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Report

Variable intrinsic sensitivity of human tumor cell lines to raltitrexed (Tomudex®) and folylpolyglutamate synthetase activity

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The cytotoxic effects of Tomudex® (TX) were investigated on a panel of 15 human tumor cell lines expressing a spontaneous sensitivity to the tested agent. We determined the basal cellular amount of relevant cellular factors potentially related to the cytotoxic efficacy of or resistance to TX. We selected thymidylate synthase (TS) as the target for TX, basal reduced folates (RF), because RF may compete with TX for a common site on the TS molecule. We also tested folylpolyglutamate synthetase (FPGS) because this is the enzyme which transforms the drug into its active polyglutamated form. Results were as follows. There was a wide inter-cell line variability in IC_{50} values for TX and there were marked differences between cell lines for all tested biochemical parameters. No link was observed between basal cellular TS activity and TX cytotoxic efficacy. There was an inverse relationship between reduced folate cellular content and TX IC_{50} values; this relationship did not, however, reach statistical significance. The only significant relationship was found between basal cellular FPGS activity and TX IC_{50} $r = -0.56$, $p = 0.03$. Tumor cells with a relatively high FPGS activity were more sensitive to TX cytotoxic effects and vice versa. Along with previous results which showed that acquired resistance to TX is accompanied by a decrease in FPGS activity, the present data are strongly indicative of a prominent role played by FPGS activity in the intrinsic sensitivity to TX. Means to up-regulate FPGS activity with pharmacological or tumor-specific genetic approaches are recommended so as to optimize TX antitumor activity.

Key words: Folylpolyglutamate synthetase, raltitrexed, Tomudex®.

Introduction

Pre-clinical and clinical results concerning the 5-fluorouracil (5-FU)–folinic acid (FA) combination have highlighted thymidylate synthase (TS) as a critical target whose inhibition results in cell death. This led to the development of a new generation of TS inhibitors which specifically interact with the TS molecule.[1,2] Among them, Tomudex® (TX), a water-soluble non-nephrotoxic quinazoline, demonstrated activity in colorectal, breast and pancreatic cancer. The results of a recently published large phase II study of TX in previously untreated advanced colorectal cancer have indicated substantial antitumor activity.[3] In addition, TX has an acceptable toxicity profile and a convenient dosing schedule (single i.v. injection every 3 weeks).[3] Phase III trials comparing TX to FU-FA in advanced colorectal cancer were recently completed and some were published.[4] Similar efficacy and an acceptable safety profile was obtained with TX in comparison to the 5-FU-FA regimen.[4]

In comparison to 5-FU which in the form of FdUMP acts at the nucleotide binding site of TS, TX acts at the folate binding site. Preclinical investigations with TX have shown that this drug requires intracellular polyglutamation to achieve better interaction with TS and an optimal cytotoxic effect.[5] The main objectives of the present study were to determine the cytotoxic effects of TX on a large panel of human cancer cell lines expressing a spontaneous sensitivity to the tested agent (absence of resistance mechanisms induced *in vitro*) and to determine the basal cellular amount of relevant cellular factors potentially related to the cytotoxic efficacy of or resistance to TX. These investigations were undertaken keeping in mind a potential clinical application. For this reason, we used our standard panel of 15 human cancer cell lines[6] representative of the clinical activity of the tested drug (colon, breast, and head and neck).[7] We selected TS as the target for TX, basal reduced folates (RF) because RF may compete with TX for a common site on the TS molecule[8] and finally folylpolyglutamate synthetase

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(FPGS) because it is the enzyme which transforms the drug into its active polyglutamated form.

Materials and methods

Chemicals

TX was kindly provided by Zeneca (Macclesfield, UK). [^{3}H]FdUMP labeled at position 6 (23 Ci/mmol) and [^{3}H]dUMP labeled at position 5 (16 Ci/mmol) were from Moravek Biochemicals (Brea, CA). [^{14}C]Glutamic acid tetralabeled (264 Ci/mol) was obtained from Amersham (Les Ulis, France). CH_2FH_4 was prepared from FH_4 supplied by Fluka Biochemika (Buchs, Switzerland) in accordance with the procedure provided by Dr Priest (University of South Carolina). Purified TS (3.7 unit/mg protein) from *Lactobacillus casei* was also from Dr Priest. Sephadex G25 fine gel was from Pharmacia Biotech (Uppsala, Sweden). Folic acid-free Dulbecco's modified Eagle's medium (DMEM) and glutamine were from Gibco (Paisley, UK), and FBS was from Dutscher (Brumath, France). Penicillin and streptomycin were from Merieux (Lyon, France). All other chemicals including aminopterin, MTT and *dl*-5-methyltetrahydrofolate were obtained from Sigma (St Quentin Fallavier, France). Cytosolic proteins were measured according to the Bradford assay[9] using the BioRad (Munich, Germany) protein assay kit with human purified albumin as standard.

