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Characterization of Drug Resistance to Topoisomerase II Poison

Chun-Mao Lin[1], Leng-Fang Wang[1], Shih-Hao Huang[2], and Yih-Chih Tung[1]

ABSTRACT

Human ovarian carcinoma A2780 cells resistant to VM-26 were cloned. Difference in two dimensional gel electrophoresis patterns between parental and resistant cells might imply an alternative pathway of cell-killing process developed in resistant cells. DNA strand passing activity of topoisomerase II affected by VM-26 was measured by K-SDS precipitation, The results showed that VM-26 influence on topoisomerase II cleavable activity was much less in resistant cells. Alteration of drug targeting site in topoisomerase II might be a factor contributing to VM-26 drug resistance in our resistant cells.

Key words: topoisomerase, drug resistance, VM-26

DNA topoisomerases have been identified as important therapeutic targets in cancer chemotherapy[1-3]. Camptothecin is a topoisomerase I-targeting antitumor drug, and VM-26 (teniposide) is a topoisomerase II-targeting antitumor drug[4].The identification of topoisomerases as antitumor therapeutic targets has offered new insights into the possible cell killing mechanisms of anticancer drugs such as VM-26. The cytotoxic action of VM-26 and VP-16 is initiated by trapping the topoisomerase II-DNA cleavable complex, followed by induction of a possible SOS-like response that leads to cell death [5]. Although treatment with these drugs has resulted in the amelioration of some types of human cancer, yet cancer patients always develop multiple drug resistance after a certain period of drug treatment. The development of drug resistance appears to be a major impediment to the successful chemotherapy of human tumor. Thus, identifying the mechanisms that confer drug resistance would help us to resolve problems in cancer chemotherapy.

The purpose of this study was to isolate and characterize VM-26 resistant cells, and through the study of a variety of characteristic changes between parental and drug-resistant cells, we hope to uncover the possible mechanisms of cellular resistance to VM-26.

MATERIALS AND METHODS

Materials. VM-26 (Figure 1) is a gift from Dr. Jaulang Hwang. Cell culture medium, serum,

1. Department of Biochemistry, School of Medicine, Taipei Medical College
2. Department of Sea Food processing, Junior College of China Marine Technology
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and chemicals (penicillin, streptomycin, and glutamine) were purchased from Gibco. Radioisotopes (^{3}H-thymidine and ^{35}S-methionine) were from Amersham. Other chemicals or buffer reagents were from either Merck or Sigma.

Cell culture. Human ovarian A2780 cells were cultured using RPMI-1640 medium containing 10% fetal bovine serum, penicillin /streptomycin (50 IU/ml and 50 μg/ml, respectively), and 1 mM glutamine in the presence of 5% CO_2 at 37°C.

Selection of VM-26 (teniposide) resistant cells. 10 ng/ml of VM-26 (M.W.=656.67) was supplemented in culture medium at the initial stage until resistant colonies were formed. The resistant colonies were trypsinized and enriched, then stepwise increased VM-26 concentration in medium to 40 ng/ml.

Two-dimensional gel electrophoresis. Cells were plated at a density of 1.0 X 10^{6} cells/60mm dish in fresh RPMI medium containing 10% fetal bovine serum. The following day, cells were labeled in 2 ml of methionine-free modified Eagles' medium containing 10μCi/ml of ^{35}S-methionine and 5% FBS for 4-6 hours. The cells were collected and lysed in isoelectric focusing (IEF) lysis buffer (9.2 M urea, 2.0 % Nonidet P-40) and a mixture of ampholines (0.8% of pH 5-8 and 0.2% of pH 3.5-10). The radioactivity was determined by trichloroacetate precipitation and scintillation counting. 2.0 X 10^{6} cpm of lysate was loaded for each sample. Two dimentional electrophoresis was carried out according to O'Farrel[(6-7)]. SDS-PAGE was performed for second dimensional electrophoresis. The electrophoresed gel was soaked in PPO solution (20% 2,5-diphenyl oxazole in dimethyl sulfoxide) for 15 min after treatment with DMSO for three time to remove water in gel. Gel was washed three time in water for 20 min each before drying and then autoradiographed.

