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Biomodulation of 5-Fu cytotoxicity by folinic acid and its stereoisomers: in vitro experiments with different cell lines of prostatic cancer

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Abstract The results of cytotoxic chemotherapy for advanced, hormone-escaped prostate cancer have been disappointing. Evaluation of the effect of new drugs or new combinations with already known ones is required. The antimetabolite 5-fluorouracil (5-FU) has been shown to be active in prostate cancer, acting via inhibition of thymidylate synthase, an essential enzyme in DNA de novo synthesis. Experiments with cell lines of different tumors have shown that 5-FU activity can be modulated by addition of the coenzyme tetrahydrofolic acid (folinic acid). We investigated the effect of folinic acid and its stereoisomers on 5-FU action in different cell lines of prostate cancer. It was found that addition of non-toxic folinic acid led to a two- to fourfold better antiproliferative effect of 5-FU. The unnatural 6R isomer, which is a compound of chemically synthesized folinic acid, inhibited the modulatory effect of the natural 6S isomer. Our results indicated that a combination of folinic acid and 5-FU may result in a better response of patients with hormone-resistant prostate cancer than of patients treated with 5-FU alone.

Key words Prostate cancer · Chemotherapy · 5-Fluorouracil · Folinic acid · Cell culture · Tumor spheroids

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The results of cytotoxic chemotherapy of advanced, hormone-resistant prostatic cancer have not been impressive [5], with remission rates ranging between 10% and 20%. Some protocols have, however, revealed a good palliative analgesic effect. One of the first substances used in such protocols was the antimetabolite 5-fluorouracil (5-FU). After its uptake into the cell, 5-FU undergoes extensive metabolism [3, 6, 13, 18, 21], being converted to its active metabolite deoxyfluorouridine monophosphate (FdUMP). FdUMP, acts via inhibition of the enzyme thymidylate synthase (TS), which catalyzes an essential step in DNA synthesis: the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is incorporated into the DNA. The coenzyme of thymidylate synthase is methylentetrahydrofolic acid (mTHF). A ternary complex of antimetabolite, enzyme and coenzyme is formed, which binds thymidylate synthase and thus inhibits conversion of dUMP to dTMP. Stability of this inhibitory complex depends on the concentration of mTHF. There have been many attempts to modulate the cytotoxic effect of 5-FU by addition of so-called modulators. Reduced folates are stored in the organism as polyglutamates and there is some evidence that tumor and normal cells differ in their intracellular concentrations of these polyglutamates [15]. The assumption is that exogenous application of formyltetrahydrofolic acid (folinic acid, FA) will lead to increased intracellular levels of mTHF and thus will stablize the inhibitory complex. Chemically synthesized FA exists in equimolar amounts of two diastereoisomers: the unnatural 6R and the natural 6S isomer, which differ in chirality at carbon 6 of the molecule. Only the levorotatory 6S-FA is biologically active and converted to mTHF. 6R-FA accumulates in vivo by up to a factor of 20 and is excreted unchanged via the kidneys [18]. Unnatural 6R isomer might inhibit the modulatory effect of natural 6S-FA. The longer the duration of TS inhibition, the more dUMP that accumulates; finally FdUMP is replaced by dUMP in

the ternary complex. In this way DNA synthesis is restored.

Modulation of 5-FU cytotoxicity by folinic acid has been shown for various cell lines of colorectal cancer [3, 8]. In clinical trials remission rates have been improved by the addition of folinic acid to 5-FU treatment of patients with advanced colorectal cancers, head and neck tumors and breast cancers [10, 11, 20]. We were interested to see whether this biomodulation of 5-FU cytotoxicity could also be found in different human cell lines of prostate cancer.