Cell lines

Culture conditions. Fifteen human cancer cell lines of different origin (six breast, five colon, and four head and neck) were investigated (Table 1). All of them expressed a spontaneous sensitivity to anticancer agents (the cells had never been previously exposed to FU or TX). Cell doubling times were between 1.3 and 4.7 days (median 2.5 days). Cells were grown in a humidified incubator (Sanyo, Tokyo, Japan) at 37°C in an atmosphere containing 8% CO_2. Cells were routinely cultured in a regular DMEM medium supplemented with 10% FBS (concentration of active folates in the FBS was 10 nM accounting for 1 nM in the culture medium), 2 mM glutamine, 50 000 U/l penicillin and 80 μM streptomycin. In order to remain as close as possible to the physiological folate concentration in humans, cells were grown in a folate-controlled medium for 10 days before starting the experiments (i.e. folate-free DMEM medium supplemented with 40 nM of *dl*-5-methyltetrahydrofolate plus 0.1 mM of *l*-ascorbic acid for folate stabilization). During this period, this folate-controlled medium was renewed after 5 or 6 days. For cytotoxicity experiments, cells were grown in 96-well microtitration plates (0.32 cm^2/well) with the folate-controlled medium. In addition, cells were cultured for 5 days in 175 cm^2 plates with the folate-controlled medium for measurement of cellular parameters (basal conditions): TS, FPGS and RF. After 5 days of growth, cells were harvested, washed 3 times in phosphate-buffered saline at 4°C and cell pellets containing approximately 50×10^6 cells were stored in liquid nitrogen. In a preliminary experiment, different cell concentrations were tested and we kept the cell concentration of the inoculate which gives the longest exponential growth. In all cases, confluence was not reached after 5 days (70–80% of confluence). All investigations (cytotoxicity and biochemical determinations, including measurement of biochemical parameters) were performed during three independent experiments.

Evaluation of cytotoxicity. Cells were plated in 96-well microtitration plates in order to obtain exponential growth for the whole duration of the experiment (initial cell density 2000–7000 cells/well depending on the cell line). Twenty-four hours later, cells were exposed for 8 h to various drug concentrations. The time-exposure to the drug, 8 h, was chosen taking into account the rather long elimination half-life of TX in treated patients. Experimental conditions were tested in sextuplicate. Growth inhibition was assessed by the MTT test 112 h after the end of the drug exposure.[10] Results were expressed as the percentage of absorbance compared to controls without drug.

Biochemical investigations

RF assay. On the day of the assay, cell suspensions (50×10^6 cells/ml) were placed in buffer A: 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA and 50 mM L-ascorbic acid. The homogenates were then sonicated on an ice-bed 3 times (5 s each time) at 10 s intervals. After centrifugation of the homogenates for 5 min at 15 000 *g* (4°C), the supernatants were boiled for 3 min in order to denature the enzymes responsible for the cycling of folates.

RF (FH_4+CH_2FH_4) were measured using to the entrapment assay described by Bunni *et al.*[11] This assay is based on the stoichiometric formation of a stable ternary complex between CH_2FH_4, TS and FdUMP. Each cytosol was assayed at three different volumes (between 10 and 70 μl) in the presence or absence of formaldehyde which allows the chemical

conversion of FH_4 into CH_2FH_4. The measurement of FH_4 was obtained from the difference between formaldehyde-containing tubes and those without formaldehyde. The cytosol was incubated in the presence of an excess of purified TS (0.225 μM final concentration) and an excess of [^{3}H]FdUMP (0.35 μM final concentration) in a total volume of 100 μl (in buffer A) containing 6.66 mM final concentration of formaldehyde or not. Controls containing increasing known concentrations of monoglutamate CH_2FH_4 (0.5–50 nM final concentrations) were included in each series, as well as blanks without cytosol for quantitation of the binary complex formed between TS and [^{3}H]FdUMP. Results were expressed as pmol of folates (monoglutamate-equivalent) per mg of cytosolic proteins. The recovery calculated from the controls was 90% on average [coefficient of variation (CV) = 8%]. The limit of sensitivity was 0.3 pmol/mg protein. The coefficient of variation for the intra- and inter-assay reproducibility, determined on cell aliquots obtained from a pool of cell pellets, was 9.4 and 25.0%, respectively.

