Attempts at improving fluoropyrimidine chemotherapy by biochemical modulators or by biomarker measurements with laser scanning cytometry

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<tr>
<td>5’-DFUR</td>
<td>5’-deoxy-5-fluorouridine, doxyfluridine, Furtulon®</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ANC</td>
<td>absolute neutrophil count</td>
</tr>
<tr>
<td>capecitabine</td>
<td>pentyl 1-(5-deoxy-beta-D-ribofuranosyl)-5-fluoro-1,2-dihydro-2-oxo-4-pyrimidinecarbamate, Xeloda®</td>
</tr>
<tr>
<td>CDHP</td>
<td>5-chloro-2,4-dihydroxypyridine</td>
</tr>
<tr>
<td>CFUc</td>
<td>Colony Forming Unit in culture</td>
</tr>
<tr>
<td>CH2-THF</td>
<td>5,10-methylene tetrahydrofolate</td>
</tr>
<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>CYP450</td>
<td>cytochrome P 450</td>
</tr>
<tr>
<td>DHFU</td>
<td>5,6-dihydro-fluorouracil</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPD</td>
<td>dihydropyrimidine dehydrogenase</td>
</tr>
<tr>
<td>dR-1-P</td>
<td>2-deoxyribose-1-phosphate</td>
</tr>
<tr>
<td>dTMP</td>
<td>deoxythymidine-5’-monophosphate</td>
</tr>
<tr>
<td>dTDP</td>
<td>deoxythymidine-5’-diphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine-5’-triphosphate</td>
</tr>
<tr>
<td>dUMP</td>
<td>deoxyuridine-5’-monophosphate</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine-5’-triphosphate</td>
</tr>
<tr>
<td>EDU</td>
<td>5-ethyl-2’-deoxyuridine</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FdUDP</td>
<td>5-fluoro-2’-deoxyuridine-5’-diphosphate</td>
</tr>
<tr>
<td>FdUMP</td>
<td>5-fluoro-2’-deoxyuridine-5’-monophosphate</td>
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<tr>
<td>FdUTP</td>
<td>5-fluoro-2’-deoxyuridine-5’-triphosphate</td>
</tr>
<tr>
<td>FPGS</td>
<td>folylpolyglutamate synthase</td>
</tr>
<tr>
<td>FUDP</td>
<td>fluorouridine diphosphate</td>
</tr>
<tr>
<td>FUdR</td>
<td>fluorodeoxyuridine</td>
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<tr>
<td>FUMP</td>
<td>fluorouridine monophosphate</td>
</tr>
<tr>
<td>FUR</td>
<td>fluorouridine</td>
</tr>
<tr>
<td>FUTP</td>
<td>fluorouridine triphosphate</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor, filgrastim</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GM-CFU</td>
<td>Granulocyte Macrophage Colony Forming Unit</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IL-3</td>
<td>interleukin-3</td>
</tr>
<tr>
<td>LSC</td>
<td>Laser Scanning Cytometer or Laser Scanning Cytometry</td>
</tr>
<tr>
<td>LV</td>
<td>leucovorin, 5′-formyltetrahydrofolate</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>OPRT</td>
<td>orotate phosphoribosyl transferase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PD-ECGF</td>
<td>platelet-derived endothelial cell growth factor</td>
</tr>
<tr>
<td>PN401</td>
<td>code for an acetylated prodrug of uridine, 2′,3′,5′-tri-O-acetyluridine</td>
</tr>
<tr>
<td>PRPP</td>
<td>5-phosphoribosyl-1-pyrophosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RR</td>
<td>ribonucleotide reductase</td>
</tr>
<tr>
<td>S-1</td>
<td>a combination of tegafur, CDHP and potassium oxonate</td>
</tr>
<tr>
<td>TAS-102</td>
<td>combination of TFT and TPI</td>
</tr>
<tr>
<td>tegafur</td>
<td>5-fluoro-1-(tetrahydro-2-furyl)-uracil, Ftorafur®</td>
</tr>
<tr>
<td>TFT</td>
<td>α,α,α-trifluorothymidine</td>
</tr>
<tr>
<td>TF-thymine</td>
<td>trifluorothymine</td>
</tr>
<tr>
<td>TF-TMP</td>
<td>trifluorothymidine-monophosphate</td>
</tr>
<tr>
<td>TF-TDP</td>
<td>trifluorothymidine-diphosphate</td>
</tr>
<tr>
<td>TF-TTP</td>
<td>trifluorothymidine-triphosphate</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>TP</td>
<td>thymidine phosphorylase</td>
</tr>
<tr>
<td>TPI</td>
<td>thymidine phosphorylase inhibitor</td>
</tr>
<tr>
<td>TS</td>
<td>thymidylate synthase</td>
</tr>
<tr>
<td>UDPG</td>
<td>uridine diphosphoglucose</td>
</tr>
<tr>
<td>UFT®</td>
<td>a combination of tegafur and uracil in 1:4 molar ratio</td>
</tr>
<tr>
<td>UK</td>
<td>uridine kinase</td>
</tr>
<tr>
<td>UP</td>
<td>uridine phosphorylase</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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1 INTRODUCTION

In spite of their more than 40-year history, fluoropyrimidine antimetabolites are still widely used for cancer therapy, especially in advanced colorectal and breast cancer. Although the fluoropyrimidine based chemotherapy e.g. in advanced colorectal cancer is still among the most active chemotherapeutic protocols, it is relatively ineffective, often resulting in objective responses for less than a third of the patients without significant benefit in terms of overall survival. These characteristics lead to extensive research aimed at improving the efficacy of fluoropyrimidines.

5-fluorouracil (5-FU), the first fluoropyrimidine drug, was designed rationally. Studies in the early 1950s showed that uracil was taken up and incorporated to a much greater extent in cancer tissues than in normal tissues (Rutman et al. 1954). This led to the synthesis of uracil analogs of which 5-FU was the most active against cancer models in rodents.

In order to exert its cytotoxic effect, 5-FU has to be converted to one of its nucleotides. This activation is catalyzed by the enzymes participating in the normal pyrimidine metabolism of cells. The existing (mostly only quantitative) differences between cancer cells and normal cells in the amount/activity of these enzymes and their substrates provide the basis of 5-FU’s tumor selective cytotoxic activity. The complex anabolism and catabolism of 5-FU in the cells offers possibilities for modulation of its efficacy and/or toxicity by other drugs influencing these processes. Additionally, the level/activity of metabolizing enzymes in the tumor cells/tissues may predict the response to fluoropyrimidine therapy, which would be a major contribution to the individualization of chemotherapy.

Over the past twenty odd years several modulation strategies have been developed to increase the anticancer activity of 5-FU, to overcome clinical resistance and to protect or rescue normal cells from 5-FU-induced damage. The main strategies that have been explored to modulate the anticancer activity of 5-FU include reduction of the degradation or increasing the activation of 5-FU, and enhancing the binding of 5-fluoro-2’-deoxyuridine-5’-monophosphate (FdUMP), the main active metabolite of 5-FU, to thymidylate synthase (TS), the main target of fluoropyrimidines. Of these, the last approach has lead to intensive studies and wide-spread clinical application of leucovorin (LV, 5’-formyltetrahydrofolate) as a biochemical modulator of 5-FU. As regards the selective protection of normal tissues from 5-FU-induced damage, uridine was examined because of the association between the incorporation of 5-FU into RNA and cytotoxicity. More recent approaches to improve the clinical efficiency of fluoropyrimidines have focused on (i) the development of prodrugs of
5-FU that can be given orally and (ii) the use of inhibitors of enzymes involved in the catabolism of 5-FU. The two most successful results of these recent developments already in clinical use are tegafur-uracil (UFT\textsuperscript{TM}) and capecitabine (Xeloda\textsuperscript{TM}). The development of UFT\textsuperscript{TM} addressed both of the above-mentioned criteria at once. Doxifluridine [(5’-deoxy-5-fluorouridine (5’-DFUR) Furtulon\textsuperscript{TM}], an oral prodrug of 5-FU, has been used for more than twenty years in Japan but its toxicity due to the extratumoral liberation of 5-FU restricted its benefits. To overcome this problem, capecitabine, a prodrug of doxifluridine was developed which, for these reasons, is often referred to as a pre-prodrug of 5-FU.

Unfortunately, the response rates to these new therapies are still not satisfactory, and the overall survival is not prolonged. One of the reasons for the relative ineffectiveness is most probably the large heterogeneity of individual tumors at the molecular level. Various factors may be responsible for the determination of tumor response to 5-FU and the quantitative differences may result in fundamentally different responses. Additionally, the differences might not affect every cell in the tumor tissue and specific histological patterns in the distribution of markers, which cannot be detected by traditional methods using homogenized tissues, might also influence the tumor response (Kobayashi et al. 2005). Further improvement in the response rates and survival can be expected from the careful selection of patients based on objective measurements of selected molecular markers. It is also expected that increasing the number of measured molecular determinants and considering their histological localization would provide a better way for the selection of those subpopulations who will respond to chemotherapy. Indeed, various investigators showed (Salonga et al. 2000 and Ishikawa et al. 1998) that measurement of more than one marker correlated better with response to fluoropyrimidine therapy and markedly enhanced the ability to predict tumor response to fluoropyrimidine based chemotherapy.

It is hoped that the revolutionary new method of laser-scanning cytometry – or other similar techniques sometimes collectively referred to as “slide-based cytometry” – would be suitable for the quantitative measurement of more than one such markers together at a previously unprecedented speed and accuracy, as well as provide a tool to connect these data with conventional histology. Another potential advantage of laser-scanning cytometry is the feasibility of detecting cells expressing fluorescent proteins (e.g., green fluorescent protein, GFP) even if they amount only to a small minority (<0.1%) of cells in a tissue or organ. The identification of cells expressing fluorescent proteins coupled with the simultaneous quantitative estimation of various other markers in the same cells may provide a useful method in experimental cancer research to study the early phases of homing of cells which play a major role in the spread of malignant cells.
2 OVERVIEW OF THE LITERATURE

2.1 Pharmacokinetics and mechanisms of action of 5-FU

Absorption of 5-FU after oral administration is incomplete and unpredictable leading to erratic bioavailability. This is why 5-FU is mostly given intravenously, either as bolus or a continuous infusion. Rapid intravenous administration of usual doses results in peak plasma concentrations of 0.1 to 1.0 mM, whereas, if given by continuous intravenous infusion for 24 to 120 hours, 5-FU achieves steady-state plasma concentrations in the range of 0.5 to 8.0 µM. Unfortunately, there is no evidence that plasma drug concentrations can predict antitumor effect or host cell toxicity. Following parenteral administration of 5-FU, the drug is rapidly distributed to tissues and extracellular fluid, including intestinal mucosa, bone marrow, liver, brain, cerebrospinal fluid, and neoplastic tissues.

The elimination of 5-FU is rapid, with an apparent terminal half-life of approximately 5 to 20 minutes. This is due primarily to a swift catabolism, the first and rate-limiting step of which is catalyzed by dihydropyrimidine dehydrogenase (DPD) present mainly in the liver but also in many other tissues, including intestinal mucosa and tumor cells. DPD reduces 5-FU to 5,6-dihydro-fluorouracil, a therapeutically inactive derivative ultimately degraded to α-fluoro-β-alanin. Renal excretion of unchanged 5-FU in 24 hours amounts to only 5 to 20% of a single intravenous dose. As mentioned in the Introduction, 5-FU is an analog of uracil with a fluorine atom at the C-5 position in place of hydrogen (Fig. 2.1.). The van der Waals radius of the fluorine atom resembles that of hydrogen which allows the molecule to mimic uracil biochemically (Chabner et al. 2005). 5-FU rapidly enters the cell using the same facilitated transport mechanism as uracil and intracellularly undergoes the same anabolic and catabolic reactions as uracil, with the exception of the methylation at position 5, catalyzed by thymidylate synthase (TS, see later).

There are three main active metabolites of 5-FU: 5-fluorouridine-5’-triphosphate (FUTP), 5-fluoro-2’-deoxyuridine-5’-monophosphate (FdUMP) and 5-fluoro-2’-deoxyuridine-5’-triphosphate (FdUTP, Fig. 2.2.).

For the formation of the 5’-monophosphate nucleotide (fluorouridine monophosphate, FUMP), various routes are available in animal cells: (i) 5-FU may be converted to fluorouridine by uridine phosphorylase and then to FUMP by uridine kinase, or (ii) it can be
directly converted to FUMP by orotate phosphoribosyl transferase (OPRT) using 5-phosphoribosyl-1-pyrophosphate (PRPP) as a cofactor (Fig. 2.2.). FUMP then can be further phosphorylated to FUDP and FUTP and incorporated into RNA. Similarly, at least two metabolic pathways are available for the conversion of 5-FU to FdUMP: (i) 5-FU may be transformed to the deoxyriboside fluorodeoxyuridine (F UdR) by thymidine phosphorylase (TP) which, in turn, can be phosphorylated to FdUMP by thymidine kinase. (ii) An alternative – and more important – route is the conversion of fluorouridine diphosphate (FUDP) to fluorodeoxyuridine diphosphate (F dUDP) by the enzyme ribonucleotide reductase which, in turn, can be dephosphorylated to FdUMP. FdUDP can also be phosphorylated to form FdUTP which then can be incorporated into DNA instead of deoxythymidine-5’-triphosphate (dTTP, Fig. 2.2.).

In all of the above mentioned enzyme reactions, fluorouracil or its metabolites substitute uracil and, using the “false substrates”, “false products” are produced, which can be used by further anabolic processes, e.g. incorporated into RNA or DNA, but the metabolic routes will not be blocked. The only exception is the reaction catalyzed by TS. This enzyme catalyzes the methylation of uracil at position 5 in 2’-deoxyuridine-5’-monophosphate (dUMP) to deoxythymidine-5’-monophosphate (dTMP, thymidylate) and provides the sole of de novo source thymidylate. However, the fluorine-carbon bond in 5-FU is much tighter than that of C-H in uracil and prevents the methylation of FdUMP by TS. Instead, in the presence of the physiological cofactor 5,10-methylene tetrahydrofolate (CH₂-THF), FdUMP locks the enzyme in an inhibited state. The TS protein is a dimer, both subunits of which contain a nucleotide binding site and a binding site for CH₂-THF. When FdUMP binds to the nucleotide binding site, a stable ternary complex is formed with the enzyme and CH₂-THF, thereby blocking the binding of the normal substrate dUMP and inhibiting dTMP synthesis.

Eventually, inhibition of TS will lead to the inhibition of DNA synthesis after depletion of dTMP and subsequently of dTTP. In addition, since the methylation of dUMP to dTMP is blocked, dUMP and dUTP accumulate, and the abundance of the latter coupled
with a deficiency in dTTP lead to the incorporation of dUTP into DNA. Imbalance in the pools of deoxyribonucleoside-triphosphates and inhibition of DNA synthesis and repair will result in single- and double-strand DNA breaks. Induction of DNA damage will activate downstream genes and proteins that initiate the processes of cell cycle arrest and cell death (Chabner et al. 2005, Milano et al. 2005).

Figure 2.1 Chemical structure and molecular weight of 5-fluorouracil and some of its derivatives used in the clinics
Although inhibition of TS is considered the most important mechanism of the cytotoxicity of 5-FU, after conversion to FUTP and FdUTP, 5-FU can also be incorporated...
into RNA (Peters et al. 1995) and DNA (Lönn & Lönn 1988), respectively. Incorporation of FUTP into RNA influences the normal RNA processing and function, and significant correlations were shown between 5-FU misincorporation into RNA and loss of clonogenic potential in human colon and breast cancer cell lines (Kufe & Major 1981 and Glazer & Lloyd 1982). Misincorporated 5-FU may influence the RNA functions at several levels, e.g. inhibition the processing of pre-rRNA to mature rRNA (Kanamaru et al. 1986 and Ghoshal & Jacob 1994) and disruption of the post-transcriptional modification of tRNAs (Santi & Hardy 1987 and Randerath et al. 1983). An indirect evidence for the importance of 5-FU incorporation into RNA is the fact that uridine reduced the incorporation of 5-FU to RNA and this was associated with the reduction of the side-effects of 5-FU (Peters et al. 1988).

Obviously, the above effects are expected to occur primarily in cells which grow faster and take up fluorouracil more rapidly.

### 2.2 Biochemical modulation of fluoropyrimidine activity

Although a host of compounds has been suggested to improve the clinical effects of 5-FU and oral fluoropyrimidines (Weckbecker 1991, Labianca et al. 1996, Connolly and Duley 1999, Grem 2000, Chabner et al. 2001, Longley et al. 2003, Milano et al. 2004), our discussion here is confined to those with direct relevance to the topic of this Thesis.

#### 2.2.1 Leucovorin

Probably the most intensively studied and currently widely used biochemical modulator of 5-FU is leucovorin (LV, 5′-formyltetrahydrofolate).

LV enhances the inhibitory effect of 5-FU (more precisely of 5FdUMP, the active metabolite of 5-FU) on TS, which is the key enzyme in the de novo synthesis of thymidine and probably the most important target of 5-FU in tumor cells (see above). LV is transported through the cell membrane by the reduced folate carrier and metabolized to CH$_2$-THF, the increased intracellular concentration of which results in stabilization of the ternary complex formed by CH$_2$-THF, TS and FdUMP; this is the generally accepted molecular mechanism of the above effect of LV. Although intermediates of the metabolic pathway of LV to CH$_2$-THF can also support the formation of the ternary complex, CH$_2$-THF is the most active substrate (van der Wilt et al. 1993). Additionally, polyglutamates of CH$_2$-THF, which are
formed by the action of folylpolyglutamate synthase (FPGS), will also enhance the inhibition of TS. According to *in vivo* studies (van der Wilt et al. 1992), the potentiation of the antitumor activity of 5-FU by LV given before 5-FU was associated with prevention of “rebound” TS induction occurring after 2-3 weeks of a dose of 5-FU alone (van Laar et al. 1996, Codacci-Pisanelli et al. 1995) and with a more pronounced inhibition of TS.

