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ORIGINAL ARTICLE

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Molecular characterization of human acute leukemia cell line resistant to ZD9331, a non-polyglutamatable thymidylate synthase inhibitor

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Abstract ZD9331 is a non-polyglutamatable, potent quinazoline antifolate inhibitor of thymidylate synthase (TS). In an effort to clarify the exact mechanism of resistance to this novel TS inhibitor, we examined the molecular alterations in its target enzyme TS, the transport protein (reduced folate carrier, RFC), and folylpolyglutamate synthetase (FPGS) in a human acute lymphoblastic leukemia cell line, MOLT-3, made resistant to ZD9331. A 310-fold resistant subline was established after 6 months exposure to the drug at concentrations up to 7 μ*M*, and was designated MOLT-3/ZD9331. MOLT-3/ZD9331 showed crossresistance to CB3717 (4.8-fold), raltitrexed (63-fold) and methotrexate (MTX) (120-fold), but retained sensitivity to trimetrexate (0.88-fold). The resistant cells demonstrated impaired initial cellular uptake and low accumulation of [^{3}H]MTX in accordance with a decreased expression of *RFC1*, suggesting the downregulation of RFC. However, Southern blot analysis demonstrated no change in gene copy number nor gross rearrangement of *RFC1* in the resistant cells. In addition, MOLT-3/ZD9331 showed amplification of the *TS* gene with a concomitantly increased level in the gene expression. In contrast, the expression of *FPGS* did not alter. These results demonstrate that continuous exposure of the cells to ZD9331 leads not only to a decreased expression of *RFC1* but also to *TS* gene amplification and overexpression. The resistant mechanisms are likely to be regulated both at a genetic and a transcriptional level for different resistance phenotypes in the ZD9331-resistant MOLT-3 cells.

Key words Thymidylate synthase inhibitor · ZD9331 · Reduced folate carrier

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Introduction

ZD9331 is a water-soluble potent quinazoline antifolate inhibitor of thymidylate synthase (TS) (Ki ~0.4 n*M* for human TS). This drug exploits the reduced folate carrier (RFC) for cell entry but, unlike raltitrexed (Tomudex), is not a substrate for folylpolyglutamate synthetase (FPGS) [7, 9]. ZD9331 is currently in phase I evaluation [17, 18]. Since resistance to chemotherapeutic agents remains one of the major obstacles in the treatment of cancer, the mechanisms involved in the resistance to a newly developed anticancer drug should be thoroughly investigated. There are three major mechanisms of resistance to the selective TS inhibitors; (1) overexpression of the target enzyme, TS [4, 10, 12], (2) impaired RFC-mediated membrane drug transport [10, 21], and (3) diminished polyglutamation [10, 22]. The development of a particular resistance phenotype would be expected to be affected by the biochemical properties of the drug. Therefore, in the case of ZD9331, alterations in RFC function and TS production probably contribute to the resistance. In an effort to clarify the mechanisms of the resistance to this novel TS inhibitor, the human acute lymphoblastic leukemia cell line, MOLT-3 [13], was cultured in the continuous presence of ZD9331 to establish a drug-resistant subline. A 310-fold resistant subline was established after 6 months exposure to the drug at concentrations up to 7 μ*M*, and was designated as MOLT-3/ZD9331.

In order to elucidate the molecular mechanisms of the resistance, we examined the alterations in TS, RFC and FPGS at the gene levels in the resistant subline established.

Materials and methods

Drugs and radioactive material

Raltitrexed (ZD1694; Tomudex), ZD9331 and CB3717 were generously provided by Zeneca Pharmaceuticals, Macclesfield, UK. Trimetrexate (TMQ) acetate was kindly supplied by Dr. T. Ohnuma, Mount Sinai School of Medicine, New York. Methotrexate (MTX) was purchased from Lederle, Tokyo, Japan. Raltitrexed and ZD9331 were dissolved in Dulbecco's phosphate-buffered saline, passed through a 0.22-μm filter, then diluted to the desired concentrations. Stock solutions of CB3717 (2 m*M*) and TMQ (0.5 m*M*) were prepared by dissolving the compounds in 0.1 *N* NaOH and pure ethanol, respectively.

[3′,5′,7-^{3}H]MTX sodium salt (8.8 Ci/mmol) was purchased from Amersham International (Amersham, UK).

Cell culture

MOLT-3 cells were maintained in RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% (v/v) fetal bovine serum (Gibco).

