Summary

Chapter 1 provides a general introduction on the metabolism of folates and antifolates. The main mechanisms of acquired resistance to MTX are described and an overview of other antifolates is given. Beyond this, 5-fluorouracil and other fluoropyrimidines are introduced only briefly as several features of these drugs are extensively discussed in chapter 6.

Chapter 2 describes the validation of the in situ TS inhibition assay as a tool to assess the ex vivo activity of methotrexate and other antifolates against cells from patients with childhood leukemia. In human leukemia cell lines we found a good correlation between antifolate sensitivity data obtained with this TS inhibition assay and cell growth inhibition data. Next, using the TS inhibition assay, we were able to obtain reproducible sensitivity data in blast cells of children with acute leukemia. By using both short term and long term drug exposure periods, differences in antifolate drug retention could be assessed that were indicative for the level of polyglutamylation. This study set the stage for follow-up studies from our laboratory exploring resistance mechanisms to methotrexate in childhood leukemia.

Chapter 3 is an original report on the identification of a mutation in the human reduced folate carrier (RFC) gene that confers resistance to methotrexate and other antifolates. Previously only N-terminal premature translation termination mutations in the RFC gene had been reported that resulted in disrupted RFC protein expression. We were the first to identify a RFC gene mutation that results in marked changes in the kinetic properties of RFC-mediated transport of folates and antifolates. Notably, this mutation results in a structurally altered RFC that has an unchanged affinity for MTX but displays a highly increased affinity for transport of both folic acid and reduced folates. Under standard cell culture folate conditions cells harboring this RFC mutation can accumulate intracellular folates to such a high level that it confers resistance to methotrexate and several other polyglutamylation dependent antifolates, as well as lipophilic DHFR inhibitors.

In chapter 4 we characterized the mechanisms underlying resistance of human leukemia cell lines that were selected for acquired resistance to a panel antifolates using clinically relevant drug exposure schedules. Intermittent exposure of leukemia cell lines to methotrexate, raltitrexed, pemetrexed and lomtrexol resulted in a remarkably rapid development of resistance due to diminished formation of polyglutamate forms of the antifolates. This rapid evolution of resistance might be caused by the selection of (pre-existing) clones with decreased activity of folylpolyglutamate synthetase (FPGS). Cells selected by continuous exposure to GW1843U89 were characterized by defective RFC transport resulting in high levels of resistance to methotrexate and other RFC-transport dependent antifolates. The molecular basis for this resistant phenotype was the expression of a structurally altered RFC protein with decreased methotrexate
transport capacity along with enhanced transport of folic acid, resulting in expanded folate pools.

**Chapter 5** focuses on the dynamics of membrane transport of a series of antifolates via the reduced folate carrier and/or membrane folate receptor in both *in vitro* and *in vivo* murine leukemia models. The potency of the antifolates, determined by measuring the level of *in situ* TS inhibition, appeared to be closely related to their relative affinities for two dominant transport systems; RFC and MFR. The DHFR inhibitors methotrexate and edatrexate displayed potent antileukemic activity against mice inoculated with RFC expressing leukemia cells and this effect was markedly enhanced by dietary folate restriction, most likely due to less competition for cellular uptake and polyglutamylation by circulating plasma folates.

**Chapter 6** reviews the pharmacogenetic aspects of colon cancer with emphasis on potential implications for treatment with 5-fluorouracil and analogues.

In **chapter 7** we investigated the effect of 5-fluorouracil exposure on mRNA levels of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase in tumor tissues of patients with colorectal cancer. While TS induction *in vitro* is not related to changes in mRNA expression, our data on clinical samples revealed a rise in TS mRNA levels after exposure to 5-fluorouracil *in vivo*. Furthermore, we showed that exposure to 5-fluorouracil results in a down regulation of dihydropyrimidine dehydrogenase, which is in line with previous reports although the mechanism underlying this feature remains unclear.

Finally, **chapter 8** describes a study in clinical tissue samples of patients with colorectal cancer on polymorphisms in the TS gene that have previously been related to in vitro TS expression. We found no correlation between TS genotype and TS mRNA and TS protein levels in malignant tissues. In contrast, normal tissues harboring a TS polymorphism were characterized by higher TS protein levels and higher TS catalytic activity compared with the wild type genotype.
Key points of this thesis

- The in situ TS assay can be used for rapid (ex vivo) screening of antifolate sensitivity in childhood leukemia cells.
- Resistance to methotrexate (and other antifolates) can be associated with a structurally altered RFC protein with decreased MTX transport capacity but markedly increased transport of natural folates. Consequently, high extracellular folate conditions will provoke markedly increased intracellular folate pools and establish resistance to several antifolates.
- Multifactorial mechanisms of resistance to antifolates in CEM cells have a differential impact on cellular folate homeostasis: decreased polyglutamylation and transport defects lead to folate depletion, whereas a structurally altered RFC protein can facilitate expanded intracellular folate pools.
- Administration of 5-FU to colorectal cancer patients influences TS and DPD gene expression in vivo.
- TSER polymorphisms predict TS levels in normal tissues but not TS levels in malignant colon cancer tissues. Consequently, these polymorphisms could be valuable for predicting toxicity of TS-targeted therapy.