The clinical evaluation and validation of leucovorin (LV) in the early 1980s demonstrated that in **advanced colorectal cancer** it can improve the response rate to 5-FU from approximately 10% to 30%. A meta-analysis involving 1381 advanced colorectal cancer patients confirmed an increased response rate with 5-FU/LV (23%) compared to that of 5-FU alone (11%) (Anonymous 1992). However, there was no significant improvement in overall survival, and the treatment was associated with significant toxicities (especially mucositis, diarrhea and leucopenia). In the **adjuvant therapy** of Dukes B2 and C stage colorectal cancer LV+5-FU combination improved the event-free and overall survival (Zaniboni et al. 1998). Combination of LV+5-FU with other anticancer drugs can further improve the clinical results. This was demonstrated in a randomized clinical trial involving 2246 patients who had undergone curative resection for stage II or III colon cancer: the rate of disease-free survival at three years was significantly better in the LV+5-FU plus oxaliplatin group than in the LV+5-FU only group (78.2% and 72.9%, respectively; Andre et al. 2004).

### 2.2.2 Uridine and its prodrugs

The incorporation of FUTP into RNA is supposed to be a major cause of the toxic effects of 5-FU to normal tissues such as bone marrow or gastrointestinal epithelium, whereas inhibition of TS may mainly be responsible for killing tumor cells. This difference is corroborated by the clinical success of the combination of 5-FU with LV as described above. Another potential way of improving the selectivity of 5-FU would be to counteract its effects on RNA-synthesis with the aim of providing selective protection to normal cells without compromising the antitumor effects. Such a protection, or rescue of normal cells may enable us to increase the dose of 5-FU and improve the clinical results. Although preclinical experiments and clinical trials with uridine have supported the validity of this concept, it has not yet reached wide-spread clinical use.
The above concept was supported by **preclinical studies** in which administration of uridine after 5-FU reduced the FUTP incorporation into RNA, but did not affect the 5-FU-induced TS inhibition in cell culture (Peters, 1988) and in murine tumors (Nord, 1992). Furthermore, uridine-rescue improved the effect of LV+5-FU combination *in vivo* in the relatively 5-FU resistant murine colon cancer cell line Colon 26 (Nadal, 1989).

As damage to hematopoiesis was known to be a major factor limiting the dose of 5-FU (Lohrmann and Schreml, 1982), the question arose how the myelotoxicity of 5-FU was modified by uridine. Peters et al. (1988) reported that 5-FU-induced leukocytopenia and reduction in hematocrit were both mitigated by uridine. As for the mechanism of this protection at the level of cell-populations, it was shown by our group that (i) the 5-FU-induced damage to the population of granulocyte-macrophage progenitors in murine bone marrow (identified by their ability to form colonies in soft gel cultures, GM-CFU or CFUc) was mitigated by uridine-rescue, and (ii) pretreatment with LV did not abolish this beneficial effect (Kovács et al. 1992a). In these studies, CFUc were estimated only two and four days after the administration of 5-FU, when the effects of treatment on the CFUc-population could be clearly demonstrated. However, longitudinal studies were required to reach the nadir of neutropenia, as well as to follow the full course of the regeneration of the bone marrow and ANC. The author of this Thesis joined the group when the studies were being extended to follow the process of damage and regeneration, as the function of time, for 20 days after the administration of 5-FU.

Uridine concentrations in human plasma, bone marrow and cerebrospinal fluid range approximately from 3 to 8 µM and similar values were reported in rat and mouse plasma (Connolly and Duley 1999). Unfortunately, the high *in vivo* dose of uridine (>3000 mg/kg), necessary to reach and sustain the high plasma concentration of uridine (~75 µM) required to protect normal cells, was associated with unwanted effects: fever was observed in rabbits (Cradock et al. 1986, Peters et al. 1987a) while in mice high dose uridine caused hypothermia (Peters et al. 1987b).

**Clinical trials** using **parenteral uridine rescue** showed that unwanted effects (e.g. phlebitis, pyrogenic reactions) of large doses of uridine (which were required for effective rescue) limited the usefulness of this modulation therapy in humans (Leyva et al. 1984, van Groeningen et al. 1986, 1992). In a phase I clinical trial, **oral uridine** administration allowed for dose-intensification of 5-FU in a modified 5-FU-doxorubicin-methotrexate regimen (Schwartz et al. 1996). However, the use of oral uridine in patients is also limited by its poor
oral bioavailability, short plasma elimination half-life and the severe diarrhea occurring after the required large amounts of oral uridine.

To avoid the toxicity of uridine, PN-401 (an oral prodrug of uridine), uridine diphosphoglucose (UDPG) or, more recently, inhibitors of uridine catabolism (Al Safarjalani et al. 2005 and 2006) have been used in preclinical and/or clinical studies with similar results to uridine but with a better safety profile.

PN401\(^1\) is an orally active, lipophilic, acetylated prodrug of uridine, which is efficiently absorbed from the gastrointestinal tract and deacetylated by nonspecific esterase, yielding uridine and free acetate. It had superior uridine bioavailability compared to oral uridine tablets in a phase I clinical trial in combination with 5-FU (Kelsen et al. 1997). In the same study, PN401 was well tolerated and almost completely blocked the non-hematologic toxicities when given 24 hours following the administration of 5-FU. In another phase I clinical trial, PN401 allowed the safe administration of 5-FU on a weekly schedule in the dosing range of 1000 to 1250 mg/m\(^2\)/week, which is at least two-fold higher than the maximum tolerated dose of 5-FU without biochemical modulation (Hidalgo et al. 2000). PN401 was also well tolerated in this study, the qualitative and temporal nature of the toxicities of the 5-FU-PN401 regimen suggested that PN401 itself did not induce clinically significant toxicity. The principal dose limiting toxicity of the 5-FU-PN401 combination was severe neutropenia while non-hematologic effects were uncommon even with high doses of 5-FU. Unfortunately, no data were reported on the antitumor activity of the 5-FU-PN401 regimen and there was no unequivocal conclusion regarding the optimal timing of PN401 or uridine treatment relative to administration of 5-FU to protect normal tissues maximally without protecting malignant tissues (Hidalgo et al. 2000).

UDPG is a physiological substance normally used in biochemical reactions as a donor of phosphorylated sugars or as a precursor of glucuronic acid; it can also be converted to uridine. From the point of view of its potential clinical use instead of uridine, UDPG is particularly attractive because it is an approved drug available in some countries (e.g. Toxepasi™ in Italy) as an intravenous formulation for the treatment of various liver diseases (e.g. chronic hepatitis, viral hepatitis, cirrhosis).

It was shown that intravenous bolus injection of UDPG in tumor bearing mice resulted in an increase of uridine nucleotide content in liver, but not in tumor tissue (Colofoiure et al. 1989). These results suggested the possibility of selectivity of UDPG in

\(^{1}\) 2',3',5'-tri-O-acetyluridine
protecting normal tissues. Later Codacci-Pisanelli et al. investigated the modulatory effect of UDPG on 5-FU in mice. Delayed UDPG rescue reduced the systemic and hematological toxicity of 5-FU while did not affect 5-FU’s antitumor activity. The hematological toxicity in these experiments was determined by the measurement of the white blood cell count, the platelet count and the hematocrit but neither granulocyte numbers nor any effects at the myeloid progenitor cell level were investigated (Codacci-Pisanelli et al. 1997).

Contrary to the experiences with uridine, no evidence of alterations of body temperature was observed in mice treated with UDPG (Codacci-Pisanelli et al. 1997), suggesting a better safety profile for UDPG in comparison to uridine. In a pilot pharmacokinetic study in oncological patients, administration of 2 g UDPG by a 30 min infusion resulted in substantial increase in plasma uridine levels, which was comparable to what uridine caused. In contrast with the experiences with the infusion of uridine, no adverse effects were reported (Tognella 1992).

### 2.2.3 Ethyl-deoxyuridine

5-ethyl-2’-deoxyuridine (EDU) had originally been designed as a potential antiviral agent (Szabolcs et al. 1975; Prusoff and Ward 1976; Gauri 1981) but later its ability to inhibit the *in vitro* proliferation of L5178Y and L-1210 leukemic cells as well as Ehrlich ascites tumor cells was also discovered. Earlier studies suggested that the mechanism of action was the phosphorylation of EDU to its monophosphate form, which subsequently inhibited thymidylate synthase (TS) or was incorporated into DNA. *In vivo*, however, Ehrlich ascites tumor failed to respond to EDU. This discrepancy is probably due to the fast elimination of EDU by catabolism to ethyluracil, a reaction mediated by pyrimidine nucleoside phosphorylases in the liver and gut. In this reaction, EDU competes with the catabolism of thymidine, which results in increased thymidine level, which, in turn, reduces the consequences of the EDU-caused blockade of TS and prevents cytotoxicity (Jeney et al. 1986).

Although the lack of *in vivo* cytotoxicity prevented the development of EDU as an antitumor agent, the potent DPD-inhibitory effect of ethyluracil, its metabolite, made EDU an attractive compound to be used for the modulation of the action of 5-FU. In fact, EDU potentiated the antitumor effect of 5-FU against human colorectal cancer xenografts (Kopper et al. 1984, Kralovanszky et al. 1999) and increased the cytotoxic effect of 5-FU *in vitro* on
COLO-1 and SW620 human colon cancer cell lines (Katona et al. 1999, Katona et al. 2004). EDU enhanced the antitumor action of 5-FU most effectively when it was given 0.5-1 hour before the administration of 5-FU, but its application simultaneously with or following 5-FU resulted in a less effective potentiation of the tumor growth inhibition. EDU-pretreatment increased the therapeutic index of 5-FU (Kralovanszky et al. 1999) and this increased antitumor effect was associated with (i) increased intratumoral FdUMP, which, in turn, enhanced the inhibition of TS, (ii) inhibition of 5-FU-catabolism through the inhibition of DPD, probably by ethyluracil, one of EDU’s metabolites, and (iii) prolonged cytotoxic plasma levels of 5-FU. Additionally, the measured uridine concentrations after EDU were high enough (more than 50 µM for longer than 6 hours) to provide a supposedly selective uridine rescue of normal cells as discussed in the previous section. Kralovánszky et al. (1999) reported that the potentiation of 5-FU’s antitumor action was accompanied by some signs of toxicity, including a loss of body weight, but they did not study any possible effects of EDU and its combination with 5-FU on the hematopoietic system.

2.3 New oral fluoropyrimidine prodrugs

In an attempt to improve the oral bioavailability of 5-FU, a number of fluoropyrimidine prodrugs have been synthesized since the 1960s. In the 1970s two prodrugs of fluorouracil, 5-fluoro-1-(tetrahydro-2-furyl)-uracil (tegafur, Ftorafur™) and doxifluridine (5'-deoxy-5-fluorouridine, 5'-DFUR, Furtulon™; Fig. 2.1) were introduced into the clinic in some countries. While the advantage of these drugs was that they could be administered orally, producing a slow release of 5-FU, there were a number of disturbing toxic effects including gastrointestinal and central nervous system toxicities. More recently, new oral fluoropyrimidines and combinations of oral fluoropyrimidines and orally available inhibitors of DPD (the rate limiting enzyme of the catabolism of 5-FU) have been investigated in clinical trials (Rooseboom et al. 2004). Two of these, tegafur+uracil (UFT) and capecitabine have been accepted for clinical use in numerous countries.

2.3.1 UFT

UFT is a combination of tegafur and uracil in 1:4 molar ratio. The development of UFT addressed two mechanisms at once: (i) Although the CYP450 system in the liver is mainly
responsible for the conversion of **tegafur** to 5-FU, TP also contributes to this activating process. As the activity of TP is higher in some tumors than in the corresponding normal tissues, this was supposed to confer some degree of selectivity. (ii) **Uracil** is a competitive inhibitor of DPD, the rate limiting enzyme of 5-FU catabolism responsible for approximately 85% of 5-FU degradation. Both agents have excellent bioavailability following oral administration. The optimal 1:4 molar ratio of tegafur:uracil was determined in preclinical studies. The combination produced substantially higher plasma and tissue levels of 5-FU than tegafur alone and increasing the proportion of uracil further increased the 5-FU concentrations but also resulted in increased toxicity. It is important to note that following UFT administration the increase in the concentration of 5-FU was higher in the tumors than in the surrounding normal tissues. This was attributed to the fact that tumors have higher levels of TP and lower levels of DPD than normal tissues. Higher TP level results in increased local activation of tegafur while lower DPD level means almost complete block of local elimination by uracil. Thus, tegafur and uracil administered in combination have resulted in lowering the tegafur dosage and therefore toxicity, while at the same time maintaining the antitumor efficacy (Blijham 2003 and Rustum 1997).

Originally UFT was developed for use as a single agent; however, recently the clinical development has been focused on the combination of UFT/LV. This is due to the facts that: (i) the positive biomodulatory effect of LV was confirmed in experimental models where UFT/LV was compared to UFT alone and (ii) the availability of LV as an oral form which made possible an oral regimen of UFT/LV. Based on the results of two Phase III clinical trials (Carmichael et al. 2002 and Douillard et al. 2002) it was concluded that the response rate, the median time to progression and the median survival were similar in the UFT/LV and 5-FU/LV groups, however, the safety profile was significantly better with UFT than with 5-FU. The oral UFT/LV treatment had the additional advantage that, as expected, the majority of patients preferred the oral formulations.

### 2.3.2 Capecitabine

As mentioned above, the development of capecitabine\(^2\) (Fig. 2.1.), an oral “pre-prodrug” of 5-FU, was based on studies with a prodrug of 5-FU, namely with 5'-deoxy-5-fluorouridine

\(^2\)(pentyl 1-(5-deoxy-beta-D-ribofuranosyl)-5-fluoro-1,2-dihydro-2-oxo-4-pyrimidinecarbamate, Xeloda®)
(5’-DFUR, doxifluridine, Furtulon\textsuperscript{TM}). 5’-DFUR is metabolized to the active form, i.e. to 5-FU by thymidine phosphorylase (TP) preferentially located in tumors (Takebayashi et al. 1996). From doxifluridine, however, some 5-FU is liberated already in the gut and this leads to intestinal toxicity after high oral doses of this drug.

Capecitabine was designed to overcome this problem by the production of a prodrug of doxifluridine, which is not significantly converted to 5-FU in the gut. Capecitabine is converted to 5’-DFUR, through non-cytotoxic intermediates, by two subsequently acting enzymes: (i) carboxylesterase which is not present in the gut but located in the liver, and (ii) by cytidine deaminase which can be found primarily in the liver but also in many solid tumors (Fig. 2.3.). Consequently, it can be safely given at higher doses without serious intestinal toxicity (Miwa et al. 1998). Compared to 5-FU, capecitabine generated much higher levels of 5-FU in tumors and showed improved antitumor effects. The Food and Drug Administration (FDA) has approved capecitabine in the first line treatment of metastatic colorectal carcinoma and as a monotherapy in metastatic breast cancer resistant to both paclitaxel and anthracycline-containing chemotherapy regimens, or as a combination therapy with docetaxel in the treatment of patients with metastatic breast cancer after failure of prior anthracycline-containing chemotherapy.

### 2.3.3 S-1

S-1 is a triple combination consisting of the prodrug tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate in a molar ratio of 1:0.4:1, respectively. CDHP is a potent reversible inhibitor of DPD and significantly potentiated the antitumor effect of tegafur in preclinical models and provided sustained fluorouracil levels (Shirasaka et al. 1996b). Potassium oxonate is an inhibitor of orotate phosphoribosyl transferase that catalyzes the conversion of 5-FU to FUMP. It has been shown in preclinical studies to inhibit this activating process mainly in the gastrointestinal tract but not in tumors, and to reduce the injury of gastrointestinal tissues and/or severe diarrhea without influencing the antitumor effect (Shirasaka et al. 1993). In a preclinical study, the antitumor activity of S-1 was superior to that of UFT (Shirasaka et al. 1996a). In several phase II clinical trials, the antitumor effect of S-1 was at least equivalent to that of continuous or IV bolus 5-FU, or of the other oral fluoropyrimidines (Schoffski 2004).
TAS-102 is a new, orally available antimetabolite agent composed of α,α,α-
trifluorothymidine (TFT) and a thymidine phosphorylase inhibitor (5-chloro-6-(2-
iminopyrrolidine-1-yl)methyl-2,4(1H,3H)-pyrimidinedione hydrochloride; TPI) in 2:1 molar
ratio. The combination is in early phase of clinical investigation but it was shown in
preclinical studies that it exhibits good antitumor activity against 5-FU resistant human
cancer cells (Emura et al. 2004a, Emura et al. 2004b, Emura et al. 2005). TFT is a thymidine
analog which exerts its antitumor and antiviral activity through inhibition of thymidylate
synthase and incorporation into DNA (Fig. 2.2). However, TFT is rapidly hydrolyzed to

**Figure 2.3. Conversion of capecitabine to 5-FU.** Carboxylesterase is not present in the gut
but located in the liver, cytidine deaminase can be found primarily in the liver but also in many
solid tumors and thymidine phosphorylase level/activity increased in many solid tumors.

