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Preclinical study

FCE 24517-resistant MCF-7 human breast cancer cell line: selection and characterization

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We have developed a stable line of the human breast carcinoma cell line MCF-7 by *in vitro* continuous exposure to increasing concentrations of the antitumoral alkylating agent FCE 24517 (tallimustine). The selected line, MCF-7/24517$_1$, was resistant to the selecting agent (RI=10) and to a lesser degree to melphalan, MEN 10710 (a related dystamycin analog), doxorubicin and etoposide, but not to *m*-AMSA. MCF-7/24517$_1$ cells did not express the multidrug-resistant phenotype, evaluated in terms of mRNA for *mdr*-1 and gp170 glycoprotein. A significant, albeit modest, increase in the cellular content of glutathione was measured and therefore other resistance mechanism(s) should be operative. We conclude that the MCF-7/24517$_1$ line is a valuable model to investigate the mechanisms of resistance of FCE 24517 and its derivatives. [© **1999 Lippincott Williams & Wilkins.**]

Key words: Alkylating agents, distamycin, drug resistance, glutathione, multidrug resistance.

Introduction

Alkylating agents, the oldest and perhaps the most important class of anticancer drugs, play a major role in the therapeutic treatment of both early and advanced breast cancer.[1] A new alkylating agent, FCE 24517,[2,3] has been introduced in clinical trials and recently discontinued because of high bone marrow toxicity in patients.[4,6] FCE 24517 is chemically characterized by the insertion of a benzoyl mustard group on an oligopyrrole skeleton and it exhibits a broad spectrum of antineoplastic activity against a series of human tumors xenografted in nude mice, including tumors resistant to conventional cytotoxic agents.[7] In our laboratory, a series of analogs of FCE 24517 have been synthesized. Among these the distamycin derivative MEN 10710, possessing four pyrrolic rings and a bis-(2-chloroethyl)-aminophenil moiety linked to the oligopyrrole backbone by a flexible butanamido chain, has been reported to have enhanced cytotoxicity and antitumor activity comparable to FCE 24517.[8] See Figure 1.

The efficacy of antitumoral agents is often impaired by the occurrence of resistance mechanism(s) such as reduced drug accumulation and active drug extrusion, increased metabolic breakdown,[9] and enhanced DNA repair.[1] The cytotoxicity of classical alkylating agents is considerably reduced by cellular glutathione (GSH), an important intracellular nucleophile, which can conjugate alkylating agents and thereby prevent their reaction with DNA.[10] Increased GSH levels have been found in tumor cells resistant to these agents.[11,12] As of now, the mechanism(s) of resistance to FCE 24517 have not yet been clarified. Both increased GSH levels[13,14] and overexpression of the multidrug-resistant (MDR) gene[15] have been detected in cell lines resistant to this drug, coupled with decreased phosphatase activity in resistant cell membranes.[16]

Since the cytotoxic activity of FCE 24517 was markedly reduced in LoVo/DX and MCF/DX cells (with overexpression of the *mdr*-1 gene), it could be hypothesized that treatment with this compound would select a MDR population.

We have isolated a human breast adenocarcinoma cell line selected after repeated *in vitro* treatment with FCE 24517 (MCF-7/24517$_1$). All the characterization experiments with this cell line have been carried out in comparison with the doxorubicin (Doxo)-resistant