MOLT-3/ZD9331 was established by continuously exposing MOLT-3 cells initially to 30 n*M* ZD9331 and then to gradually increasing drug concentrations. Cells resistant to ZD9331 were selected as a pool through stepwise dose escalation up to 7 μ*M* after 6 months. All the experiments were carried out after cells were grown in a drug-free medium for 2 weeks.

Drug sensitivity studies

The in vitro sensitivity of the parent and resistant cells to antifolates was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, Mo.) colorimetric assay with minor modifications as previously described [11, 15]. The cells were incubated at 37 °C in a humidified 5% CO_2/95% air atmosphere for 72 h in the continuous presence of drug, and then harvested for the MTT assay.

Initial uptake of radiolabeled drug in the sensitive and resistant cells

The Vortex-Finnpipette procedure [20] was used to measure the uptake of [^{3}H]MTX by the cells sensitive and resistant to ZD9331. An aliquot of [^{3}H]MTX was added to a cell suspension (2×10^6 viable cells/ml) in folate-free RPMI-1640 medium (Gibco) at a final concentration of 0.1 μ*M*, and the mixture was incubated at 37 °C or 0 °C. At specified times, 0.5 ml of reaction mixture was removed from a culture tube and layered on a mixture of mineral oil (Sigma) and silicon oil (Fluka Japan, Tokyo, Japan), the final density of which was adjusted to 1.031 g/ml. This was followed immediately by centrifugation at 12 000 *g* for 1 min to separate the cells from the supernatant under the oil layer. After removal of the supernatant and most of the oil layer, the cell pellet was solubilized by incubation with Solvable (New England Nuclear, Boston, Mass.) at 50 °C for 3 h, then the solution was transferred to a counting vial. Radioactivity was measured with a liquid scintillation counter after the addition of 10 ml liquid counting scintillant (ACS-II; Amersham). The results were calculated as net uptake by subtracting the diffusion value at 0 °C from the total radioactivity at 37 °C for each incubation period.

For determination of the transport kinetics of MTX, the parent and ZD9331-resistant cells were washed twice and resuspended in folate-free RPMI-1640 medium at a concentration of 2×10^6/ml. After preincubation of the cells for 15 min at 37 °C, [^{3}H]MTX at graded concentrations (final concentrations of MTX 5, 10, 20 and 40 μ*M*) was added to the medium to initiate the reaction. At various times (0 to 5 min), the reaction was terminated by layering the reaction mixture on the oil and centrifuging as described above. The net uptake value (the total uptake minus the diffusion value at 0 °C) was taken as the average from three separate experiments performed in duplicate. In this assay, the uptake of MTX was found to be linear up to 5 and 2.5 min in the parent and resistant cells, respectively. The initial uptake values at 2.5 min for MTX were determined graphically and were plotted as a Lineweaver-Burk plot.

Nucleic acid isolation and blotting

The cDNA for the human *RFC1* (pHuMtxT4, *Bam*H I digestion) was kindly provided by Dr. W.F. Flintoff, University of Western Ontario, Canada [24]. The cDNA for human *FPGS* (pTZ18U, *Eco*R I digestion) was a generous gift from Dr. B. Shane, University of California, Berkeley [6]. The cDNA for the human *TS* (pcHTS-7, *Xho* I digestion) was kindly supplied by Dr. T. Seno, National Institute of Genetics, Japan [1]. The cDNA for the human *GAPDH* (pHcGAP, *Pst* I-*Xba* I fragment) was obtained from American Type Culture Collection and used as an internal control probe. Total cellular RNA was isolated using TRIzol Reagent (Gibco). Total RNA (8 μg per lane) was size-fractionated by electrophoresis in 0.41 *M* formaldehyde/1% agarose gels. RNA was transferred onto Maximum Strength Nytran nylon membranes using Turboblotter Rapid Downward Transfer Systems (Schleicher and Schuell, Keene, N.H.) as described by Chomczynski [2]. DNA was purified using a QIAGEN Blood & Cell Culture DNA Midi Kit (Qiagen, Valencia, Calif.). For Southern hybridization, DNA was digested with *Sac* I. The digested DNA (10 μg) was size-fractionated by electrophoresis in 0.8% agarose gels and transferred onto Maximum Strength Nytran nylon membranes. The membranes were hybridized with ^{32}P-labeled cDNA probes. The intensities of specific signals were measured using a scanning densitometer and normalized as the ratio of gene or mRNA values in the resistant subline to those in the parent cell line.

