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GENISTEIN RESISTANCE IN HUMAN LEUKAEMIC CCRF-CEM CELLS: SELECTION OF A DIPLOID CELL LINE WITH REDUCED DNA TOPOISOMERASE II β ISOFORM*

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Abstract—Genistein, an isoflavonoid derivative initially described as an *in vitro* protein tyrosine kinase inhibitor, also inhibits mammalian DNA topoisomerase II both *in vitro* and *in vivo*. From a human leukaemic T cell line (CCRF-CEM), two genistein-resistant cell lines, which grow in the presence of 50 and 150 μM genistein, respectively, were selected and designated CEM/GN$_{50}$ and CEM/GN$_{150}$. Flow cytometry and karyotype analyses revealed that more than 95% of the parental cells were tetraploid whereas both resistant sublines were essentially diploid and were likely derived from the diploid fraction in the initial population. The CEM/GN cells were 3- to 4-fold resistant to genistein, and highly cross-resistant to certain metabolic inhibitors such as cytosine-arabinoside (50-fold) and 5-fluoro-2′-deoxyuridine (5000-fold). This resistance was associated with a markedly decreased uptake of thymidine and a 10-fold reduction in thymidine kinase activity. The CEM/GM cells were also 15- to 30-fold cross-resistant to topoisomerase inhibitors (etoposide, m-AMSA, 2-Me-9-OH-ellipticinium). Comparison of topoisomerase II activities in the sensitive and resistant cells showed: (i) an approximatively 2-fold reduced decatenation activity in nuclear extracts from the resistant cells; (ii) an approximate 30% reduction in DNA-protein cross-links in etoposide-treated resistant cells; and (iii) a markedly reduced expression of the topoisomerase II β isoform. These data, consistent with our previous results, indicate that the cytotoxicity of genistein is at least in part related to its capacity to inhibit DNA topoisomerase II.

Key words: CCRF-CEM cells; genistein; resistance; topoisomerase II; thymidine kinase

Tyrosine phosphorylation plays a crucial role in cell proliferation and cell transformation, suggesting that tyrosine kinase specific inhibitors might be considered for use as anticancer agents [1, 2]. Genistein, an isoflavonoid derivative isolated from the fermentation broth of *Pseudomonas* sp., was one of the first specific tyrosine kinase inhibitors to be described. Genistein was initially reported as an *in vitro* specific inhibitor of the tyrosine kinase activities of the EGF‖ receptor, pp60$^{v\text{-}src}$ and p110$^{gag\text{-}fes}$ [3]. Inhibition was competitive with respect to ATP and non-competitive with respect to the phosphate acceptor [3]. In NIH-3T3 cells, Linassier *et al.* [4] showed that genistein was able to block EGF and insulin-mediated mitogenic effects at similar concentrations (IC_{50} = 12 and 19 μM, respectively). However, although the thrombin receptor does not involve a protein tyrosine kinase activity, thrombin-mediated mitogenic effects were also inhibited by genistein at approximately the same concentration. Furthermore, c-*myc* transcription, an event associated with the EGF receptor activation by EGF, was not inhibited by genistein. These data indicated that the cytostatic effect of genistein on NIH-3T3 cells did not appear to be mediated by EGF receptor tyrosine kinase inhibition.

When genistein toxicity on various other cell lines was examined, it was found that 9-OH-ellipticine resistant Chinese hamster lung cells (DC-3F/9-OH-E) were markedly more resistant to this drug than the parental line (DC-3F) [5]. Cross-resistance of the DC-3F/9-OH-E cells to various DNA topoisomerase II inhibitors, such as m-AMSA and etoposide, was associated with an altered topoisomerase II activity. *In vitro* studies showed that genistein inhibited the decatenation activity of DNA topoisomerase II and stabilized the cleavable complex, an intermediate product in the topoisomerase II catalysed reaction in which the enzyme is covalently bound to the 5′ termini of the broken DNA molecule [5]. All these data demonstrate that genistein is an inhibitor of both protein tyrosine kinase and topoisomerase II activities. The presence of a highly homologous sequence in the ATP binding

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‖ Abbreviations: EGF, epidermal growth factor; m-AMSA, 4′-(9-acridinylamino)methanesulphon-*m*-anisidide; VP-16, etoposide; 5-FUdr, 5-fluoro-2′-deoxyuridine; 5-FUr, 5-fluorouridine; 2-Me-9-OH-E, 2-methyl-9-hydroxy-ellipticinium; 5-FU, 5-fluorouracil; ara-C, cytosine arabinoside; SDS, sodium dodecyl sulphate; 5-FdUMP, 5-fluorodeoxyuridine monophosphate; DPC, DNA–protein cross-links.

site of tyrosine kinase and topoisomerase II could account for the existence of common inhibitors of both enzymes [5]. DC-3F/9-OH-E cross-resistance to genistein indicates that the interaction with topoisomerase II is involved in the mechanism of toxicity of this drug.

Isolation and characterization of resistant cells has often proven to be a successful approach to analyze a drug mechanism of action. This work describes the selection and biochemical characterization of genistein-resistant sublines (CEM/GN cells) selected from the human leukaemic CCRF-CEM cell line. Flow cytometry analysis showed that the resistant cells derive from a diploid fraction initially present in the tetraploid parental CEM population. Unexpectedly, CEM/GN cells display a very high cross-resistance to 5-FUdr, which is associated with a markedly reduced thymidine kinase activity. CEM/GN cells are also cross-resistant to various topoisomerase II interactive drugs. This resistance is associated with a decreased expression of the topoisomerase II isoform β.

