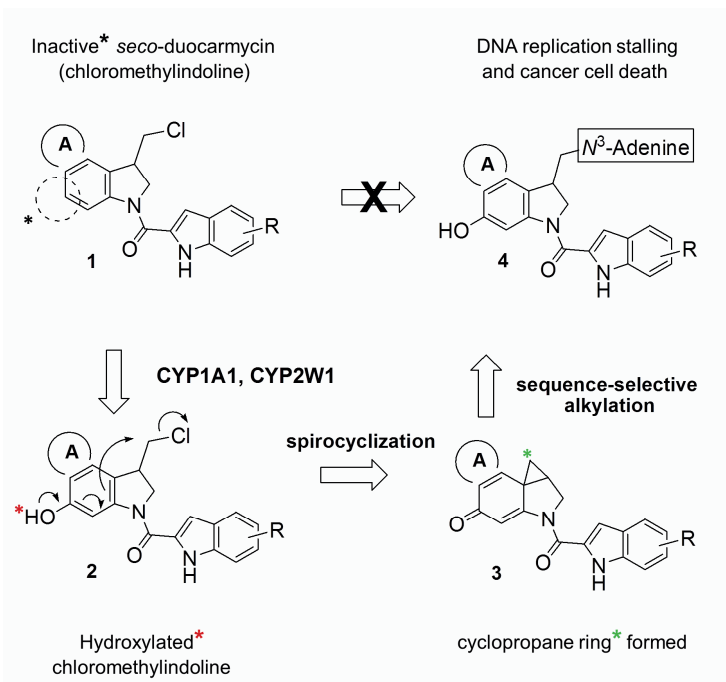


**Figure 6.** Proposed mechanism for CYP activation of duocarmycin (chloromethylindoline) prodrug. A - any heterocycle or aromatic ring.

By redesigning the duocarmycin molecules, we have synthetized a library of chloromethylindolines as candidate prodrugs for specific activation by CYP1A1 or CYP2W1. Chemosensitivity screens performed using a panel of cell lines have identified several chloromethylindolines as substrates for these enzymes, where two compounds, designated ICT2705 and ICT2706 (IDs 20 and 21 in Paper II), have demonstrated very high affinity for activation by CYP2W1. This finding, spawned from viability analyses in transfected HEK293 cells, has revealed first duocarmycin prodrugs that can be selectively activated by CYP2W1 into potent cytotoxins. In addition, we found a novel high-affinity substrate for CYP2W1, whose metabolism does not generate toxic species, rendering this compound (ICT2726) an elegant tool for assessing the enzyme's activity. Since CYP2W1 is expressed in one third of colon tumors (Karlgren et al., 2006), ICT2726 has a valuable potential as a biomarker for identifying the subset of cancer patients that would benefit from CYP2W1-targeted therapy.

In order to evaluate their potential as anticancer therapeutics, we have analyzed the effects of chloromethylindolines ICT2705 and ICT2706 in colon cancer cell lines. We showed that in the presence of CYP2W1, these compounds cause detrimental DNA damage and consequent loss of viability, while no effects are exerted on CYP2W1-negative cells (Figure 7). In addition, using LC/MS analyses, we have identified the active metabolite of the most potent chloromethylindoline, ICT2706.