Material and methods

Cell lines

Three cell lines of the American Type Culture Collection (ATCC, Rockville, USA) were used: *PC3* [9], which was originally derived from a bone metastasis, with 62 chromosomes and a doubling time of 26 h; *DU145* [12], isolated from a brain metastasis with a doubling time of 28 h and 64 chromosomes; and *LnCaP* [7], derived from a lymph node metastasis, with 76 chromosomes and a doubling time of 68 h. Cells were stored in liquid nitrogen and cultured for 7 days before use at 37 °C in microtiter plates (Costar) in Dulbeccos minimal essential medium (LnCaP), minimal essential medium and Earl's salt (DU145) and RPMI 1640 (PC3). All media contained 10% fetal calf serum, antibiotics, nonessential amino acids and pyruvate. Cells were harvested by trypsinization. Tests performed to screen for the presence of mycoplasms gave negative results throughout this study.

Monolayer proliferation assay

For the monolayer proliferation assay (MPA) 1×10^4 cells were transferred to 24-well microtiter plates. 5-FU (Ribosepharm company, Haan, FRG) was added to cells in different concentration in 0.5 ml medium. In modulation experiments, folinic acid (Ribosepharm company, Haan, FRG) and 6R and 6S folate (Sapec, Lugano, Switzerland) was added 30 min before application of 5-FU. Folinic acid and 5-FU were washed out 3 h after application. Media were changed every 3 days. At the same time, cell count was determined by means of a culture counter. Cells were cultured for a total of 10 days. The viability test by means of the trypan blue dye exclusion assay revealed viability after cell collection by trypsinization of 96–98% in all our experiments. Each experiment was performed 4 times simultaneously and repeated 2–3 times. Cell number in each well was determined 4 times. The concentration of 5-FU (μM) able to inhibit 50% of cell proliferation at day 10 was referred to as the inhibitory concentration 50 (IC_{50}).

Multicellular tumor spheroids

We used a modified "liquid overlay culture" technique to produce multicellular tumor spheroids (MTS) [2]. Cells were cultured on agarose-coated 24-well plates (Costar) in different concentrations (PC3 3000 cells, Du145 2000 cells, LnCaP 4000 cells). These numbers were chosen on the basis of the normal growth curves of the MTS. The media contained 10% fetal calf serum during all procedures. Within 4 days of incubation, cells formed tight aggregates. Individual spheroids were gently transferred with Pasteur pipettes in medium containing either 5-FU alone or in combination with folinic acid (one spheroid per well). After a 3-h incubation period, MTS were retransferred to medium without 5-FU or folinic acid. Spheroids were washed prior to the transfer. The diameter of each individual MTS was measured every 3–4 days under the microscope. The volume (in cubic micrometers) of spheroids was determined according to the formula for an ideal sphere.

There was no statistically significant difference between spheroid volumes after retransfer to normal medium after the 3-h incubation time. MTS were cultured for 30–35 days after incubation. The concentration of 5-FU (μM) capable of reducing spheroid volume by 50% – as compared with the normal growth curve – at day 30 was referred to as inhibitory concentration 50 (IC_{50}). Each experiment was performed twice. A minimum of ten spheroids was evaluated.

Statistical methods

Data were analyzed with an ANOVA test (repeated measures) on an Apple MacIntosh computer using the software program Statview II.

Results

We found that the antimetabolite 5-FU was able to inhibit cell growth in all three lines of prostate cancer tested. The antiproliferative effect depended on the concentration added. In modulation experiments, we found that folinic acid was able to enhance the antiproliferative effect of 5-FU in all three cell lines (e.g., results for PC3 in Fig. 1). Table 1 shows the IC_{50} of treatment with 5-FU alone and in combination with folinic acid. Folinic acid alone was not toxic for the cells over the concentration range tested (0.1–100 μM). As shown in Fig. 1, there was no significant difference between normal growth curves and cell proliferation in the presence of folinic acid or 5-FU in the concentration listed. A combination of folinic acid and 5-FU resulted in significantly reduced cell proliferation in all our experiments compared with treatment with 5-FU alone. Antimetabolite concentration could be reduced 4–5 times without loss of antiproliferative effect.

