Thesis for the Degree of Doctor of Philosophy (Ph.D.)

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

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Debrecen, 2006.

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, the results are frequently far from being satisfactory. These characteristics lead to extensive research aimed at improving the efficacy of fluoropyrimidines.

Active nucleotide analogs should be formed intracellularly from 5-FU to exert their cytotoxic effect and 5-FU is also converted into inactive metabolites. This complex anabolism and catabolism offers possibilities for modulation of its efficacy and/or toxicity by biochemical modulators. The different levels of metabolizing enzymes in the tumor and normal cells/tissues are responsible for the selective action of biochemical modulators and, additionally, may provide a possibility for predicting the response to fluoropyrimidine based therapy, which would be a major contribution to the individualization of chemotherapy.

Of the various approaches, enhancing the binding of 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) to thymidylate synthase (TS) by administration with leucovorin (LV) significantly improved the response rate and gained wide-spread clinical application in the chemotherapy of colorectal cancers. On the other hand, for 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, but was not introduced into the clinical practice due to the adverse effects associated with the high doses required for effective blood levels. Recently

preclinical and clinical trials investigated prodrug forms of uridine and uridine catabolism inhibitors for continuous maintenance of the high plasma uridine level necessary for the effective rescue of normal cells without the adverse effects related to the administration of high uridine doses. The positive results of these studies suggest that uridine rescue may have an important role in the escalation of 5-FU doses for better chemotherapeutic efficacy. Other recent approaches to improve the clinical efficacy 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^{TM}) and capecitabine (Xeloda^{TM}).

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. 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.

It is hoped that the new method of laser-scanning cytometry - or other similar techniques sometimes collectively referred to as “slide-based cytometry”- would be suitable for the parallel and quantitative measurement of more than one such markers at a previously unprecedented speed and accuracy while preserving the histological structure of the tissue – thus in effect provide a tool to connect these data with conventional histology. Obviously, these features are useful for not only the investigations of the enzymes playing a role in the fluoropyrimidine metabolism but might prove to be useful in those systems where the tissue localization of more than one antigen is important in the characterization of the process.

Another potential advantage of laser-scanning cytometry is the feasibility of detecting and enumerating cells expressing fluorescent proteins (e.g., GFP) even if they amount only to a small minority (<0.1%) of cells in a tissue or organ. In contrast with laser-scanning confocal microscopy, laser-scanning cytometry can detect and analyze much higher numbers of cells more rapidly thus providing data useful for statistical analysis, too. The identification of cells expressing fluorescent proteins coupled with the simultaneous quantitative estimation of various other markers in the same or surrounding 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 development and spread of malignant cells.

2. GOALS

Based on the above reasons our overall goal was to study the possibilities for the improvement of the efficacy of fluoropyrimidine based chemotherapy. Our interest primarily focused on two aspects: (i) effects of biochemical

modulators on the myelotoxicity of 5-fluorouracil at the level of bone marrow progenitor cells and (ii) exploring the possibilities of the new laser-scanning cytometry based method for objective measurement of multiple antigens playing role in the determination of tumor sensitivity. Additionally, we wanted to investigate the potential applicability of laser-scanning cytometry in investigation of metastasis formation.

In general, we wanted to study the effects of four established or potential modulators – LV, U, UDPG and EDU – on the myelopoiesis of mice treated with 5-FU. Specifically, we were particularly interested in answering the following questions: (i) How does LV pretreatment influence the 5-FU caused myelotoxicity? (ii) Is there any beneficial effect of U on the bone marrow damaging effect of 5-FU if it is combined with LV to enhance the blockade of TS? Further, we wanted to know whether the combination of modulators protecting hematopoietic cells and granulocyte colony stimulating factor (G-CSF), which promotes their recovery, may further mitigate the damage to bone marrow and the consequent neutropenia. As the high doses of U were not well tolerated in preclinical or in clinical studies we also aimed to study whether a prodrug of U, namely UDPG, which is a licensed drug for clinical use in some countries, produced similar effects to U on the 5-FU caused myelotoxicity. Another potential biochemical modulator – 5-ethyl-2'-deoxyuridine (EDU) – was shown to improve the antitumor action of 5-FU. However, this effect is beneficial only if the potentiation of cytotoxicity selectively affects the malignant cells and does not occur, at least not to the same extent, in normal cell renewal systems. According to our knowledge, earlier this effect had not been studied at the level of bone marrow progenitor cells so our goal was to

characterize the effect of EDU and its combination with 5-FU on the myelopoiesis of mice.