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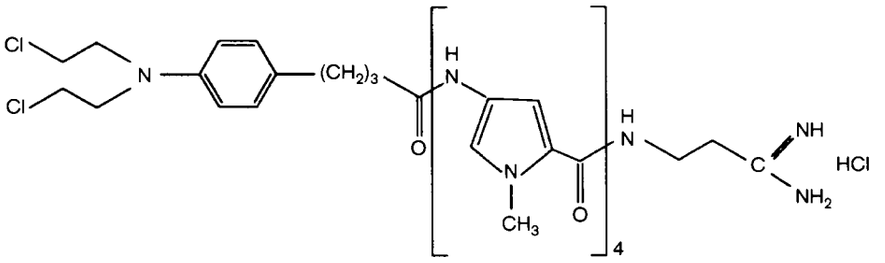
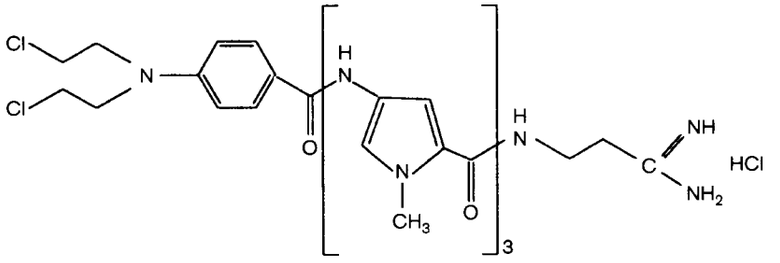


MEN 10710



FCE 24517

Figure 1. Chemical structures of MEN 10710 and FCE 24517.

cell line, MCF-7/DX,[9] which presents the classical MDR phenotype, with overexpression of *mdr*-1 mRNA and strong positivity of monoclonal antibodies directed against gp170.

Materials and methods

Drugs and reagents

FCE 24517 (3-[1-methyl-4-[1-methyl-4-[1-methyl-4-[4-[*N*,*N*-bis (2-chloroethyl) amino]benzene carboxamido]-pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]-propionamidine hydrochloride) and MEN 10710 (3-[1-methyl-4-[1-methyl-4-[1-methyl-4-[1-methyl-4-[4-[*N*,*N*-bis(2-chloroethyl) amino]benzene butanamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propionamidine hydrochloride) were synthesized in the Chemistry Department of Menarini Ricerche (Pomezia, Italy).

Their chemical characterization and purity were assessed by comparing spectral data for FCE 24517 with those reported in the literature[7] and by NMR for the new compound. Etoposide, *m*-AMSA and melphalan were purchased from Sigma (St Louis, MO). Doxo was commercially available.

RPMI 1640, L-glutamine, Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS) and colcemid were obtained from Gibco (Grand Island, NY). Fetal bovine serum was purchased from ICN-Flow Laboratories (Paisley, UK). NADPH, DTNB (5,5'-dithiobis-2-nitrobenzoic acid), Glutathione reductase, oxided glutathione, crystal violet stain and hematoxylin were purchased from Sigma. Giemsa was purchased from Carlo Erba (Milan, Italy). RNAzol B kit was from Biotech (Houston, TX).The pHDR5A plasmid was kindly provided by Dr Ira Pastan (Bethesda, MD). Hybridization solutions and nylon membranes were from Oncor (Gaithersburg, MD).

Cell lines

The human mammary carcinoma MCF-7 and its related resistant line MCF-7/DX were kindly provided by Dr Zunino (Istituto Nazionale Tumori, Milan). All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and 1% L-glutamine 200 mM.

MCF-7/DX cells were maintained in the presence of 100 ng/ml Doxo. Doxo was removed from the culture medium at least one passage before the experiment.

Growth rate

Doubling times were evaluated by seeding 1×10^4 cells into 60 mm plastic wells. Cell growth was monitored daily for 7 days by counting the cells starting 24 h after plating.

Cytotoxicity (colony-forming efficiency)

Cells were seeded in 60 mm plastic dishes at a concentration of 1.5×10^3 cells dish. After 24 h of incubation, cells were treated with the drugs (or vehicle) for an additional 24 h, washed with PBS and fresh medium was added. Colonies were coloured with crystal violet stain after 10–13 days incubation at 37°C. Colonies with 50 or more cells/colony were counted by eye by colony counter quartz (PBI). Cytotoxicity of Doxo, FCE 24517 and MEN 10710 was tested in the range 0.8–100 nM; melphalan was tested in the range 12.5–800 nM. The concentration (nM) inhibiting the growth of colonies by 50% (IC_{50}) was calculated from concentration-response curves.