Using the pure diastereoisomers, we found that the unnatural 6R molecule did not affect 5-FU cytotoxicity at all. The natural 6S isomer did not show a significantly better modulating effect in two cell lines than did the racemate in equimolar concentrations (Table 2).

In vivo the 6 R isomer accumulated by up to a factor of 20 and it may be possible that high concentrations of the unnatural molecule inhibit modulation of 5-FU by the 6S isomer. We incubated the cells in monolayer culture with a mixture of the two isomers in which the 6R concentration exceeded the concentration of the 6S molecule ten fold. We then added 5-FU and found that a high concentration of 6R-FA inhibited the modulation of 5-FU by 6S-FA (Fig. 2), indicating – in order to achieve the same inhibitory effect – higher concentrations of 5-FU were necessary in combination with the modified racemate (ten fold excess of 6R-FA) than with the active 6S-FA.

The concentration of folinic acid used for preincubation did not play an important role in the monolayer

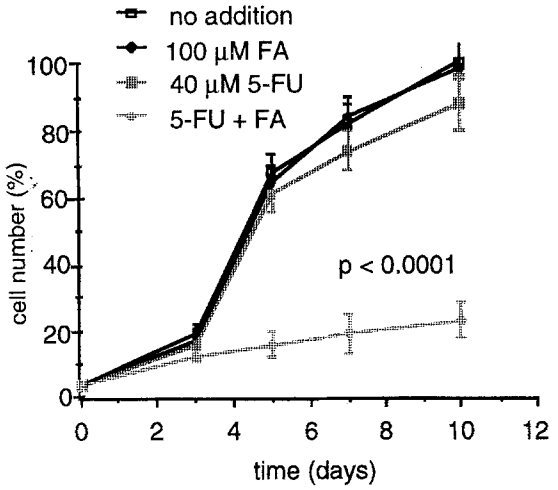

Fig. 1 Inhibition of cell proliferation by the combination of 5-FU and folinic acid in monolayer cultures of PC3 cells. *Error bars* indicate standard deviation

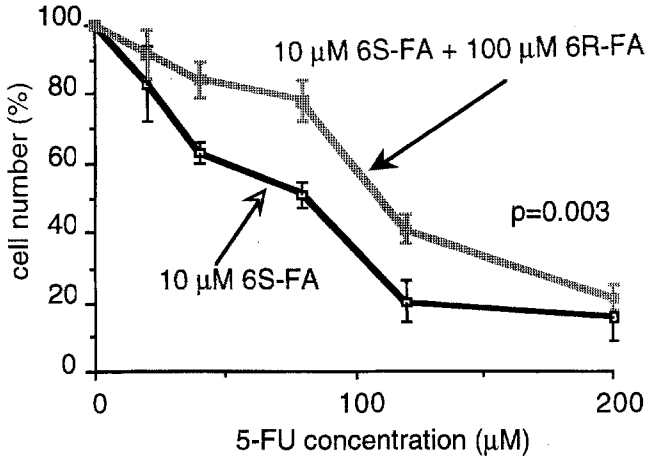

Fig. 2 Influence of 6R-folinic acid excess on the modulatory effect of 6S-folinic acid in monolayer cultures of PC3 cells. *Error bars* indicate standard deviation

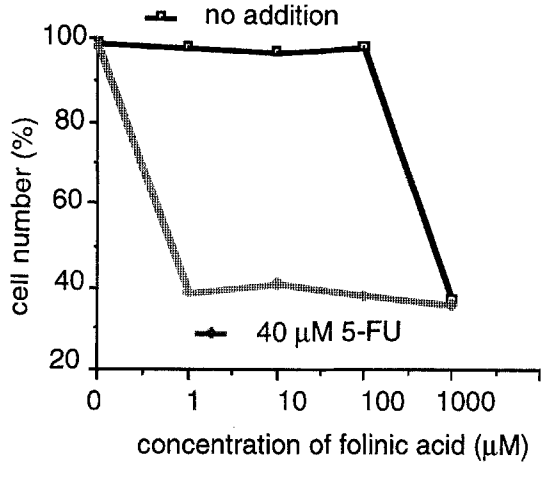

Fig. 3 Effect of folinic acid concentration on cell proliferation with or without 5-FU in monolayer cultures of PC3 cells. *Error bars* indicate standard deviation