MATERIALS AND METHODS

Drugs and chemicals. Genistein was purchased from Extrasynthese Laboratories (France). The erbstatin analogue, methyl-2,5-dihydroxycinnamate, was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). VP-16 (NSC 141540) and m-AMSA (NSC 249992) were generous gifts of Drs W. T. Bradner (Bristol-Myers Co., Syracuse, NY, U.S.A.) and Y. Pommier (National Cancer Institute), respectively. Stock solutions (20 mM) of these drugs were prepared in DMSO and stored frozen at −20° for less than 1 week. Just before use, these solutions were diluted in distilled water to the desired concentration. The final concentration of DMSO in the culture medium never exceeded 1% (v/v). 2-Me-9-OH-E, a gift from Sanofi (France), was prepared as a 10 mM stock in distilled water. 5-FU and 5-FUdr were from Sigma (St. Louis, MO, U.S.A.). Vincristine sulphate was obtained from Eli Lilly Co. (Indianapolis, IN, U.S.A.). Ara-C was from Upjohn Laboratories (France).

Cell lines and cultures. The CCRF-CEM human leukaemic cell line, kindly provided by Dr A. Bernard (Institut Gustave Roussy, Villejuif, France), was grown in suspension culture in RPMI 1640 medium (Gibco) supplemented with 10% heat inactivated foetal calf serum (Gibco), 2 mM glutamine, and 50 μg/mL gentamycine (Gentalline 80, Laboratoire Unilabo). Cultures were diluted twice weekly, maintained at 37° in a humidified atmosphere with 5% CO_2, and were periodically checked for the absence of mycoplasma. Cells were counted with a ZM Coulter counter (Coulter Electronics Inc.).

For determination of the cell doubling times, 12 mL of growth medium were inoculated in duplicate with 2×10^5 cells. In each culture, the cells were counted in duplicate every 12 hr for 4 days.

Selection of resistant cells. CEM genistein-resistant cells were selected by adding stepwise increasing drug concentrations to the cell growth medium in the presence of 20% foetal calf serum. The selection started at 10 μM. At each step during the selection, the cells were grown in the presence of the drug for two to three passages and then in drug-free medium for two to three passages. After 6 weeks, a first intermediate subline was selected at 50 μM genistein and designated CEM/GN_{50}. The CEM/GN_{50} cells were then submitted to a further selection. After approximately 13 months, a second subline was selected at 150 μM genistein and designated CEM/GN_{150}. Both resistant sublines were then grown alternatively in the presence or the absence of 50 and 150 μM genistein, respectively, and 20% foetal calf serum.

Cytotoxicity. All experiments were carried out on exponentially growing cells which had previously been grown for two to three passages in absence of genistein. Briefly, the cells, seeded in 24-well plates, were exposed to increasing drug concentrations (3 wells/dose) for 48 hr, and then counted with a Model ZM Coulter Counter. In each experiment, 6 wells containing untreated cells were used as controls.

Flow cytometry. Analysis of cellular DNA content by flow cytometry was carried out as previously described [6].

Karyotype analysis. Mitoses from the parental CEM and resistant sublines were prepared by standard cytogenetic and RHG banding techniques when the cells were in exponential phase of growth [7]. For each cell line, 19–31 mitoses were analysed and karyotypes described according to the International System for Cytogenetic Nomenclature (1991).

Tumourigenicity. Exponentially growing CEM, CEM/GN_{50}, and CEM/GN_{150} were washed in serum-free medium and 10^7 cells were injected subcutaneously into the right posterior flank of irradiated 6–7-week-old female Swiss *nu/nu* mice. The mice were examined biweekly for the development of tumours. Cells were scored as tumourigenic if a palpable nodule appeared and grew progressively at the injection site within 6–7 weeks. Animals were killed on day 46 after cell injection and tumours were removed aseptically for weighing and analysis. After the isolated tumour was minced, passed through a stainless needle and washed, the tumour cells were inoculated into culture medium. Since the tumour cells were grown in suspension, no contaminating fibroblasts from the nude mice were present. The cells were then examined for eventual changes in their ploidy, cell cycle, and uptake of macromolecule precursors.

Macromolecule syntheses. For determination of macromolecule syntheses, 0.5 μCi/mL of either tritiated thymidine, deoxyadenosine, uracil, deoxycytidine or leucine were added to 12 mL of cell suspension at 3×10^5 cells/mL. At the indicated times, the radioactivity incorporated into trichloracetic acid precipitable material was measured by liquid scintillation counting [8].

Enzyme assays. Several enzyme activities involved in pyrimidine metabolism were assayed in exponentially growing cells stored as dry pellets in liquid nitrogen. Enzyme assays were performed on 105 000 *g* supernatants of cells extracted in 50 mM Tris-HCl, 250 mM sucrose, pH 7.5. Detailed assay procedures for thymidine kinase, thymidylate synthetase, uridine

kinase and uridine phosphorylase have been previously described [8].

Deoxycytidine kinase activity was determined in a reaction medium (30 μL) containing 50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 15 mM ATP, 10 mM $MgCl_2$, 15 mM NaF, 1.5 mM deoxyuridine and 2 μM 5-[^{3}H]deoxycytidine (21 Ci/mmol). After incubation for 30 min at 37°, an aliquot (20 μL) was loaded on a DE81 whatman filter disc. After washing once in 75% ethanol and twice in 95% ethanol for 10 min, the radioactivity remaining on the disc was measured by scintillation counting.