Cytogenetic analysis

Cells were plated 18–22 h before incubation with colcemid at 0.6 μg/ml for 1 h in culture medium and trypsinization. Following hypotonic treatment (KCl 0.56%) for 8 min at room temperature, cells were centrifuged and the cell pellet was initially fixed in 4:1 methanol:acetic acid for 10 min. The preparation was centrifuged and resuspended in fresh fixative, then dropped onto slides which were air-dried and stained with Giemsa. At least 50 metaphases were analyzed for each cell line.

mdr-1 mRNA expression

Total cellular RNA was extracted by the method of Chomczynski and Sacchi[17] by acid guanidium thiocyanate-phenol-chloroform extraction (RNAzol B kit). For Northern blot analysis 20 μg of total RNA was fractionated on 1% formaldehyde agarose gel and transferred to nylon membranes. The filters were hybridized for 16 h at 42°C in solution for hybridization, containing 10^6 c.p.m/ml of denatured ^{32}P-labeled probe. After hybridization the filters were washed sequentially twice in 1 × SSC (0.15 M sodium chloride and 0.017 M sodium citrate)/0.1% SDS (sodium dodecyl sulfate) at room temperature and once in 0.2 × SSC/0.1% SDS at 65°C and then were exposed to autoradiography for 5 days. The probes utilized were the 1.4 kb *Eco*RI insert of pHDR5A containing the human *mdr*-1 gene and the human β-actin oligonucleotide.[18]

P-glycoprotein determinations

The expression of the gp170 glycoprotein was detected with the monoclonal antibody MM 4.17 which recognizes an extracellular MDR1 P-glycoprotein epitope.[19]

Flow-cytometry determinations were performed by standard procedures using FITC-conjugated $F(ab')_2$ goat anti-mouse IgG to reveal monoclonal antibody binding.[20] After staining, cells were fixed in 1% formaldehyde in PBS pH 7.2, and analyzed on a bench-top flow cytometer (FACSscan; Becton Dickinson, Mountain View, CA). Fluorescence signals were collected in logarithmic mode, relative cell numbers per channel in linear mode.

GSH determination

Exponentially growing cells (1×10^6 cells) were gently harvested, counted, centrifuged and resuspended in 1 ml of sulfosalicylic acid (0.6%), and sonicated at low power.

A crude cytosolic fraction was obtained by centrifugation at 6000 r.p.m. for 5 min in an Eppendorf microcentrifuge.

The total GSH content was measured as described by Tietze[21] mixing in the spectrometer cuvette 50 μ of sample, 100 μl of 0.6 mM of Ellman reagent (DTNB), then adding 10 μg of glutathione reductase and 700 μl of 0.3 mM NADPH. Components were dissolved in phosphate-EDTA buffer (pH 7.5). The rate of reaction at 25°C was expressed as the change in absorbancy at 9 min at 412 nm and the GSH concentration was determined with the appropriate calibration scale.

Results

Development of drug resistance

MCF-7 cell line was cultured continuously with FCE 24517 at increasing concentrations of 11 nM (two passages), 44 nM (two passages), 80 nM (two passages), 160 nM (four passages) and 320 nM (three

passages). When resistance was obtained, the MCF-7/24517_1 line was selected after cloning by the limiting dilution method.[22]

The line was 10-fold resistant to the selecting agent. The resistance index (RI) remained unchanged up to 18 passages without the drug (Figure 2). MCF-7/24517_1 cells were maintained in 10 nM FCE 24517. One passage before the experiment, the drug was removed from the culture medium.

Biological characteristics of MCF-7/24517_1 clone

MCF-7/24517_1 clone was studied for its morphological appearance, growth pattern and chromosomal characteristics. The resistant clone preserved an epithelial morphology (not shown).