### 2.3.4 TAS-102

TAS-102 is a new, orally available antimetabolite agent composed of α,α,α-
trifluorothymidine (TFT) and a thymidine phosphorylase inhibitor (5-chloro-6-(2-
iminopyrrolidine-1-yl)methyl-2,4(1H,3H)-pyrimidinedione hydrochloride; TPI) in 2:1 molar
ratio. The combination is in early phase of clinical investigation but it was shown in
preclinical studies that it exhibits good antitumor activity against 5-FU resistant human
cancer cells (Emura et al. 2004a, Emura et al. 2004b, Emura et al. 2005). TFT is a thymidine
analog which exerts its antitumor and antiviral activity through inhibition of thymidylate
synthase and incorporation into DNA (Fig. 2.2). However, TFT is rapidly hydrolyzed to
inactive trifluorothymine by TP and/or UP and its half life is approximately 12-18 minutes when used alone. The combined use of TPI reduced this inactivation and increased the bioavailability (de Bruin et al. 2003). As TP was shown to be identical to platelet-derived endothelial cell growth factor (PD-ECGF), TAS-102 can be expected to have not only cytotoxic anti-tumor effects but also antiangiogenesis activity and may inhibit, e.g., liver metastasis development in colorectal cancer.

2.4 Individualized cancer chemotherapy by biomarker measurements in tumors

2.4.1 Tumor specific metabolism of fluoropyrimidine prodrugs

For many drugs the most important site of metabolism is the liver. However, in the case of prodrugs developed for cancer chemotherapy, one of the goals was to increase the selectivity by producing agents which are metabolized to their active forms selectively in the tumor tissue, thus preventing or at least reducing the systemic toxicity to normal cells/tissues (Rooseboom et al. 2004). Even 5-FU can be viewed as such a prodrug as it requires intracellular transformations to its active metabolites to exert its effects (for details see above). However, the selectivity of 5-FU metabolism in tumor cells is based only on the faster growth of the tumor cell population as compared to most normal cells and, not surprisingly, the major adverse effects damage the fastest growing normal cell populations such as the myelopoietic system and the epithelial lining of the gastrointestinal tract. The better tumor selectivity of the prodrugs doxifluridine and capecitabine is probably due to the fact that the level of TP, which catalyzes their activation, is higher in many tumors compared to normal tissues (Takebayashi et al. 1996, Mori et al. 2000).

2.4.2 Biomarkers may predict the efficacy of fluoropyrimidines

Three enzymes have been intensively studied as possible biomarkers for the prediction of the sensitivity of tumors to various fluoropyrimidines. Two of these, namely TP and DPD, mediate metabolic transformations of the drug molecules whereas the third one, TS, is the main target of fluoropyrimidines. The expression of these enzymes may be independent variables, at least this was observed in a set of colorectal tumors by Salonga et al. (2000).
2.4.2.1  Thymidine phosphorylase (TP)

**Doxifluridine and capecitabine**, as mentioned above, are prodrugs requiring activation by TP, so the intratumoral level of this enzyme was expected to play a pivotal role in the clinical efficiency of these drugs. Indeed, some clinical data support the usefulness of the estimation of this enzyme in a variety of surgically removed tumors to predict the response to postoperative doxifluridine or capecitabine (Koizumi et al. 1999, Sadahiro et al. 2001, Tominaga et al. 2002, Takiguchi et al. 2003, Noguchi et al. 2003, Grem 2005, Toi et al. 2005). Furthermore, the difference between normal and tumor tissues with respect to TP levels/activities suggests that the tumor-selectivity of these drugs, a main determinant of therapeutic success, can be predicted individually by comparing the TP-levels in normal and malignant tissues (Miwa et al. 1998, Mori et al. 2000).

As regards **5-FU**, TP mediates only the first step of one of the three known pathways of the activation. TP catalyses the reversible phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate (dR-1-P) (de Bruin et al. 2003). The conversion of 5-FU to F UdR follows the opposite direction and, therefore, requires dR-1-P as a cofactor. Although F UdR can be phosphorylated in a single step to FdUMP, the main active metabolite of 5-FU, this pathway seems to play only a minor role in comparison with the two other activation mechanisms of 5-FU, probably because of the low level of dR-1-P (Weckbecker 1991). So it is not surprising that a major role of TP in the sensitivity of tumors to 5-FU, contrary to doxifluridine and capecitabine, has not been unequivocally established. Actually, the role of intratumoral TP-levels in determining the sensitivity of tumors to 5-FU is controversial, as shown by the conflicting results of studies addressing this question (Schwartz et al. 1995, Fox et al. 1997, Metzger et al. 1998, Ishii et al. 2001, Marchetti et al. 2001, Yang et al. 2002, de Bruin et al. 2003, Hirano et al. 2002, Hirano et al. 2003, Toi et al. 2005). Other factors contributing to the conflicting nature of these results may include the following.

(i) There are species differences in the substrate-specificity of uridine phosphorylase (UP). Murine UP, unlike the corresponding human enzyme, cleaves thymidine (Haraguchi et al. 2002) and can be supposed to contribute to the conversion of 5-FU to F UdR in this species.

(ii) TP and the originally independently described **platelet-derived endothelial cell growth factor** (PD-ECGF) are now considered identical. TP, by means of its PD-ECGF-activity, promotes angiogenesis and may accelerate the growth of tumors.
and thereby impair the prognosis (Metzger et al. 1998, Hirano et al. 2002, Jakob et al. 2004, Toi et al. 2005). In patients treated with 5-FU, the relative importance of facilitating a minor pathway of the activation of 5-FU and the promotion of angiogenesis in the tumor, both mediated by high levels of TP, may be variable (Ciccolini et al. 2004). This variability may be due, in addition to the origin and histologic type of the primary tumor, also to the stage of the disease in which 5-FU is used.

(iii) Sensitivity of various malignant cells (both cell lines and primary cultures of surgically removed human tumors) is often estimated *in vitro* under experimental conditions not permitting angiogenesis to occur and, therefore any angiogenic activity of TP fails to contribute to the results.

Realization of the contradictory effects of high levels of TP on the sensitivity of tumors to 5-FU may help to counteract the angiogenic effects associated with TP by at least two methods. The first of these would be the application of inhibitors of angiogenesis, one of which, namely the anti-VEGF drug bevacizumab, has recently been approved by the FDA for clinical use. Another possibility is to use more specific inhibitors of the angiogenic effect of TP. Although currently no specific TP inhibitor drug is available in the clinics, several such drugs have already been tested clinically or preclinically. A potential candidate is TAS-102 described in Chapter 2.3.4. For 5-FU prodrugs, such as doxifluridine and tegafur, the inhibition of TP is not desirable as it would also decrease the activation of these agents. 2-deoxy-D-ribose is supposed to be a downstream mediator of the angiogenic effects of TP and the inhibition of its effects would be a preferable target in the case of these prodrugs. In fact, 2-deoxy-L-ribose (the stereoisomer of 2-deoxy-D-ribose) reportedly prevented various effects of 2-deoxy-D-ribose and is under detailed investigations (Akiyama et al. 2004, Nakajima et al. 2004, Toi et al. 2005).

2.4.2.2 Dihydropyrimidine-dehydrogenase (DPD)

High expression of DPD in tumor tissue was reported to result in poor response to 5-FU (Beck et al. 1994, Ishikawa et al. 1999, Nita et al. 1998).

As regards the fluoropyrimidines whose activation requires TP, Ishikawa et al. found that the capecitabine sensitivity of human xenograft tumors in mice correlated much better with the ratio of TP/DPD than either TP or DPD alone (Ishikawa et al. 1998). This finding later was supported by other investigators in clinical studies. Terashima et al. showed that, in
gastric cancer patients, a high TP/DPD ratio was associated with significantly better survival rates after adjuvant doxifluridine administration (Terashima et al. 2002). Furthermore, in a recent study the TP/DPD ratio in tumor tissues was a good predictor of tumor response to doxifluridine-based chemotherapy in metastatic gastric cancer (Nishina et al. 2004).

In addition to the role played by DPD in the determination of the response of tumors to fluoropyrimidines, DPD-levels may also profoundly influence the toxicity of these drugs. As the main way of elimination of 5-FU is mediated by DPD, an inherited deficiency of this enzyme leads to increased sensitivity to the drug and the rare person who totally lacks DPD may experience profound toxicity after conventional doses of 5-FU. DPD deficiency can be detected either by enzymatic or molecular assays using peripheral white blood cells or by determining the plasma ratio of 5-FU to its metabolite 5-6-dihydrofluorouracil (Chabner et al. 2001, Ezzeldin and Diasio, 2004).

2.4.2.3 Thymidylate synthase (TS)

Thymidylate synthase (TS) activity and protein and gene expression in human colorectal cancer has been investigated by several authors as a predictor of response for fluoropyrimidine based chemotherapies. These studies have suggested that high TS expression in advanced colorectal cancers, determined by several methods (immunohistochemical staining, enzyme activity, and reverse transcription PCR), is followed by non-response to 5-FU and poor prognosis (Johnston et al. 1995, Aschele et al. 1999, Cascini et al. 1999, Davies et al. 1999, Kralovanszky et al. 2002). It seems clear that high TS levels in the metastases of patients with advanced disease predict non-responsiveness to 5-FU, but it is far less certain that TS levels measured in local or locally advanced primary colorectal cancer predict clinical benefit resulting from 5-FU–based adjuvant treatment. This may be explained in part by the finding from several reports suggesting that TS expression levels measured in primary colorectal cancer may not be an accurate reflection of TS levels in metastatic or micrometastatic deposits, which represent the mechanism of relapse in patients with local and locally advanced disease (Aschele et al. 2000, Findlay et al. 1997). Conflicting data were published recently about the correlation between TS level and the outcome of adjuvant 5-FU based chemotherapy. Edler et al. (2002) observed that those patients whose tumors expressed the highest TS had a significantly longer disease-free survival if they were treated with adjuvant 5-FU based therapy compared with surgery alone, whereas patients whose tumors expressed low TS levels had an impaired outcome after
adjuvant therapy. However, other studies described either beneficial effect of 5-FU based adjuvant chemotherapy in patients with low intratumoral TS levels (Iacopetta et al. 2001) or no clear correlation was described between TS levels and response to adjuvant chemotherapy to discern groups of individuals who would be predicted to derive greater or lesser benefit from the use of adjuvant chemotherapy (Allegra et al. 2003).

These observations also underline that while single-marker investigations have been the mainstay of molecular diagnostics and proved to be useful in some cases, the development and introduction of technologies allowing parallel determination of multiple markers might help in more clearly defining individuals who are most likely to benefit from specific therapeutic interventions (Danesi et al. 2001).

2.4.3 Laser-scanning cytometry (LSC) for biomarker determinations

To determine the activities/levels of enzymes of interest, the most direct approach is biochemical measurement of enzyme activities, but this requires fairly large samples of fresh or freshly frozen tissue and cannot take into account the proportion of normal tissue in a tumor sample. At least three indirect assays have been developed to estimate the levels of these proteins in tumor and normal tissues: (i) immunohistochemical (IHC) assays for examining expression of the enzymes; (ii) quantitative PCR assays for measuring mRNA levels; (iii) ELISA assays, also for measuring protein levels. While measurements of the enzyme activity, as well as quantitative PCR and ELISA assays provide objective and quantitative results, they do not provide information about the regional or cellular localization of the molecules and they usually require fresh or frozen tissues or cells. The IHC assay can be done with paraffin embedded and fixed tumor tissues and gives information about the localization of the antigens but it is a subjective assay depending on the judgment of the person reading the slides and is, at best, only semi-quantitative. Additionally, the parallel measurement of two or more enzymes/antigens is practically not possible with these methods.

2.4.3.1 Laser-scanning cytometry

Flow cytometry has made possible the analysis of large populations of cells by rapid measurements, allowing the detection of rare cells and distinguish subpopulations of cells according to their different characteristics. Also attractive is the possibility of
multiparametric analysis and cell sorting. Flow cytometry with cell sorting is now indispensable e.g. in immunology, molecular and cell biology and cytogenetics, however, it has some limitations which restrict its usefulness in certain applications.

These limitations include: (i) individual cells are measured only once which means that it is not possible to analyze time-resolved events on the same cells, e.g. to measure enzyme kinetics; (ii) although in theory, sorting and examining of the morphology of single cells is possible, it is cumbersome and rarely used; (iii) flow cytometry cannot provide information on the spatial distribution of fluorochromes within the cell, e.g. nuclear vs. cytoplasmic localization, uniformity of distribution, co-localization with another fluorochrome etc.; (iv) it is impossible to re-stain the already measured cells with another probe and merge the results on a cell by cell basis; (v) solid tissues can be analyzed only after dissociation of cells and the isolation procedure produces a plethora of undesirable effects. Additionally, the information on tissue topography is lost (e.g. relationship of tumor cells to normal stromal or infiltrating cells, blood vessels, presence of localized inhomogeneities of tumor (or normal) cells which otherwise seem to be homogenous; (vi) because of the significant cell loss occurring during the sample preparation, analysis of samples containing only a small number of cells (e.g. fine needle aspirate or cerebrospinal fluid) in practice is very error prone, consequently these small size samples are seldom analyzed by flow cytometry (Darzynkiewicz et al. 1999).

Laser-scanning cytometry integrates the capabilities of flow cytometry with optical microscopy and offers many of the advantages of flow cytometry while eliminates most of the limitations listed above. A prototype laser-scanning cytometer was first described in 1991 (Kamentsky & Kamentsky, 1991) and a refined model entered commercial production in 1996. During the last decade, numerous reports and reviews have been published describing the capabilities of the LSC and its various applications. For the details of the instrument itself, its concept and analytical capabilities the reader is referred to the numerous excellent reviews (Kamentsky et al. 1997, Darzynkiewicz et al. 1999, Kamentsky 2001, Tarnok and Gerstner, 2002). It should be noted that, because of similarity in name, the laser-scanning microscope, a confocal microscope illuminated by a scanning laser beam, and other instruments having a scanning laser as an illumination source are sometimes confused with the LSC.

The most characteristic feature of laser-scanning cytometry distinguishing it from flow cytometry is that cell analysis is done on a slide. This offers the possibility of visual
examination to assess morphology and correlate it with the measured parameters. It provides the same possibility for multiparametric analysis as flow cytometry and, in addition, more than one cytofluorometric analysis can be done on the same cells using different sets of markers. The results of the sequential measurements can then be integrated using the merge capability of the instrument’s software. Re-staining and reanalysis of cells is possible not only with a new set of fluorescent markers but also with conventional stains used in pathology and the results of the fluorescent measurements can be easily compared with the morphological picture because the position of each detected object is recorded by the instrument and later can be relocated. As not only the position of detected cells (objects) is recorded but also the time at the moment of measurement, sequential measurements can be done on the same cells providing information about the kinetics of a process at the level of individual cells. Right angle (side) light scatter, common to flow cytometry, cannot be measured by LSC but individual pixel fluorescence intensities are measured which is not possible with a flow cytometer. This parameter reflects the inhomogeneity of fluorescence distribution within the analyzed cell/object and allows e.g. the characterization of cells with respect to the maximum pixel intensity (generally called the “max pixel value”) which represents the maximal fluorochrome concentration per area imaged on a single pixel. This proved to be especially useful in cell cycle analysis as the maximal pixel DNA-associated fluorescence correlates well with chromatin condensation (Luther and Kamentsky 1997, Kawasaki et al. 1997).

2.4.3.2 Tissue investigations with laser-scanning cytometry

The LSC has the capability to perform cytometric analyses of tissue sections with multiple colors. This will add valuable information to routine histopathology because it combines both objective quantification of cell subtypes with distinct characteristics and analysis of their spatial distribution within a tissue section. Manual histopathology allows only qualitative and subjective interpretations. Standardized analysis of sections could be a major application of LSC, but this potential has not been exploited yet. Only a few publications are available on tissue section analysis by LSC.

The first studies were performed on human breast cancer tissue sections for the quantification of estrogen receptors and Ki-67 expression (Gorczyca et al. 1998, Gorczyca et al. 2001, Hendricks 2001). Grace et al. analyzed tumor xenografts in mice for viral vector effectiveness in gene therapy by detection of induced apoptosis (Grace et al. 1999). Mackler
et al. investigated the activation and distribution of macrophages in tissue sections from murine uterus (Mackler et al. 2000). Immunophenotyping of leukocytes in tissue sections requires multicolor analysis to define and quantify leukocyte subsets. Three-color immunophenotyping of tissues has been performed with confocal laser-scanning microscopy (Steiner et al. 2000) and the LSC recently was applied to immunophenotype lymphatic tissue (Gerstner et al. 2004). Virtually all of the above mentioned studies used normal contouring for the detection of cells which is based on triggering on nuclear DNA staining (propidium iodide in all cases). This approach seems to be reasonable in thin tissue sections of <10 µm thickness if the majority of nuclei are separated and they can be detected as single objects. However, e.g. in various malignant tumors and in lymph nodes or tonsils, the cell density could be high and the detection of individual cells becomes difficult or impossible. Gerstner et al. (2004) recently developed a method named “multiple thresholding” to overcome the limitations of high cell density in sections of lymph nodes. They scanned propidium iodide stained sections repeatedly with step-wise increases in the threshold level of the triggering parameter (i.e. propidium iodide fluorescence in this case). Depending on the applied threshold levels, different cells in different microanatomical regions could be detected during each scan. The acquired data files then were merged, and objects triggered at the same x-y-coordinates were condensed to a single object (Gerstner et al. 2004). Although this approach seems to be applicable to detect virtually all cells of a section even in case of high cell density, it is time consuming when larger specimens are measured.

### 2.5 Colony stimulating factors in cancer chemotherapy

The above approaches to improve the therapeutic benefits of fluoropyrimidines are specific for this group of anticancer drugs. As damage to bone marrow is a major dose-limiting factor not only for fluoropyrimidines but also for the majority of other anticancer agents, any drugs protecting the hematopoietic tissues from cytotoxic insults or promoting their restitution from damage may find a much broader application in the therapy of malignant diseases. The discovery of colony-stimulating factors seems to be an important step to this goal.