Decatenation of kinetoplast DNA. Trypanosoma cruzi, grown in the presence of [methyl-^{3}H]thymidine (2 μCi/mL) and guanosine (350 μg/mL), were lysed with sarkosyl and the labelled kinetoplast DNA was extracted as previously described [9]. Kinetoplast DNA decatenation was carried out at 30° for 15 min in a reaction mixture (50 μL) containing 40 mM Tris-HCl pH 8, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM Na_3-EDTA, 0.5 mM DTT, 1 mM ATP, 30 μg/mL BSA, k[^{3}H]DNA (specific activity: 32700 cpm/μg) and 4–25 μg/mL of nuclear extract proteins. The reaction was stopped by adding 50 μL of a 2 M NaCl, 10 mM Na_3-EDTA solution. After centrifugation at 13000 rpm for 10 min in a Biofuge A (Heraeus) microcentrifuge, 50 μL of supernatant containing decatenated minicircles were carefully removed and the amount of DNA determined by liquid scintillation counting.

One unit of topoisomerase II activity is defined as the amount of enzyme which releases 1 μg of kDNA minicircles in 15 min at 30°, in 1 mL of reaction mixture.

Measurements of DNA damage by alkaline elution. The determination of protein-associated DNA strand breaks was carried out as previously described [10].

RNA extraction and Northern blot analysis. Total RNAs were extracted by the thiocyanate technique [11]. Polyadenylated RNAs, purified from total RNA using the mRNA purification kit from Pharmacia-LKB Biotechnology (Uppsala, Sweden), were fractionated by electrophoresis in 1.2% (w/v) agarose gel containing 7% formaldehyde and transferred to nylon Hybond-N membrane (Amersham, U.K.) in 150 mM ammonium acetate. After prehybridization for 2 hr at 42° in 40% formamide, 5 × SSC (SSC: 0.15 M NaCl, 15 mM Na citrate), 50 mM phosphate buffer pH 6.8, 5 × Denhardt, and 0.1% SDS, hybridization was performed for 20 hr in the same buffer containing the ^{32}P labelled probe. The membrane was washed twice for 15 min at room temperature in 0.1% SDS, 2 × SSPE (SSPE: 10 mM HPO_4, 0.13 M NaCl, 1 mM EDTA) and twice for 30 min at 50° in 0.1% SDS, 1 × SSPE. After autoradiography at −70°, the autoradiograms were obtained on Fuji RX film with a Dupont Cronex Lighting-Plus screen.

Nuclear extracts and Western blot analysis. Nuclear extract preparation, topoisomerase II α antibody, and Western blot analysis have been described previously [12].

RESULTS

Selection of genistein-resistant cells

Genistein-resistant CCRF-CEM cells were selected by adding stepwise increasing drug concentrations to the cell growth medium, starting at 10 μM. During selection, the cells were alternatively grown in the presence or absence of the drug. After 6 weeks, a first subline was selected at 50 μM genistein and designated CEM/GN_{50}. These cells were further exposed to increasing concentrations of the drug and, after 13 months, a second subline, named CEM/GN_{150}, was selected in the presence of 150 μM genistein. Exposure to higher drug concentrations was not possible because of the limited water solubility of genistein. From the IC_{50} values determined on Fig. 1, the CEM/GN_{50} and CEM/GN_{150} were found to be 2.5- and 4-fold resistant to genistein, respectively. In both resistant sublines, the level of resistance remained stable when the cells were grown for 14 weeks in absence of selective agent. Both resistant cell lines grew more slowly than the parental cells. Table 1 shows that the doubling time of the CEM/GN_{150} cells was approximately 26 hr as compared to 19 hr for the CEM cells. The resistant cells were found by laser scanning microscopy to be smaller than the parental cells, with average diameters of approximately 8 μm and 12 μm, respectively (Table 1).

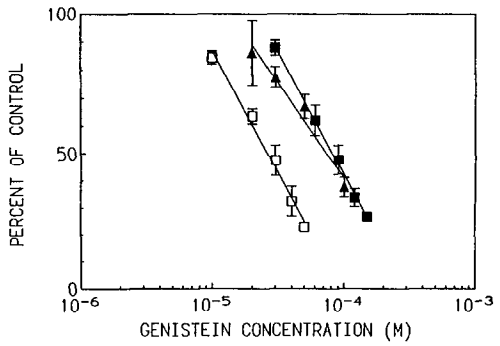

Fig. 1. Toxicity of genistein on CEM (□), CEM/GN_{50} (▲), and CEM/GN_{150} (■) cells. After treatment with the drug at the indicated concentrations for 48 hr at 37°, the cells were counted as described in the Materials and Methods section.

Tumourigenicity of genistein-sensitive and resistant cells

For determination of their oncogenic potential, 10^7 cells from each cell line were inoculated subcutanously into pre-irradiated female nude mice. Tumors appeared as solid lymphoid masses at the site of injection approximately 14 days after injection, and the three cell lines grew at approximately the same rate (not shown). In two independent experiments, the incidence of tumors was similar for both the resistant and parental cells (Table 1). Necrosis was observed in approximately 50% of the tumours developed from the CEM cells but not in tumours from the resistant cells. These data indicated that the oncogenic potential in the genistein-resistant cells remained similar to that in the parental cells.

Table 1. Properties of genistein-resistant CEM cells

	Resistance*	Cell size (μM)	Doubling time (hr)	Tumourigenicity†	Number of chromosomes‡
CEM	1	12–12.6	19	7–8/10	88–90
CEM/GN_{50}	2.5	ND§	ND	5–9/10	46–47
CEM/GN_{150}	3–4	8–9	26	8–10/10	46–49

* Ratio of the IC_{50} values.
† Tumour incidence in nude mice after injection of 10^7 cells. Results of two independent experiments.
‡ Chromosome numbers (range) determined for each cell line on 19–31 mitoses. According to International System for Cytogenetic Nomenclature (1991).
§ ND, not determined.