FPGS assay. Basal FPGS activity was measured on cells grown in the folate-controlled medium, at 70–80% of confluence. Cell pellets were homogenized (50×10^6 cells/ml) in buffer B: 10 mM Tris-HCl buffer, pH 7.5, containing 1.5 mM EDTA, 0.5 mM dithiothreitol and 10 mM sodium molybdate supplemented with 0.2 mg/ml trypsin soybean inhibitor, freeze-thawed 3 times and sonicated on an ice-bed 4 times (10 s each time) at 10 s intervals. Cytosols were obtained after centrifugation of the homogenates for 30 min at 105 000 *g* (4°C). FPGS activity was measured according to a method derived from that of Montero and Llorente[12] based on the incorporation of an additional [^{14}C]glutamic acid residue into the glutamate chain of aminopterin. Each cytosol was assayed in duplicate. The assay consisted of incubating 100 μl of cytosol with [^{14}C]glutamic acid (isotopic dilution, 250 μM final concentration) and aminopterin (250 μM final concentration) in a total volume of 250 μl (final concentrations of the buffer, pH 8.9 are 100 mM Tris-HCl, 20 mM $MgCl_2$, 20 mM KCl, 10 mM ATP and 100 mM β-mercaptoethanol). Results were expressed as pmol/min/mg protein. The limit of sensitivity was 0.4 pmol/min/mg protein. The intra- and inter-assay reproducibility, determined on cell aliquots obtained from a pool of cell pellets, gave coefficients of variation of 7.2 and 9.4%, respectively.

TS assay. TS activity was measured according to the tritium-release assay described by Spears and Gustavsson.[13] The assay consisted of incubating 25 μl of cytosol with [^{3}H]dUMP (1 μM final concentration) and CH_2FH_4 (0.62 mM final concentration) in a total volume of 55 μl. The sensitivity limit was 10 fmol/min/mg protein. Inter-assay reproducibility was evaluated through repeated analysis of single-use aliquots of a pooled cytosol: n=5, mean=1109.95 fmol/min/mg protein, SD=78.59 fmol/min/mg protein, CV=7.08%.

Analysis of data

For *in vitro* investigations, curve fittings were done on Graph Pad software (ISI, Philadelphia, PA). For each studied parameter, the mean values of three separate experiments were calculated. For RF, analyses were performed on the concentration of CH_2FH_4+FH_4 (sum). The analyzed parameters were the following: the logarithm of TX concentration causing a 50% growth inhibition as compared to controls without drug; the FPGS value and the TS value; the CH_2FH_4+FH_4 values.

Results

There was a wide inter-cell line variability in IC_{50} values for TX (Table 1). In both colon (five cell lines), breast (six cell lines), and head and neck (four cell lines) there were marked differences in intrinsic cell sensibility to TX with, for each tumor type, a 30-fold variation in IC_{50} values between the extreme values.

Basal cellular activities of TS, FPGS and the cellular amount of RF are given in Table 1 for all investigated cell lines. There were marked differences between cell lines for all tested biochemical parameters. Between investigated cell lines, there was a 8-fold variation for TS activity and a 5-fold variation for FPGS activity. According to the cell culture conditions (folate-free medium supplemented with 40 nM of 5-methyltetrahydrofolate), the basal intracellular levels of reduced folates (FH_4+CH_2FH_4) were low and detectable in seven of 15 of the investigated cell lines.

An analysis was performed of the correlation between the level of basal expression of each of these parameters and TX IC_{50} values (Table 2). There was no link between basal cellular TS activity and TX cytotoxic efficacy. There was an inverse relationship between reduced folate cellular content and TX IC_{50} values; this relationship did not, however, reach statistical significance. Cell doubling time was not significantly related to TX cytotoxic effects. Interestingly, the only significant relationship was found between basal cellular FPGS activity and TX IC_{50}