Results

Crossresistance pattern to various antifolates in the MOLT-3 cells sensitive and resistant to ZD9331 (Table 1)

The MOLT-3/ZD9331 subline (310-fold resistant to ZD9331) showed a crossresistance to other TS inhibitors (raltitrexed, CB3717), but the degree of resistance to CB3717, which has a lower affinity for RFC than does raltitrexed [3], was relatively low (4.8-fold). MOLT-3/ZD9331 cells showed crossresistance not only to other TS inhibitors but also to MTX, which also utilizes RFC for cell entry. In contrast, the resistant cells showed little or no crossresistance to TMQ, a lipophilic dihydrofolate reductase (DHFR) inhibitor which does not use RFC for cell entry [5].

The phenotype described above was stable in a ZD9331-free medium for more than 3 months. All the dose-response curves were steep (data not shown), suggesting that the resistant cells pooled through the

Table 1 Crossresistance pattern to folate analogues in the MOLT-3 sublines sensitive and resistant to ZD9331.

Cell Line	IC_{50} values (μM) (Relative resistance)				
	ZD9331	Raltitrexed	CB3717	MTX	TMQ
MOLT-3	0.032	0.0038	1.1	0.0013	0.0040
MOLT-3/ ZD9331	10 (310)	0.24 (63)	5.3 (4.8)	0.16 (120)	0.0035 (.88)

IC_{50} values were obtained from dose-response curves of each cell line after exposure of the cells for 86 or 72 h to MTX or the other drugs, respectively. Each value represents the mean of at least two independent quadruplicate experiments, and all experimental data points were within 15% of the mean

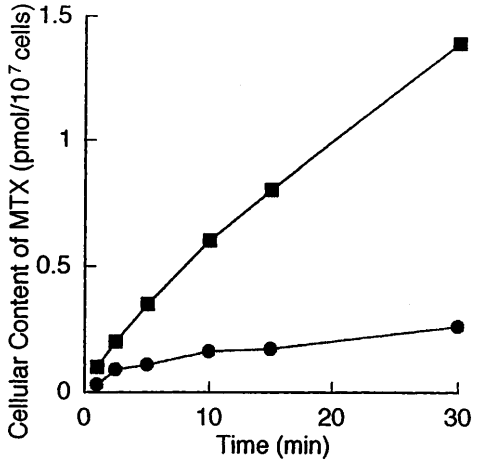

Fig. 1 Initial uptake and subsequent accumulation of [^{3}H]MTX in MOLT-3 (■) and MOLT-3/ZD9331 (●). Data shown are from one representative experiment out of three independent experiments

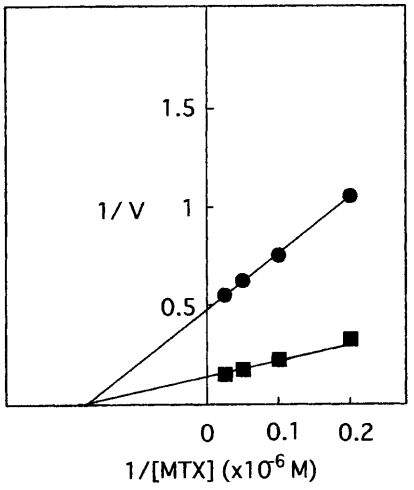

Fig. 2 Uptake kinetics of [^{3}H]MTX from MOLT-3 (■) and MOLT-3/ZD9331 (●). V is nanomoles MTX uptake per 2.5 min per 10^7 cells. Each time point represents the average from three separate experiments carried out in duplicate

selection procedure were uniformly resistant to TS inhibitors and MTX.