The formation of colonies of hematopoietic cells in soft gel in vitro cultures was first described forty years ago (Pluznik and Sachs 1965, Bradley and Metcalf 1966). The cells giving rise to such colonies were shown to constitute a dividing transit population between
pluripotent stem cells and morphologically recognized precursors of granulocytes and macrophages and the term “progenitor cells” was coined to express their intermediate position in hematopoiesis (Metcalf 1977). They are frequently referred to by the operational terms Colony Forming Unit in culture (CFUc) or Granulocyte-Macrophage Colony Forming Unit (GM-CFU).

It was postulated that some chemical entities were necessary to stimulate hematopoietic progenitor cells to proliferate and differentiate in vitro, hence the name colony stimulating factors (CSF). To cut the long and fascinating history of CSFs short, up to now four human CSFs – all of them glycoproteins – were identified, cloned, produced and subjected to intensive studies including clinical trials. The various CSFs received their names after the type of cells in the colonies the growth of which they stimulate in soft gel cultures: the prefixes used are G for granulocytic, M for monocyte-macrophages, GM for a mixture of granulocytic and monocyte-macrophage cells, whereas multi-CSF stimulates the formation of colonies containing all the major myelopoietic lineages; this latter factor was found identical to interleukin-3. The CSFs stimulate the commitment, proliferation and differentiation of progenitor cells and their progeny, possess anti-apoptotic effects and enhance some functions of the mature cells the production of which they promote. In addition to the invaluable contribution of the discovery of CSFs to our understanding of the regulation of the hematopoietic system, two of them (G-CSF and GM-CSF) have been approved for clinical use by the FDA in the United States and are also widely used in other countries, whereas M-CSF is approved for therapeutic use in Japan (Metcalf 1984, Mertelsmann and Herrmann 1994, Morstyn and Dexter 1994).

It is beyond the scope of our work to give a detailed discussion of the clinical indications and benefits of CSFs. It should be noted, however, that one of the main indications of G-CSF is the reduction of the extent and duration of chemotherapy-induced neutropenia, which is still a major limiting factor in the treatment of neoplastic diseases. As expected, both G-CSF and GM-CSF possess such effects, but the former is the preferred agent because of its better safety-profile. It is generally accepted and proved by clinical trials that G-CSF reduces episodes of febrile neutropenia, requirement for broad-spectrum antibiotics, and days of hospitalization (Crawford et al. 1991, Trillet-Lenoir et al. 1993). Unfortunately, to date no clinical trial has shown improved survival in cancer patients treated with any of the colony stimulating factors.
2.6 Green fluorescent protein labeling in cancer research

The formation of metastases is a series of complex interactions between cancerous cells and host cells or tissues. Many points of it are still undefined and characterization of their molecular basis is crucial in the development of new cancer therapies. A major problem in studying the metastatic process is difficulty in visualizing micrometastases, especially in living animals. Currently, mostly histological or immunohistological analyses of target tissues are used to detect metastases. The sensitivity of the current methods are low and other problems with older methods, e.g. expression of Escherichia coli lacZ gene or optical imaging via luciferase reporter, have led to the increasing use of the green fluorescent protein (GFP) as a marker for cancer cells.

Initially isolated from the jellyfish *Aequoria victoria* (Shimomura et al. 1962), the GFP gene was cloned in 1992 by Prasher et al. (Prasher et al. 1992). It is a compact, acid and globular 27-kDa protein, composed of 238 amino acids (Yang et al. 1996), with an excitation peak of 488 nm and an emission peak of 508 nm. Its fluorescent properties are acquired by an autocatalytic mechanism giving rise to the fluorophore, which does not require any biochemical transformation, contrast agent or the use of ionizing radiation in order to be visualized (Cody et al. 1993, Hoffmann 2002). The GFP has been expressed in a variety of cells and organisms: bacteria (Feilmeier et al. 2000), yeasts (Shulga et al. 2000), eukaryotic cell lines (Chalfie et al. 1994, Cheng et al. 1996, Cheng et al. 1997) and transgenic mice (Okabe et al. 1997).

One of the main advantages of GFP labeled tumor cells lies in the possibility of *in vivo* detection of single cancer cells. At present, a wide variety of human tumor cell types, including brain, breast, colon, lung, skin, pancreas, prostate and stomach, can be tracked using the GFP-expression system. The *in vivo* detection of metastatic tumor cells and the quantitative characterization of metastases are very important but it does not allow molecular level resolution. Confocal laser-scanning microscopy has already been used for molecular characterization of the events in metastasis formation (Mook et al. 2003, Jaggi et al. 2005). Similarly, the ability to relocate cells and to quantitatively measure multiple fluorescent markers in samples mounted on slides make laser-scanning cytometry an appealing tool in quantifying the molecular determinants of the metastatic process and homing of tumor cells. Obviously, not only solid tumors can be investigated with this method, but it makes possible the quantitative characterization of the process of homing of GFP-transfected leukemic cells at the molecular level.
3 GOALS

Our main goal was to study some possibilities to improve fluoropyrimidine-based cancer chemotherapy. It is described in the previous chapter that, because of the complex intracellular metabolism of 5-FU, the most promising approaches are: (i) increasing the selectivity of 5-FU for tumor cells by using biochemical modulators or (ii) predicting a patient’s response to the fluoropyrimidine-based chemotherapy before the treatment starts, thus eliminating the unnecessary drug therapy in those patients who most probably would not respond to chemotherapy.

Leucovorin is widely used in the clinical practice as a modulator of 5-FU and it is supposed to increase the cytotoxicity of 5-FU predominantly on the tumor cells. Rescue protocols, which primarily decrease the damage to the most sensitive normal cell renewal systems, e.g. to the bone marrow and the gastrointestinal epithelium, have remained investigational. Because of the possibility of improved selectivity of a combination of a rescue protocol with leucovorin modulation, our aim was to study how the effects of LV pretreatment, uridine “rescue” and the combination of these two modulators affected the hematopoiesis in mice, especially the granulocyte-macrophage progenitor cell population. Unfortunately, the clinical use of uridine for this indication does not seem feasible because of its serious side effects. For this reason, prodrugs of uridine with better safety profile are searched for, and we wanted to see whether uridine diphosphoglucose (UDPG, a uridine prodrug commercially available for human use) may serve as an alternative for uridine in our murine model.

Recombinant human colony stimulating factors (e.g. granulocyte colony stimulating factor; filgrastim; G-CSF) are used in the clinical practice to reduce the duration of post-chemotherapy neutropenia. However, the effect of G-CSF on the hematopoietic recovery after LV, 5-FU and uridine had not been investigated. We combined these three modulators (LV, uridine, G-CSF) with 5-FU in mice to study their effects on myelopoiesis.

5-ethyl-2’-deoxyuridine (EDU) was shown by other investigators to increase the antitumor action of 5-FU. Since this is beneficial only if not accompanied by a similar enhancement of the toxic effects of 5-FU on normal cell renewal systems, we wanted to study the effects of EDU and its combination with 5-FU on the myelopoiesis of mice.
Despite efforts to predict the tumor response of patients to fluoropyrimidine-based chemotherapy considering various pharmacokinetic parameters of patients or biological characteristics of tumors, to date these attempts did not result in a breakthrough in forecasting tumor responses. The currently evolving methods of slide based cytometry might provide a better way for analysis of the relevant parameters by preserving the structure of tissues and allowing quantitative multi-parametric measurements. Our broad aim was to explore the possibilities and limitations of the laser-scanning cytometer (LSC) for studying the level of fluoropyrimidine-metabolizing enzymes in archived paraffin-embedded tissue sections prepared for routine histology. Both preclinical and clinical studies suggested the high importance of the measurement of the ratio of thymidine phosphorylase and dihydropyrimidine dehydrogenase in the determination of the tumor’s response to capecitabine therapy. According to these observations, we were particularly interested in the parallel and quantitative measurement of the ratio of these two enzymes in archived paraffin-embedded tissue sections.

In most cases, the metastatic property of malignant tumors is the feature which makes them fatal. Theoretically, a single “appropriate” cell is enough to give rise to a metastasis, and studying the early stages of the formation of metastases requires both the detection and characterization of rare tumor cells in various tissues. The relocalizing and multiparametric analytic capabilities of LSC appear attractive in these investigations and may be particularly useful if coupled with the potential of identifying rare tumor cells labeled appropriately, e.g. by GFP. As GFP-transfected cells and GFP-transgenic animals are promising new tools to study the homing of tumor cells and the subsequent development of metastases, we wanted to investigate the sensitivity of laser-scanning cytometry to detect rare GFP-positive cells in a GFP-negative population. This, coupled with the capacity of LSC to estimate various molecular markers in the identified cancer cells, would facilitate studies on the earliest phase of the formation of metastases.
4 MATERIALS AND METHODS

4.1 Modulation of the myelotoxicity of 5-fluorouracil in mice

4.1.1 Drugs and treatment schedules

4.1.1.1 Drugs

Uridine (U) was purchased from Sigma (St. Louis, MO, USA) or from Reanal (Budapest, Hungary), whereas 5-ethyl-2'deoxyuridine (EDU) was synthesized at the Central Chemical Research Institute of the Hungarian Academy of Sciences (Budapest, Hungary).

The following drugs were commercial pharmaceutical preparations:

- **5-fluorouracil (5-FU):** Fluoro-uracil Roche, Hoffmann-La Roche, Basel, Switzerland or Fluorouracil-TEVA, Pharmachemie BV, Haarlem, Netherlands
- **uridine diphosphoglucose (UDPG):** Toxepasi, Roche S.p.A, Milan, Italy
- **recombinant human Granulocyte Colony-Stimulating Factor (G-CSF) filgrastim:** Neupogen, Hoffman-La Roche, Basel, Switzerland
- **leucovorin (LV):** Calciumfolinat-Ebewe, Ebewe, Unterach, Austria or Leucovorin-TEVA, Pharmachemie BV, Haarlem, Netherlands

4.1.1.2 Administration of drugs

All drugs were given intraperitoneally in a volume of 10 ml/kg, except U and UDPG, where 20 ml/kg was used because of the unusually high doses needed. Control animals were treated according to the schedule of the drug-treated animals but only with the solvent of the drugs.
4.1.1.3 Doses

5-FU, 100 mg/kg in a single-dose, or 5x20 mg/kg;
LV, 100 mg/kg;
U, 2x3500 mg/kg;
UDPG 2x2000 mg/kg;
filgrastim (G-CSF), 100 µg/kg daily;
EDU, 200 or 400 mg/kg.

4.1.1.4 Dose schedules

The effects of 5-FU were studied after various combinations of LV, U, UDPG, G-CSF or EDU. In all experiments, the moment of the administration of 5-FU (or, if given repeatedly, that of the last dose of 5-FU) was considered as time zero and all dose-schedules and the results are presented on this time-scale.

a.) LV+5-FU+U+G-CSF
LV was given one hour before (at -1h) and U 2 and 20 hours after 5-FU, whereas G-CSF was applied every 24 hours after 5-FU.

b.) LV+5-FU+UDPG+G-CSF
The schedule was the same as used for LV+5-FU+U+G-CSF, except that UDPG was given 2 and 24 hours after 5-FU.

c.) EDU+5-FU
In these experiments, 5-FU and EDU were given according to either of two schedules: (i) the single-dose schedule consisted of a single 100 mg/kg dose of 5-FU preceded by 200 or 400 mg/kg of EDU as shown in Table 4.1; (ii) in the fractionated-dose schedule the same total dose of 5-FU was given in five equal parts (i.e. 5x20mg/kg) 24 hours apart, and each dose of 5-FU was preceded by 200 mg/kg EDU (Table 4.2).
Table 4.1. Combination of 5-FU and EDU: *Single-dose schedule*

<table>
<thead>
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<th>groups</th>
<th>time of administration* (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1</td>
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<tr>
<td>1x control</td>
<td>S</td>
</tr>
<tr>
<td>1x FU100</td>
<td>S</td>
</tr>
<tr>
<td>1x EDU200</td>
<td>EDU200</td>
</tr>
<tr>
<td>1x EDU400</td>
<td>EDU400</td>
</tr>
<tr>
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<td>EDU200</td>
</tr>
<tr>
<td>1x EDU400 + 1x FU100</td>
<td>EDU400</td>
</tr>
</tbody>
</table>

*The time of the administration of 5-FU was considered as time zero

S: 10 ml/kg 0.9 % NaCl
FU100: 100 mg/kg 5-fluorouracil
EDU200: 200 mg/kg 5-ethyl-2’-deoxyuridin
EDU400: 400 mg/kg 5-ethyl-2’-deoxyuridin
Table 4.2. Combination of 5-FU and EDU: Fractionated-dose schedule

<table>
<thead>
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<th>groups</th>
<th>time of administration* (hours)</th>
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<tbody>
<tr>
<td></td>
<td>-97</td>
</tr>
<tr>
<td>5x control</td>
<td>S</td>
</tr>
<tr>
<td>5x FU20</td>
<td>S</td>
</tr>
<tr>
<td>5x EDU200</td>
<td>EDU200</td>
</tr>
<tr>
<td>5xEDU200+5xFU20</td>
<td>EDU200</td>
</tr>
</tbody>
</table>

*The time of the administration of the last dose of 5-FU was considered as time zero

S: 10 ml/kg 0.9 % NaCl

FU20: 20 mg/kg 5-fluorouracil

EDU200: 200 mg/kg 5-ethyl-2’-deoxyuridin

4.1.2 Experimental animals

(BALB/c x CBA)F1 or BDF1 mice of both sexes at least 8 weeks old were given standard rodent diet and fresh drinking water ad libitum. The number of mice in each treatment group at each time of observation was 8-12 (typically 9) if not indicated otherwise.

4.1.3 Cell counts

Blood was drawn from the retroorbital plexus; bone marrow cells were washed out of femoral shafts with culture medium under aseptic conditions. Leucocytes and nucleated bone marrow cells were counted visually in a counting chamber in Türk’s solution. Differential cell counts were done on smears stained according to May-Grünwald-Giemsa. Generally 100 cells on each smear were counted but even in case of severe leucopenia a visual count of a minimum of 50 cells were done. The cellularity of the bone marrow is defined and will be presented in the Results as the number of nucleated bone marrow cells found in one femur.
4.1.4 Colony assay for hematopoietic progenitor cells

The number of progenitor cells with colony forming ability (CFUc = Colony Forming Unit in culture = GM-CFU = Granulocyte-Macrophage Colony Forming Unit) was estimated in semi-solid agar gel cultures free of the drugs used for the in vivo treatment of mice. The source of colony stimulating factor was either L-cell-conditioned medium or WEHI-3B-conditioned medium. McCoy’s 5A modified medium (Gibco, Grand Island) was supplemented with amino acids, vitamins, Na-pyruvate, NaHCO₃, penicillin and streptomycin according to Pike and Robinson (Pike and Robinson, 1970) as well as with horse serum, 5×10⁻⁵ M 2-mercaptoethanol (Loba, Fischamend, Germany) and 0.3% agar (Ionagar No3, Oxoid, London, or Noble Agar, Difco). Usually 1x10⁵ cells were plated in each dish; higher numbers of cells were, however, plated when the expected frequency of CFUc among nucleated bone marrow cells was low (at the first days after the administration of 5-FU). Plastic Petri dishes with a diameter of 35-mm were used (Greiner, Nürtingen, Germany), and the cultures were incubated for 7 days at 37°C in a humidified atmosphere containing 5% (v/v) CO₂.

Colonies, defined as groups of at least 50 cells, were counted under a dissecting microscope (SZ6045; Olympus, Hamburg, Germany) at the end of the incubation period. At least three parallel Petri dishes were prepared for each animal. The results will be given in terms of (i) frequency of CFUc defined as the number of colonies growing from 10⁵ nucleated bone marrow cells plated, and (ii) CFUc-content of the femoral bone marrow, calculated by multiplying the frequency of CFUc by the cellularity of the femoral marrow.

4.1.5 Statistical analysis

For comparisons of multiple treatment groups, non-parametric methods were used: first the Kruskal-Wallis test was performed, then – if indicated by this non-parametric analysis of variance – paired comparisons were done according to Conover (Conover 1980) or Dunn (Dunn 1964, Motulsky 2003). If there were only two groups to be compared, the non-parametric Mann-Whitney test was used. The incidences of neutropenia were compared by Fisher’s exact test.
4.2 Measurements of fluoropyrimidine metabolizing enzymes in single cells and tissue sections

4.2.1 Preparation of single cells for LSC measurements

Two human breast cancer cell lines (MDA-MB-231 and ZR-75-1), and one human bladder cancer cell line (T-24) in which the activity of TP and DPD had been well characterized (Ishikawa et al. 1998) were chosen as controls. These cell lines represented the extreme low (T-24 and MDA-MB-231) and the extreme high (ZR-75-1) TP/DPD ratios as determined by enzyme activity measurements (Ishikawa et al. 1998).

4.2.1.1 Cell cultures

Cancer cell lines were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) supplemented with amino acids, vitamins, penicillin, streptomycin, α-thioglycerol and 5% FBS in humidified air containing 5% CO₂.

4.2.1.2 Slide preparation

The cells were harvested from cultures by trypsinization, washed, and suspended in phosphate buffered saline (PBS) at a concentration of 10⁵ cells per ml. One ml of this cell suspension was deposited on silanized slides using a Hettich Universal 16 cytospin centrifuge (Hettich, Tuttlingen, Germany). The slides were air-dried and kept at -20°C until stained.