KCl/SDS precipitation assay of trapped topoisomerase II-DNA complex by VM-26 treatment. The in vivo formation of covalent topoisomerase II-DNA complex was measured using the K-SDS precipitation assay [(8-9)]. Cellular DNA was labeled by adding ^{3}H-thymidine into the medium to a final concentration of 10 μCi/ml. After an overnight incubation, cells were plated to a density of 1 X 10^{5} cells/well in a 24-well plate for another overnight incubation, and treated with various concentrations of VM-26 for 60 min. The medium was removed from each well and cells were washed with PBS and lysed with 1 ml of prewarmed (65°C) lysis solution (1.25% SDS, 5 mM EDTA, 0.4 mg/ml salmon sperm DNA). Lysate was transferred to a centrifuge tube and sheared using a 21-gauge

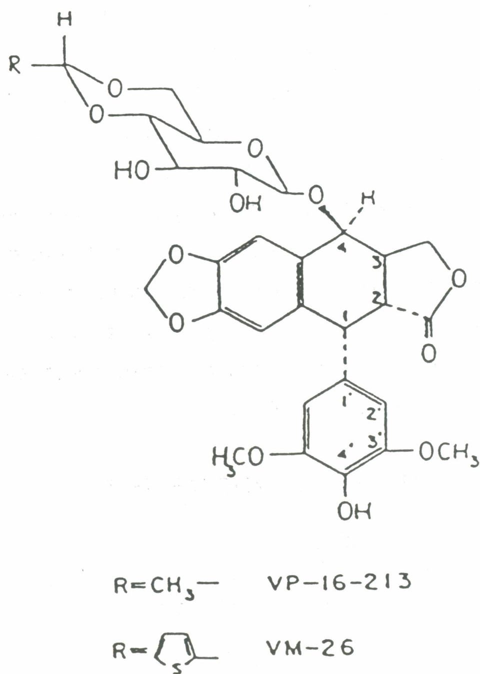

Fig. 1. Structure of antitumor drug VM-26 and VP-16

needle. The samples as background control were performed as the above procedure but with proteinase K (400μg/ml) in the lysis buffer, and incubated at 50°C for 2 hours.250 μl of KCl (325 mM) was added to each sample, vortexed vigorously, cooled on ice for 10 min, and centrifuged at 2500 rpm in a Beckman RT6000 centrifuge for 10 min at 4°C. The pellet was washed twice in 1 ml wash solution (10 mM Tris-HCl, 100 mM KCl, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA) and incubated at 65°C for 10 min, cooled on ice, then centrifuged at 2500 rpm for 10 min. The pellet was resuspended in 400 μl prewarmed H_2O (65°C), combined with 4 ml scintillation liquid, and radioactivity counts determined.

RESULTS

So far we have obtained sublines resistant up to concentration of 40 ng/ml of VM-26. The RNA and protein levels of topoisomerase II remain fairly constant in resistant cells. The data might imply that topoisomerase II had been mutated, therefore VM-26 could no longer recognize its target.

Difference in Two Dimensional Gel Electrophoretic Patterns between Parental and Resistant Cells. After the trapping of topoisomerase II-DNA complex by VM-26, certain responses such as DNA repair, SOS-like response, stress shock protein expression, and increase in GST might be induced, and subsequently cell death would occur. If defect in these procedures should occur in resistant cells, then VM-26 would fail to induce cell death. We, therefore, carried out two dimensional gel electrophoresis to compare the total protein patterns between parental cells, parental cells treated with VM-26, resistant cells, and resistant cells treated with VM-26, in order to see if new proteins were synthesized. The acidic protein patterns (equilibrium electrophoresis) did not show any change upon VM-26 treatment for 48 hr between parental and resistant cells (Figure 2). But, the basic protein patterns (non-equilibrium electrophoresis) showed some changes as indicated by arrows. After VM-26 treatment, in the parental cells, a set of proteins on the left hand side of the gel almost disappeared, and another set of proteins on the lower right hand side of the gel increased drastically (Figure 2A, B). However, no such noticeable changes in protein expression could be observed in the VM-26 resistant cells (Figure 2C, D). These results alone cannot enable us to conclude that these protein changes are involved in the above mentioned cell-killing process. However, we did show that, after treatment with VM-26, changes in protein expression pattern did occur; but the same changes did not occur in the resistant cells.