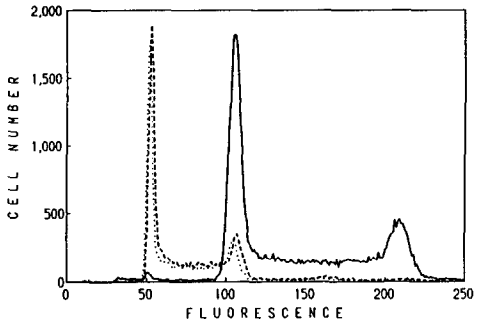

Fig. 2. Cell cycle distribution of parental CEM cells (solid line), CEM/GN_{50} (dashed line) and CEM/GN_{150} (dotted line). DNA histograms were obtained as described in Ref. 6.

Cytogenetic characterization of the genistein-resistant cells

Cytofluorimetric analysis revealed that the cellular DNA content in the parental CEM cells was approximately twice that in the CEM/GN_{150} (Fig. 2). Similar results were obtained with the CEM/GN_{50} cells. These observations also suggested that the initial CEM cell line was pseudo-tetraploid and the resistant cells pseudo-diploid. A microsatellite DNA polymorphism study (data not shown) revealed the same fragment pattern in the CEM/GN_{150} cells and the parental CCRF-CEM cells, thus demonstrating that both cell lines have the same origin.

Karyotype analysis (Table 2) indeed showed that most of the parental CEM cells were hypotetraploid (N = 88–90). However, a subpopulation of hypodiploid cells (N = 45), representing approximately 2% of the total, was also detected. Chromosome banding analysis, carried out on 24 metaphases of the tetraploid CEM cells (Table 2), showed the del(9)(p13) specific of this cell line [13], the loss of 2 number X, 2 number 10, 2 number 13 and the presence of four to six non-identified markers (Fig. 3). The CEM hypodiploid mitosis revealed the loss of one number X, the del(9p), and one add(10)(p14) (Table 2).

Both the CEM/GN_{50} and CEM/GN_{150} cells had a paradiploid modal number of chromosomes (N = 45–47 and 46–49, respectively) (Table 2). Chromosome banding analysis showed the same basic chromosomal abnormalities in these resistant cells as in the diploid parental CEM cells: X, del(p9), add(10)(p14) (Table 2 and Fig. 3). During the different steps of the selection, a clonal evolution was observed through the acquisition of normal chromosomes, the most frequent being the presence of trisomies 21, 20 or 19, and, in the CEM/GN_{150}, trisomy 7. Moreover, we also observed that a fraction of the CEM/GN_{150} cells (approximately 13%) recovered a tetraploid karyotype with a chromosome pattern resulting from the duplication of the diploid CEM/GN_{150} (Table 2).

Cross-resistance to other antitumour agents

Cross-resistance of the CEM/GN_{150} cells to several antitumour drugs, endowed with various mechanisms of action, is shown in Table 3. All turned out to be more cytotoxic on the parental CEM cells than genistein. The CEM/GN_{150} cells were only approxi-

Table 2. Composite karyotypes of tetraploid CEM cells and genistein sublines

Cell line	Number of mitoses	Composite karyotypes
CEM	24	88–90, XX, −X, −X, del(9) (p13),del(9) (p13) −10, −10, −13, −13, +4 to 6 mar.
GEM/GN_{50}	5	45, X, −X, del(9p), add(10) (p14)
	17	46–47, X, −X, del(9p), add(10)(p14), +19, +20, +21
GEM/GN_{150}	31	46–49, X, −X, +7, del(9p), add(10p), +19, +20, +21
	4	92–98, XX, −X, −X, +7, +7, del(9p) × 2, add(10p) × 2) +19, +19, +20, +20, +21, +21

