Summary

View from the sky. Chicago, USA 2003.
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Pyrimidine antagonists belong to the group of antimetabolite anti-cancer drugs and show structural resemblance with naturally occurring nucleotides. Their action is accomplished through the incorporation as false precursors in DNA or RNA or through inhibition of proteins involved in nucleotide metabolism. The most commonly used pyrimidine antagonists are 5-fluorouracil, gemcitabine and cytarabine. Newer oral variants of 5-fluorouracil are capecitabine and tegafur. 5-Fluorouracil and its analogues are used e.g. in the treatment of colorectal-, breast- and head and neck cancer, whereas gemcitabine is especially prescribed for non-small cell lung cancer and pancreatic cancer. Cytarabine is used in the treatment of leukemia. All pyrimidine antagonists are prodrugs and intracellular conversion into cytotoxic nucleosides and nucleotides is needed to produce cytotoxic metabolites. Proteins involved in pyrimidine metabolism handle these synthetic drugs, as if they were naturally occurring substrates. The extensive metabolism of pyrimidine antagonists implies that the intracellular concentrations of cytotoxic metabolites, largely depend on intracellular metabolic enzyme activity. Therefore, understanding of the genetics of metabolizing enzymes and the range of (iso)enzyme kinetics involved is essential for the optimal subscription of these anticancer drugs.

In this thesis, a number of pharmacokinetic and pharmacogenetic aspects of pyrimidine antagonist chemotherapy are evaluated in relation to safe use of these agents. Better knowledge of factors that have critical impact on pharmacokinetics and/or pharmacodynamics may help to reduce the incidence of side effects.

Genetic factors at least partly explain interindividual variation in anti-tumor efficacy and toxicity of pyrimidine antagonists. In chapter 2, proteins relevant for the efficacy and toxicity of pyrimidine antagonists are described. In addition, the role of germ-line polymorphisms, tumor specific somatic mutations and protein expression levels in the metabolic pathways and clinical pharmacology of these drugs are discussed. With respect to the 5-fluorouracil metabolic pathway, germ-line polymorphisms have been reported in uridine monophosphate kinase (UMPK), orotate phosphoribosyl transferase (OPRT), thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), and methylene tetrahydrofolate reductase (MTHFR). The impact of most germ-line polymorphisms is not yet clear. Mutations in the dihydropyrimidine dehydrogenase gene are however highly associated with DPD enzyme deficiency and can result in life threatening toxicity after 5-fluorouracil chemotherapy due to reduced 5-fluorouracil catabolism. Furthermore, the impact of a triple tandem repeat polymorphism in the promoter enhancer zone of the thymidylate synthase gene has been studied in a number of small trials, but remains to be established.

In addition, intratumoral gene expression levels of uridine monophosphate kinase, orotate
phosphoribosyl transferase, thymidylate synthase, dihydropyrimidine dehydrogenase, uridine phosphorylase, uridine kinase, thymidine phosphorylase, thymidine kinase, and dUTP nucleotide hydrolase have been studied in relation to 5-fluorouracil efficacy. Most interesting results have been reported with respect to dihydropyrimidine dehydrogenase, thymidylate synthase, thymidine phosphorylase, and orotate phosphoribosyl transferase expression levels. High intratumoral expression levels of both dihydropyrimidine dehydrogenase and thymidylate synthase may be associated with decreased sensitivity of tumor cells to 5-fluorouracil, but this remains to be established.

Regarding the gemcitabine and cytarabine metabolic pathway, germ line polymorphisms in cytidine deaminase (CDA) and 5′-nucleotidase (5NT) have been reported, but the impact of these polymorphisms on the efficacy of these drugs is hardly explored. Interesting results were obtained with regard to intratumoral gene expression levels of cytidine deaminase, 5′-nucleotidase and deoxycytidine kinase in relation to cytarabine efficacy, but these need to be further explored in larger clinical trials.

In section A, involving chapters 3, 4.1, 4.2 and 5, several pharmacokinetic and pharmacogenetic aspects of 5-fluorouracil in colorectal cancer patients are explored. A simple and sensitive high performance liquid chromatographic method with UV detection was developed for the determination of both 5-fluorouracil and its metabolite 5,6-dihydrofluorouracil in small volume plasma samples. The method is presented in chapter 3. It is characterized by isocratic elution at ambient temperature on a C18 5 µm column and subsequent ultraviolet diode array detection. The limits of quantification in plasma were 0.040 µg/mL for 5-fluorouracil and 0.075 µg/mL for 5,6-dihydrofluorouracil. The method appeared linear over a range from 0.04-15.90 µg/mL for 5-fluorouracil, and from 0.075-3.84 µg/mL for 5,6-dihydrofluorouracil. Compared to previous methods, the extraction procedure was simplified and the required sample size was reduced to only 100 µL.

The impact of liver metastases and liver function on 5-fluorouracil pharmacokinetics has long remained unclear. In most clinical trials, only patients with good performance status and adequate organ functions are included. However in actual practice, many patients with compromised performance status and/or inadequate renal or liver function are treated with chemotherapy.