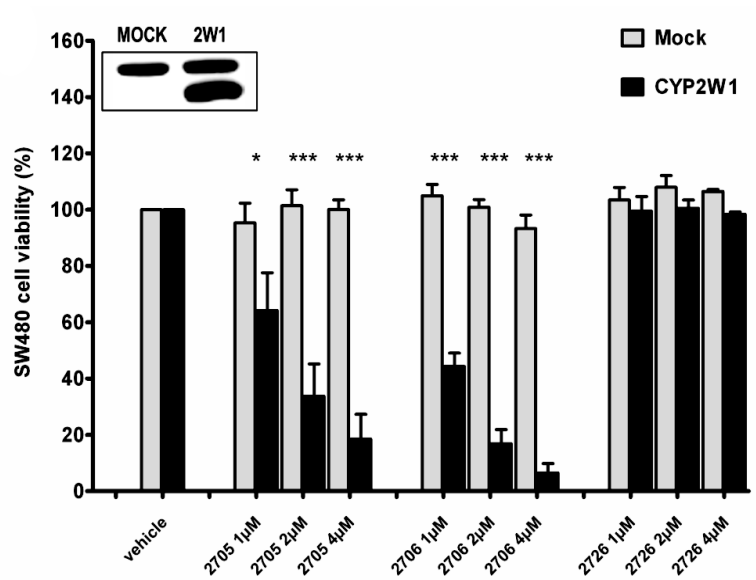


**Figure 7**. The effect of chloromethylindolines ICT2705 (2705), ICT2706 (2706) and ICT2726 (2726) on the viability of human colon cancer cell line SW480 stably transfected with CYP2W1 or control plasmid (mock). Insert, the immunodetection of CYP2W1 protein in transfected SW480 cells.

In order to elucidate the antitumor potential of ICT2706 *in vivo*, we have employed human colon cancer xenograft murine model, generated by subcutaneous injections of CYP2W1-expressing SW480 colon cancer cells (Figure 8). This approach has demonstrated the inhibition of tumor growth throughout the daily i.p. ICT2706 dosing (Figure 8A) by continuous eradication of CYP2W1-positive cells. We also showed that the requirement of CYP2W1 enzyme was absolute for the antitumor effect observed, as the xenografts negative for the enzyme displayed unrestrained growth during the prodrug treatment (Figure 8B).

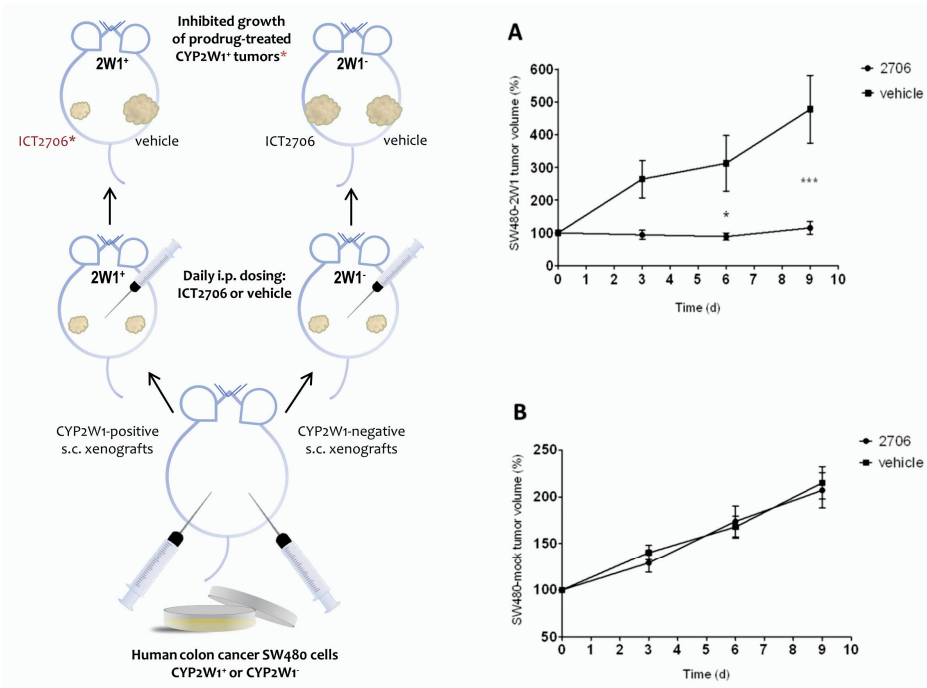


**Figure 8.** CYP2W1-dependent antitumor effect of daily dosed ICT2706 (100 mg/kg) in mice baring human colon cancer SW480 xenografts; A. Tumors expressing CYP2W1 ($2W1^+$); B. Tumors negative for CYP2W1 ($2W1^-$, mock).