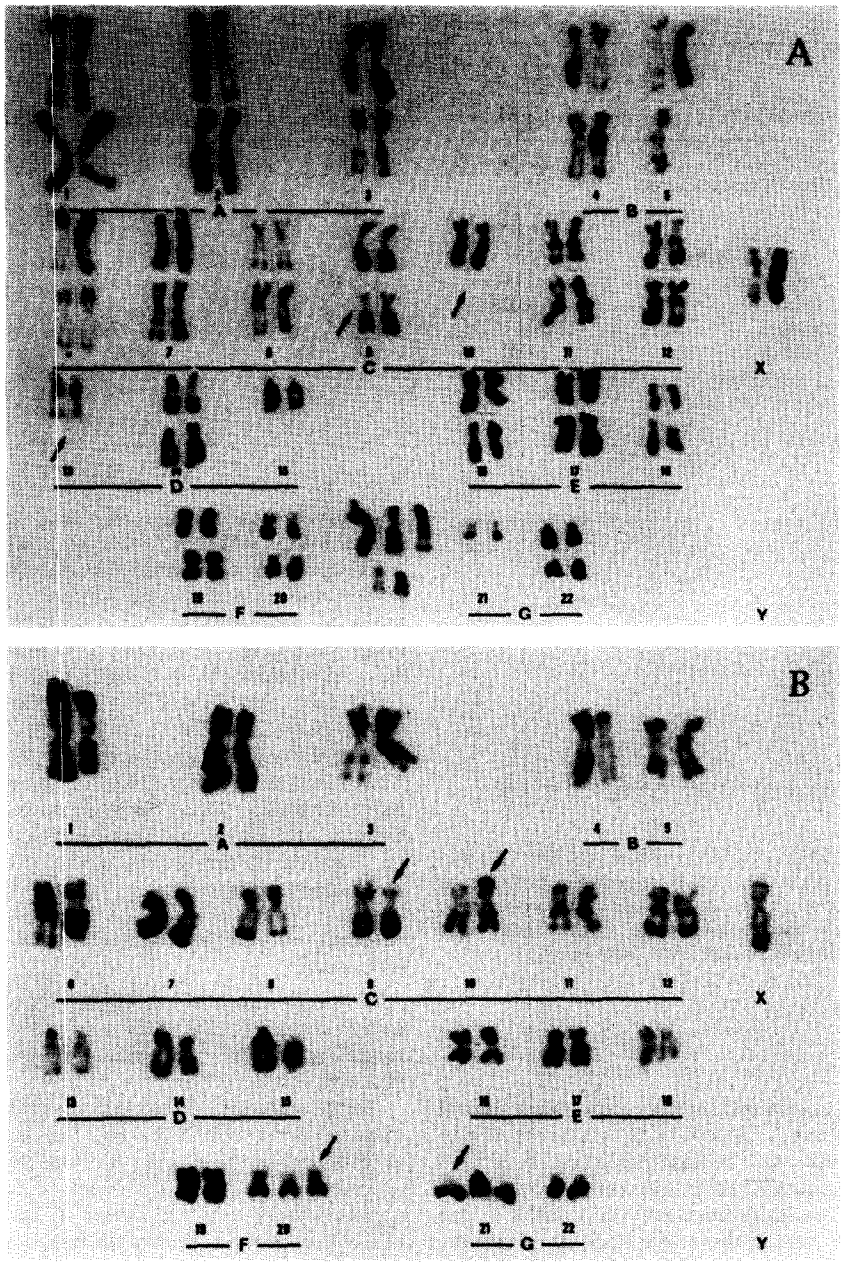

Fig. 3. Karyotype analysis of parental CEM cells (part A) and CEM/GN_{150} (part B). Mitoses from both cell lines were prepared and analysed by RHG banding as described in the Materials and Methods section. Arrows indicate the lack of chromosomes and the prominent structural alterations.

mately 2-fold cross-resistant to the protein tyrosine kinase inhibitor erbstatin analogue. In contrast, they are approximately 15- to 30-fold cross-resistant to the three topoisomerase II inhibitors VP-16, m-AMSA and 2-Me-9-OH-E. Cross-resistance to vincristine, the most cytotoxic compound in the series, was approximately the same as that to genistein. However, Northern blot analysis of the MDR1 gene did not reveal any expression of the multidrug resistance phenotype (data not shown).

Unexpectedly, the CEM/GN_{150} cells were also found to be resistant to some metabolic inhibitors, including 5-FU (approximately 7-fold), ara-C (approximately 50-fold), and 5-FUdr (approximately 5000-fold). This unexpected cross-resistance pattern led us to analyse some of the enzyme activities involved in the pyrimidine metabolic pathway.

Analysis of 5-FUdr metabolism enzymes

Rates of incorporation of different macromolecule

Table 3. Cross-resistance of CEM/GN_{150} cells

Drug	IC_{50} (μM) CEM	CEM/GN_{150}	Degree of resistance
Genistein	20–30	70–80	3.5–4
Erbastatin analogue	13	27	2
VP-16	0.19	2.7	14
m-AMSA	0.077	1.8	23
2-Me-9-OH-E+	1.3	39	30
Vincristine	0.002	0.007	3.5
Ara-C	0.028	1.5	53
5-FU	10	75	7.5
5-FUdr	0.01	50	5000

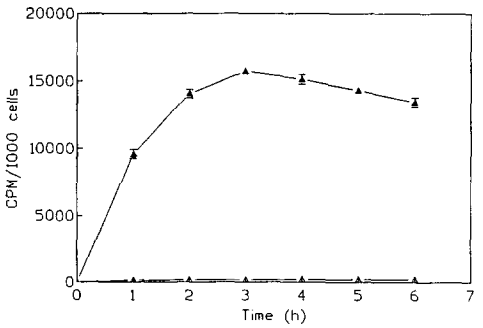

Fig. 4. Accumulation of [^{3}H]thymidine in CEM (▲) and CEM/GN_{150} (△) cells. The cells were grown in the presence of the radioactive precursor. At the indicated times, the radioactivity incorporated into trichloracetic acid precipitable material was determined.

synthesis precursors into acid-precipitable material were first examined. Uptake of tritiated leucine and uridine was decreased by approximately 20–30% in the resistant as compared to the sensitive cells (data not shown). This difference may be related to the slower growth rate of the resistant cells. However, as shown in Fig. 4, the incorporation rate of [^{3}H]-thymidine, in both CEM/GN_{50} and CEM/GN_{150} cells, was almost completely inhibited. In contrast, deoxyadenosine as well as deoxycytidine (not shown) were incorporated into acid insoluble material at approximately the same rate in both sensitive and resistant cells. These data indicate that the genistein-resistant cells present an alteration in the thymidine pathway, which might also account for the high cross-resistance of these cells to 5-FUdr.

In mammalian cells, 5-FU can be engaged in two metabolic pathways: (i) conversion to 5-FUr by uridine phosphorylase, and then to 5-FU monophosphate by uridine kinase; (ii) alternatively, FU may be converted to 5-fluorodeoxyuridine by thymidine phosphorylase and then to 5-FdUMP by thymidine kinase. 5-FdUMP is a potent inhibitor of thymidilate synthetase and further phosphorylation of 5-FUMP and 5-FdUMP to di- and triphosphate derivatives would eventually lead to their incorporation into RNA or DNA, respectively.

Comparison of thymidylate synthetase, uridine kinase and deoxycytidine kinase activities in the parental and resistant cells only revealed small differences (Fig. 5). Uridine phosphorylase activity was approximately 2- and 3-fold higher in the CEM/GN_{50} and CEM/GN_{150} cells, respectively, than in the parental CEM cells. In contrast, thymidine kinase activity was approximately 10-fold lower in both resistant cell lines than in the sensitive cells. Since thymidine kinase activity is essential for the toxicity of 5-FUdr, an important reduction in this enzyme activity might then account for a high cross-resistance to this drug.