The currently evolving methods of slide-based cytometry might provide a better way for analysis of the enzymes involved in the fluoropyrimidine metabolism 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 (TP) and dihydropyrimidine dehydrogenase (DPD) in determination of the tumor response to capecitabine therapy. According to these observations we were particularly interested in the simultaneous and quantitative measurement of the ratio of these two enzymes in archived paraffin-embedded tissue sections.

Based on the experiences with the LSC for the measurement of fluoropyrimidine metabolizing enzymes it turned out that its capabilities (quantitative measurements of cells in statistically valuable numbers with preserved tissue structure) makes the LSC a valuable tool in the investigation of tumor metastasis formation. However studying the growth of metastases requires the ability of detecting rare tumor cells in various tissues. As green fluorescent protein (GFP) transfected cells and GFP-transgenic animals are promising new tools to study the process of metastasis development and homing of tumor cells, first we wanted to investigate the sensitivity of laser scanning cytometry to detect rare GFP-positive cells in a GFP-negative population.

3. MATERIALS AND METHODS

3.1. Biochemical modulators of the myelotoxicity of 5-fluorouracil

3.1.1. Drugs

The various drugs investigated as modulators of the myelotoxicity of 5-FU were used according to multiple schedules and doses which are described in details in the appropriate part of the thesis. 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 other drugs were commercially available pharmaceutical preparations (5-fluorouracil, uridine diphosphoglucose (UDPG), recombinant human Granulocyte Colony-Stimulating Factor (rhG-CSF, filgrastim), leucovorin).

All drugs were given intraperitoneally in a volume of 10 ml/kg, except U and UDPG which were used in 20 ml/kg 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.

3.1.2. Experimental animals

(BALB/c x CBA)F1 or BDF1 mice of both sexes at least 8 weeks old were used in the experiments. The number of mice in each treatment group at each time of observation was 8-12 (typically 9) if not indicated otherwise.

3.1.3. Cell counts

From mice, 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 was defined as the number of nucleated bone marrow cells found in one femur.

3.1.4. 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, $NaHCO_3$, penicillin and streptomycin 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 $1x10^5$ 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 (e.g. 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) CO_2.

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 10^5 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.

3.1.5. 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 or Dunn. If there were only two groups to be compared, the non-parametric Mann-Whitney test was used. The prevalences of neutropenia were compared by Fisher's exact test.

3.2. Evaluation of LSC for fluoropyrimidine metabolizing enzyme level estimation

3.2.1. Cell lines

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). The cancer cell lines were cultured at 37°C 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% CO_2. The cells harvested from the cultures were deposited on silanized slides using a Hettich Universal 16 cytospin centrifuge (Hettich, Tuttlingen, Germany) and were air-dried and kept at -20°C until stained.

3.2.2. Tissue sections

Either "artificial tissues"– prepared from the previously mentioned established cancer cell lines – or standard formalin fixed paraffin embedded tumor tissues were used. For the preparation of "artificial tissues" a tight cell pellet was prepared after harvesting large number of cultured tumor cells. Following fixation in neutral buffered formalin the cell pellets were first embedded in HistoGel™ (Richard-Allan Scientific, Kalamazoo, MI, USA) and then in paraffin according to standard pathology protocols. The paraffin-embedded cell lines and tissue blocks were cut into 4-5 μm-thick sections and, in some 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.

3.2.3..Indirect immunofluorescent labeling of enzymes

For the parallel indirect labeling it was essential to use primary antibodies from different species to avoid cross-reaction with the secondary antibodies. According to this a mouse anti-human-TP (1C6-203) and a rat anti-human-DPD (2H9-1b) monoclonal antibody (both were from Roche Diagnostics, Indianapolis, IN, USA) were applied for the primary labeling. 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 fluorescently labeled secondary antibodies were purchased from Molecular Probes, Eugene, OR, USA). To avoid overlapping of emission spectra of various fluorescent dies, on cytospin slides with well separated cells, TO-PRO-3 DNA dye (Molecular Probes, Eugene, OR, USA) was used for cell detection. On the tissue sections no nuclear dye was used, and generally Alexa 488 and Alexa 647 labeled secondary antibodies were applied for the double labeling.