In chapter 4.1 the influence of liver metastases on the pharmacokinetics of 5-fluorouracil and its metabolite 5,6-dihydrofluorouracil is described in 33 patients with gastrointestinal cancers. Patients were assigned to two different groups based on the presence or absence of liver metastases. Chemotherapy consisted of 5-fluorouracil plus leucovorin according to the Mayo Clinics schedule. Blood sampling was carried out on the first day of the first chemotherapy cycle. A multi-compartment Michaelis-Menten model was developed for simultaneous analysis of 5-fluorouracil and 5,6-dihydrouracil pharmacokinetic data. Extensive hepatic replacement due to liver metastases did not influence
the clearance of 5-fluorouracil and its metabolite. From the study it was concluded that there is no need for dose adjustment of 5-fluorouracil as a standard procedure in patients with liver metastases and mild to moderate elevations in liver function tests. One of the patients who participated in the liver metastases study experienced more than expected toxicity. Further research was subsequently initiated to elucidate the cause of this and results are presented in chapter 4.2. 5-Fluorouracil pharmacokinetics, dihydropyrimidine dehydrogenase-activity and DNA sequence analysis were compared between an index patient with extreme 5-fluorouracil induced toxicity and six control patients with normal 5-fluorouracil related symptoms. The 5-fluorouracil area under the curve (AUC) in the index patient was about 2.5 timer higher, and the clearance 2.5 times lower than in control patients. The activity of dihydropyrimidine dehydrogenase in blood mononuclear cells of the index patient was 50% of that in controls. Sequence analysis of the dihydropyrimidine dehydrogenase gene revealed that the index patient was heterozygous for a IVS14+1G→A point mutation. These results indicate that the inactivation of one dihydropyrimidine dehydrogenase allele can result in a strong reduction in 5-fluorouracil clearance, causing severe 5-fluorouracil induced toxicity.

The case of the dihydropyrimidine dehydrogenase deficient patient illustrates the impact of dihydropyrimidine dehydrogenase deficiency on 5-fluorouracil pharmacokinetics. The prevalence of dihydropyrimidine dehydrogenase deficiency has been estimated 1-2% in the Caucasian population. During the last decade, several methods have been proposed for early detection of dihydropyrimidine dehydrogenase deficiency, including genotyping, measurement of endogenous uracil/dihydouracil plasma levels and measurement of dihydropyrimidine dehydrogenase activity in peripheral mononuclear blood cells. For general large scale purposes however, these methods are too expensive and/or lack sensitivity.

Therefore, the pharmacokinetics of uracil after oral administration was studied with the aim to develop a cheap and easy oral Uracil Challenge Test. Chapter 5 contains a preliminary report about the potential clinical use of such a test for dihydropyrimidine dehydrogenase phenotyping. The pharmacokinetics of uracil and its metabolite 5,6-dihydouracil after oral administration of an uracil challenge dose were studied in 12 human volunteers and in one patient with dihydropyrimidine dehydrogenase deficiency. All subjects ingested 500 mg/m² uracil as an oral solution on an empty stomach. Blood sampling was carried out during 4 h after oral intake. All volunteers had dihydropyrimidine dehydrogenase activities within normal range, but in the patient it was reduced due to heterozygosity for missense mutation D949V in exon 22 and I543V polymorphism in exon 13 of the dihydropyrimidine dehydrogenase gene. Uracil plasma concentrations at 1 and 2 h and the uracil AUC were increased in the patient and differed more than 2 standard deviations from mean values in volunteers. These preliminary results indicate that further research in a larger number of previously characterized, partially dihydropyrimidine dehydrogenase
deficient patients is warranted, to determine its use in pre-chemotherapy phenotyping for early detection of dihydropyrimidine dehydrogenase deficiency.

In section B, involving chapters 6, 7, 8, and 9, focus is placed on clinical pharmacological, pharmacokinetic and pharmacogenetic aspects of gemcitabine in the treatment of patients with non-small cell lung cancer.

Gemcitabine has been recognized as a potent radiosensitizer, and as such, an interesting candidate for pre-radiotherapy radiosensitization in non-small cell lung cancer patients. The mechanism of gemcitabine mediated radiosensitization is yet poorly understood. Inhibition of DNA double-strand break repair by nonhomologous end-joining was previously excluded as a means of radiosensitization. In chapter 6 is explored whether gemcitabine affects either homologous recombination-mediated double strand break repair or base excision repair. Gemcitabine-mediated radiosensitization in cell lines deficient in base excision repair or deficient in homologous recombination were compared with that in their base excision repair-proficient and homologous recombination-proficient parental counterparts. Sensitization to mitomycin C was also investigated in cell lines deficient and proficient in homologous recombination. Mitomycin C is known to cause DNA double strand breaks by interstrand cross-links and repair of these cross-links is especially dependent on homologous recombination. In addition, the effect of gemcitabine on Rad51 foci formation after irradiation was studied. Rad51 foci are thought to represent homologous recombination. Gemcitabine did induce radiosensitization in base excision repair-deficient cells; however, the respective mutant cells deficient in homologous recombination did not show gemcitabine-mediated radiosensitization. In homologous recombination-proficient, but not in homologous recombination-deficient cells, gemcitabine also induced substantial enhancement of the cytotoxic effect of mitomycin. Finally, it was observed that gemcitabine interferes with Rad51 foci formation after irradiation. From this study was concluded that gemcitabine causes radiosensitization by specific interference with homologous recombination.

