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Etoposide-resistant human colon and lung adenocarcinoma cell lines exhibit sensitivity to homoharringtonine

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Abstract. Human colon (HCT116/VP48) and lung (A549B/VP29) adenocarcinoma cell lines selected for resistance to etoposide exhibited modified patterns of multidrug resistance (MDR) that included a differential sensitivity to other DNA topoisomerase II inhibitors and to the plant alkaloids homoharringtonine, vinblastine, and vincristine. The resistance and cross-resistance drug phenotype of the A549B/VP29 cell line was different from that of the HCT116/VP48 cell line. The HCT116/VP48 cell line was 50-fold resistant to etoposide and 30-fold resistant to teniposide. The degree of resistance to other DNA topoisomerase II inhibitors was of a lower magnitude: Adriamycin, 9-fold; daunomycin, 3-fold; 4′-[(9-acridinyl)-amino]-methanesulfone-*m*-anisidide (m-AMSA), 3-fold; and actinomycin D, 6-fold. The HCT 116/VP48 cell line exhibited a 7-fold resistance to vincristine and a 2-fold resistance to vinblastine but was sensitive to homoharringtonine. The A549B/VP29 cell line was 5-fold resistant to etoposide and 2-fold resistant to teniposide. The A549B/VP29 cell line exhibited a 2-fold resistance to Adriamycin but was sensitive to daunomycin and showed a 3-fold resistance to m-AMSA. This cell line was sensitive to actinomycin D. The A549B/VP29 cell line was 2-fold resistant to vinblastine and sensitive to homoharringtonine. Both cell lines (HCT116/VP48 and A549/VP29) exhibited no amplification of the human *mdr1* DNA sequence, the 4.3-kb P-glycoprotein transcript, or the membrane P-glycoprotein. The sensitivity of cells exhibiting an MDR phenotype not mediated by P-glycoprotein suggests a potential use for homoharringtonine in treating tumors with this type of drug resistance.

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Introduction

Clinical activity reported from China prompted the National Cancer Institute to assess homoharringtonine in the United States [22]. Homoharringtonine has antitumor activity that is equal to that of harringtonine and occurs in greater abundance among the active cephalotaxines [22]. Homoharringtonine has shown antineoplastic activity in leukemia and solid tumors in a number of experimental and clinical studies [2, 5, 21, 29, 33].

The observation that cross-resistance to currently available antileukemic agents may not be complete [33] may be consistent with homoharringtonine's unique structure and mechanism of action [22]. Homoharringtonine is a potent inhibitor of eukaryotic protein synthesis [8, 10, 28]. However, Chou et al. [5] reported that sublines of murine leukemia L1210 resistant to vincristine or Adriamycin appeared insensitive to harringtonine in chemotherapy studies in vivo; on the other hand, a subline of L1210 cells resistant to 1-β-D-arabinofuranosylcytosine was sensitive to harringtonine. Wilkoff et al. [34] reported that mice bearing murine leukemia P388 tumors resistant to vincristine or Adriamycin were cross-resistant to optimal treatment with homoharringtonine. Furthermore, mice bearing leukemia P388 tumors resistant to 1-β-D-arabinofuranosylcytosine were collaterally sensitive to optimal treatment with homoharringtonine. Proliferating cultured cell populations derived from these tumors exhibited either significant cross-resistance to homoharringtonine (P388 tumors resistant to Adriamycin or vincristine) or collateral sensitivity to this agent (P388 tumors resistant to 1-β-D-arabinofuranosylcytosine). These results indicated that cross-resistance or collateral sensitivity to homoharringtonine was primarily a function of the cellular properties of the target P388 cell populations that was independent of host factors. Shoemaker et al. [26] have reported that murine leukemia P388 tumors resistant to Adriamycin or vincristine are multidrug-resistant and express P-glycoprotein. Tebbi et al. [30] reported that a neuroblastoma cell line resistant to homoharringtonine was cross-resistant to Adriamycin, vincristine, vinblastine, melphalan, and lomustine. The ho-

moharringtonine-resistant line exhibited an increased expression of P-glycoprotein and decreased cellular accumulation of homoharringtonine. Treatment with cyclosporin A and dipyridamole completely restored the cytotoxic effect of homoharringtonine and Adriamycin.