Analysis of topoisomerase activities in the CEM/GN_{150} cells

Cross-resistance of the CEM/GN cells to various topoisomerase II inhibitors suggested that these cells might display some alteration of their topoisomerase II activities.

Topoisomerase II catalytic activity in nuclear extracts from sensitive and resistant cells was determined by measuring the rate of decatenation of kinetoplastic DNA. This activity was found to be approximately two-fold lower in the CEM/GN_{150} (110 U/mg protein) than in the parental cells (229 U/mg protein).

DNA damage provoked by topoisomerase II inhibitors in different cell lines may be compared by measuring the frequencies of DPC induced by these drugs in the cellular DNA. Filter elution assays were performed on sensitive and resistant cells treated with VP-16, one of the most potent DPC inducers, at increasing concentrations for 1 hr. In both cell lines the amount of DNA damage increased as a function of drug concentration and reached a plateau at concentrations above 10 μM. However, the average frequency of DPC was approximately 25–30% lower in the CEM/GN_{150} cells than in the parental CEM cells (Fig. 6).

To study the disappearance of these protein associated DNA breaks, CEM and CEM/GN_{150} cells were then incubated after the VP-16 treatment for different lengths of time in drug-free medium. In order to increase the initial amount of DPC in the

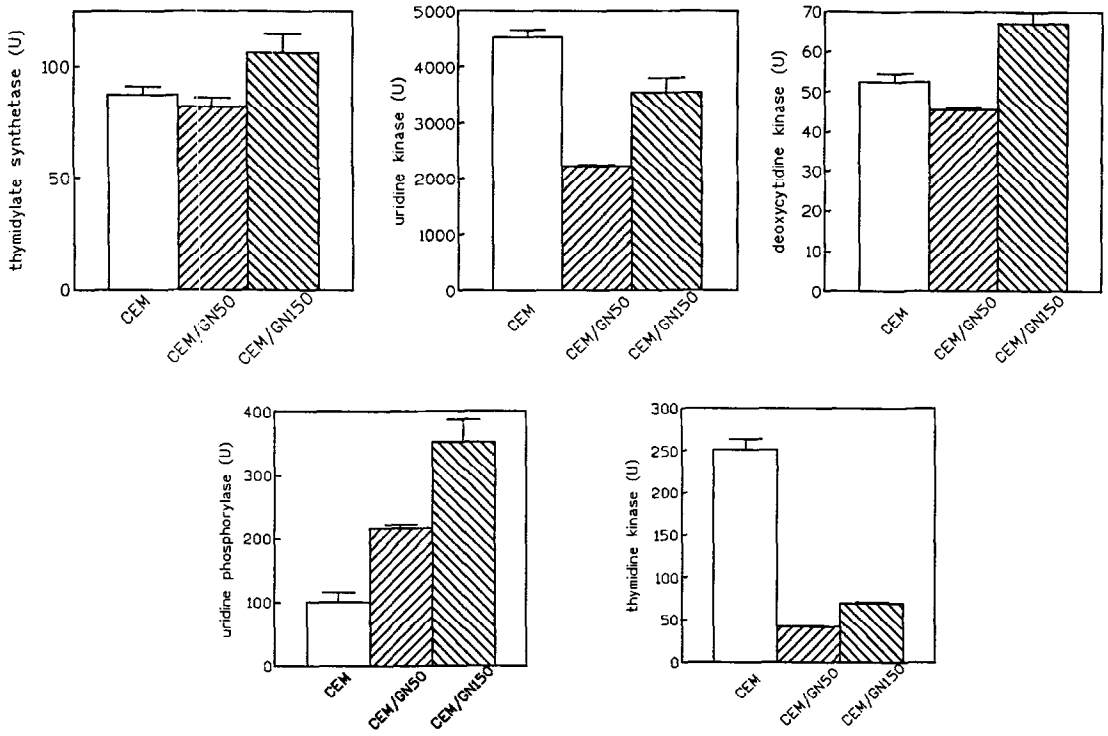

Fig. 5. Comparative activities of thymidylate synthetase, uridine kinase, deoxycytidine kinase, uridine phosphorylase and thymidine kinase in CEM, CEM/GN_{50}, and CEM/GN_{150} cells. Each determination was carried out in duplicate and standard deviations were calculated from two independent experiments. One unit of enzyme activity corresponds to the formation of 1 pmol of reaction product/mg of protein/min.

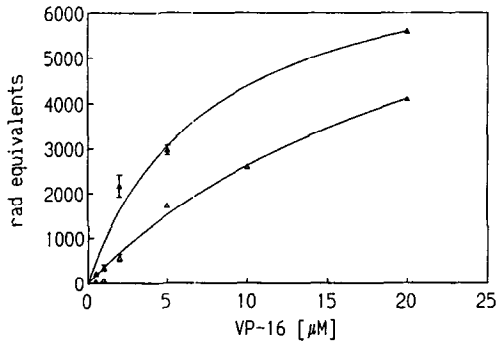

Fig. 6. DNA-protein cross-links (DPC) induced by VP-16 in CEM (▲) and CEM/GN_{150} (△) cells. Cells were exposed to the indicated drug concentrations for 1 hr at 37°. The drug was removed by washing the cells twice with HBSS at 0°. DPC were measured by alkaline elution as described in the Materials and Methods section. Standard deviations were calculated from three independent experiments.

resistant cells, the concentration of VP-16 was raised to 5 μM. The kinetics of DPC reversion was then determined by filter elution assay at 0, 20, and 60 min following drug treatment. Table 4 shows that the rate of reversion of DNA damage was identical in both sensitive and resistant cells. Therefore the reduced frequencies of DPC in the resistant cells

Table 4. Reversion of DNA protein cross-links (Rad-equivalents)

Incubation time (min) after VP-16 treatment	CEM cells	CEM/GN_{150} cells
0	2870	2034
20	1008	1028
60	633	635

were not due to a shorter life time of the damage but to a reduced rate of formation of these lesions.

