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Lack of cross-resistance to fostriecin in a human small-cell lung carcinoma cell line showing topoisomerase II-related drug resistance

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Summary. Cells exhibiting decreased topoisomerase II (Topo II) activity are resistant to several drugs that require Topo II as an intermediate. These drugs are cytotoxic due to the formation of a cleavable complex between the drug, Topo II and DNA. Fostriecin belongs to a new class of drugs that inhibit Topo II without inducing the formation of this cleavable complex. We tested fostriecin in three human small-cell lung carcinoma cell lines. GLC_4 is the parent line. GLC_4/ADR is the P-glycoprotein-negative multidrug-resistant subline, which is resistant to several Topo II inhibitors due to its decreased Topo II activity. GLC_4/cDDP is the cisplatin-resistant subline, which displays increased Topo II activity. Topo II activity proved to be 100% in GLC_4, 35% in GLC_4/ADR and 130% in GLC_4/cDDP. The fostriecin concentration causing inhibition of the growth of 50% of the cells (IC_{50}) in the microculture tetrazolium assay following continous incubation was 11.2, 4.1 and 14.9 μM, respectively. After 1-h incubations, the IC_{50} was 117.8, 101.3 and 219.8 μM, respectively. Our results indicate a relationship between Topo II activity and fostriecin sensitivity in these closely related cell lines. At least in vitro, fostriecin displayed the capacity to kill cells showing resistance to drugs due to decreased Topo II activity. There was no relationship between this capacity and an increase in the activity of the reduced-folate carrier system, the proposed machanism for cellular entry of fostriecin, since we found no correlation between the cytotoxity of fostriecin and that of methotrexate.

Introduction

The existence or development of drug resistance is a major clinical problem. Basic research has revealed several mechanisms by which tumor cells are capable of surviving exposure to chemotherapeutic drugs [4, 17, 18]. One of these mechanisms is centered around topoisomerase II (Topo II) [9, 16, 25].

Together with topoisomerase I (Topo I), Topo II is a nuclear enzyme that is responsible for the topological handling of DNA [24]. Topo II binds covalently with DNA, cleaves both DNA strands, passes another part of double-stranded DNA through the break and reseals the latter [19, 26]. Several drugs exhibit their cytotoxicity in conjunction with Topo II [20]. Epipodophyllotoxins, anthracyclines, acridines and other agents stabilize the cleavable complex between Topo II and DNA when the breaks are formed. This complex is called the cleavable complex because under protein-denaturing conditions in vitro, it disintegrates, enabling the quantitation of DNA strand breaks. It is the formation of this complex rather than the inhibition of the catalytic activity of Topo II that is cytotoxic [20]. Therefore, cells with decreased Topo II content are less susceptible to Topo II-mediated toxicity. This defense mechanism has been identified in several cell lines [5, 6, 9, 13, 16, 22]. To date, no countermeasures antagonizing this type of resistance have been identified. In some cells, Topo II activity can be increased by estrogens or tumor necrosis factor resulting in an increase in drug sensitivity [1, 8], but this increase respresents an enhancement of sensitivity rather than a reversal of resistance.

In theory, decreased Topo II activity could lead to collateral sensitivity. In most functions, Topo II can be substituted by Topo I, the exception being the segregation of DNA strands at mitosis, which is vital for dividing cells [11]. At least dividing cells that exhibit decreased Topo II activity should therefore be more sensitive to drugs that inhibit the catalytic activity of Topo II. To date, no drug of this class has been described. Cleavable complex-forming drugs inhibit catalytic activity only at high concentrations [25]. Only recently have fostriecin and merbarone been reported to exhibit cytotoxicity and to inhibit Topo II without forming a cleavable complex [2, 7, 14, 21].

To investigate whether decreased Topo II activity renders cells more susceptible to Topo II inhibitors that do not

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form such cleavable complexes, we tested the cytotoxicity of fostriecin in three human small-cell lung cancer (hSCLC) cell lines displaying differing Topo II contents. We used a variant-type cel line, GLC_4, the atypical multidrug-resistant subline GLC_4/ADR and the cisplatin-resistant subline GLC/cDDP [12, 25]. An increase in the sensitivity of the atypical multidrug-resistant subline, which shows decreased Topo II activity, to fostriecin might indicate a new class of cytotoxic drugs. We also tested these cell lines for the cytotoxicity of methotrexate (MTX) because both fostriecin and methotrexate enter the cell via the same reduced-folate carrier [10].