A frequently prescribed chemotherapy schedule for the treatment of stage IIIIB/IV non-small cell lung cancer is the combination of gemcitabine with cisplatin. Unfortunately, cisplatin can cause nephro-, neuro-, and ototoxicity. Moreover, cisplatin administration requires pre- and post hydration. The combination of gemcitabine plus epirubicin has been proposed as an alternative treatment schedule, that can be administered in outpatient setting. This schedule was well tolerated in phase 1/2 clinical trials. Mucositis was reported as the main non-hematological toxicity. Therefore the excretion of gemcitabine and epirubicin in saliva was studied in non-small cell lung cancer patients treated with both drugs, in order to estimate the relative contribution of salivary excretion to total drug exposure of mucosal cells in the oral cavity and upper gastrointestinal tract. The high performance liquid chromatography (HPLC) method used for determination of
epirubicin and epirubicinol in plasma and saliva is described in chapter 7. Preparation of saliva and plasma samples was performed by extraction in organic extraction fluid. The chromatographic analysis was carried out by reversed-phase isocratic elution on a C18 5 µm column. The detection was performed with spectrofluorimetry. The method appeared linear over a concentration range of 5 to 1000 µg/L for epirubicin and 2 to 400 µg/L for epirubicinol in both saliva and plasma. The lower limit of quantification was 5 µg/L for epirubicin and 2 µg/L for epirubicinol. The method proved to be precise and accurate.

The excretion in saliva of gemcitabine and epirubicin and their main metabolites dFdU and epirubicinol was studied in 12 patients and is described in chapter 8. Gemcitabine was detectable in saliva only during the first hour after infusion. The $C_{\text{max}}$ in saliva was 0.66 ± 0.61 mg/L and the saliva/plasma ratio was 0.038 ± 0.037. The epirubicin concentration in saliva 6 h after administration was 55 ± 27 µg/L and decreased to 28 ± 14 µg/L at 22 h. The corresponding saliva/plasma ratios were 1.28 ± 0.73 and 1.72 ± 1.00. The absolute amount of drug excreted in saliva was for both anticancer agents estimated under 0.2% of the administered dose. It was therefore concluded that, although gemcitabine and epirubicin as well as their main metabolites dFdU and epirubicinol are excreted in detectable amounts in saliva, their absolute amounts in saliva remain relatively low.

Another issue that was explored in this thesis concerns the possible pharmacokinetic interaction of gemcitabine and epirubicin when both drugs are used concurrently. Only few reports are available with respect to the pharmacokinetics of this particular drug combination. Information on pharmacokinetic and pharmacodynamic interactions of anticancer drugs is essential for optimizing the efficacy-toxicity ratio of a treatment schedule. Therefore a study was initiated to determine the pharmacokinetics of gemcitabine in gemcitabine-epirubicin compared to gemcitabine-cisplatin treated non-small cell lung cancer patients. Additionally, the effect of a common A79C polymorphism in the cytidine deaminase gene in relation to gemcitabine pharmacokinetics was explored. The results of this study are presented in chapter 9. Patients were treated with gemcitabine followed by epirubicin (n=12) or cisplatin (n=10). Plasma was collected during 3 h after administration of gemcitabine. The pharmacokinetics of gemcitabine was similar in both treatment groups. The half-life of 2',2'-difluorodeoxyuridine was larger in epirubicin- compared to cisplatin co-treated patients. Although the renal function was about 20% better in gemcitabine-cisplatin treated patients, we only found a weak correlation between 2',2'-difluorodeoxyuridine clearance and serum creatinine ($R=0.39$) or glomerular filtration rate (GFR; $R=0.40$) in a spearman rank correlation analysis. This suggests that, apart from renal function, another factor contributes to the observed difference.

We hypothesized that this might be due to increased 2',2'-difluorodeoxyuridine excretion and/or tissue disposition, as a result of pre-hydration measures, necessary for cisplatin administration. Another possibility could be an interaction of epirubicin or cisplatin with
2',2'-difluorodeoxyuridine. Since 2',2'-difluorodeoxyuridine has no cytotoxic potential, the observed difference was considered as clinically irrelevant.

The influence of the cytidine deaminase A79C genotype was studied in 20 patients. PCR plus DNA sequencing was performed to determine the genotype. A trend towards slightly higher AUC (+16%) and little lower clearance values (-11%) was observed in individuals heterozygous or homozygous for the C-genotype (C/C; A/C; n=12), compared to homozygotes for the A-genotype (A/A; n=8). Based on these data, it can be calculated that at least 120 patients would be required to elucidate the precise role of the A79C polymorphism. It was concluded that the pharmacokinetics of gemcitabine is similar in epirubicin- compared to cisplatin co-treated patients. There was a small 10-15%, not significant influence of a common A79C polymorphism in the cytidine deaminase gene on gemcitabine pharmacokinetics.