4.2.1.3 Indirect immunofluorescent labeling of enzymes

For the primary labeling of the enzymes, a mouse anti-human-TP (1C6-203) and a rat anti-human-DPD (2H9-1b) monoclonal antibody were used (both were from Roche Diagnostics, Indianapolis, IN, USA). For the parallel indirect labeling it was essential to use primary antibodies from different species to avoid cross-reaction with the secondary antibodies. As secondary antibodies, Alexa Fluor 488 goat anti-rat IgG (H+L) was used for labeling the rat anti-DPD antibody, and either Alexa Fluor 546 goat anti-mouse IgG (H+L) or Alexa Fluor 647 goat anti-mouse IgG (H+L) was used for labeling the mouse anti-TP antibody (all labeled secondary antibodies were purchased from Molecular Probes, Eugene, OR, USA). To avoid overlapping of emission spectra of various fluorescent dyes, TO-PRO-3 (Molecular
Probes, Eugene, OR, USA) was used for detecting the cells with Alexa 488 and Alexa 546 double labeling. TO-PRO-3 is a monomeric cyanine nucleic acid stain, which is excited by the 633 nm wavelength HeNe laser and its emission is detected in the long red channel. After blocking non-specific binding sites with Superblock (ScyTek, Logan, UT, USA), the slides were incubated at room temperature for 90 minutes with optimized concentrations of the primary antibodies (1:200 for the anti-TP and 1:500 for the anti-DPD, the stock concentration was 1 mg/ml for both). Following a washing step in PBS and 30 minutes incubation with the secondary antibodies at room temperature, the slides were washed again in PBS and incubated for 15 minutes with TO-PRO-3 at a concentration of 1 µM. The saturating concentrations for the primary and secondary antibodies were determined using single primary-secondary pairs and for the double staining protocol, equal volumes of 2x concentrations of the antibodies were mixed. Finally, the slides were rinsed with distilled water and cover-slipped with Prolong antifade solution (Molecular Probes, Eugene, OR, USA).

4.2.2 Preparation of tissue sections for LSC measurements

4.2.2.1 Preparation of “artificial tissues” from established cancer cell lines

The cultured tumor cells were trypsinized, washed three times in PBS, and a tight pellet was prepared by centrifuging the cells at 1500 g for 10 min. The cell pellet was incubated in 10% neutral buffered formalin overnight at 4°C. The cell pellets were first embedded in HistoGel™ (Richard-Allan Scientific, Kalamazoo, MI, USA) and then in paraffin according to standard pathology protocols.

4.2.2.2 Slide preparation

The paraffin-embedded cell lines and tissue blocks were cut into 4-5 µm-thick sections and, in most cases, one section of each control cell line sample was mounted on the same slide with the tumor tissue section. Before staining, sections were deparaffinized with xylene, rehydrated with 95% ethanol, placed in preheated DAKO Target Retrieval solution (DAKO Corporation, Carpinteria, CA) and antigen retrieval was performed at 95°C for 25 min by heating in a vegetable steamer.
4.2.2.3  Indirect immunofluorescent labeling of enzymes

The staining procedure was the same as described above for single cells, except that TO-PRO-3 was not used in sections for contouring. This made it possible to use Alexa 647 goat anti-mouse IgG (H+L) (Molecular Probes, Eugene, OR, USA) which was excited by the HeNe laser, instead of the Alexa 546.

4.2.3  Laser-scanning cytometry on single cells

After staining, slides were analyzed using a Laser Scanning Cytometer (LSC, CompuCyte Corp, Cambridge, MA, USA). The LSC was equipped with an Ar-ion and a HeNe laser (wavelength 488 and 633 nm, respectively). The Ar-ion laser line was used for excitation of the Alexa 488 and 546 dyes, and the HeNe was used for the DNA dye TO-PRO-3. For the measurements on single cells, contouring was based on the TO-PRO-3 fluorescence detected in the long red channel (>650 nm). In this case the fluorescence of the Alexa 488 and 546 dyes were detected in the green and the orange channel, respectively.

4.2.4  Laser-scanning cytometry on tissue sections

The phantom contouring feature of the LSC was chosen to detect the fluorescence intensity in tissue sections. Phantom contouring is a stereologic, random sampling technique where fixed size circular contours are randomly generated by the LSC (Luther 2002). The size and density of phantom contours are determined by the operator. They are analyzed by the software in the same way as contoured cells. In our studies, 10x objective was used, the radius of the phantom contours was set to 10 µm, the minimal distance between the phantom centers to 20 µm (to avoid overlapping of contours), and the number of contours was set to the maximum possible (950 per field). The Alexa 488 dye was excited and its fluorescence was detected as described above for the single cells, and the Alexa 647 was excited by the HeNe laser and its emission was detected in the long red channel.
4.2.5 Data analysis

4.2.5.1 Parameters used for characterizing the enzyme levels

In single cells identified by contouring on fluorescence of a nuclear stain, the integral fluorescence related to each enzyme divided by the area of the contour was used to describe the enzyme levels. This corrects for differences in cell size. In sections of cell lines, the integral alone was used, because the area of each phantom contour is constant.

4.2.5.2 Normalization

The measured fluorescence intensities were normalized to those of the ZR-75-1 cell line, which had the highest ratio of TP/DPD enzyme activities out of a number of cancer cell lines studied previously (Ishikawa et al. 1998).

4.2.5.3 Contour mapping of enzyme levels and ratios of enzyme levels in tumor sections

The standard proprietary software of the LSC (WinCyte 3.4) is capable to draw scatter plots of the X and Y positions of the detected cells or phantoms, and it is also possible to choose regions on, e.g., the fluorescence integral histograms and assign a color to those points. However, WinCyte 3.4 graphs can only be transferred using screen capture, resulting in poor quality reproductions for publication purposes. Additionally, the data files produced by WinCyte 3.4 use the user-defined ‘OTHER’ segment of the FCS 3.0 data file standard for storing the list mode data of cells/contours in a proprietary format. Thus, to get these data for the individual contours the data files had to be exported as ASCII text files from within WinCyte 3.4. The list mode data then were used for creating contour maps by using the “filled contour plot” graph style of Sigma Plot 8.0 software (SPSS Inc., Chicago, IL, USA) after smoothing the raw data with the built-in 3D data smoother.

4.3 Estimating the sensitivity of tumor cell lines to 5’-DFUR with the MTT assay

Sensitivities of the tumor cell lines to 5’-DFUR were determined using the MTT assay (Eliason et al. 1990). Tumor cells were plated into triplicate wells of a 96-well plate at cell concentrations for each line that had been previously determined to be optimal. Two different
cell concentrations were plated for each cell line (1600 and 800 cells/well for ZR-75-1 cells; 800 and 400 cells/well for T-24 cells; 400 and 200 cells/well for MDA-MB-231 cells). After 24 hours incubation at 37°C in a fully humidified atmosphere of 5% CO₂ in air, increasing concentrations of 5′-DFUR were added. After 72 hours of incubation 50 µl of MTT (3 mg/ml) were added and the plates were incubated for 4.5 hours before addition of 50 µl of 25% SDS at pH 2.0 to stop the further transformation of MTT to its blue formazan derivative. The plates were incubated overnight, and the absorbance was measured at 595 nm using a Tecan Ultra ELISA reader (Tecan Inc., Durham, NC, USA).

4.4 Estimating the sensitivity of laser-scanning cytometry to detect cells expressing green fluorescent protein

4.4.1 Cell sample collection and preparation

Adult female C57BL/6-TgN(ACTbEGFP)1Osb mice hemizygous for ‘‘enhanced’’ GFP (EGFP) cDNA under the control of a chicken beta-actin promoter and cytomegalovirus enhancer were mated with adult male wild-type C57BL/6J mice. The test progeny were bled (< 0.1 ml/mouse) from the retro-orbital plexus at 21–35 days after birth. Blood was also obtained from adult parental GFP-hemizygous (GFP+/–) mice and wild-type C57BL6/J (GFP–/–) mice, as positive and negative controls, respectively. After dilution with 1 ml of normal saline containing 10 units/ml of heparin, the mononuclear cells were separated with density gradient centrifugation using Nycoprep 1.077A density gradient media (AXIS-SHIELD PoC, Oslo, Norway). The separated cells were fixed in 1% paraformaldehyde for 20 min at room temperature, deposited on slides using a Hettich Universal 16 cytospin centrifuge (Hettich, Tuttlingen, Germany) and a coverslip was placed on top with 50% glycerol. For experiments detecting rare cells, known numbers of GFP+/– mononuclear cells were mixed with GFP–/– cells at a ratio of 1:1000.

4.4.2 Fluorescence measurements

The 488-nm wavelength Ar-ion laser line of the LSC (CompuCyte, Cambridge, MA, USA) was used as the excitation light source and the emitted fluorescence was detected in the green
channel. During phenotype determination, forward scatter was used for cell contouring and the fluorescence intensity of cells was measured by the green fluorescence max pixel parameter, which is the fluorescence intensity of the brightest pixel inside a detected cell. For estimating rare cell detection sensitivity, green fluorescence was selected as the contouring parameter and to ensure high specificity, the threshold level was set to a value that did not detect cells in the negative control sample. This threshold was used during the scanning of the slides containing mixed GFP positive and GFP negative cells.
5 RESULTS

5.1 Effects of LV, U or UDPG and G-CSF on the hematotoxicity of 5-FU

5.1.1 Effect of a single ip. dose of 5-FU or the combination of LV+5-FU on the myelopoiesis of mice

As expected, the myelotoxic effect of 5-FU was proven by all measured parameters. After 5-FU treatment, the cellularity (total number of nucleated bone marrow cells in a femur) was significantly below the control from Day 2 to Day 10 (Fig. 5.1A) and the nadir was reached on day 4, when the median in the 5-FU treated group was only 19% of the control.

The frequency of CFUc (number of colonies grown per $10^5$ nucleated bone marrow cells) was significantly below the control on day 1, 2 and 3; the nadir occurred on the first day, when the median of the 5-FU group was less than 0.1% of the control. On Day 5, 6, 7, 8, 10 and 12 this parameter was above the control, although statistical significance was detected only on Day 10 ($p<0.001$, Fig. 5.1B).

The shape of the femoral CFUc content vs. time curve was similar, but the CFUc content in the 5-FU-only group was still significantly below the control on day 4. Similarly, the CFUc-content was higher than the control from Day 7 to Day 12, and the difference was significant only on Day 10 ($p<0.05$, Fig. 5.1C).

The absolute neutrophil count (ANC) in the 5-FU group was significantly below the control from day 4 to day 10, and the nadir was measured on day 6 (Fig. 5.1D) when the median was only 4% of the control group. The prevalence of neutropenia (percentage of mice with ANC below $0.3\times10^9$/liter, the limit of neutropenia) was significantly higher in the mice given 5-FU than in the controls on Days 4, 5, 6, 7 and 8 ($p = 0.0058$, $p<0.0001$, $p<0.0001$, $p = 0.0001$ and $p = 0.0294$, respectively, Fisher’s exact test). The nadir of the median ANC was observed on Day 6, when 17 of the 18 mice (94.4%) were neutropenic.

Pretreatment with LV appeared to enhance the myelotoxicity of 5-FU. Although a day-for-day comparison failed to reveal statistical significance, the median values in the group given LV before 5-FU were mostly lower than those in the 5-FU-only group. The prevalence of neutropenia also supported an increase in the toxicity after LV pretreatment, as on Day 5 it was significantly higher in the LV+5-FU group than in the 5-FU only group (100% and 76.2%, respectively; $p = 0.0239$, $n = 21$ in each group).
Since the combination of LV and 5-FU was at least as myelotoxic as 5-FU alone, the presentation of our further results shall be confined to experiments describing our efforts to mitigate the myelotoxicity of this combination, which is widely used in the treatment of colorectal cancer.

Fig. 5.1. Effect of 100 mg/kg 5-FU or a combination of LV and 5-FU on the number of nucleated bone marrow cells per femur (cellularity) (A), on the number of colonies formed by $10^5$ nucleated bone marrow cells (B), on the femoral CFUc content (C) and on the absolute neutrophil count (ANC) in the peripheral blood of mice (D). The limit of neutropenia was considered $0.3 \times 10^9/l$ (shown as a dotted line on D).
5.1.2 **Uridine post-treatment reduced the bone marrow damage caused by LV+5-FU**

The median cellularity of the bone marrow in the LV+5-FU+U group was higher than in the mice given only LV+5-FU from Day 2 to Day 10, *i.e.* at eight consecutive observations. Day-for-day comparisons showed statistical significance on Day 4 and Day 5, when the median cellularity in the LV+5-FU+U group was two and three times, respectively, as high as in the other group (*p*<0.05, Fig. 5.2A). U-rescue seemed to allow for an earlier and less deep nadir (Day 3, 5x10⁶ cells/femur as compared with Day 4, 3x10⁶ cells/femur without U) as well as for an earlier recovery.

In the first six days, the median values for the frequency of CFUc and for the CFUc-content were higher in the U-rescue group than those in the other group, and the ratio of the values observed simultaneously in the two groups approximated or even exceeded an order of magnitude; these differences were statistically significant on Day 3 and 4 (*p*<0.001, Fig. 5.2B and C).

One day after 5-FU, uridine resulted in significantly increased ANC (Fig. 5.2D). The mobilization of mature neutrophil granulocytes into the bloodstream may explain this early response. No significant increase was detected in ANC after uridine "rescue" in the LV+5-FU group from Day 2, but on Day 7 and 8 the median of ANC was 3.7 and 7.9 times higher (Fig. 5.2D). The median ANC was reduced below the limit of neutropenia on Day 5 for four consecutive days in the LV+FU group but only for two days in the mice treated also with U. In this group the ANC returned to the control by Day 8, when the median ANC was still below the limit of neutropenia without U-treatment. U-rescue reduced the prevalence of neutropenia on Day 5 from 100% (21/21) to 71% (12/17, *p* = 0.012) and on Day 8 from 67% (6/9) to 0% (0/9, *p* = 0.0045). The nadir of the median ANC was observed on Day 6 in both groups.
Fig. 5.2. Effect of U on the myelotoxicity caused by LV+5-FU as measured by the number of nucleated bone marrow cells per femur (cellularity) (A), by the number of colonies formed by $10^5$ nucleated bone marrow cells (B), by the femoral CFUc content (C) and by the absolute neutrophil count (ANC) in the peripheral blood of mice (D). The limit of neutropenia was considered $0.3 \times 10^9$/l (shown as a dotted line on D).

5.1.3 Effect of G-CSF on the myelotoxicity of LV+5-FU

Daily administration of G-CSF after LV+5-FU was associated with higher cellularity on Days 5-7, but these differences were not significant statistically (Fig. 5.3A). Neither the frequency of CFUc nor the CFUc-content of the femoral marrow was significantly different in the two groups (Fig. 5.3B and C). The beneficial effect of G-CSF-treatment, however,
manifested itself in the ANC, which—after a nadir on Day 5 (median: 0.0905x10^9/liter)—began to recover from Day 6, one day earlier than without G-CSF (Fig. 5.3D), so that on Day 7 the ANC was not lower than the control value and significantly higher than without G-CSF (p<0.05). G-CSF-treatment reduced the prevalence of neutropenia on Day 5 from 100% (21/21) to 75% (9/12, p = 0.04) and on Day 7 from 67% (12/18) to 0% (0/9, p = 0.0011). However, it should be noted that on Day 4 the median ANC in the G-CSF-group was lower than without G-CSF; although this difference was not significant statistically, the prevalence of neutropenia was increased on this day by G-CSF from 38% (9/24) to 75% (12/16, p = 0.022). As for the duration of neutropenia, the median ANC in the G-CSF-group was lower than the limit of neutropenia for 3 days, as compared to 4 days without G-CSF (Fig. 5.3D).

5.1.4 The combination of G-CSF and uridine was synergistic in mitigating the LV+5-FU-induced neutropenia

Daily administration of G-CSF after LV+5-FU+U to further mitigate the myelotoxicity of LV+5-FU or promote recovery did not produce dramatic effects on the three bone-marrow parameters (Fig. 5.4A, B and C). However, the ANC indicated the benefits of this combination, which seemed to surpass those of either U or G-CSF given alone: the median ANC remained above the limit of neutropenia even at its nadir (Day 4, 0.316x10^9/liter). On Day 6, when the nadir of ANC in both of the LV+5-FU and LV+5-FU+U groups occurred (with values of 0.0085 x10^9/liter and 0.145x10^9/liter, respectively), the median ANC in the LV+5-FU+U+G-CSF group amounted to 0.85x10^9/liter, almost three times as high as the limit of neutropenia and only slightly below the normal median ANC (about 1.3 x10^9/liter in our control mice). On Day 6, the prevalence of neutropenia was also significantly lower in the LV+5-FU+U+G-CSF (11%, 1/9) group than in the mice treated with LV+5-FU+U (78%, 14/18; p = 0.0016). The duration of neutropenia in the LV+5-FU+U+G-CSF group was zero day, since the median ANC in this group was never below the limit of neutropenia. For comparison, the median ANC was below the limit of neutropenia on four, two and three days in the groups treated with LV+5-FU, LV+5-FU+U, and LV+5-FU+G-CSF, respectively.
Fig. 5.3. Effect of G-CSF on the myelotoxicity caused by LV+5-FU as measured by the number of nucleated bone marrow cells per femur (cellularity) (A), by the number of colonies formed by $10^5$ nucleated bone marrow cells (B), by the femoral CFUc content (C) and by the absolute neutrophil count (ANC) in the peripheral blood of mice (D). The limit of neutropenia was considered $0.3 \times 10^9/\text{l}$ (shown as a dotted line on D).
Fig. 5.4. Effect of G-CSF on the myelotoxicity caused by LV+5-FU+U as measured by the number of nucleated bone marrow cells per femur (cellularity) (A), by the number of colonies formed by $10^5$ nucleated bone marrow cells (B), by the femoral CFUs content (C) and by the absolute neutrophil count (ANC) in the peripheral blood of mice (D). The limit of neutropenia was considered $0.3 \times 10^9/l$ (shown as a dotted line on D).