Materials and methods

Drugs and DNA substrates. Fostriecin was generously donated by Parke Davis (Ann Arbor, Mich.). Etoposide (VP16) was obtained from Bristol-Myers SAE (Madrid, Spain) and methotrexate, from Cyanamid (Mont-Saint-Guibert, Belgium). Form I kinetoplast DNA (kDNA) was isolated from the mitochondria of *Crithidia fasciculata* by CsCl/ethidium bromide centrifugation as previously described [13]. A supercoiled dimer of plasmid pBR 322 DNA was prepared from *Escherichia coli* strain HB 101 using the alkaline lysis method and CsCl/ethidium bromide centrifugation [13].

Cell lines. GLC_4 ist an hSCLC cell line that was derived from a pleural effusion and kept in continuous culture in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum. GLC_4/ADR, a subline of the parental line, was made resistant by treatment with stepwise increasing concentrations of Adriamycin [25]. GLC_4/ADR exhibits an atypical multidrug-resistant phenotype involving resistance to vincristine, vindesine, VP16, amsacrine (m-AMSA) and Adriamycin in the absence of overexpression of mdr_1 mRNA and gp170 [13]. GLC_4/cDDP was derived from GLC_4 by long-term continuous incubation with cisplatin until a stable resistance factor of 6.4 was obtained. This cell line has been extensively described elsewhere [12]. The doubling times of GLC_4, GLC_4/ADR and GLC_4/cDDP are 15, 18 and 24 h, respectively.

Drug sensitivity assay. The microculture tetrazolium assay (MTA) was performed as previously described [23]. In brief, exponentially growing cells were incubated in 96-well culture plates with various concentrations of drugs for 4 days. For 1-h experiments, cells were incubated with drug and washed three times with PBS before being seeded in the wells. Incubations involved 5,000 cells/well for GLC_4; 12,500 cells/well for GLC_4/ADR and 15,000 cells/well for GLC_4/cDDP. After 4 days, 20 µl MTA solution [5 mg MTA/ml phosphate-buffered saline (PBS)] was added to each well for 3.5 h. The plates were washed and the formazan crystals were dissolved in 100% dimethyl sulfoxide. The extinction was read using a scanning microtiter-well spectrophotometer (Titertek Multiskan Flow Lab, Irvine, UK). At least four separate experiments were performed in quadruplicate at each concentration tested.

Preparation of nuclear enzyme extracts. Nuclei were isolated as described elsewhere [13]. Briefly, log-phase cells ($3-8 \times 10^5$ cells/ml) were pelleted by centrifugation at 150 *g* for 10 min and washed three times with ice-cold PBS for 10 min at 4° C. The cell pellets were resuspended in 1 ml nucleus buffer [150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM dithiothreitol and 1 mM PMSF, pH 6.4] at 4° C and then mixed with an additional 9 ml nucleus buffer containing 0.3% Triton X-100. The cell suspension was mixed gently by rotation for 10 min at 4° C and then centrifuged at 150 *g* for 10 min at 4° C. The nucleus pellet was washed once with Triton-free nucleus buffer and resuspended in fresh Triton-free nucleus buffer. The nuclei were extracted for 30 min at 4° C after the addition of an equal volume of extraction buffer [50 mM TRIS-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.55 M NaCl, 1 mM dithothreitol, 1 mM PMSF].