After blocking the non-specific binding sites with Superblock (ScyTek, Logan, UT, USA), the labeling with optimized concentrations of the primary and secondary antibodies were separated by gentle but thorough washing. The nuclear staining with TO-PRO-3 followed these steps but only in the case of well separated cells on cytospin slides. Finally, the slides were rinsed with distilled water and cover-slipped with Prolong antifade solution (Molecular Probes, Eugene, OR, USA).

3.2.4. 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.

3.2.5. Laser-scanning cytometry on tissue sections

The phantom contouring feature of the LSC was chosen to measure the fluorescence intensity in tissue sections. The 10x objective of the microscope 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.

3.2.6. Data analysis

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 corrected for differences in cell size. In sections of cell lines, the integral alone was used because the area of each phantom contour is constant.

For comparisons, 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).

3.2.7. Contour mapping of enzyme levels and ratios of enzyme levels in tumor sections

The standard proprietary software of the LSC (WinCyte 3.4) is also 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, these graphs can only be transferred using screen capture, resulting in poor quality reproductions for publication purposes so we exported the list mode data and created 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.

3.2.8. Estimating the sensitivity of tumor cell lines to 5'-DFUR

Sensitivity of tumor cells to 5'-DFUR was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Eliason et al. 1990). Tumor cells were plated into triplicate wells of a 96-well plate. Two different cell concentrations were plated for each cell line and after 24 hours of incubation at 37°C in a fully humidified atmosphere of 5% CO_2 in air, increasing concentrations of 5'-DFUR were added. After further 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).

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

Mononuclear cells were separated with density gradient centrifugation using Nycoprep 1.077A density gradient media (AXIS-SHIELD PoC, Oslo, Norway) from the blood of GFP-hemizygous (GFP+/–) mice and wild-type C57BL6/J (GFP–/–) mice. The separated cells were fixed and deposited on slides for the LSC measurements. For experiments detecting rare cells, known numbers of GFP+/– mononuclear cells were mixed with GFP–/– cells at a ratio of 1:1000.

For the fluorescent 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.

4. RESULTS AND DISCUSSION

4.1. Effects of biochemical modulators on the myelotoxicity of 5-FU

To describe the effects of 5-FU and its combination with modulators and/or G-CSF on myelopoisis, 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.

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

As expected, the severe myelotoxicity of 5-FU was clearly detectable with respect to all of the four variables which, actually, was a prerequisite to study any effects supposed to mitigate the myelotoxicity of 5-FU. Pretreatment with LV, which supposed to selectively increase the toxicity of 5-FU to tumor cells by prolonging the blockade of TS, seemed to result in a slight enhancement of 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. Even if our results suggest that the selectivity of LV for tumor cells 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.

The beneficial effect of U in mitigating the myelotoxicity of 5-FU was clearly demonstrated by our results. With regard to the median cellularity of the bone marrow, U-rescue seemed to allow for an earlier and less deep nadir as well as for an earlier recovery, while the time period of median ANC below the level of neutropenia was decreased from 4 consecutive days to only two days. However, 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, as concluded from the slopes of the curves, did not seem to be higher in the U-rescue group. 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.

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. The beneficial effect of G-CSF-treatment, however, manifested itself in the ANC which showed a less deep and earlier nadir as well as an earlier recovery to the control range. The duration of neutropenia, i.e. the days on which the median ANC was below the level of neutropenia, was decreased by one day (from 4 to 3 days). These results showed that, as expected, G-CSF promoted the recovery from neutropenia. A possible explanation for this discrepancy between the obvious effect on ANC and minor effects, if any, on the cellularity and CFUc-content of bone marrow might be that the CFUc in the bone marrow constitute a dividing transit-population, in which an increased output can compensate for the increased production of cells. Another possibility is that the effect of G-CSF occurred mainly on the spleen which is supported by the following observations: (i) it is known that the murine spleen is an important site of increased hematopoiesis in case of an increased demand induced e.g. by a cytotoxic agent and (ii) in mice G-CSF induces migration of stem- and progenitor cells from the bone marrow to the spleen.