Cellular uptake of [^{3}H]MTX as a phenotype of RFC function in the MOLT-3 cells sensitive and resistant to ZD9331

To assess RFC function in the sensitive and resistant cells, the initial uptake of [^{3}H]MTX in the cells was determined by measuring cellular ^{3}H for up to 30 min. To examine the influence of folate receptor-α [19] on the uptake of MTX, we carried out uptake experiments with [^{3}H]MTX not only in folate-free medium but also in the regular RPMI-1640 medium containing 2.2 μM folic acid, which has been reported to result in complete saturation of the folate receptor but to have no effect on RFC-mediated transport [23]. In both MOLT-3 and MOLT-3/ZD9331, the uptake rates of [^{3}H]MTX in regular RPMI-1640 medium containing 2.2 μM folic acid were virtually the same as those in folate-free medium, suggesting that RFC is the preferential route of entry for MTX in these cell lines (data not shown). As shown in Fig. 1, the decrease in the cellular uptake of [^{3}H] MTX in MOLT-3/ZD9331 indicated impairment of RFC function. When the initial uptake of MTX was plotted as a Lineweaver-Burk plot (Fig. 2), there was an obvious decrease in the velocity of the initial uptake of MTX in MOLT-3/ZD9331 (the Vmax relative to sensitive cells was 30%). The Km values were similar, however, for both sensitive and resistant cell lines, with a concentration of approximately 6 μM.

Northern and Southern blot analysis of the *RFC1* gene in the MOLT-3 cells sensitive and resistant to ZD9331

In the Northern blot analysis for *RFC1* mRNA (Fig. 3A), MOLT-3/ZD9331 revealed decreased expression of *RFC1* (30% as compared with MOLT-3), which was comparable with diminished cellular uptake of [^{3}H]MTX (Figs. 1, 2). There were no changes in gene copy number nor gross rearrangement in MOLT-3/ZD9331 as compared with the parent cells, as shown in Fig. 3B.

Northern and Southern blot analysis of *TS* gene in the MOLT-3 cells sensitive and resistant to ZD9331

We examined the molecular alteration of the target enzyme, TS. Figure 4 compares the amounts of *TS*

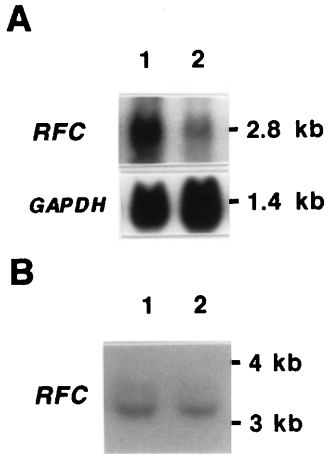

Fig. 3A,B Nucleic acid analysis of the *RFC1* gene in the MOLT-3 sublines sensitive and resistant to ZD9331. **A** Northern blot analysis (*lane 1* MOLT-3, *lane 2* MOLT-3/ZD9331). The signal intensity in MOLT-3/ZD9331 (normalized to *GAPDH* expression) relative to that in MOLT-3 is 0.3. **B** Southern blot analysis *lane 1* MOLT-3, *lane 2* MOLT-3/ZD9331)

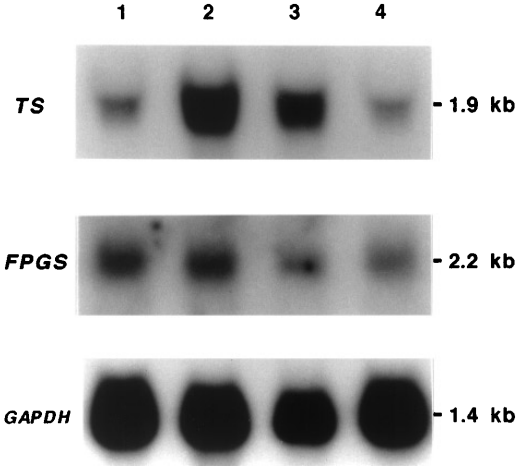

Fig. 4 Northern blot analysis of *TS* and *FPGS* in the MOLT-3 sublines sensitive and resistant to ZD9331. *Lane 1* MOLT-3 (6 μg total RNA), *lane 2* MOLT-3/ZD9331 (6 μg total RNA), *lane 3* MOLT-3/ZD9331 (3 μg total RNA), *lane 4* MOLT-3 (6 μg total RNA). The signal intensity of *TS* and *FPGS* in MOLT-3/ZD9331 (normalized to *GAPDH* expression) relative to that in MOLT-3 is 4.4 and 1, respectively

mRNA, and shows a 4.4-fold increased expression of *TS* mRNA in MOLT-3/ZD9331 as compared with that in MOLT-3. There was a significant (20-fold) amplification of the *TS* gene in MOLT-3/ZD9331 by Southern blot analysis (Fig. 5), although no major rearrangements of the gene were noted.

The expression of FPGS in the MOLT-3 cells sensitive and resistant to ZD9331

The chemical structure of ZD9331 would make it chemically impossible for the FPGS reaction to proceed.