Even though the CYP2W1-positive cells were nearly completely eliminated from mice tumors at the end of the ICT2706 dosing, we found that the adjacent CYP2W1-negative tumor tissue also suffers substantial levels of DNA damage. In the absence of the suicide enzyme, the sensitivity of this residual tissue to DNA alkylation can be plausibly explained as bystander toxicity, a crucial effect behind the efficacy of most prodrug strategies. One example, the cytosine deaminase/5-fluorocytosine GDEPT system, has shown to trigger colon cancer xenograft regression *in vivo* with only 4% of successfully transfected cells (Duarte et al., 2012). Furthermore, toxic metabolites of the CPA prodrug, which are generated by CYP enzymes, are efficiently disseminated

killing the surrounding tumor cells deprived of CYP activity (Doloff et al., 2010). Our *in vitro* analyses suggested that a similar bystander mechanism might be augmenting the antitumor effect of ICT2706. Colon cancer cells which do not express CYP2W1 die if incubated with conditioned medium from enzyme-expressing cells treated with ICT2706. Taking into account the variable expression of CYP2W1 in human colon tumors (Gomez et al., 2007), this prospect of bystander toxicity speaks in favor of achievable therapeutic effect.

Next, we wanted to analyze the route of administered prodrug in mouse tissues. We found higher concentration of ICT2706 in xenografts than in plasma, which indicated preferential accumulation of prodrug in the target tissue. Furthermore, the prodrug cleared from the animals' body within 24 hours, inferring that on each day of treatment the tumors were exposed to an equal concentration of ICT2706. The predominantly higher concentration of the prodrug detected in liver was consistent with hepatic elimination of highly lipophilic compounds (Figure 9).

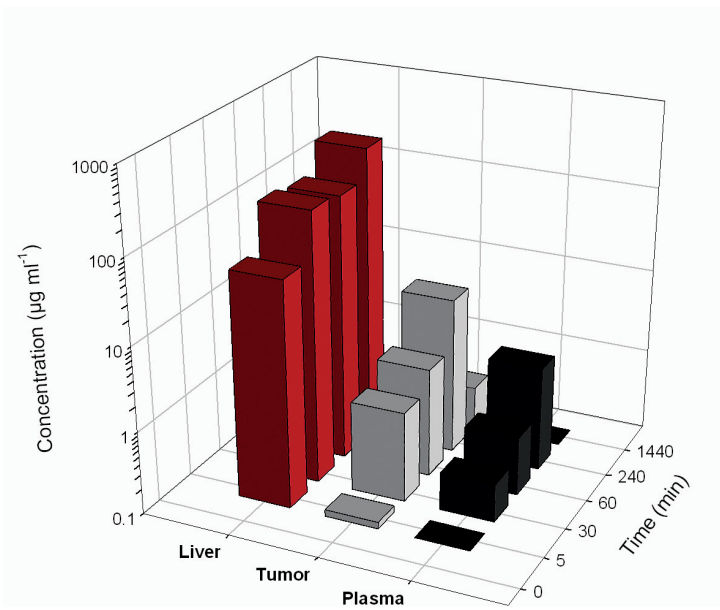


**Figure 9**. The distribution of ICT2706 in the tissues of mice baring CYP2W1-expressing xenografts.

In all xenograft experiments, no signs of unwanted host toxicity were apparent. Although a partial overlap in specificity toward chloromethylindoline substrates exists between CYP2W1 and CYP1A1, there were no indications that ICT2706 prodrug has undergone any metabolism by systemic CYPs *in vivo*. Supporting this notion, our analyses of a CYP1A1-targeting chloromethylindoline ICT2803 (ID 29 in Paper II), have shown that although this compound undergoes hepatic transformation, its active metabolite is formed exclusively by CYP1A1, with no other enzyme contributing to the resulting cytotoxicity. Furthermore, we have demonstrated that subtle changes in the

structure of the chloromethylindoline DNA-binding motif strongly affect their susceptibility to activation by a specific CYP, suggesting that further re-engineering of the *seco*-duocarmycin prodrugs can lead to even greater selectivity for CYP2W1 over CYP1A1-activation (or vice-versa), providing with the ultimate tumor-selective drug.