5.1.5 UDPG-rescue reduced the LV+5-FU-induced damage to bone marrow

Because of the limited availability of endotoxin-free UDPG in Hungary, the effects of UDPG-rescue were studied only on Day 2, 4, 5 and 6, which included the time of the nadirs
of bone marrow cellularity (Day 4) and of ANC (Day 6) in the LV+5-FU group, whereas Day 2 seemed to be suitable to detect effects on the CFUc-population (Fig. 5.1). The median cellularity of the bone marrow in the LV+5-FU+UDPG group was higher than in the mice given only LV+5-FU from Day 4 to Day 6 \textit{i.e.} at three consecutive observations (Fig. 5.5A). The ratio of the medians of these two groups was about 2 on Day 5 and 6 (p < 0.01 and p < 0.05, respectively). In other words, UDPG-rescue—similarly to the results with U-rescue (Fig. 5.2)—seemed to allow for an earlier recovery. The median number of colonies per $10^5$ nucleated bone marrow cells in the UDPG treated group was higher than in the LV+5-FU group on day 2, 4 and 5, and the difference was significant on Day 2 and 5 (p < 0.001 and p < 0.01, respectively; Fig. 5.5B). The femoral CFUc content was higher in the LV+5-FU+UDPG-group at each day of our observations (p < 0.001 for both Day 2 and 5, Fig. 5.5C). The ANC after UDPG post-treatment was slightly below (p < 0.05) the LV+5-FU group on day 4 and above it on Day 5 and 6 (p < 0.05 for both days; Fig. 5.5D).

Daily administration of G-CSF after LV+5-FU+UDPG to further mitigate the myelotoxicity of LV+5-FU, or promote recovery, produced results (Fig. 5.6) similar to those described (Chapter 5.1.4) when the rescue-agent was uridine. The effects were not conspicuous in the three bone marrow parameters, and the significant benefits of the combination of a rescue agent, in this case UDPG, with G-CSF manifested themselves in the ANC. The median ANC remained above the limit of neutropenia even at its nadir (Day 4), with a value (0.32x10⁹/liter) close to that observed after the administration of LV+5-FU+U+G-CSF. After Day 4, the ANC began to rise and on Day 6, when the ANC-nadir was observed in all groups not given G-CSF, the median ANC in the LV+5-FU+UDPG+G-CSF group was 1.59x10⁹/liter, well within the normal range of ANC. On this day, none of the 9 mouse given this regimen was neutropenic. For comparison, the prevalence of neutropenia on Day 6 was 89% (8/9) in the LV+5-FU+UDPG-group (p = 0.0002).

It should be noted that the prevalence of neutropenia was significantly decreased by any of the “double rescue treatments” (U+G-CSF or UDPG+G-CSF) on day 6 after the LV+5-FU combination. This supported the previously described observation that LV pretreatment did not prevent the beneficial effect of uridine.
Fig. 5.5. Effect of UDPG on the myelotoxicity caused by LV+5-FU as measured by the number of nucleated bone marrow cells per femur (cellularity) (A), by the number of colonies formed by $10^5$ nucleated bone marrow cells (B), by the femoral CFUc content (C) and by the absolute neutrophil count (ANC) in the peripheral blood of mice (D). The limit of neutropenia was considered $0.3 \times 10^9/l$ (shown as a dotted line on D).
Fig. 5.6. Effect of G-CSF on the myelotoxicity caused by LV+5-FU+UDPG as measured by the number of nucleated bone marrow cells per femur (cellularity) (A), by the number of colonies formed by $10^5$ nucleated bone marrow cells (B), by the femoral CFUc content (C) and by the absolute neutrophil count (ANC) in the peripheral blood of mice (D). The limit of neutropenia was considered $0.3 \times 10^9/l$ (shown as a dotted line on D).
5.2 Effect of 5-ethyl-2’-deoxyuridine (EDU) on the bone marrow toxicity of 5-FU

As described under Materials and Methods two dose-schedules were applied in these experiments: 100 mg/kg of 5-FU was administered either in a single dose or in five equal parts given 24 hours apart (i.e. 5 x 20 mg/kg 5-FU). Sixty minutes before each dose of 5-FU, 200 mg/kg of EDU was given; in the single-dose experiments EDU was applied also at a dose of 400 mg/kg. In the next three figures, the results are presented as a function of time after the administration of the last dose of 5-FU.

5.2.1 Fractionated dosing of 5-FU reduced its myelotoxicity (Fig. 5.7)

As seen in our previous experiments (Fig. 5.1.), a single 100 mg/kg dose of 5-FU produced severe myelotoxicity; this was, however, dramatically mitigated by the fractionated administration (Fig. 5.7). The median cellularity of the bone marrow, although it was significantly lower than the control 4 hours, 1 and 2 days after the last dose of 5-FU (p<0.05), remained above 60% of the control even at its nadir on Day 1, when it was 12.3×10^6 per femur, nine times as high as the nadir after a single dose of 100 mg/kg (1.36×10^6 per femur, Fig. 5.7A). The frequency of CFUc in the bone marrow was not significantly lower than the control (Fig. 5.7B), whereas the CFUc-content of the femoral marrow was moderately but significantly below the control only 4 hours after the last dose of 5-FU (Fig. 5.7C). For comparison, both of these variables were reduced to approximately 0.1% of the controls when 100 mg/kg 5-FU was given in a single dose. The ANC was significantly lower than the control from Day 1 to Day 3, but returned to the control by Day 5; the nadir of median ANC was 0.2×10^9/L (Day 2), just below the 0.3×10^9/L limit of neutropenia. For comparison, after a single dose of 100 mg/kg of 5-FU the median ANC at its nadir was 0.05 ×10^9/L (Day 6, see Fig. 5.7D)
**Fig. 5.7.** Comparison of the myelotoxicity of 5x20 mg/kg and 1x100 mg/kg 5-FU by measuring the number of nucleated bone marrow cells per femur (cellularity) (A), the number of colonies formed by $10^5$ nucleated bone marrow cells (B), the femoral CFUc content (C) and the absolute neutrophil count (ANC) in the peripheral blood of mice (D). The limit of neutropenia was considered $0.3 \times 10^9$/L (shown as a dotted line on D).

### 5.2.2 Effect of EDU on the myelopoiesis in mice

EDU alone did not result in biologically significant changes in any of the measured hematological parameters either given according to the single-dose schedule or according to
the fractionated dosing schedule. Although after 5x200 mg/kg EDU the ANC was consistently below the control at all investigated times, the difference was not statistically significant (data not shown).

5.2.3 EDU increased the myelotoxicity of 5-FU

5.2.3.1 Single-dose schedule (Fig. 5.8)

EDU pretreatment aggravated the effect of 5-FU. This effect appeared to be conspicuous in the frequency of CFUc among nucleated bone marrow cells and in the CFUc-content of the femoral marrow on Day 1 and 2, as well as in the cellularity of the femoral marrow on Day 5 and 6. The difference in the cellularity between the 5-FU only group and the EDU+5-FU-treated animals was significant on Day 5 and 6 (p<0.01). In both groups treated with 5-FU+EDU the median CFUc-content of the femoral marrow was lower than in the 5-FU-only group all through the six day observation period and this was statistically significant on each day from Day 2 to Day 6 (p<0.05 on Day 2, 4 and 6; p<0.01 on Day 3 and 5). The difference associated with the administration of EDU approached or even exceeded an order of magnitude on almost all days of observation. The ANC in the group treated with either dose of EDU was lower than in the mice given 5-FU-only on Day 5 and 6, but this difference was not statistically significant (p = 0.13, Fig. 5.8D).
5.2.3.2 Fractionated-dose schedule (Fig. 5.9)

Pretreatment with 200 mg/kg EDU one hour before each dose of 5-FU given according to the fractionated-dose schedule (20 mg/kg daily for 5 days) severely increased the myelotoxicity with respect to all of the measured parameters (Fig. 5.9).
The **cellularity** remained as low as 13-25% of the 5-FU-only group all through the observation period (Fig. 5.9A) and both variables characterizing the **CFUc-content** were several orders of magnitude lower than in the 5-FU-only group (Fig. 5.9B and 5.9C) till Day 4. Actually, few if any CFUc were found in the bone marrow of the EDU+5-FU group from 4h to 3 days after the last dose of 5-FU, whereas the frequency of CFUc in bone marrow cells and the total femoral CFUc-content were normal or, at most, moderately subnormal in the 5-FU-only group. In the EDU+5-FU group, a rapid increase in the frequency of CFUc was observed from Day 3 to Day 5, indicating the beginning of the regeneration. The disadvantageous effect of EDU was supported by the **ANC**, the median of which was always below that of the 5-FU-only group and decreased almost steadily without unequivocal signs of reaching its nadir till the end of the 5 day observation period. For comparison, the ANC in the 5-FU-only group reached its nadir on Day 2 and returned to the control by Day 5; as a consequence, the difference between the two groups increased with time (Fig. 5.9D). The prevalence of neutropenia, a variable with obvious clinical relevance, was always higher in the 5×[EDU+5-FU] group than in the mice given only 5-FU, and this difference was highly significant on Days 1, 4 and 5 (Table 5.1).

**Table 5.1.** Prevalence of neutropenia in mice treated with 5×20 mg/kg 5-FU or 5×[200 mg/kg EDU + 20 mg/kg 5-FU]

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*Fisher’s test for the difference between the two groups
Fig. 5.9. Effect of 200 mg/kg EDU pretreatment on the myelotoxicity of 5x20 mg/kg 5-FU as measured by the number of nucleated bone marrow cells per femur (cellularity) (A), by the number of colonies formed by $10^5$ nucleated bone marrow cells (B), by the femoral CFUc content (C) and by the absolute neutrophil count (ANC) in the peripheral blood of mice (D). The limit of neutropenia was considered $0.3 \times 10^9$/L (shown as a dotted line on D).
5.3 Measurements of fluoropyrimidine metabolizing enzymes with Laser Scanning Cytometry (LSC)

5.3.1 TP and DPD measurements in single cells and “artificial tissue sections”

Three cancer cell lines were used to establish conditions for measuring immunoreactive enzyme levels in tumor sections, a bladder cancer cell line (T-24) and two breast cancer cell lines (MDA-MB-231, ZR-75-1). Initial experiments were performed with cells prepared using a cytocentrifuge. In these experiments the numbers of cells used for preparing the slides was limited so that the majority of individual cells were isolated from each other. The nuclei of the cells were contoured using the DNA dye TO-PRO-3, which appeared to produce good results with the fluorochromes applied in preliminary studies so it was used for all further work.

5.3.1.1 TP in single and double stained single cells

The results of measurements of TP obtained with cells stained only with anti-TP are compared to those obtained with cells stained with both anti-TP and anti-DPD (Fig. 5.10A and 5.10B). Results are expressed as integral fluorescence of the orange channel divided by the contour area to correct for the variability in cell sizes. The relative TP levels in the cell lines measured were similar whether stained with anti-TP alone, or both antibodies, with T-24 having the lowest level of fluorescence and ZR-75-1 the highest.

5.3.1.2 TP in double stained “artificial tissue sections”

During the course of these experiments, it was clear that there was considerable overlap between the green and orange channels that could not be completely compensated for by the WinCyte program. Further, experiments with tumor sections indicated that establishing optimal threshold levels for nuclear contouring was extremely difficult due to the close proximity of the cells and the random orientation of the nuclei in the sections as seen in Fig. 5.11A. There are clusters of multiple nuclei that are contoured and areas where none are contoured. Setting higher thresholds for the contours lead to diminishing numbers of cells that were detected. Therefore, the phantom contouring capability of the LSC was used to provide unbiased random sampling of areas in the sections so that all areas had an equal probability of being sampled (Fig. 5.11B).
Fig. 5.10. Comparison of staining for TP in single cells and sections using different fluorochromes. **A:** Single cells stained with Alexa-546 labeled secondary antibody to detect binding of anti-TP. **B:** Single cells double-stained with Alexa-546 labeled secondary antibody for detection of anti-TP and Alexa 488-labeled secondary antibody to detect binding of anti-DPD. **C:** Sections double-stained with Alexa 546-labeled secondary antibody for detection of anti-TP and Alexa 488-labeled secondary antibody to detect binding of anti-DPD. **D:** Sections double-stained with Alexa 647-labeled secondary antibody for detection of anti-TP and Alexa 488 labeled secondary antibody to detect binding of anti-DPD. Solid lines represent results obtained with T-24 cells, dotted lines represent results with MDA-MB-231 cells and dashed lines represent results with ZR-75-1 cells.
The results of the double stained “artificial tissue sections” using the same fluorochromes as used in the experiment with single cells are shown in Fig. 5.10C. Because the area of the phantom contours is constant, the results are expressed as integral fluorescence. The histograms are narrower than those in Fig. 5.10B, appearing similar to those shown for the single labeled, isolated cells (Fig. 5.10A).

**Fig. 5.11.** Scan data display images of standard contouring (A) and phantom contouring (B) on sections of an esophageal tumor.
Because TO-PRO-3 was no longer needed for contouring the nuclei with the stereological analysis, the far red channel was used for detecting TP as shown in Fig. 5.10D where Alexa 647 is used instead of Alexa 546 as the secondary label for TP in cell line sections. The Alexa 488 secondary label remained the same for staining DPD. The use of the two lasers for these measurements, which scan sequentially, means that there is no overlap between the two labels.

5.3.1.3 Results of DPD measurements

The DPD measurements in single cells stained with only anti-DPD (Alexa 488) and in sections stained with both TP and DPD antibodies indicated that both protocols gave similar results with respect to the relative enzyme levels in the three cell lines (Fig 5.12A and 5.12B). We measured the lowest DPD level in the T-24 cells and the highest in the MDA-MB-231 cells.

**Fig. 5.12.** Comparison of single staining in single cells and double staining in sections for detection of DPD. **A:** Single cells were stained with Alexa 488 labeled secondary antibody to detect binding of anti-DPD (see Fig. 14A). **B:** Sections were double-stained with Alexa 647 labeled secondary antibody for detection of anti-TP and Alexa 488 labeled secondary antibody to detect binding of anti-DPD. Solid lines represent results obtained with T-24 cells, dotted lines represent results with MDA-MB-231 cells and dashed lines represent results with ZR-75-1 cells.
5.3.1.4 TP/DPD ratio measurements

The ratios of TP fluorescence integral (as measured with either Alexa 546 or Alexa 647) to the DPD fluorescence integral (Alexa 488) were calculated directly by WinCyte. Representative histograms are shown in Fig. 5.13 (A and B) for the ratio measurements in single cells with Alexa 546/488 and in cell line sections with Alexa 647/488. The highest TP/DPD ratio was seen in ZR-75-1 cells while T-24 and MDA-MB-231 cells showed a much lower ratio of the TP/DPD levels.

![Graph showing TP/DPD ratios in single cells and sections](image)

**Fig. 5.13.** Comparison of TP/DPD ratios in single cells and in sections. **A:** Single cells were stained with Alexa 488-labeled secondary antibody for detection of anti-DPD and Alexa 546-labeled secondary for detection of anti-TP. **B:** Sections were stained with Alexa 488-labeled secondary antibody for detection of anti-DPD and Alexa 647-labeled secondary antibody for detection of anti-TP. Solid lines represent results obtained with T-24 cells, dotted lines represent results with MDA-MB-231 cells and dashed lines represent results with ZR-75-1 cells.

Based on the results of several studies with these three cell lines, the differences in fluorescence intensities for the T-24 and ZR-75-1 cell lines were reproducibly distinct and well separated. Thus, these cells appear to be useful standards for comparing the levels of TP and DPD in tumor sections.
5.3.2 Correlation between 5’-DFUR sensitivity and immunofluorescence measurements

To demonstrate that the immunofluorescence measurement of the enzyme levels is related to biological activity, results were compared to 5’-DFUR cytotoxicity measurements determined with the MTT assay. ZR-75-1 cells were the most sensitive to 5’-DFUR (50% inhibitory concentration [IC₅₀] = 38 µM), whereas T-24 cells were least sensitive ([IC₅₀] = 200 µM). The MDA-MB-231 cell line showed an intermediate level of sensitivity ([IC₅₀] = 120 µM). There was good correlation between the TP/DPD ratios measured with the LSC (normalized to the ZR-75-1 cell line) and the sensitivities of the cell lines (log IC₅₀) as depicted in Fig. 5.14.

![Graph showing correlation between 5’-DFUR IC₅₀ and TP/DPD ratios](image)

**Fig. 5.14.** Correlation between the TP/DPD ratios measured with the IF-LSC method in single cells and the 5’-DFUR sensitivity of ZR-75-1, MDA-MB-231 and T-24 cell lines measured by the MTT assay. The median of the ratio of the integral fluorescence measured for TP and DPD
was normalized to the ZR-75-1 cells. IC50 and normalized fluorescence intensity values are the averages of at least three separate experiments. (SEM: standard error of mean)

5.3.3 Estimation of enzyme levels in a tumor section

Fig. 5.15 shows the analysis of TP/DPD ratio in a tumor section mounted on the same slide as sections of the three standard tumor cell lines. Scan areas were selected for each section and scanned using both the Ar-ion and the HeNe lasers. (Fig. 5.15A) The scanned areas were not set exactly to the size of the sections, so the green fluorescence was used for gating out areas where there was no tissue (Fig. 5.15B and 5.15C). The histograms of ratio of long red and green fluorescence (the ratio of TP/DPD) show that the TP/DPD ratio in the ZR-75-1 cell line is higher than in the other two cell lines, as expected, and that the heterogeneity of the TP/DPD ratio in the tumor sample is much higher than in the sections of the cell lines (Fig 5.15D), as expected.