Table 1 Inhibitory concentration 50 (IC_{50}) of 5-FU (µM) with or without folinic acid for cells growing in monolayer culture

Cell line	5-FU Mono	5-FU + 100 µM FA
PC3	200	40
DU145	40	10
LnCaP	80	20

Table 2 Inhibitory concentration 50 (IC_{50}) of 5-FU (µm) after incubation of cells with the combination of 5-FU, folinic acid and its diastereoisomers

Cell line	FA (0 µM)	6R-FA (100 µM)	6S-FA (50 µM) 6R-FA (50 µM)	6S-FA (100 µM)
PC3	200 (± 30)	200 (± 35)	45 (± 12)	40 (± 17)
Du145	35 (± 10)	35 (± 7)	17 (± 5)	7 (± 2)
LnCaP	65 (± 15)	67 (± 12)	15 (± 4)	15 (± 6)

Table 3 Inhibitory concentration 50 (IC_{50}) of 5-FU (µM) with a without folinic acid in the MTS system depending on the concentration of folinic acid

Cell line	5-FU Mono	5-FU + 10 µM FA	5-FU + 100 µM FA
PC3	35	35	35
DU145	160	135	100
LnCaP	210	195	200

experiments. In all three cell lines 1-µM concentrations showed nearly the same modulating effect as 100-µM concentrations. FA itself was toxic for cells only in concentrations above 100 µM (Fig. 3, results for PC3).

We incubated tumor spheroids with different concentrations of 5-FU alone or in combination with folinic acid. The IC_{50} values are listed in Table 3. Interestingly we found that for PC3 cells the IC_{50} for MTS was lower than obtained in monolayer culture. For DU145 and LnCaP cells the IC_{50} was higher in the spheroid system than in the MPA. The effect of pereincubation with folinic acid was not as impressive as in MPA, but we found statistically significant differences in PC3 and DU145 cells (e.g., results for PC3 in Fig. 4). In one cell line (LnCaP) a modulatory effect of FA was not observed. As shown in Table 3, the concentration of folinic acid plays an important role in the modulation efficacy in the MTS system.

With prolonged preincubation times with folinic acid (1, 2 and 3 h instead of 30 min), we found no modulatory effect. The IC_{50} values were exactly the same as those with 5-FU monotreatment. A 30-min preincubation time therefore seems to be the optimal schedule in the system described.

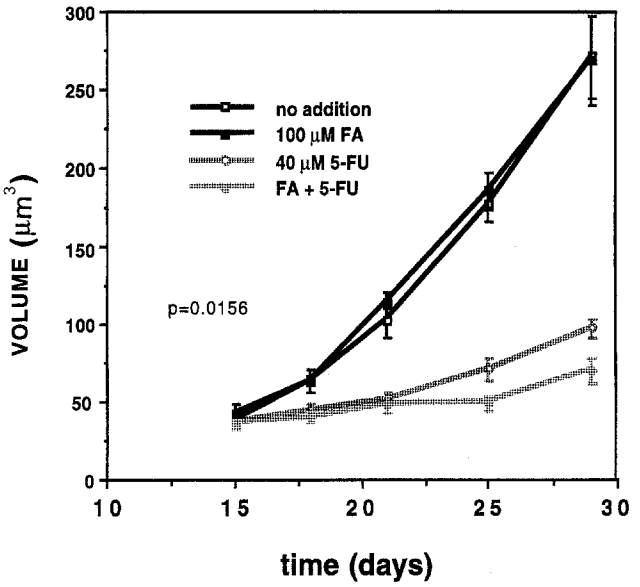

Fig. 4 Growth inhibition of multicellular tumor spheroids (PC3) by a combination of 5-FU with folinic acid. *Error bars* indicate standard deviation