Table 1. Investigated tumor cell lines

Tumoral origin	Name	Doubling time (days)	TX IC_{50} (μM)	TS activity (pmol/min/mg prot)	FPGS activity (pmol/min/mg prot)	Reduced folates (FH_4+CH_2FH_4) (pmol/mg prot)
Breast	MCF 7	1.7 (0.2)	0.11 (0.008)	18.1 (3)	5.2 (0.8)	1.3 (0.52)
	ZR 75	2.6 (0.8)	0.027 (0.007)	14.4 (1)	10.6 (0.5)	1.2 (0.3)
	T 47 D	4.7 (0.6)	0.009 (0.005)	29.1 (1)	3.7 (0.2)	ND
	CAL 51	1.3 (0.2)	0.33 (0.17)	48.6 (5.6)	2.5 (0.1)	ND
	CAL 85-2	1.6 (0.1)	0.16 (0.017)	41.6 (0.9)	4.5 (0.6)	ND
	CAL 120	2.8 (0)	0.29 (0.07)	17.1 (1.5)	2.0 (0.3)	ND
Head and neck	CAL 33	2.8 (0.8)	0.008 (0.0017)	34.3 (0.9)	7.6 (0.17)	ND
	CAL 27	4.5 (1.4)	0.05 (0.01)	38.4 (5.8)	6.4 (0.6)	2.4 (0.9)
	Hep 2	2.2 (0.3)	0.08 (0.03)	58.3 (3.9)	5.3 (0.3)	ND
	DETROIT 562	1.8 (0.2)	0.27 (0.43)	18.6 (2.0)	5.3 (0.1)	0.9 (0.1)
Colon	WIDR	1.7 (0.8)	0.02 (0.008)	9.5 (3.0)	11.1 (1.6)	1.4 (0.2)
	CAL 14	2.4 (0.7)	0.17 (0.0035)	11.6 (2.1)	7.0 (3)	0.7 (0.34)
	CAL 124	1.6 (0.2)	0.11 (0.02)	7.2 (0.7)	5.8 (0.8)	ND
	COLO 205	1.9 (0.5)	0.007 (0.002)	19.9 (3.8)	8.3 (1.5)	5.7 (3.2)
	SW 403	1.9 (0.4)	0.024 (0.008)	6.7 (0.2)	5.2 (0.3)	ND

Mean value (standard deviation with three independent experiments); ND, not detectable (below 0.3 pmol/mg protein).

Table 2. Correlation between sensitivity to TX (log IC_{50}) and biochemical parameters of the cell lines

Log IC_{50} (μM)	FPGS (pmol/min/mg prot)		TS (pmol/min/mg prot)		Folates (pmol/mg prot)	
	r	*p*	*r*	*p*	*r*	*p*
TX	−0.56	0.03	0.13	0.65	−0.41	0.12

Statistic analysis, linear correlation. *r*=correlation coefficient; *p*=probability value.

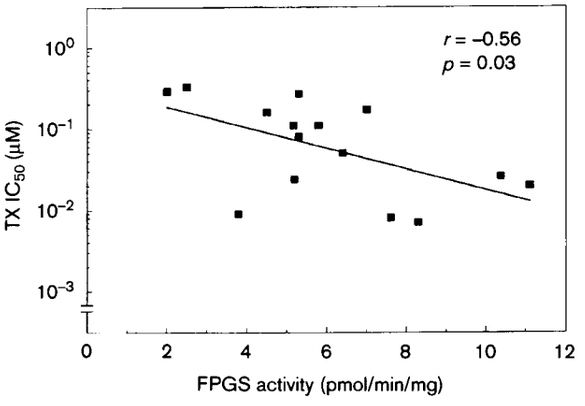

Figure 1. Relationship between basal FPGS activity and cytotoxic effects of TX (IC_{50}). Each point represents one individual cell line.

(Table 2 and Figure 1): $r=-0.56$, $p=0.03$. Tumor cells with a relatively high FPGS activity were more sensitive to TX cytotoxic effects and vice versa.