Given the high level of genomic instability and mutations seen in tumors, therapy resistance mechanisms are likely to be complex and multifactorial, allowing the cancer cell many escape routes to survival. Accordingly, a duocarmycin-based prodrug must not only demonstrate efficient activation by cancer-specific CYP, but once activated it must evade the drug resistance associated with DNA repair and efflux mechanisms. It was previously shown that the functionality of alkylating antineoplastic agents can be limited in the presence of the DNA-repair enzyme O-6-methylguanine-DNA methyltransferase (MGMT), whose promoter methylation in gliomas is a useful predictor of the responsiveness to alkylation therapy (Esteller et al., 2000). Within our chloromethylindoline library, compound ICT2700 (ID 15 in Paper II) was the first one explored as a CYP1A1-activated prodrug (Pors et al., 2011; Sutherland et al., 2013). *In vitro* analyses of both ICT2700 and its active metabolite have shown that their cellular uptake is equal, and that the cytotoxicity is not affected by either overexpression of the efflux pumps or deficiency in the DNA-repair (Pors et al., 2011). Even though detailed explorations of possible ICT2706 tolerance await, these findings suggest that the evasion of resistance to chloromethylindoline therapy can be achieved.

In summary, these studies present newly designed chloromethylindolines as substrates or prodrugs for cancer-specific CYP enzymes, and demonstrate the first example of CYP2W1 anticancer targeting both *in vitro* and *in vivo*. We have identified ICT2706 as a potential therapy which could be trialled in colon cancer to further prevent growth or metastasis, or to assist tumor shrinkage or ablation prior to surgery. The endogenous cancer-confined CYP2W1 expression in combination with ICT2706 prodrug therefore represents a novel candidate system for selective colon cancer treatment without the risk of systemic toxicity.

## 3.3 CYP2W1 POLYMORPHISM: FUNCTIONAL ASPECTS AND RELATION TO RISK FOR COLORECTAL CANCER

### Paper IV

Polymorphisms of CYP genes that alter the functional activity of the enzymes have been shown to affect the interindividual susceptibility to various types of tumor (Rodriguez-Antona et al., 2010; Rodriguez-Antona and Ingelman-Sundberg, 2006). Given that the extent of CYP2W1 expression affects the prognosis of colon cancer (Edler et al., 2009) it is reasonable to assume that the yet unknown endogenous function of the enzyme may contribute to the aggressive tumor phenotype or even to carcinogenesis *per se*. Furthermore, such effects might be modified by the expression of different CYP2W1 variants with presumably altered catalytic function.

Recently, the *CYP2W1*2* genotype was associated with decreased risk for CRC (OR 0.42), based on the analysis of genomic DNA from 150 cancer patients and 263 controls (Gervasini et al., 2010). This proposed relationship could be explained by the altered CYP2W1 metabolic function that is determined by the *CYP2W1*2* genotype. Up to date however, the catalytical properties of the CYP2W1 variant enzymes could not have been explored due to the lack of specific high-affinity substrates.

Our discovery of the chloromethylindolines ICT2706 and ICT2726 has provided a unique opportunity to analyze the impact of the amino acid substitutions on the function of the CYP2W1 enzyme, from the standpoints of both susceptibility to colon cancer and the pertinence of therapeutic targeting. Employing cytotoxicity assays and HPLC analysis in colon cancer cells transfected with equivalent amounts of *CYP2W1*1* (wtCYP2W1), *CYP2W1*2*, and *CYP2W1*6*, we found that all of the three corresponding enzymes have comparable catalytic capacity in activation of ICT2706 to a cytotoxic agent (Figure 10) and in the metabolism of ICT2726 to a non-toxic product.