All these data indicated that resistance to genistein and other topoisomerase II inhibitors in the CEM/GN_{150} cells was associated with a decreased ability of these drugs to induce topoisomerase II mediated DNA damage. In order to further characterize topoisomerase II activities in the CEM/GN_{150} cells, we examined the expression of topoisomerase II genes in these cells. Using the SP1′ and SP12′ probes [12] which specifically hybridize with the α and β transcripts respectively, Northern blot analysis (Fig. 7A) revealed that the expression of the α enzyme was not modified in the resistant as compared to the sensitive cells. In contrast, the beta transcripts were almost undetectable in the resistant cells.

The amounts of topoisomerases II α and β present

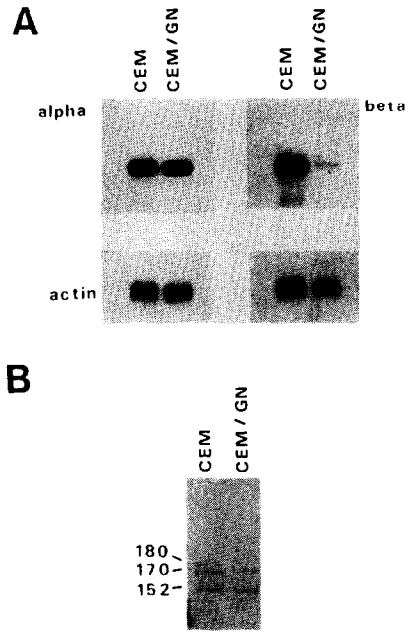

Fig. 7. Expression of topoisomerases II α and β in CEM and CEM/GN_{150} cells. Panel A: Northern blot analysis. In a duplicate experiment, total RNAs were fractionated by electrophoresis (1.2% agarose) and after transfer to Hybond-N membrane, hybridized either with the SP1 (topo II α) or SP12 (topo II β) probe. Loading of the gel was controlled by hybridization with a β-actin probe. Panel B: Immunoblot analysis. 0.35 M NaCl nuclear extracts from CEM and CEM/GN_{150} cells were fractionated by 7.5% PAGE and after transfer to a nitrocellulose membrane, the blot was probed with the A6 antibody. The immunoreactive bands were detected by light emission using an enhanced chemiluminescence detection system as described in Ref. 12.

in the nuclear extracts from the sensitive and resistant cells were then analyzed by immunoblot using the A6 antibody. This antibody, raised against an 837 amino-acid peptide from the human topoisomerase II α [12], recognizes both topoisomerase II isoforms. Figure 7B shows that in the CEM cells this antibody recognizes three proteins of 180, 170 and 152 kDa. In the CEM/GN_{150} cells, the intensity of the bands at 170 and 152 kDa were similar to that in the CEM cells, whereas the 180 kDa band was hardly detectable. The origin of the 152 kDa protein, which might be a degradation product from either the alpha or the beta enzyme, is not presently established.

In conclusion, both Northern and immunoblot analyses show that the resistance to genistein in CEM/GN_{150} is associated with a decreased expression of topoisomerase II β, whereas the α enzyme is apparently not modified.

DISCUSSION

Genistein has been shown to inhibit the function of protein tyrosine kinases *in vitro* in a manner which is competitive with respect to ATP [3]. However, *in vivo* genistein antiproliferative effects do not appear to be specifically mediated by the inhibition of protein tyrosine kinase activities, but also to result from eventual interactions of the drug with other potential intracellular targets, such as the DNA topoisomerases II [4, 5]. In this paper, our approach to a better understanding of the mechanism of action of genistein was based on the selection and characterization of a drug-resistant CCRF-CEM subline.

An unexpected trait of the CEM/GN cells was the hypodiploid karyotype which appeared in the early steps of the selection, whereas the parental CEM cells were hypotetraploid. Chromosome numbering in a large number of metaphases showed that the tetraploid parental population in fact contained a minor diploid subpopulation (approximately 2%). In the karyotypes of both populations, chromosome banding analysis revealed the presence of several structural abnormalities, listed in Table 2. With the exception of the del(9)(p13), common to both populations, variations in these structural abnormalities made it possible to differentiate the tetra- and diploid parental cells and to show that the resistant cells exhibited the same structural abnormalities as the diploid parental cells from which they most likely derived. In the CEM/GN_{50}, which represent an early step in the selection process, the diploid cells already accounted for approximately 90% of the cell population. The karyotypes of the resistant cells differed from those of the parental diploid cells by the acquisition of various chromosomes, the most frequent being 19, 20, and 21 (Table 2). The trisomy of chromosome 7 was observed in some subclones from CEM/GN_{150}. These karyological features allowed us to show that the tetraploid CEM/GN_{150} cells, which tend to develop when the resistant cells are maintained in culture, derive from the diploid CEM/GN_{150} cells and not from the parental tetraploid population.

The mechanism of this preferential selection for diploid resistant cells, which was also observed by Patel and Fisher [14] during the selection of etoposide resistant CEM cells, remains unclear. One possibility is that the initial diploid population was already resistant to the drug. Consistent with the view was the rapid selection (4–6 weeks, reproduced in two independent experiments) of the CEM/GN_{50} cells which were already diploid. On the other hand, further exposure of the cells to higher concentrations of genistein for several months did not result in a major increase in resistance to the selecting agent. Alternatively, if resistance to genistein was a recessive phenotype, a lower gene copy number in the diploid cells would facilitate the selection of the resistant cells from the diploid rather than from the tetraploid population. Presently, the karyological abnormalities identified in the diploid CEM cells do not explain the preferential selection of diploid genistein-resistant cells.