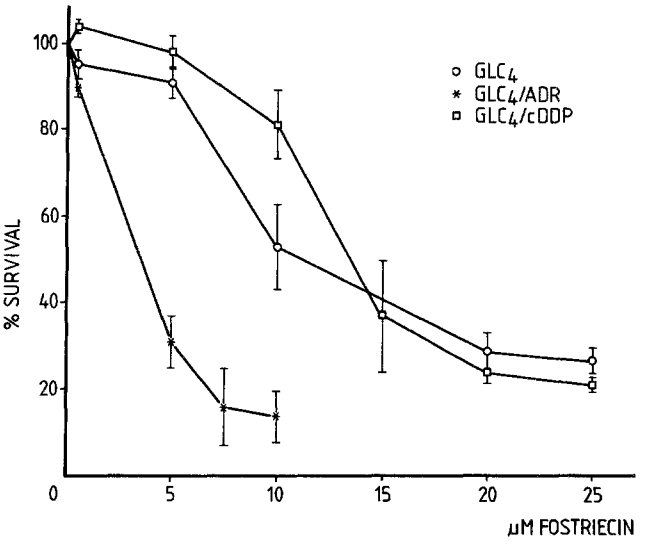

Fig. 1. MTA of fostriecin after continuous incubation. Bars represent SE

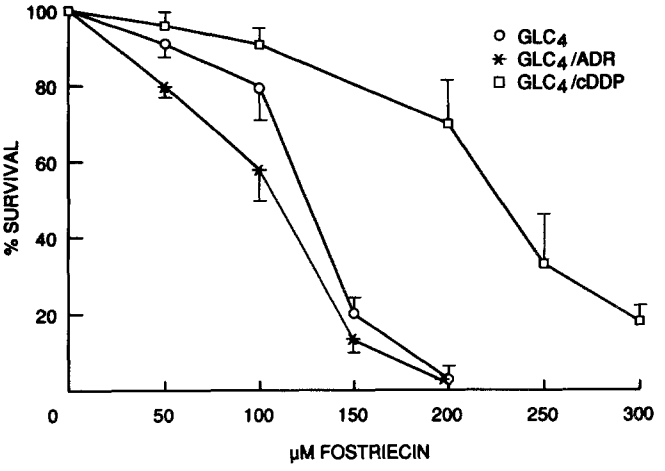

Fig. 2. MTA of fostriecin after 1 h incubation. Bars represent SE

Protein concentrations were determined by the method of Bradford [3]. The enzyme solution was diluted with an equal volume of 87% glycerol and then stored at –20° C.

Decatenation assay. Topoisomerase II activity was determined using the decatenation assay [13]. The standard reaction mixture for the decatenation assay was 50 mM TRIS-HCl (pH 7.5), 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, bovine serum albumin (0.03 mg/ml) and 1 mM adenosine 5′-triphosphate (ATP). Decatenation of kDNA was carried out by incubating 5 µl of a serial dilution of a nuclear extract with 0.1 µg kDNA in a final volume of 25 µl standard reaction mixture for 30 min at 378°C. Reactions were terminated by the addition of 5 µl sodium dodecyl sulfate (SDS), 0.3% bromophenol blue and 30% glycerol. Samples were then electrophoresed in 1% agarose in 89 mM TRIS-borate and 2 mM EDTA (pH 8.3) at 35 V for 4 h. Gels were stained with ethidium bromide (1 µg/ml) for 45 min and destained for 2–3 h in H_2O. DNA bands were visualized by transillumination with UV light and were phtotographed using Polaroid type 665 positive/negative films [13]. Relative Topo II activity was estimated by scanning the photographic negatives (LKB Ultro Scan XL Laser densitometer) and determining the dilution at which 50% of the minicircles were released. This was corrected for the protein concentration. The relative decatenating activity of GLC_4 was considered to be 100% [9, 13].

Table 1. Cytotoxicity of fostriecin and VP16 and relative Topo II activity in three hSCLC cell lines

Cell line	Fostriecin IC_{50} (μM)[a]	Fostriecin IC_{50} (μM)[b]	VP16 IC_{50} (μM)	MTX IC_{50}	Relative Topo II activity
GLC_4	11.2 (3.8)	117.8 (4.3)	0.159 (0.025)	24.7 (12.9)	100%
GLC_4/ADR	4.1 (0.8)	101.3 (12.6)	10.2 (2.4)	63.7 (16.9)	35% (9%)
GLC_4/cDDP	14.9 (3.7)	219.8 (32)	0.106 (0.02)	35.8 (9.5)	130% (11%)

[a] Continuous incubation
[b] 1-h incubation
Standard deviation are shown in parentheses

Statistical analysis. All P values were calculated using Student's t-test. Avalue of $P < 0.05$ was considered to be significant.