Topoisomerase II-DNA Complex Trapping Activity in Resistant Cells. KCl/SDS can precipitate protein, but not DNA except when it is linked to protein, Therefore, the amount of precipitated DNA reflects the VM-26 trapping activity[8-9]. ^{3}H-thymidine labeled cells were treated with VM-26, and in vivo K-SDS precipitation was performed. In parental cells, the DNA trapped by VM-26 was approximatly 7% at 15.0 μg/ml of the drug. In two sublines of resistant cells, 20 ng/ml and 40 ng/ml resistant towards VM-26, the DNA trapped by VM-26 decreased to 3% (Figure 3). These results indicate that the ability of VM-26 causing the formation of topoisomerase II-DNA complex has decreased in the VM-26 resistant cells. Therefore, it is likely that the VM-26 target site in the topoisomerase II of resistant cells has been mutated, and hence, a decrease in binding

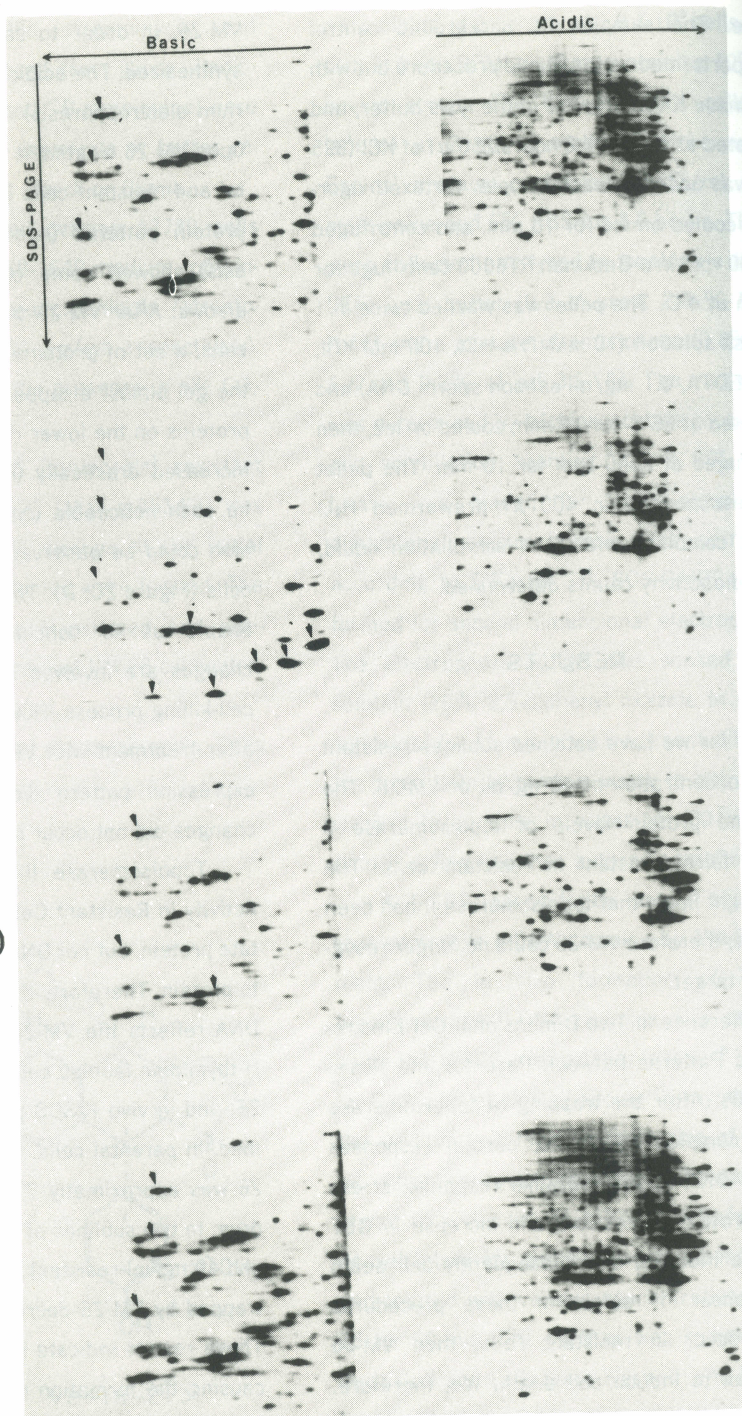

Fig. 2. Two-dimensional gel electrophoresis patterns in parental and resistant cells with or without VM-26 treatment. (A) Parental A2780 cells. (B) Parental cells after treatment with 80 ng/ml of VM-26 for 48 hr. (C) VM-26resistant cells. (D) VM-26 resistant cells after treatment with 80 ng/ml of VM-26 for 48 hr. Arrows highlight the differences in protein pattern between parental and resistant cells.