Discussion

The experimental design of the present study was established so as to minimize the unavoidable gap existing between *in vitro* investigations and the reality of the clinical situation. The concentration of reduced folates in the culture medium (40 nM 1,5-methyltetrahydrofolate) was adopted so as to reflect the physiological situation.[14] The spectrum of human cell lines investigated was representative of TX clinically responsive cancers.[7] One of the main objectives was to mimic the clinical conditions of previously untreated tumors spontaneously expressing different degrees of drug sensitivity. The time-exposure to the drug, 8 h, was adopted for taking into account both the rather long elimination half-life of TX in treated patients[15] and to allow a sufficient amount of polyglutamates to be formed.[16]

Thus far all experimental studies which concern the elucidation of cellular resistance mechanisms for TX have been focused on acquired resistance with stepwise escalation of the drug concentration resulting

in TX-resistant sub-lines.[17-22] These experimental conditions are very useful for allowing potential resistance mechanisms to emerge but they did not reflect the clinical situation where a drug-naive tumor must be treated. From the previously published studies it appears that acquired resistance to TX has multifactorial origins and the mechanism which is the most often encountered involves a loss of FPGS activity. Based on the present experimental conditions, an inverse and significant correlation was shown between cellular basal FPGS activity and intrinsic cell sensitivity to TX reflected by IC_{50} values. This FPGS activity plays a determinant role in both acquired and *de novo* resistance to TX. This role of FPGS in TX resistance can be explained by assuming that TX must be activated into polyglutamate forms beforehand to exert an optimal effect on TS[23] and that FPGS is the enzyme which catalyzes the linkage of glutamic acid residues to the carrier. It has been shown that the addition of one, two or three glutamate residues to TX resulted in 17-, 56- and 89-fold reduction, respectively, in the K_i values for TS.[23] Thus, even a relatively modest amplitude of variability in FPGS activity, as found in the present study, may have a strong impact on the interaction of TX with target. It would thus appear justified to investigate tumor FPGS activity in patients being treated by TX so as to compare drug efficacy and pretreatment enzyme levels as has previously been done for TS with 5-FU.[24] The high inter-patient variability we previously found in FPGS activity measured in colorectal tumors strongly suggests a potential role for FPGS in the clinical activity of TX.[25]

Because TX competes with RF for a common site on TS protein, it was decided to check whether differences in basal cellular levels of RF were accounting for cell-cell variations in TX cytotoxic efficacy. The relevance of this investigation was underlined by the observation that folinic acid was able to antagonize cell growth inhibition by TX.[26] No significant link was found between TX IC_{50} and cellular RF concentrations. One possible explanation is that these basal RF concentrations are too low for competing with TX in targeting the specific acceptor site on TS. Data concerning RF concentrations in human tumors are very scarce and show marked inter-patient variability.[27] As RF concentrations in human tumors[27] are much higher than those found in tumor cell lines in the present paper, the relevance of RF as a biochemical determinant for TX activity should be confirmed by specific testing in the clinical situation.

Using a similar strategy with 5-FU tested on a panel of several human tumor cell lines expressing and intrinsic sensitivity to the drug, we were able to confirm the direct link between TS cellular activity and 5-FU efficacy.[6] In the present study we were unable to demonstrate such a relationship between TS activity and TX cytotoxic effects (Table 2). Data concerning the change in TS following acquired resistance to TX are controversial; some investigators described an increase in TS protein levels, mRNA levels or activity associated with TX resistance,[17,19,22] whereas others failed to find a noticeable modification in TS activity.[20] In the present study there was only a 8-fold variation in TS activity between investigated cell lines; the amplitude of change in TS levels following acquired resistance was much higher.[17,19,22] The relatively low amplitude of variation in TS activity in the present tumor cell line panel may explain the absence of a significant link between this cellular factor and TX cytotoxic efficacy. An alternative explanation can be the fact that following the interaction between TX and TS protein itself there is a 10- to 40-fold rise in TS protein which can modulate the final cytotoxic effect of TX.[26]

Note that the purpose of the present study was not to undertake an exhaustive investigation covering the cellular parameters potentially responsible for variability in TX cytotoxic efficacy. Our study was centered on the drug's target, TS, and the cellular parameters which are susceptible to modulate the interaction of the active drug with its target, i.e. RF and FPGS activity. Another cellular factor which would certainly merit deeper investigation is the RF membrane transport carrier protein (RFC) which controls the cellular penetration of TX. Previous investigations have shown that a relative loss in RFC was associated with acquired resistance to TX.[17,22]

In conclusion, along with previous results showing that acquired resistance to TX is accompanied by a decrease in FPGS activity, the present data are strongly indicative of a prominent role played by FPGS activity in the multifactorial intrinsic sensitivity to TX. Additional investigations are required to check whether tumor-to-tumor variation in TX efficacy is not only associated with changes in FPGS activity but also in opposite modifications in γ-glutamyl-hydrolase activity. Means to up-regulate FPGS activity with pharmacological or tumor-specific genetic approaches are recommended so as to optimize TX antitumor activity.

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