Results

Cytotoxicity

The results of five idependent MTAs using continuous incubation of drug are shown in Fig. 1. The concentrations required for cytotoxicity lay in the same range previously described by other ivenstigators [7, 9, 21]. The results of four independent MTAs using 1-h incubations are shown in Fig. 2. The drug concentrations causing inhibition of the growth of 50% of the cells (IC_{50} values) shown in Table 1 were determined by intrapolation of the individual experiments. As compared with GLC_4, GLC_4/ADR displayed an increased IC_{50} for VP16 ($P < 0.001$). GLC_4/cDDP exhibited a slight but significant decrease in IC_{50} for VP16 ($P < 0.02$) and was therefore collaterally sensitive to VP16. As compared with GLC_4, GLC_4/ADR showed sensitivity to fostriecin ($P < 0.001$). In contrast, GLC_4/cDDP displayed cross-resistance to fostriecin as compared with GLC_4 ($P < 0.01$) following continuous incubation. This cross-resistance was also found after 1 h incubation with fostriecin ($P < 0.001$; GLC_4/cDDP vs GLC_4). However, the collateral sensitivity of GLC_4/ADR did not reach significance in the 1-h incubation assay ($0.05 < P < 0.1$). The GLC_4/ADR subline exhibited increased ($P < 0.005$) MTX resistance as compared with GLC_4 and GLC_4/cDDP following continuous incubation.

Quantification of Topo II activity

The results of three idependent isolation and Topo II-activity determination procedures are shown in Table 1. As compared with GLC_4, GLC_4/cDDP displayed increased Topo II activity ($P < 0.05$) and GLC_4/ADR exhibited decreased Topo II activity ($P < 0.001$).

Discussion

In the hSCLC cell line and sublines used in the present study, fostriecin was cytotoxic at concentrations comparable with those previously found to be toxic in other cell lines [7, 9, 21]. This model seems to support our assumption that cells with decreased Topo II activity should be more suspectible to fostriecin. The line with the lowest Topo II activity was found to be most sensitive to fostriecin. This relationship is the opposite of that between VP16 cytotoxicity and Topo II activity. It therefore appears that the mechanism of action does not involve the transformation of Topo II into a cellular poison [15]. In the 1-h experiments, the same pattern of fostriecin sensitivity was found. However, the significance of the increase in sensitivity to fostriecin observed in GLC_4/ADR is unclear. It is possible that Topo II inhibition, like fostriecin cytotoxicity, requires a longer incubation period to reach its optimal level. This might reflect another cytotoxic mechanism. In contrast to cleavable complex-forming drugs, which induce an irreversible cascade leading to cell death, enzyme inhibition might be necessary for a given period. The results are compatible with fostriecin's acting as an inhibitor of the enzyme. The step in the enzymatic pathway that is inhibited remains to be elucidated.

Resistance mechanisms are complex and many different factors are involved. For instance, the doubling time of a cell line has an influence on its sensitivity to Topo II inhibitors. It has also been reported that fostriecin enters the cell via the reduced-folate carrier [10]. The well-known methotrexate-resistance mechanism based on a decrease in the level of carrier would therefore also result in fostriecin resistance. In the present study, both of these mechanisms could be excluded based on the estimated doubling times and the methotrexate sensitivity exhibited by the cells, respectively. However, numerous other resistance mechanisms are known that may also play a role in fostriecin resistance, which renders the extrapolation of the present results to other cell lines difficult. Studies on larger group of closely related cell lines that ideally differ only in Topo II activity could confirm the reverse sensitivity/resistance relationship between fostriecin and other Topo II-directed drugs.

A different mechanism of action for fostriecin that would result in collateral sensitivity could prove to be of value for the treatment of tumors exhibiting acquired or intrinsic Topo II-related resistance. Fostriecin is a promising compound that deserves further pharmacological and clinical evaluation.

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