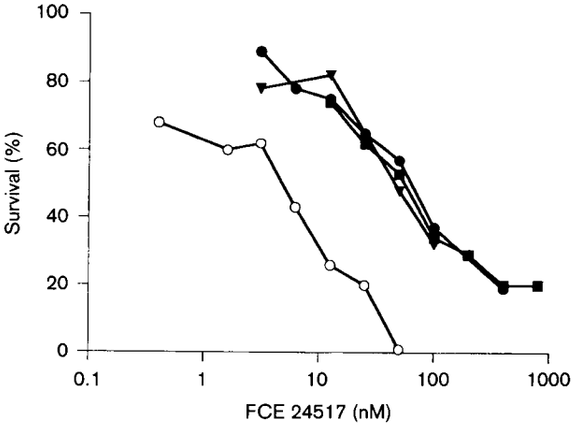


Figure 2. Concentration–response curve for the cytotoxic activity of FCE 24517 on the wild-type MCF-7 cell line (○) and on the selected clone MCF-7/24517_1 after 7 (▲), 15 (■) and 18 (●) passages without the drug. Each value is the mean of two experiments.

The doubling time was 20 h for MCF-7 cells, and 24 h for MCF-7/24517_1 and MCF-7/Dx. The colony-forming efficiency was 25, 16 and 17% for MCF-7, MCF-7/24517_1 and MCF-7/DX cells, respectively. The average number of chromosomes per metaphase was 57 (range 33-68) in MCF-7 cells, 79 (range 46-111) in MCF-7/DX cells and 107 (range 55-130) in MCF-7/24517_1 cells (data not shown). In the parent line metaphases were always hypotriploides ($<3n$); both resistant cell lines had hypertetraploid metaphases.

Sensitivity of MCF-7/24517_1 cells to cytotoxic agents

The sensitivity of MCF-7/24517_1 cells to the cytotoxic action of several anticancer compounds is shown in Table 1. The highest RI was observed for the selecting agent itself (RI=10), but a moderate resistance was detected for its related compound MEN 10710 (RI=4), the classical alkylating agent melphalan (RI=2) and for topoisomerase inhibitors Doxo and Etoposide (RI=3). No resistance was observed to *m*-AMSA. MCF-7/DX cells were highly resistant to MDR-sensitive drugs such as Doxo and etoposide (RI=140 and 31, respectively), as well as to FCE 24517 (RI=19). Conversely MEN 10710 presented marginal resistance index on MCF-7/24517_1 and MCF-7/DX cells.

mdr-1 mRNA expression

The levels of *mdr*-1 mRNA expression in the three cell lines are shown in Figure 3. The autoradiographic signals indicate that both MCF-7 and MCF-7/24517_1 cells do not express *mdr*-1 mRNA, while a significant expression of this transcript is evident in MCF-7/DX cells.

Table 1. Cytotoxic effect of different drugs on MCF-7, MCF-7/24517_1 and MCF-7/DX

Compound	IC_{50} (nM)[a]				
	MCF-7	MCF-7/24517_1	RI[b]	MCF-7/DX	RI
Doxo	9+0.8	30+5	3	1250+2	140
FCE 24517	6+0.5	60+8	10	136+25	19
m-AMSA	5516+644	4000+1201	0.76	5100+2600	0.8
Etoposide	230+8.5	640+86	3	7100+100	31
MEN 10710	2+0.5	9+1	4	5+2	2
Melphalan	632+168	1316+283	2	1516+100	2.4

[a]Colony-forming efficacy 24 h exposure, IC_{50}+SEM=concentration inhibiting 50% colony formation.
[b]RI=resistance index=IC_{50} on resistant cells/IC_{50} on sensitive cells.

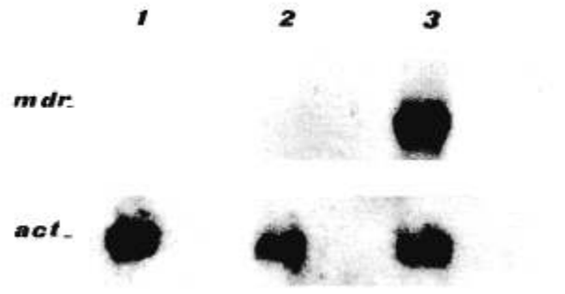


Figure 3. Northern blot analysis of *mdr*-1 mRNA expression, in MCF-7 (line 1), MCF-7/24517$_1$ (line 2) and MCF-7/DX (line 3). The filter was subsequently hybridized to the actin probe to normalize the amount of RNA loaded in each line.