The combination of U, which increased the survival of progenitor cells, with G-CSF, a stimulator of granulocytopoiesis, further improved the outcome. This combination 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.

We also tried to use UDPG, which had been reported to have a better safety profile than U, as a rescue agent after LV+5-FU to substitute for U.

The benefits of UDPG after LV+5-FU were quite similar to those observed with U and included the excellent synergism with G-CSF, 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.

4.1.2. Effect of 5-ethyl-2'-deoxyuridine (EDU) on the bone marrow toxicity of 5-FU

We investigated the effects of 5**-ethyl-2'-deoxyuridine (EDU)** on murine myelopoiesis as a modulator of 5-FU because it showed promising in vivo data against human colon tumor xenografts in mice. In these experiments 5-FU was administered either in a single dose (100 mg/kg) or in five equal parts given 24 hours apart and EDU (200 or, in the case of single dose, 400 mg/kg, too) was always given one hour before each dose of 5-FU.

The fractionated administration of the same total dose of 5-FU dramatically mitigated its myelotoxicity, while 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. While the toxicity enhancing effect of EDU was also observed with the single dose schedule, it was particularly serious in the fractionated-schedule experiments. Both variables characterizing the CFUc-content were several orders of magnitude lower in the 5×[EDU+5-FU] group than in the 5-FU-only group. The disadvantageous effect of EDU was supported by the ANC and the prevalence of neutropenia, a variable with obvious clinical relevance. The median ANC 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. Similarly, the prevalence

of neutropenia throughout the whole observation period was higher in the 5×[EDU+5-FU] group than in the mice given only 5-FU and the difference was highly significant on Days 1, 4 and 5. Actually, all of the four hematologic 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.

Kralovánszky et al., who used the same dose-schedules, also observed that enhancement of the toxicity of 5-FU by EDU was more severe after repeated administration. These authors observed that the increment in tumor killing effect 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.

Several factors might be involved in the explanation of the observed conspicuous difference in the toxicity-enhancing effect of EDU in the single-dose versus fractionated-dose experiments. The total dose of EDU was higher and the exposure of the repeatedly damaged and continuously regenerating bone marrow to EDU was prolonged. Cell-kinetic factors might also be important as 5-FU, a cell-cycle-specific antimetabolite, is known to be more potent against rapidly proliferating than against normal bone marrow. Additionally, changes in the stem cell compartments after repeated exposure to 5-FU may be associated with an increased sensitivity to effects caused by modulating agents such as EDU.

Whatever was the cause of the serious enhancement of the myelotoxicity 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.

4.2. Measurements of fluoropyrimidine metabolizing enzymes with Laser Scanning Cytometry

Initially, to investigate the applicability of double staining, we did comparisons of TP measurements in single stained and double stained cells using normal contouring. The relative TP levels in the cell lines measured were similar whether stained with anti-TP alone, or both antibodies. Amongst the used three established cancer cell lines T-24 showed the lowest level of fluorescence and ZR-75-1 the highest.

Because of the difficulties in establishing optimal threshold levels for nuclear contouring in preliminary experiments in tissue sections, we used the phantom contouring capability of the LSC to provide unbiased random sampling of areas in the sections so that all areas had an equal probability of being sampled. The measurements using the same fluorochromes in "artificial" tissue sections of the same cell lines showed similar relative differences in the fluorescence intensity. To improve the signal to noise ratio the fluorescent marker for TP detection was switched to Alexa 647 which has similar excitation/emission characteristics to TO-PRO-3. This change of the fluorescent dyes did not result in alteration in the relative differences in the fluorescence intensity.

The DPD and TP/DPD ratio measurements in single cells and in sections with single or double staining protocols consistently gave similar results with respect to the relative enzyme levels in the three cell lines. We

measured the lowest DPD level in the T-24 cells and the highest in the MDA-MB-231 cells and 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. 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 appeared to be useful standards for comparing the levels of TP and DPD in tumor sections.

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 [IC_{50}] = 38 μM), whereas T-24 cells were least sensitive ([IC_{50}] = 200 μM). The MDA-MB-231 cell line showed an intermediate level of sensitivity ([IC_{50}] = 120 μM). There was good correlation (r^2 = 0.8948) between the TP/DPD ratios measured with the LSC and the sensitivities of the cell lines.