Discussion

Our experiments showed that the antimetabolite 5-FU was able to inhibit cell proliferation in all three cell lines of human prostate cancer, depending on the concentration added. This antiproliferative effect could be enhanced by preincubating cells with folinic acid. In the presence of folinic acid the effective dose of 5-FU could be reduced by a factor of 4–5 in the MPA. The unnatural 6R molecule was not able to modulate 5-FU cytotoxicity. But at high concentration it inhibited the action of the natural 6S isomer in our experiments. This high concentration occurred in vivo because of accumulation of 6R-folinic acid. These findings indicate that it may be interesting to use pure 6S folinic acid in clinical trials. White and coworkers reported that the inhibition of the 6S effect may be due to competition for uptake by cells [22]. Sato and Moran [17] found that even 6R-FA is a substrate of folyl polyglutamate synthase, an essential enzyme in folate-induced modulation of fluoropyrimidines [16]. In colonic cancer 6S-FA was not superior to 6RS-FA in the modulation of 5-FU toxicity, but in methotrexate rescue 6S-FA was more effective than 6RS-FA [23].

Concentration of folinic acid by itself seems not to play an important role with respect to its modulatory effect in experiments with monolayer cultures. In sensitive cell lines, dissociation of the ternary complex of thymidylate synthase, FdUMP and mTHF is suppressed in concentrations of 2–5 μM reduced folate. Clinical data concerning the efficacy of high (500 mg/m^2), medium (200 mg/m^2) or low (20 mg/m^2) doses of folinic acid are contradictory, so that an optimal dose in clinincal trials has not yet been established [1].

In monolayer cell cultures each cell is in direct contact with the medium and its compounds. In vivo active transport and passive diffusion play an important role since not every cell is in contact with capillary vessels. So a multicellular tumor spheroid model may reflect the situation in vivo better than a monolayer culture. It is comparable to small metastatic, avascular cancer cell complexes in blood circulation. One would expect the IC_{50} values to be higher in the spheroid model than in monolayer experiments. This is true for DU145 and LnCaP. In spheroids of PC3 cells, a lower 5-FU concentration leads to a 50% growth inhibition compared with the MPA. The higher susceptibility of this cell line in the MTS system cannot be explained.

In experiments with MTS the concentration of FA is important. This may be due to longer diffusion times of the antimetabolite to the inner layer of the spheroid. In general the modulatory efficacy of FA in the MTS system is not as pronounced as in the MPA. Ehrlichman and Wu [4] investigated the modulation of 5-FU by FA in MTS and monolayers of MGH-U1 cells and found no increase of 5-FU cytotoxicity by FA in the MTS system, whereas in monolayer culture a significant enhancement was observed. Possible mechanisms of relative resistance of MTS to cytotoxic drugs may be the different phases of the cell cycle in different layers of the spheroid, pH gradients and cell-to-cell interaction in a three-dimensional complex [14, 19].

With both test systems different results for the IC_{50} were obtained for the different cell lines and in the different test systems. This may reflect the situation in vivo. Only a minority of the patients with prostate cancer responded to 5-FU treatment, which may have been due to a concentration problem. It is possible that the remission rates could have been increased by adding folinic acid to the treatment. However, as indicated in our in vitro experiments, some patients do not benefit from the combination therapy. This may be dependent on individual intracellular folate or polyglutamate concentrations.

We are well aware that results of in vitro experiments cannot easily be transferred into patient treatment. Nevertheless our result have encouraged us to start a prospective randomized clinical trial in which we are comparing monotherapy with 5-FU (600 mg/m^2) with the combination of folinic acid (400 mg/m^2) and 5-FU (400 mg/m^2).

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