Flow cytometric analysis of *mdr*-1/gp170 expression

The expression of the gp170 glycoprotein was evaluated in the three cell lines by means of flow cytometry and by using an antibody which recognizes an external epitope[19] (Figure 4). The MCF-7/DX[9] and CEM-VBL-100[23] cell lines were employed as positive controls. The results indicate that MCF-7 and MCF-7/24517$_1$ cells do not present the expression of gp170.

GSH determination

Increased levels of GSH have been reported to play a role in the resistance of tumor cells to alkylating agents and anthracyclines.[10-12] It was therefore of interest to determine whether modifications of GSH cellular concentrations existed in the MCF-7/24517$_1$ clone. GSH levels were 8 ± 0.9 nmol/1 $\times 10^6$ cells in MCF-7/DX, 10.6 ± 1.8 nmol/1 $\times 10^6$ cells in MCF-7/24517$_1$ and 4.4×0.4 nmol/1 $\times 10^6$ cells in the parental cell line.

Discussion

We report for the first time the selection, isolation and characterization of a MCF-7 breast adenocarcinoma cell line, selected for resistance to the new antineoplastic alkylating agent, FCE 24517.

A stable clone with a 10-fold resistance to the cytotoxic action of FCE 24517 was obtained and maintained in drug-free medium for more than 18 passages. MCF-7/24517$_1$ cells proved to be only marginally resistant to Doxo (3-fold), etoposide (3-fold) and the classical alkylating agent melphalan (2-fold), but proved to be sensitive to *m*-AMSA. A sizeable resistance (although lower than for FCE 24517) was also detected for the other distamycin derivative MEN 10710 (RI=4).

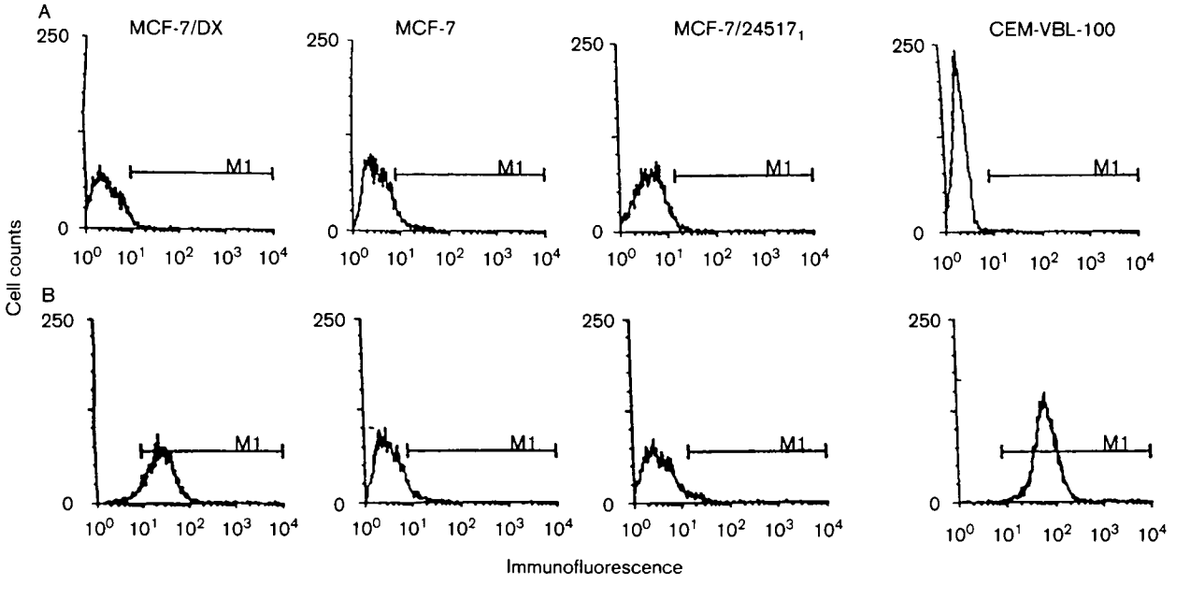


Figure 4. Immunofluorescence distribution of monoclonal antibody *mdr*. MM4.17 in the MCF-7/DX, MCF-7 and MCF-7/24517$_1$ cell lines. Line A represents the negative controls. As a positive control, the immunoreactivity measured in VBL100 cell line is also reported.