These results supported the applicability of the LSC based approach using double staining for the direct measurement of the ratio of TP/DPD. The difficulties with the normal contouring method on individual cells in tumor sections with high cell density led us to investigating the phantom contouring feature of the LSC. Phantom contouring represents an ideal design-based, unbiased sampling protocol which can provide much more accurate estimates of differences of fluorescence intensity 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. However it should be noted that

phantom contouring does not measure individual cells, rather the fluorescence intensity differences (corresponding to enzyme levels) between various microanatomical regions (such as tumor, stroma, necrotic tissue, inflammatory cell infiltrated regions) are characterized.

We also investigated this method for the characterization of differences in TP, DPD and TP/DPD ratio in a tumor section. Sections of the three standard tumor cell lines were mounted on the same slide as the tumor section allowing for more accurate staining and thus comparisons. The histograms of the ratio of long red and green fluorescence (the ratio of TP/DPD) showed that the TP/DPD ratio in the ZR-75-1 cell line was higher than in the other two cell lines, and that the heterogeneity of the TP/DPD ratio in the tumor sample is much higher than in the sections of the cell lines, as expected. To improve the analysis and the quality of images of the topological distribution of the enzyme levels and ratios in the tumor sample a contour-mapping method was also developed.

Although our experiments were focused on TP and DPD, which are relevant in the metabolism of fluoropyrimidines like capecitabine, in principle this method can be used for any drug target/metabolic system where the key components are known and for which suitable antibodies are available.

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

To investigate the sensitivity of the LSC for detecting GFP positive cells first we established a method for the determination of the phenotype of GFP transgenic mice from minute amount of peripheral blood. If the limit of

green fluorescence level was set so that approximately only 3.0–4.0% of the peripheral blood mononuclear cells (PBMC) in the negative control were above this level the differentiation between the GFP+/- and GFP-/- mice was unambiguous. The mean fluorescence intensities in positive control samples were at least 4.4 times higher than that of the negative control samples which proved to be high enough for the clear separation of the two phenotypes.

For the sensitivity measurements we mixed PBMCs from GFP+/– and GFP–/– mice at 1:1000 ratio. Contouring was set using only green fluorescence as described in Methods. The cytocentrifuge slides were prepared with $5x10^5$ total cells. Approximately 35–50 cells with strong green fluorescence were identified, representing 5–10% of the theoretically detectable GFP+/– cells on the slides. Considering this 5-10% hit probability and that the maximum number of cells on a cytospin area without significant overlapping is approximately 10^6 cells it can be estimated that if 100 GFP+/- cells can be found in the population of $1x10^6$ GFP-/- cells than approximately 5-10 cells would be identified resulting in at least $1:10^4$ sensitivity. This relatively low sensitivity can be increased e.g. by amplifying the GFP signal or by decreasing the autofluorescence but it still 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.

5. SUMMARY OF OUR NEW RESULTS

5.1. 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.

5.1.1. 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.

5.1.2. 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.

5.1.3. 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.

5.2. 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.

5.3. 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.

6. ACKNOWLEDGMENT

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 advices 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 Institue of Oncology, Budapest) for their valuable advices and help.

I also would like specifically mention Dr. Zsolt Bacso (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 Benko (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.

ABBREVIATIONS

5'-DFUR	5'-deoxy-5-fluorouridine, doxifluridine, Furtulon®
5-FU	5-fluorouracil
ANC	absolute neutrophil count
CFUc	Colony Forming Unit in culture
DPD	dihydropyrimidine dehydrogenase
EDU	5-ethyl-2'-deoxyuridine
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FdUMP	5-fluoro-2'-deoxyuridine-5'-monophosphate
G-CSF	Granulocyte Colony Stimulating Factor, filgrastim
GFP	green fluorescent protein
GM-CFU	Granulocyte-Macrophage Colony Forming Unit
LSC	Laser Scanning Cytometer or Laser Scanning Cytometry
LV	leucovorin, 5′-formyltetrahydrofolate
MTT	3-(4,5-dimethylthiazol-2yl)-2,3-diphenyltetrazolium bromide
RNA	ribonucleic acid
TP	thymidine phosphorylase
TS	thymidylate synthase
UDPG	uridine diphosphoglucose

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