To examine the topological distribution of the TP/DPD ratios in the tumor sample, different colors were assigned to different ranges of the TP/DPD ratios on the histogram with the WinCyte software (Fig. 5.15E). A scattergram of X position vs. Y position was made to show the topological expression of the TP/DPD ratios (Fig. 5.15F). To improve the quality of this map, we used the SigmaPlot program, as described in the Materials and Methods to make a contour map of the tumor section (Fig 5.15G). The scattergram produced by the WinCyte software and the contour map made by SigmaPlot show similar distributions of the high and low ratios in the section.
Fig. 5.15. Contour mapping of the ratio of fluorescence intensities in a tissue section. A. Distribution of all phantom contours showing the actually scanned areas. Three cell line sections and a tissue section were mounted on the same slide (ZR-75-1 – upper left, MDA-
MB-231 – lower left, T-24 – lower right and the tissue section – upper right). B. Separating the non-tissue containing areas from the tissue containing parts based on the higher green fluorescence max pixel values of the tissue containing areas. C. The distribution of gated phantom contours show the shape of the tissue pieces mounted on the slides. D. Histograms of the ratio of fluorescence integrals characterizing TP and DPD. The colors of curves correspond to the colors of the regions on the previous scattergrams. E. The cells in five regions on the histogram were colored with different colors. F. X-Y scattergram produced by WinCyte software showing the two dimensional distribution of the colored cells G. Contour map of the tissue section as made by SigmaPlot (see chapter 4.2.5.3). Scaling is in percent of the median of control ZR-75-1 cells.

5.4 Estimating the sensitivity of laser scanning cytometry for detection of rare GFP positive cell in a GFP negative population

5.4.1 Separation of GFP-/- and GFP+/- samples

Fig. 5.16A shows a typical histogram demonstrating the difference in green fluorescence max pixel values between the GFP-/- and GFP+/- cells. The limit for the fluorescence level was set so that approximately 3.0–4.0% of the cells in the negative control were above this level. This level was used as a reference level for comparing the GFP+/- control (positive control) and the unknown samples. The results of three independent experiments are combined in Fig. 5.16B. The majority of cells in the positive control samples were above the reference level (80–98%). The mean fluorescence intensities in positive control samples were at least 4.4 times higher than that of the negative control samples. Among the unknown samples, two distinct groups could be distinguished. The first group was clearly GFP-/- with 0.0–6.7% of the cells above the GFP-/- reference level and mean fluorescence intensities were 0.7–1.0 times that of the negative control. The second group was GFP+/- with 81–98% of the cells above the reference level and mean fluorescence intensities were 3.8–8.44 times higher than the negative controls.
Fig. 5.16. Determination of GFP expressing phenotype of mice by measuring the green fluorescence max pixel in peripheral blood mononuclear cells. (A) Typical histograms demonstrating the difference in green fluorescence max pixel values between the cells of GFP-/- and GFP+/- mice. (B) Separation of GFP+/- and GFP-/- mice based on the mean of max pixel intensity in cells and percentage of cells above a reference level. There was no overlap between the different phenotypes with respect to these two parameters.
5.4.2 Detection of rare GFP+/- cells mixed with GFP-/ cells

In mixing experiments with a dilution of 1 GFP+/– cell per 1000 GFP–/– cells, contouring was set using only green fluorescence as described in Methods. The cytocentrifuge slides were prepared with $5 \times 10^5$ total cells. Approximately 35–50 cells with strong green fluorescence were identified, representing 5–10% of the theoretically detectable GFP+/– cells on the slides. The results of three separate experiments are detailed in Table 5.2.

**Table 5.2** Sensitivity of laser scanning cytometry for detection of GFP+/– cells in an artificial mixture of GFP+/– and GFP–/– white blood cells

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Ratio of GFP+/– to GFP–/– cells$^a$</th>
<th>Contours detected$^b$ (%) of maximum</th>
<th>Cells detected$^c$ (%) of maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1000</td>
<td>18.0 ± 2.9</td>
<td>8.8 ± 1.9</td>
</tr>
<tr>
<td>2</td>
<td>1:1000</td>
<td>22.0 ± 9.6</td>
<td>10.4 ± 4.1</td>
</tr>
<tr>
<td>3</td>
<td>1:1000</td>
<td>26.1 ± 6.3</td>
<td>10.5 ± 4.2</td>
</tr>
</tbody>
</table>

$^a$ 500000 cells were cytopun onto an approximately 3.1 cm$^2$ area

$^b$ Number of detected contours using a threshold value which did not detect any contours on GFP–/– control slides; mean of three parallel slides ± standard deviation of the mean; expressed in percent of the theoretical maximum of 500

$^c$ A contoured event was considered cell if its area was less than 500 µm$^2$; mean of three parallel slides ± standard deviation of the mean; expressed in percent of the theoretical maximum of 500
6 DISCUSSION

Improvement in the clinical results of fluoropyrimidine-based chemotherapy can be expected from biochemical modulation or from prediction of the patient’s response.

6.1 Effects of some biochemical modulators on the myelotoxicity of 5-FU

Biochemical modulators are drugs with no significant antitumor effects but capable of increasing the therapeutic index of fluoropyrimidines. During the last three decades, a number of such agents have been investigated in preclinical and/or clinical studies. In our experiments, we focused on the effects of four established or potential modulators – LV, U, UDPG and EDU – on the myelopoiesis of mice treated with 5-FU; LV and EDU are known to enhance the cytotoxic effects on tumor cells, whereas U and UDPG are supposed to protect normal cells (see Chapter 2.2). G-CSF is known to promote the recovery from neutropenia induced by various cytotoxic drugs (see Chapter 2.5). We supposed that combination of modulators protecting hematopoietic cells and G-CSF, which promotes their recovery, may further mitigate the damage to bone marrow and the consequent neutropenia, and thus possibly contribute to the more effective clinical use of fluoropyrimidines.

To describe the effects of 5-FU and its combination with modulators and/or G-CSF on myelopoiesis, we used four variables. The first three of these are related to bone marrow whereas the fourth one is the number of neutrophils in the peripheral blood:

(i) **cellularity**, defined as the number of nucleated bone marrow cells in a femur;
(ii) **frequency of CFUc**: number of colonies per $10^5$ bone marrow cells plated;
(iii) **CFUc-content** of one femur, actually the product of the two previous variables;
(iv) **ANC** in the peripheral blood.

Estimation of CFUc has a special significance: it measures the reproductive integrity of a cell type which is actively proliferating under physiologic conditions, is regarded to be a major target of cytotoxic agents damaging bone marrow, and is supposed to play a basic role in the recovery of hematopoiesis from damage (Lohrmann and Schreml, 1982). Furthermore, similar methods are available to estimate CFUc in various species, including *Homo sapiens*. The CFUc-content of the bone marrow appears to be more directly related to the restorative capacity of hematopoiesis from damage than the frequency of CFUc in bone marrow cells.
The latter variable, however, can be estimated in human bone marrow, whereas the quantitative measurement of the cellularity and, hence, the total CFUc-content of the bone marrow in humans is difficult, if not impossible, in the clinical practice. Whereas the first three variables mentioned above, in particular the CFUc-content, are pivotal for the recovery from damage, the ANC has direct relevance to the short-term capability of the organism to survive.

As shown in Fig. 5.1, the severe myelotoxicity of 5-FU was clearly detectable in all of the four variables used in our experimental system; actually, this was a prerequisite to study any effects supposed to mitigate the myelotoxicity of 5-FU. A single 100 mg/kg dose of 5-FU reduced CFUc to undetectable levels (approximately <0.1% of the control) as early as 24h after the administration of 5-FU, whereas the nadirs for the cellularity and for ANC occurred later, namely on Day 4 and 6, respectively (Fig. 5.1). The earliest and deepest reduction in CFUc indicates that 5-FU was most toxic to these cells, and the lag period before the manifestation of neutropenia shows the lack of any significant damage to mature neutrophils. Clear signs of regeneration appeared first in CFUc, later in cellularity, and even later in ANC. This can be interpreted in terms of a cell-renewal system, where both damage and regeneration occur first in the stem- and progenitor cells and only later in their progeny (Bond et al. 1965).

Administration of LV before 5-FU seemed to result in a slight enhancement of the myelotoxicity of 5-FU (Fig 5.1). This combination is frequently used in the clinical practice with the aim of selectively increasing the toxicity to tumor cells by prolonging the blockade of TS, which is probably mainly responsible for the effect of 5-FU in tumor cells but may play a less important role in normal cells (see Chapter 2.2.1). Although our results suggest that this selectivity is not absolute, they should not be interpreted as a counter-argument against the clinical use of the combination, the usefulness of which has been validated in the clinical practice. At any rate, the discussion of our further results shall be restricted to the mitigation of the myelotoxicity of LV+5-FU, since this combination is widely used in the therapy of colorectal cancer and seems to be at least as myelotoxic in mice as 5-FU alone.

The toxic effects of 5-FU on normal cells, contrary to those on malignant cells, were suggested to be more dependent on the RNA-directed effects of 5-FU than on TS blockade. Actually, administration of U has been shown, mainly in preclinical experiments using 5-FU without LV, to provide some degree of protection to normal tissues including hematopoiesis (see Chapter 2.2.2). To our knowledge, no studies have dealt with the effect of such a “U-
rescue” after a combination of 5-FU with LV and on the main target of cytotoxic agents in
the bone marrow: stem- and progenitor cells.

Our results clearly demonstrate that U can considerably mitigate the myelotoxicity of
5-FU even if 5-FU is combined with LV in order to enhance the inhibition of TS (Fig. 5.2).
The most conspicuous effect of U was to increase both the frequency of CFUc and the CFUc-
content of the femoral marrow from the earliest observations after the administration of
LV+5-FU by at least an order of magnitude. The rate of the regeneration of the CFUc-
content of the bone marrow, however, did not seem to be higher in the U-rescue group, as
judged from the slopes of the two curves in Fig. 5.2C. U-associated changes in the other two
variables (cellularity of bone marrow and ANC in the blood) corroborated the significant
beneficial effect of this modulator.

From the shapes of the curves shown in Fig. 5.2, the most probable interpretation of
these results is that U-rescue increased the survival of CFUc, and the higher number of
surviving CFUc provided the basis for the earlier recovery of the cellularity of the bone
marrow and of the ANC in the blood.

These results are in agreement with previous observations in colon 26 tumor bearing
and in normal non-tumor bearing mice according to which U-rescue after 5-FU resulted in
significant improvement in the leukocyte counts and the number of nucleated bone marrow
cells, respectively (Martin et al. 1982, Klubes and Cerna 1983).

In our studies, daily administration of G-CSF after LV+5-FU was not associated with
significant changes in the bone marrow as compared to the LV+5-FU group (Fig 5.3A, B and
C). However, the ANC showed a less deep and earlier nadir as well as an earlier recovery to
the control range (Fig5.3D), demonstrating that, as expected, G-CSF promoted the recovery
from neutropenia. The apparent discrepancy between the conspicuous effect on ANC and
minor effects, if any, on the cellularity and CFUc-content of bone marrow can be explained
by the following considerations. CFUc in the bone marrow constitute a dividing transit-
population, in which an increased output can compensate for the increased production of
cells, resulting in a lack of change in the population size. In addition, the murine spleen is an
important site of increased hematopoiesis in case of an increased demand induced e.g. by a
cytotoxic agent, and such an increase is supported by the migration of progenitor cells from
the bone marrow to the spleen. It is well known that G-CSF mobilizes stem cells and CFUc
from the bone marrow to the peripheral blood and, in mice, G-CSF induces migration of
stem- and progenitor cells from the bone marrow to the spleen. In fact, it was shown that
G-CSF administration without prior cytotoxic therapy reduced the number of stem- and progenitor cells in the bone marrow but concomitantly strong increases in the spleen were observed (de Haan et al. 1995).

As described above, U-rescue increased the survival of CFUc after LV+5-FU, whereas daily administration of G-CSF from Day 1 after the same cytotoxic treatment promoted the recovery from neutropenia without any conspicuous effect on the survival of

Fig. 6.1. Effect of the combination of U+G-CSF or UDPG+G-CSF on the myelotoxicity of LV+5-FU on the number of nucleated bone marrow cells per femur (cellularity) (A), on the number of colonies formed by $10^5$ nucleated bone marrow cells (B), on the femoral CFUc content (C) and on the absolute neutrophil count (ANC) in the peripheral blood of mice (D). The limit of neutropenia was considered $0.3\times10^9$/l (shown as a dotted line on D).
CFUc. It was reasonable to suppose that the combination of a rescue agent capable of increasing the survival of progenitor cells with an agent stimulating granulocytopenesis, such as G-CSF, would further improve the results. This was clearly demonstrated in our experiments (Fig. 5.4) showing that the combination of U and G-CSF was superior to either of these agents, since the improvement in the recovery of the bone marrow was accompanied by the mitigation of neutropenia surpassing the benefits of either of these agents.

The benefits of this combination are even more conspicuous, if compared to those observed after LV+5-FU without any of these protective agents (Fig. 6.1). In addition to the earlier regeneration of the bone marrow and a mitigation of neutropenia seen already after U-rescue without G-CSF (Fig. 5.2), this combination proved to be excellent in mitigating neutropenia (Fig. 6.1D): (i) The nadir of median ANC was raised from $0.0085 \times 10^9$/liter to $0.316 \times 10^9$/liter. (ii) At the nadir of the median ANC in the LV+5-FU group (Day 6), the prevalence of neutropenia$^3$ in the LV+5-FU+U+G-CSF group was only 11% (1/9) as compared to 89% (16/18) in the LV+5-FU group ($p = 0.00017$). (iii) The prevalence of neutropenia$^3$ was reduced by U+G-CSF even if compared at the respective nadirs of the median ANCs in the two groups: 47% (7/15, Day 4) versus 89% (16/18, Day 6, $p = 0.0116$). (iv) The median ANC in the LV+5-FU+U+G-CSF group was never below the limit of neutropenia, as compared to four days in the group treated with LV+5-FU.

As described in Chapter 2.2.2, the unwanted effects of U preclude its clinical application as a rescue agent, and, for this reason, prodrugs of U with a better safety profile are searched for. Among the suggested agents, UDPG seems to be particularly attractive because it is a licensed drug for clinical use in some countries and its safety profile seems to be better than that of U (Codacci-Pisanelli et al. 1997, Tognella 1992). This was the reason for which we tried to use UDPG as a rescue agent after LV+5-FU to substitute for U. Because of the limited availability of endotoxin-free UDPG in Hungary, these experiments had to be confined to a few points of time. The results (Fig 5.5 Chapter 5.1.5), however, are fully compatible with the hope that the benefits of UDPG as a rescue agent after LV+5-FU are quite similar to those observed with U and discussed in more detail in the previous paragraphs. This similarity includes the excellent synergism with G-CSF (Fig 5.6 and 6.1) supporting the hope that the combination of UDPG and G-CSF may be useful for mitigating neutropenia after 5-FU or LV+5-FU in clinical oncology. We think that our studies warrant further animal experiments and, if corroborated, suggest clinical trials. These trials would

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$^3$percentage of mice with ANC below $0.3 \times 10^9$/liter, the limit of neutropenia
involve two already licensed drugs: for UDPG (Toxepasi) the goal would be to find a new indication, whereas G-CSF (filgrastim, Neupogen) would be applied in its approved indication—but in combination with UDPG used in its new indication.

The possible use of 5-ethyl-2’-deoxyuridine (EDU) as a modulator of 5-FU was supported by promising in vivo data against human colon tumor xenografts in mice (Kopper et al. 1984, Kralovánszky et al. 1999; see Chapter 2.2.3). Since there had been no available data on the effects of EDU on hematopoiesis and on the myelotoxicity of 5-FU, we performed experiments to fill this gap. Our studies included both single-dose and fractionated-dose schedules of EDU+5-FU combinations since, in the clinical practice, 5-FU is frequently given repeatedly at intervals of 24h or in continuous infusion lasting for a couple of days.

In our studies, EDU did not produce remarkable myelotoxicity when given alone, but it enhanced the myelotoxicity of 5-FU in all of the three dose-schedules studied (Fig. 5.8 and 5.9). This enhancement was particularly serious in the fractionated-schedule experiments, i.e., if daily doses of 200 mg/kg EDU and 20 mg/kg FU were given for five consecutive days. Actually, all of the four hematological variables studied indicated that the damage to the bone marrow caused by 5x(200 mg/kg EDU+20 mg/kg 5-FU) was even more serious than that seen after a single 100 mg/kg dose of 5-FU, so that the bone-marrow sparing effect of fractionation was abolished by EDU (Fig. 5.7 and 5.9). Kralovánszky et al. (1999), who used the same dose-schedules, also observed that enhancement of the toxicity of 5-FU by EDU was more severe after repeated administration, albeit this observation was based only on loss of body weight. These authors observed that the increment in the antitumor effect (as measured by the growth delay factor) was much greater if EDU was added to the fractionated schedule than if it was used according to the single-dose schedule. Our results suggest that this effect of EDU in the fractionated-schedule is, unfortunately, not restricted to the tumor cells but includes non-malignant hematopoietic cells, too.

The strong enhancement of the myelotoxicity of 5-FU in the fractionated-dose experiments may have been the consequence of a combination of several factors. It should be taken into consideration that the total dose of EDU in the fractionated-dose experiments was 5x200 = 1000 mg/kg of body weight, 2.5 times as high as the larger one of the single doses used in our experiments. Pharmacokinetic factors also might have contributed, as the repeatedly damaged and continuously regenerating bone marrow was subjected to a prolonged exposure to EDU. An accumulation of EDU seems to be improbable, as the half-
life of EDU in mice is about 15 minutes. On the other hand, some accumulation of ethyluracil, one of EDU’s metabolites, is conceivable because its terminal half-life is as long as 760 minutes (Kralovánszky et al. 1999). As ethyluracil is supposed to substantially contribute to the modulatory effect of EDU, mainly by inhibition of DPD, its prolonged presence may have been important. The pharmacokinetic data reported by Kralovánszky et al (1999) showed a prolongation and increase in the concentration of some of the active metabolites of 5-FU as a consequence of pretreatment with EDU.