If found, the difference in catalytic properties between the enzyme variants would provide a functional explanation for the underrepresentation of *CYP2W1*2* in colorectal cancer patients, as suggested by Gervasini et al. (2010). In the early stages of colon tumor development, such altered CYP2W1 function might significantly influence the fate of the disease. Even though the cryptic endogenous CYP2W1 substrate could hypothetically be metabolized in another manner than the chloromethylindolines, our *in vitro* analyses showed no significant differences in the metabolic capacity of the three variant enzymes. Importantly, their equivalent efficacy in the activation of ICT2706 prodrug renders CYP2W1-targeted therapy applicable for all CYP2W1 carriers and circumvents the need for genotype screening.

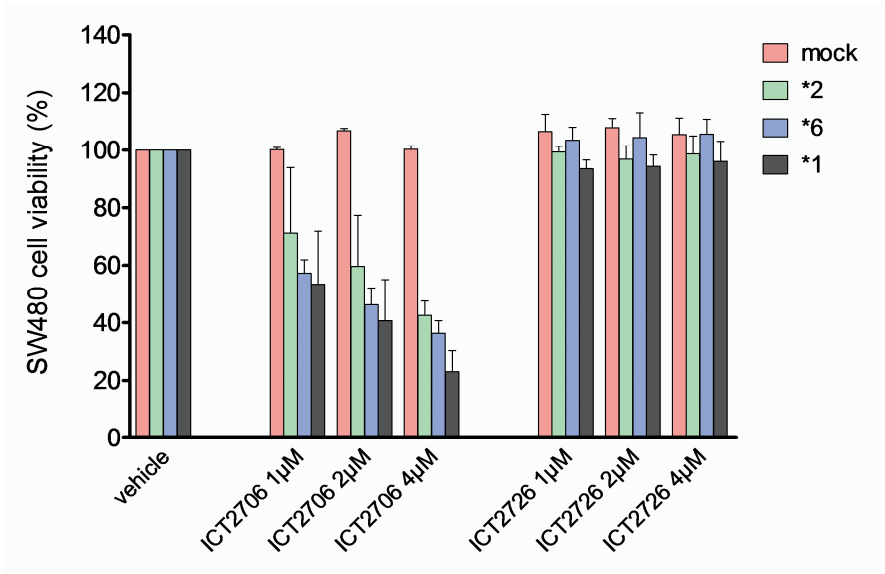


**Figure 10**. CYP2W1 variants show no difference in mediating cytotoxicity of ICT2706. Non-toxic ICT2726 was used as a control. *1, *CYP2W1*1*; *2, *CYP2W1*2*; *6, *CYP2W1*6;* mock, control-plasmid.

In line with the absence of functional differences, we also found no differences in the CYP2W1 allele or genotype frequencies between the 1785 colorectal cancer patients and 1761 control subjects analyzed. By the means of allele-specific PCR, we have determined that *CYP2W1*1*, *CYP2W1*2*, and *CYP2W1*6* are all in Hardy-Weinberg equilibrium and that their equivalent odds ratios are showing no statistically significant association with either of the analyzed groups. This finding, based on genotyping data from more than 10-fold larger cohorts than the preceding study by Gervasini and coworkers (2010), opposes the initial assertion of lower CRC risk among the carriers of *CYP2W1*2*. The most likely explanation for such discrepancy is the statistical power of our study, whose size becomes highly relevant if the low frequency of the *CYP2W1*2* allele in Caucasians is taken into account.

In summary, we have found that the relevant CYP2W1 variant forms are equally catalytically effective in substrate turnover and do not associate with the altered risk for colon cancer, which is in contrast to results presented in a previous, smaller study. Any functional role of CYP2W1 in colon cancer remains to be established.