The resistant line preserved a normal epithelial morphology; its growth rate was lower than the parental cell line. Interestingly, the karyotype of the resistant line was consistently hypertetraploid, while the parent cell line was usually hypotriploid. The hypotriploid condition is normally present in breast cancer cells like MCF-7,[24] while the hypertetraploid condition has been detected in both types of resistant cells: MCF-7/24517$_1$ and MCF-7/DX.

Previous work proved that exposure to FCE 24517 enabled the isolation of FCE 24517-resistant cell lines in a human colon adenocarcinoma cells (LoVo)[15] and murine leukemia L1210 cells.[13] The LoVo resistant cell line was characterized by a modest (2-fold) expression of gp170; the treatment with revertant agents, such as verapamil or cyclosporin A, could partially reduce the resistance to FCE 24517.[15] Although these were indications that FCE 24517 could be recognized by gp-170-mediated extrusion mechanisms, additional mechanisms of resistance have been postulated to fully explain the high degree of resistance (56-fold) of this cell line.[15] Results presented by Ciomei *et al.*[16] show that both sublines resistant to the antineoplastic agent FCE 24517 present a modification in the tyrosyl-specific phosphatase and kinase balance: L1210/FCE 24517 cells present an increase in the phosphatase activity, whereas LoVo/FCE 24517 cells present a decrease in the kinase activity. This modification is related to a decrease in tyrosine phosphorylation levels of LoVo/FCE 24517 and L1210/FCE 24517 protein extracts.

A balance between tyrosine phosphorylating and dephosphorylating activity seems to be important for the activity of FCE 24517.

The situation in our MCF-7 resistant line to FCE 24517 does not differ from LoVo and L1210 resistant ones, at least regarding the lack of expression of the *mdr*-1 gene (neither as mRNA nor as surface protein) and a slight increase in the cellular GSH content (2.4-fold). All results observed with different resistant cell lines such as LoVo, L1210 and MCF-7 suggest that resistance to FCE 24517 falls into a different category than that most frequently involved in the cell resistance to *mdr* molecules and alkylating agents (overexpression of the *mdr*-1 gene, coding for a gp170 that acts as a drug efflux pump). It is conceivable that the drug resistance is due to a more efficient repair of the DNA damage caused by FCE 24517. In fact, FCE 24517 can alkylate adenine-N3 located in highly specific DNA sequences[24] and therefore an increase in adenine-N3 glycosylases might protect the cells. However, the significant cross-resistance to the parent compound distamycin A, which is not an alkylating agent, conflicts with the latter hypothesis.

It is interesting to note that MEN 10710 exhibits a cross-resistance with FCE 24517, but its cytotoxic potency in the MCF-7/24517$_1$ is markedly superior to FCE 24517, suggesting that this drug is less prone to resistance mechanism(s) operating in these cells. At variance with FCE 24517, MEN 10710 was cytotoxic in MCF-7/DX, suggesting a lack of recognition of this drug by the *mdr*-related pump.

Conclusions

The MCF-7/24517$_1$ clone appears to be a valuable tool for investigating the mode of action and the mechanism(s) of resistance to FCE 24517 and related compounds. This line shows a 10-fold resistance to FCE 24517 and is marginally resistant to Doxo (3-fold), etoposide (3-fold) and the classical alkylating agent melphalan (2-fold). It is characterized by the absence of expression of the MDR protein and an increase in GSH-dependent detoxification mechanism. The latter could concurr to determine the drug resistance but other important mechanisms must be operative.

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