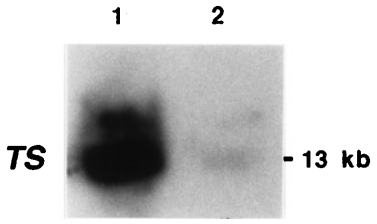

Fig. 5 Southern blot analysis of *TS* in the MOLT-3 sublines sensitive and resistant to ZD9331 (*lane 1* MOLT-3/ZD9331, *lane 2* MOLT-3)

As expected, the gene expression of this enzyme did not alter (Fig. 4).

Discussion

This study sought to determine the molecular mechanisms of resistance to a potent quinazoline-based TS inhibitor ZD9331, which is currently in phase I evaluation [17, 18]. ZD9331 utilizes the RFC for cellular entry and exerts its antitumor activity through TS inhibition (Ki ~0.4 n*M* for human TS) without metabolic activation through polyglutamation, unlike raltitrexed [7, 9]. The resistance mechanisms to this drug are likely to relate to these biochemical properties. Indeed, our experimental results can be summarized as follows: (1) decreased expression of *RFC1* was observed together with diminished cellular uptake of [^{3}H]MTX, which was responsible for the crossresistance to other antifolates transported via the RFC (e.g. MTX and raltitrexed); (2) the gene amplification and overexpression of the target enzyme, TS, emerged as a mechanism of resistance contributing to the crossresistance to other TS inhibitors irrespective of the requirement for RFC for uptake (e.g. CB3717 and raltitrexed); and (3) the expression of *FPGS* did not alter as expected, since the chemical structure of ZD9331 made the FPGS reaction chemically impossible. These experimental observations strongly suggest that the development of a particular resistance phenotype is affected by the biochemical properties of ZD9331 and is based on the molecular events.

We carried out Southern blot analysis as well as Northern blot analysis to examine the structure and copy-number of the *RFC* and *TS* genes. The fact that there was neither a change in *RFC1* gene copy number nor gross gene rearrangement observed in the Southern blot analysis despite decreased expression of *RFC1* in MOLT-3/ZD9331 indicates that the impaired RFC function was downregulated at the transcriptional level. This assumption is consistent with the decrease in the influx Vmax of MTX, which usually results from a decrease in the number of RFC. On the other hand, the gene amplification and subsequent overexpression of *TS* observed suggest the regulation of TS at the genetic level. It is likely that the process of gene amplification results in overproduction of its product, TS, which

allows the cell to overcome the effect of the drug on TS in MOLT-3/ZD9331. Although TS protein or enzyme activity was not directly measured, a 4.4-fold increased expression of *TS* in MOLT-3/ZD9331 as compared with that in the parent cells paralleled the degree of resistance to CB3717 (4.8-fold), another TS inhibitor with low affinity to RFC, suggesting that the amount or activity of TS in MOLT-3/ZD9331 would parallel the message. Our experimental observation of the amplification of the *TS* gene in MOLT-3/ZD9331 is analogous to previous findings with cell lines made resistant to other TS inhibitors, CB3717, raltitrexed, or C^2-desamino-C^2-methyl-N^{10}-propargyl-5,8-dideazafolic acid [4, 8, 10, 14, 16]. Since TS is a cell-cycle-dependent enzyme, it is possible that defects in cell-cycle checkpoints are responsible for the genomic instability resulting in the amplification.

ZD9331 would not be a substrate for FPGS; thus this drug may overcome the resistance resulting from alterations in *FPGS* expression [7]. In addition, lack of retention as polyglutamates may result in a different toxicity profile and spectrum of antitumor activity. Such a biochemical property of ZD9331 does not predict alterations in *FPGS* expression as a consequence of resistance. In fact, our results confirmed the prediction.

The phenotype found in our experiments displayed two molecular alterations described above: decreased expression and function of *RFC1*, and amplification and overexpression of *TS*. Since the dose-response curves in drug sensitivity studies were steep, it is likely that the selection procedure selected for both changes in the final population.

The results presented here suggest that a particular resistance phenotype, such as amplification of the target enzyme or impaired drug transport, is likely to be regulated at the genetic or transcriptional level, respectively, to circumvent the cytotoxic effect of the drug at least in MOLT-3/ZD9331 cells. Both regulations for resistance mechanisms worked in a single subline, MOLT-3/ZD9331.

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