# 4 CONCLUSIONS

This thesis can be concluded as follows:

- Regardless of its inverted ER membrane topology which allows for protein glycosylation at Asn177, CYP2W1 represents an active enzyme whose catalytic activity is sustained in an intact cell system
- Duocarmycin analogues can be redesigned to become specific substrates for CYP2W1 as well as for other cancer-confined CYP enzymes
- CYP2W1 can be targeted therapeutically using specific duocarmycin-based chloromethylindoline prodrugs, which cause selective colon cancer cell death *in vitro* and *in vivo*
- CYP2W1 polymorphic variants are equally efficient in their catalytic activity towards duocarmycin-based substrates and anticancer prodrugs
- CYP2W1 genotype is not associated with the risk of colon cancer development

# 5 SUMMARY AND FUTURE PERSPECTIVES

Cancer is the price we pay for life and cell division. In our existing arsenal of anticancer strategies, the antiproliferative approaches have had a major impact on some cancers, yet their efficacy against most solid tumors remains limited. The partial failure of such classic chemotherapeutic options is closely related to the narrow therapeutic windows of systemic treatments and their insufficient selectivity towards the essential cell populations. Aiming to bypass the limited efficacy and lack of specificity, the modern anticancer strategies, such as GDEPT and ADEPT, are focusing on creating differentiating features between malignant and non-malignant cells that can become determinants of targeted treatment. While simple in concept, these binary principles have proven to be challenging. In contrast, the possibility of prodrug activation by endogenous tumor-specific enzymes circumvents the need for gene delivery and eliminates the side effects associated with normal tissue exposure.

The confinement of CYP2W1 enzyme to colon cancer cells offers a unique opportunity for tumor-selective prodrug activation. Among many explored substrates, the duocarmycins have provided an avenue for the redesign of known molecules into specific, CYP2W1-activated cytotoxics, capable of significantly impeding the colon tumor progression. While the optimal prodrug formulation and its pharmacokinetics remain as issues for further explorations, the studies presented herein offer reassuring evidence that CYP2W1 targeting with ICT2706 could be feasible in clinical setting and represent a toxicologically sound approach with sparingly low risk of side effects. All CYP2W1 carriers, irrespective of the enzyme genotype, could be identified using non-toxic duocarmycin substrates (i.e. ICT2726), defining a subset of CRC patients that may benefit from the therapeutic approach. In the forthcoming years, the effectiveness of CYP2W1 targeting with duocarmycin prodrugs will be evaluated clinically, and probably guide the fine-tuning of the chloromethylindoline architecture to achieve the optimal prodrug. In addition, the ongoing efforts to resolve the CYP2W1 crystal structure may in the following years provide valuable directions for the design of new drugs.

Primary colon tumors accumulate CYP2W1 as they develop. If sustained during malignant expansion, this increment would yield even higher levels of the enzyme in metastases as compared to primary tissues. From the standpoint of targeted therapy, the CYP2W1-enriched metastatic colonies would therefore become increasingly sensitive to duocarmycin prodrugs during their propagation, at the phase where they typically acquire resistance to most available chemotherapeutics. Impending *in vitro* and *in vivo* studies will explore the efficacy of CYP2W1-targeted therapy in late-stage colon cancers and hopefully elucidate if the unleashed fetal protein has any role in sustaining the dedifferentiation of neoplastic cells. Bearing in mind the cancer stem cell hypothesis, it would be interesting to explore the presence of CYP2W1 specifically in this cell subpopulation.

The mechanistic details of CYP2W1 catalysis are presently under investigation. Further research will identify the elusive electron donor that facilitates the activity of the inversely positioned enzyme, and possibly provide with the means to manipulate the CYP2W1 functionality in cancer. If proven therapeutically valuable, the enrichment of CYP2W1 levels in disease may also be possible through induction of gene expression.

The near future will bring a clearer image of the probable endogenous CYP2W1 function, and the mechanisms that drive the gene's expression. Fetal CYP2W1 may have a significant purpose in the developing intestine, shaping yet unknown endogenic molecules into structural or signaling components. Currently under development, the CYP2W1 knock-out mouse model can help resolve these questions.

In conclusion, the CYP2W1-targeted therapy of tomorrow may offer novel means to battle the chemoresistant cell populations, achieving greater efficacy with less side effects. Pending its clinical evaluation, this promising tactic may significantly improve the outcome for many colon cancer patients in the future.

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