Genistein-resistant cells display a complex pattern of cross-resistance to other antitumor agents. They are first highly cross-resistant to several metabolic inhibitors, such as 5-FU, ara-C and 5-FdUrd. The toxicity of these agents is based on complex mechanisms which first require their phosphorylation by different kinases (reviewed in Ref. 15). These metabolic transformations can lead to precursors which may be incorporated into RNA (5-FU) or DNA (5-FU and ara-C), thus resulting in impaired RNA processing or decreased DNA stability.

Alternatively, some of these metabolites may behave as powerful inhibitors of essential enzymes, such as DNA polymerase and thymidylate synthetase which are inhibited by ara-CTP and 5-FdUMP, respectively. Although a mechanism has recently been proposed to explain the thymineless death induced by the inhibition of thymidylate synthetase [16], the links between the biochemical effects of the antimetabolites and their cytotoxicity remain in most cases unclear. Cellular resistance to these agents also involves complex mechanisms based on mutations occurring in genes coding not only for the enzymes involved in the biotransformation of these molecules, but also for other enzymes regulating the intracellular nucleotide pools which play a major role in the modulation of antimetabolite cytotoxicity. We have compared some of these enzyme activities in the genistein-sensitive and genistein-resistant cells: the levels of thymidylate synthetase, uridine kinase and deoxycytidine kinase, which is essential for the activation of Ara-C, were similar in both the sensitive and resistant cells. Thymidine kinase activity, which is required for the transformation of 5-FdUrd to 5-FdUMP is approximately 10-fold lower in the CEM/GN than in the parental CEM cells. The 5000-fold cross-resistance of the genistein-resistant cells to FdUrd is certainly in part related to this reduction in thymidine kinase activity, but other biochemical changes will have to be identified to fully account for such a resistance level and to understand the cross-resistance to the other metabolic inhibitors. At this time, the possibility that the mechanism(s) underlying the cross-resistance of the CEM/GN cells to the antimetabolites were also involved in the genistein-resistance cannot be excluded. However, we also tested the toxicity of genistein on a bromodeoxyuridine resistant variant from the R4 cell line (a BALB/c 3T3 cell line described in Ref. 17), in which the thymidine kinase activity is markedly reduced (Dr J. Belehradek, pers. comm.). This variant did not show any resistance to genistein as compared to the parental R4 cells, thus indicating that decreased thymidine kinase activity does not contribute to cellular resistance to genistein. It would now be interesting to isolate the diploid population from the parental CEM cells to test whether or not the antimetabolite resistance phenotype was already expressed in this population.

Genistein-resistant cells are cross-resistant to DNA topoisomerase II inhibitors such as VP-16, m-AMSA and 2-Me-9-OH-E. In fact, cross-resistance to these agents, ranging from 14- to 30-fold, is much higher than resistance to genistein itself (approximately four-fold). This pattern of cross-resistance is similar to the 'at-MDR' phenotype for altered topo II-associated multidrug resistance [18], except that CEM/GN cells are also approximately four-fold cross-resistant to vincristine. This resistance to vincristine, not usually observed in at-MDR cells, remains unexplained since we did not detect any difference in the expression of the *mdr1* gene between the CEM and the CEM/GN cells (data not shown). The cross-resistance of the CEM/GN cells to other topoisomerase II inhibitors further supports our previous conclusion that the toxicity of genistein was at least in part mediated by interaction with topoisomerase II [5]. The amount of DNA damage induced in the parental and the genistein-resistant cells was measured in the presence of VP-16, which is a much more potent inducer of DNA-protein cross-links than genistein. The frequency of DPC induced in the CEM/GN_{150} cells was approximately 25–30% lower than in the parental cells, indicating that resistance to topoisomerase II inhibitors in these cells was indeed associated with a decreased frequency of topoisomerase II mediated DNA damage. Northern and immunoblot analyses did not reveal any change in the amount of topoisomerase II α. In contrast, the topoisomerase II β transcripts were greatly reduced while the protein was almost undetectable in the CEM/GN_{150} cells. Other CEM variants selected for resistance to topoisomerase II inhibitors have been described previously. Teniposide resistant cells (47- and 418-fold) have reduced topoisomerase II α and β activities [19, 20]. In addition, point mutations have been identified in the alpha genes, located near the ATP [21] and the DNA [22] binding sites. Furthermore, in the most resistant subline, the alpha enzyme was almost undetectable by Western blot analysis [23]. These topoisomerase II alterations resulted in decreased drug-induced DNA damage, and are therefore thought to be responsible for resistance to teniposide. The above-mentioned VP-16-resistant cell line (15-fold) [14] was found to express both alpha and beta topoisomerase II isoforms at roughly similar levels. One of the alpha genes was shown to contain a Lys-797 → Asn codon change which lies close to the catalytic tyrosine 804 and may impair the trapping of the cleavable complex by the drug. These cells did not exhibit the mutations identified in the teniposide-resistant cells, thus indicating that multiple mutation sites may be involved in the selection of topoisomerase II inhibitor-resistant cells.

In conclusion, we suggest that the resistance to genistein in CEM/GN cells results from the same mechanism which is responsible for cross-resistance to topoisomerase II inhibitors. So far, a markedly decreased expression of the beta isoform is the only topoisomerase II alteration demonstrated in these cells which may account for the reduction in drug induced DNA damage. The search for eventual mutations in the alpha and beta genes must await the isolation of the parental diploid subpopulation.

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