The conspicuous difference in the toxicity-enhancing effect of EDU in the single-dose versus fractionated-dose experiments might be attributed also to cell-kinetic factors. We have to bear in mind that, during the five days of the fractionated doses, the bone marrow must be in active regeneration; this is supported by the strong sparing effect of the fractionated administration of 5-FU (Fig. 5.7). The regenerating bone marrow may be more sensitive to the 5-FU-potentiating effect of EDU. 5-FU is known to be more potent against rapidly proliferating than against normal bone marrow (Ogawa et al. 1973, Lohrmann and Schreml 1982). Pluripotent stem cells are resting under physiological conditions, and a single dose of 5-FU has a relatively moderate effect on the stem cell pool, whereas more primitive stem cells may be involved in the regeneration during repeated administration of 5-FU and probably this process is more sensitive to EDU than the regeneration after a single dose.

The survival of a whole mammalian organism during and after continuous or repeated low-dose exposure to ionizing radiation or cytotoxic drugs depends on the number and quality of surviving hematopoietic stem cells and progenitors, as well as on the rate at which they can proliferate to compensate for loss of cells (Kovács and Gidáli 2002). These changes in the stem cell compartments may be associated with an increased sensitivity to effects caused by modulating agents such as EDU.

In this context it seems relevant to mention the earlier studies in our laboratory which have shown that fractionated administration of some cytotoxic hexitol-derivatives, namely dibromodulcitol, dianhydrogalactitol as well as the diacetyl- and disuccinyl derivatives of the latter, clearly mitigated the damage to the bone marrow. However, the regeneration of the progenitor cell pool in the bone marrow was much slower after the fractionated doses than after single administration of the drugs, suggesting that repeated doses may disturb the steady-state of the stem-cell and progenitor-cell pool more profoundly (Hernádi et al. 1980, 1982 and 1987, Kovács et al. 1982a, 1982b, 1983, 1985, 1987 and 1992b).
Whatever was the cause of the serious enhancement of the myelotoxicity of 5-FU by EDU in the fractionated regimen, this finding of ours suggests that it would be prudent to be extremely cautious with the design and realization of any clinical trials in which EDU or similar modulators would be applied repeatedly.

6.2 Measurements of fluoropyrimidine metabolizing enzymes with Laser Scanning Cytometry

The extensive metabolism, which is essential for both the activation and elimination of fluoropyrimidines, may provide opportunity for the prediction of the patients’ response through the determination of various enzymes catalyzing these processes (e.g. TP and DPD). As described in Chapter 2, estimation of more than one of these enzymes may improve the predictive power of such tests. Furthermore, some data suggests that the histological distribution of some enzymes (e.g. DPD) might be also essential in determination the response to fluoropyrimidines. As discussed in Chapter 2, laser-scanning cytometry (or slide-based cytometry) offers the possibility for the simultaneous and quantitative estimation of multiple enzymes while preserving the histological structure of the tissues. Based on these observations, our main goal was to study the applicability of laser-scanning cytometry for quantitative multiparametric measurements of fluoropyrimidine metabolizing enzymes (primarily TP and DPD) in archival tumor sections.

In our investigations – improvement of fluoropyrimidine chemotherapy through prediction of patients’ response by measurement of metabolizing enzymes – the primary goal was to develop a method for direct measurement of the ratios of TP and DPD in sections of formalin-fixed, paraffin-embedded tissue using the LSC. The currently available assays such as enzyme activity measurements, immunohistochemical analysis, quantitative PCR, or ELISA are not able to provide quantitative estimations of this ratio in sections from formalin-fixed, paraffin-embedded tumor blocks, which are routinely used for clinical diagnoses.

One of the advantages of the LSC for this assay is that it is a microscope-based system and therefore ideal for use with tissue sections. Further, this instrument stores the X–Y location of each measured event and has the capability of relocating these events, so that slides can be re-stained for morphologic examination and the areas of interest readily found. In the past, detection of cells on slides was usually performed by contouring for the whole cell on the basis of forward light scatter or for the cell nucleus on the basis of a fluorescent
DNA dye. This contouring works best when individual cells are spatially well separated (e.g., cytospin preparations or cytology specimens), although it has been used in some studies on tissue sections (Gorczyca et al. 1998 and 2001, Hendricks 2001). For contouring epithelial tumor cells, use of a fluorescent DNA dye seems to be most suitable (Pollice et al. 2000). We used TO-PRO-3 in our initial studies with the cell lines because it is excited by the HeNe laser and its emission is detected in the long red channel. Thus, it was possible to simultaneously measure the fluorescence intensity of the Alexa 488 and 546, which are excited by the Ar-ion laser and are detected in the green and orange channels, respectively. However, because the excitation peak of Alexa 546 is far from the 488-nm wavelength of the Ar-ion laser, the absolute fluorescence intensity of Alexa 546 was relatively dim, resulting in a decreased signal-to-noise ratio and decreased accuracy.

We found it very difficult to contour cells in most tumor sections based on nuclear staining. This is because contouring on the nucleus and setting a fixed distance for the peripheral contour so that at least most of the cytoplasm of each cell could be included does not work well with the non-uniform distribution of nuclei in the cells and the general heterogeneity of cells in the sections. In our experience with epithelial tumor cell lines on cytocentrifuge preparations, increasing the cell density to greater than $3 \times 10^5$ cells/cm$^2$ made the segmentation of single cells difficult. The recently described technique for the LSC, named “multiple thresholding” uses conventional propidium iodide contouring on cells of frozen human lymphatic tissue sections with scanning the same areas at five to eight different threshold levels and merging the data (Gerstner et al. 2004). However, such procedures work well for lymphatic tissues and other tissues in which the cells have relatively uniform size and spacing, but not for most cancer tissues in which the cells are very heterogeneous.

The phantom contouring feature of the LSC provides a stereologic approach to measure features of the sections. Stereology is a group of techniques for obtaining quantitative estimates of various parameters from tissue sections and is widely applied in neuromorphology. The basis of stereology is statistical sampling of the sections, and phantom contouring represents an ideal design-based, unbiased sampling protocol (Geuna 2000). Phantom contours are placed randomly on the scanned area in user-defined number, size, and density. The fluorescence data collected from each phantom contour are stored in a list mode file together with the positional information in the same way as cells contoured in the conventional manner. Using these data, it is possible to characterize the fluorescence of different areas on sections. This approach can provide much more accurate estimates of
fluorescence such as those obtained using method-based sampling, an example of which is standard contouring on nuclei, because it is based on assumptions about the regularity of cells that may not be true for all areas of the section being examined and therefore is a biased sampling technique.

Further, use of phantom contouring allowed us to switch from Alexa 546 to Alexa 647 for one of our secondary antibodies so that we could use separate lasers to excite each of our markers. The sequential measurement of the two fluorochromes resulted in an improved signal-to-noise ratio without the need for compensation. The order of TP and DPD levels and the TP/DPD ratios measured in the cell lines using the different staining and contouring methods were comparable with the exception of the MDA-MB-231 line. One factor that might influence differences between the two measurements is the use of Alexa 488 labeling, which is measured with the green filter; thus autofluorescence of the cells and tissues could influence the results. Our results are in good agreement with the enzyme activities and ratios measured in these cell lines previously (Ishikawa et al. 1998). The LSC measurements showed good correlation with the in vitro sensitivity of the cancer cell lines to 5'-DFUR. This functional correlation appears to validate the use of an immunoreactive protein as an indirect measurement for activity of these enzymes.

The absolute values of the measured immunofluorescence are dependent not only on the levels of the target proteins in the tissue sections but also on the staining conditions and instrument settings of the LSC. For the method to be useful with clinical specimens, it is important to have internal standards that will allow comparison between assays of different tissue samples. The use of sections of well-characterized cell lines mounted on the same slide with the investigational tissue sample provides such internal standards and improves the reliability of the measurements.

The quantitative measurement of TP, DPD, and their ratio in fixed tumor tissues makes it possible to perform retrospective studies to define selection criteria for patients who most probably will respond to capecitabine therapy. The advantage of this method for clinical studies is that it can be performed with materials that are taken and processed for diagnostic purposes. Other quantitative methods for measuring levels of these enzymes, such as PCR and ELISA, require that biopsy materials be snap frozen in liquid nitrogen or assayed immediately, whereas general clinical practice is to fix materials with formalin and embed in paraffin so that the tissue structure is preserved. The LSC can also be used with needle biopsy samples because very few cells are required for the analysis as has been demonstrated
with the elegant immunophenotyping method of Clatch and Foreman (1998) for needle biopsy specimens of lymphomas.

Phantom contouring, although it provides more accurate measurement of fluorescence over the entire section, no longer provides direct cellular measurements as one can obtain with dispersed cells on cytocentrifuge slides. However, it is still possible to identify regions of interest such as those with the highest or lowest fluorescence ratios and relocate these areas after staining for morphologic assessment of the cellular composition. Thus, questions such as whether the enzymes are more highly expressed in tumor areas, stromal areas, or areas with infiltrating inflammatory cells can be addressed.

An additional advantage of this method that it is not restricted to the enzymes of the fluoropyrimidine pathway but can be used for any drug target/metabolic system where the key components are known and for which suitable antibodies are available. Obviously, to answer the question whether the measurement of TP/DPD ratio with this method is suitable for the prediction of patients’ response to capecitabine or other fluoropyrimidine containing chemotherapeutic protocol it is essential that relatively large number of tissue measurements on archived material should be correlated with the outcome data of fluoropyrimidine chemotherapy.

6.3 Estimating the sensitivity of laser scanning cytometry for the detection of rare GFP positive cells in a GFP negative population

Flow cytometry makes possible the analysis of large number of cells in relatively short time which allows the more or less sensitive identification and separation of rare cells in a large population. However, as discussed earlier, the cells should be suspended in fluid which requires the destruction of the tissue structure and results in the loss of topological information; the relative position of the elements of a tissue in vivo. Although laser-scanning cytometry is not capable to analyze cells as fast as flow cytometry, the relocalization capability makes it very attractive for quantitative investigations where the spatial distribution of cells in a tissue seems to be an important factor (e.g. in homing of metastatic tumor cells). There are data suggesting differences between the characteristics of tumor cells in the primary tumor and in the metastasis with respect to the fluoropyrimidine metabolizing enzymes which makes more problematic to predict the effect of adjuvant chemotherapy (Aschele et al. 2000). Studying the quantitative aspects of homing of tumor cells in relation
to the surrounding cellular environment might provide data to help in optimizing not only the fluoropyrimidine based chemotherapy of colorectal cancers and other solid tumors but also leukemias. However the first step of these investigations should be the determination of the sensitivity of laser-scanning cytometry for the detection of rare cells.

To assess the sensitivity of laser scanning cytometry for rare cell detection we used a GFP transgenic animal model. It was shown that minute amounts of blood is enough to quickly identify GFP +/- hemizygous mice with the LSC which is otherwise difficult by macroscopic means when hair covers the mice’s body as the hair and red blood cells are not fluorescent. We found a small overlap between the fluorescence intensity histograms between the blood cells from GFP-/- and those from GFP+/- mice, which was due, in part, to green autofluorescence in the GFP-/- cells as well as to the heterogeneity in fluorescence intensities in the GFP+/- cells. The overlap between these populations might negatively influence the sensitivity of the method for rare cell detection, but the differences were such that discrimination between GFP positive and negative samples was unambiguous. Low number of GFP +/- cells were mixed with large number of GFP-/- cells and using the above described conditions we found an overall detection level of approximately $1:10^4$ considering that the maximum number of cells on a cytospin area is approximately $10^6$ cells.

This relative lack of sensitivity means that only the brightest GFP expressing cells are detected. The sensitivity can be increased by amplifying the GFP signal or by decreasing the autofluorescence. The GFP signal might be amplified by using in situ PCR amplification of the gene sequence or by using a specific antibody against GFP. Either approach could be performed with green fluorochrome labels to amplify the green signal, or with other colors to decrease the overlap with autofluorescence. However, the later approach would limit the number of other markers that could be used to phenotype the GFP positive cells. Another possibility for decreasing the background autofluorescence in fixed and permeabilized cells is pretreatment with trypan blue dye. We did some preliminary experiments with in situ trypan blue pretreatment of cells and saw a concentration-dependent decrease in autofluorescence, while the decrease in the specific fluorescence of the GFP+/- cells decreased in a lower extent (data not shown), suggesting the usefulness of this approach.

Even the achieved relatively low sensitivity might allow the detection of metastatic tumor cells in the bone marrow or other tissues and, at the same time, makes possible the in situ investigation of the quantitative relations to other fluorescent labeled markers.
7 SUMMARY

1. We studied the effect of some biochemical modulators on the myelotoxicity of 5-fluorouracil (5-FU) in mice. To characterize the damage to and recovery of bone marrow, we estimated the cellularity and CFU-GM-content (granulocyte-macrophage progenitor cell) of the femoral marrow as well as neutrophil counts in the blood. 5-FU was used in combination with leucovorin (LV), an agent used in the clinical practice to enhance the cytotoxicity of 5-FU to tumor cells.

1.1. Uridine (U) given after LV+5-FU significantly reduced the severe myelotoxicity of this combination as shown by all of the variables mentioned above. Uridine-diphospho-glucose (UDPG), an agent capable of increasing the plasma U level and possessing a better safety profile in humans than U, produced similar beneficial effects; the significance of this observation is emphasized by the availability of UDPG as a drug licensed for clinical use.

1.2. Filgrastim, a G-CSF preparation, improved the recovery from neutropenia after LV+5-FU. A combination of U-rescue and filgrastim-treatment was more effective in counteracting the unwanted consequences of the highly myelotoxic combination of LV+5-FU than either U or filgrastim alone, almost completely eliminating the neutropenia induced by LV+5-FU; similar results were obtained if UDPG was substituted for U. Explanation: U potentiated the beneficial effect of filgrastim by increasing the number of progenitor cells surviving LV+5-FU and capable of responding to this cytokine. This warrants further studies and may contribute to the more effective clinical use of the combination of LV+5-FU.

1.3. Ethyl-deoxyuridine (EDU) had been reported by other authors to potentiate the antitumor effects of 5-FU in mice. In our studies, it increased the myelotoxicity of 5-FU in mice, especially with fractionated 5-FU dosing, supporting the idea that this effect was mainly due to delaying the recovery of myeloid progenitor cells.

2. We developed a new method for the direct and quantitative measurement of the ratio of TP and DPD, two enzymes supposed to determine the sensitivity of cells to some
fluoropyrimidines, in tissue sections by means of a laser-scanning cytometer (LSC). Originally the LSC was developed for quantitative fluorescent measurements in well separated cells on slides but the high density of cells and the various relative positions of cells to the cutting plane in tissue sections makes individual cell detection and measurement very difficult and inaccurate. Thus in tissue sections we applied the phantom contouring feature of the LSC which is an unbiased stereologic approach and can provide much more accurate estimates of fluorescence intensities from the different microanatomical regions in the sections. This method opens up new vistas to test the idea how the simultaneous estimation of various marker enzymes or other proteins in histological samples of human tumors can contribute to the individualized use of antitumor drugs in general, and fluoropyrimidines in particular. Furthermore, our method can be applied for any drug target/metabolic system where the key components are known and for which suitable antibodies can be produced.

3. The above capabilities of LSC could be extended to study the homing of tumor cells, an early event in the formation of metastases, provided that the small minority of tumor cells could be readily identified among the overwhelming majority of normal cells. Green fluorescent protein (GFP) is a useful marker for tumor cells of various origin, so we established a method capable of detecting GFP-positive cells by LSC with a sensitivity of 1:10^4. This sensitivity was reached by using a method which set the detection threshold above the highest fluorescence level detected in the GFP negative cell population and very specifically discriminates the GFP positive from the GFP negative cells which also show relatively high autofluorescence in the detection channel. Even this relatively low sensitivity is good for detection of GFP-labeled cells and at the same time quantitative measurements of multiple interesting markers in preserved anatomical structures which is not possible with other currently available methods. The combination of the identification of rare tumor cells with the capability of LSC to quantitatively estimate the amount of other labeled markers in the identified tumor cells or in surrounding cells seems to be a promising way of studying the homing of cells.
8 REFERENCES


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9 ACKNOWLEDGEMENT

First I would like to express my gratitude to Prof. Zoltán Szilvássy (Head of Department of Pharmacology, University of Debrecen) for permitting and supporting my work. I thank to my advisor Prof. Péter Kovács (Department of Pharmacology, University of Debrecen), for introducing me to research and pharmacology. His thoughts and ideas inspired and encouraged me throughout the whole work described in this thesis. Without his help and advises this dissertation could not have been written.

I am also indebted to Dr. James F. Eliason (Karmanos Cancer Institute, Detroit, MI, USA) who provided me the opportunity to expand my interest to Laser Scanning Cytometry and helped and inspired my work.

I thank Prof. Dr. András Jeney (1st Department of Pathology, Semmelweis University, Budapest) and Dr. Judit Kralovánszky (National Institute of Oncology, Budapest) for their valuable advices and help.

I also would like specifically mention Dr. Zsolt Bacsó (Department of Biophysics, University of Debrecen), whose expertise and friendly help significantly contributed to the improvement of my knowledge about the methods of cytometry, and Dr. Ilona Benkő (Department of Pharmacology, University of Debrecen), who helped me a lot during the work with the biochemical modulators.

I am also especially grateful to all those people whose work was also indispensable to reach these results, but unfortunately it would be too long to list all of them.

Finally I have to thank to my family whose tolerance and support behind the scenes greatly helped my work.