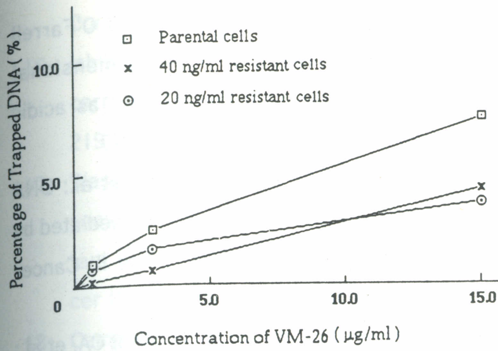

Fig. 3. Topoisomerase II-DNA complex trapping activity of VM-26. In vivo K-SDS precipitation shows that the trapped topoisomerase II-DNA complex by VM-26in resistant cells (20, 40 ng/ml) is less than that in parental cells.

of VM-26 to this site.

DISCUSSION

Several mechanisms that may contribute to drug resistance have been considered : (I) overexpression of membrane protein p170 which can pump drugs out of cell [10-12], (II) overexpression of glutathion S-transferase activity that can increase drug degradation rate[13-16], (III) alteration of drug target site in topoisomerase II[17-18], (IV) defect in cell-killing process, (V) decrease of topoisomerase expression[19-21], and (VI) gene rearrangement leads to topoisomerase alteration[22-23].

We have cloned human ovarian carcinoma A 2780 cells that are resistant to VM-26, and we concentrated most of our analyses on the resistant sublines that have developed stable resistance towards 20 ng/ml, and 40 ng/ml of VM-26. Therefore, the resistance of this subline towards VM-26 is much more specific. The mRNA and protein levels of topoisomerases II stayed very much constant in parental and resistant cells during VM-26 treatment. Therefore, we think that the reduction of transcriptional activity affected by VM-26 and other drugs cannot fully explain the resistance in our resistant cells and their specificity towards VM-26. The basic protein patterns in two dimensional gel electrophoresis of untreated parental cells, VM-26 treated parental cells, untreated resistant cells, and VM-26 treated resistant cells show that differences exist only in the VM-26 treated parental cells. The newly synthesized proteins as well as the proteins that gradually disappeared in the parental cells during treatment with VM-26 may be a consequence of the switching on of certain cellular responses such as DNA repair, or SOS-like phenomenon, which then lead to the killing of the parental cells. But, as VM-26 resistance developed, defects in the aforementioned cellular responses occurred and cell death was avoided. We cannot rule out that these defects in the cell killing process may be another possible mechanism of drug resistance in our VM-26 resistant cells.

Basically, we do not think that one single factor would give rise to drug resistance during cancer chemotherapy. Rather, a synergistic effect of multiple factors such as MDR, drug metabolism, defects in drug-target, cell-killing processes, decrease of topoisomerase activity, and gene rearrangement could result in the appearance of drug resistance. We would like to think that defects in drug-target and cell-killing processes might play important roles in the development of drug resistance during chemotherapy. A better understanding of all the mechanisms involved would help to overcome or prevent resistance to topoisomerase II - targeting drugs. Then, in the K-SDS precipitation experiment, the topoisomerase II-DNA complex trapping activity of the two VM-26 resistant sublines (20 ng/ml, 40 ng/ml) were only 50% that of the

parental cells at very high VM-26 concentration (15 μg/ml). Therefore, it is now apparent that the VM-26 resistance of our resistant sublines may not come from changes in enzyme activity decrease in drug accumulation, but from the alteration of the topoisomerase II protein itself. We propose that the VM-26 resistance in our resistant subline may come from the alteration of the VM-26 target site in the topoisomerase II protein , and this alteration may make VM-26 failed to react with topoisomerase II. The next step in this work is to locate and characterize the mutation site in the topoisomerase II protein.

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第二型拓撲異構酵素毒素之抗藥性探討

林俊茂[1]　汪棱芳[1]　黃世浩[2]　董一致[1]

人類卵巢癌細胞抗藥株已被篩選。由雙向電泳圖譜可發現抗藥株經 VM-26 處理後的細胞蛋白質表現有明顯不同。其可能係抗藥株發展出抗細胞致死機制。由 K-SDS 沈澱試驗檢測 VM-26 對第二型拓撲酵素去氧核糖核酸交錯活性之影響，結果顯示，抗藥株所受到的影響遠小於原母細胞株。因此，在我們所篩到的抗藥株中，第二型拓撲異構酵素上的 VM-26 目標位置發生改變，是產生抗藥性的因素之一。

1.台北醫學院　生化學科
2.中國海事專科學校　水產食品工業科

民國八十三年十一月十日受理