We were interested in determining if cells not expressing elevated levels of P-glycoprotein were capable of exhibiting a degree of resistance to homoharringtonine and the vinca alkaloids. Since cultured cells selected for resistance to the semisynthetic epipodophyllotoxins may not express elevated levels of P-glycoprotein [36], we obtained etoposide-resistant human colon and lung adenocarcinoma cell lines [15, 18] for these studies.

The results of the current study indicate that both human colon and lung adenocarcinoma cell lines selected for resistance to etoposide exhibited modified patterns of multidrug resistance (MDR) that included a differential sensitivity to DNA topoisomerase II inhibitors and to the plant alkaloid homoharringtonine and the vinca alkaloids. The resistance and cross-resistance drug phenotype of the human lung adenocarcinoma cell line was different from that of the human colon adenocarcinoma cell line.

Materials and methods

Cell lines. A human colon adenocarcinoma cell line resistant to etoposide (HCT116/VP48) and its parent cell line HCT116 [3, 35] as well as a human lung adenocarcinoma cell line resistant to etoposide (A549B/VP29) together with its parent cell line A549B [16, 17] were received from Dr. Michael G. Brattain, Baylor College of Medicine, Houston, Texas; the etoposide-resistant cell population was selected from the parental line HCT116 in his laboratory by repeated exposure to etoposide using the following procedure (Dr. M. G. Brattein, personal communication). HCT116 cells in log-phase growth were exposed for 1 h to 1 μM of etoposide. The treated cell population was cultured in fresh medium until surviving cells resumed log-phase growth, at which time the cells were passaged and the etoposide treatment cycle was repeated. The HCT116/VP48 cell population was derived by 48 treatment cycles of HCT116 cells carried out at weekly intervals prior to passage of the resistant cells without etoposide exposure. An etoposide-resistant human lung adenocarcinoma cell line was derived from the parental line A549B using the same procedure. The A549B/VP29 cell population was derived by 29 treatment cycles of A549B cells carried out at weekly intervals prior to passage of the resistant cells without etoposide exposure. This resistance is stable and does not diminish in continuous culture of these cells [18].

The cell lines were propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 µg gentamicin/ml. The doubling times of the cell populations (mean values ±SD) were 32.6±0.9 h (HCT116/VP48), 29.8±2.6 h (HCT116), 31.1±1.9 h (A549/VP29), and 22.0±1.6 h (A549B) under our experimental conditions.

Assay of 48-h growth inhibition. Cells were seeded at a density of 1.0×10^6 cells/5 ml medium in 25-cm^2 tissue-culture flasks at 24 h prior to the addition of the drug. At 24 h, the medium was decanted and medium containing drug was added to the cell monolayers. The solvent consisted of the appropriate concentration of solvent in the medium and was used as the cell control. The cells were harvested after 48 h with the use of 0.25% TRL (Worthington's purified trypsin)-0.2% ethylenediaminetetraacetic acid (EDTA; 2 ml/flask), sedimented, washed with culture medium, and resuspended in cell-culture medium. The cells were counted using a hemacytometer. Each drug was evaluated using a series of five to six concentrations, three flasks per concentration, and the LC_{50} value (the concentration that inhibits the growth of a test culture by 50% relative to the growth of a control culture) was determined. The mean LC_{50} value for each drug was based on three separate determinations. When the sensitivities of the HCT116/VP48 and HCT116 or the A549B/VP29 and A649B cell populations were compared, the resistant and parent (drug-sensitive) cell populations were evaluated in parallel in the same experiment. The statistical significance of the difference between the means of each population (drug-resistant and drug-sensitive) was determined using the standard Student's *t*-test.

Homoharringtonine was dissolved in 1 *N* HCl, adjusted to neutrality with 1 *N* NaOH, and diluted to selected concentrations with cell-culture medium. Vincristine, vinblastine, Adriamycin, and daunomycin were dissolved in sterile distilled and deionized water and diluted with cell-culture medium to selected concentrations. Actinomycin D, etoposide, teniposide, and m-AMSA were dissolved in a minimal volume of *N,N*-dimethylformamide (DMF) and then diluted with cell-culture medium to selected concentrations. The final DMF concentration was 0.025% in the cell-culture medium.

Hybridization of DNA and RNA. Genomic DNA was isolated from drug-resistant and -sensitive cell populations by a previously described method [20]. Total cellular RNA was isolated by the guanidinium/hot phenol method [7]. Genomic DNA was digested with *Bam*H1, loaded onto a 0.8% agarose gel and resolved by electrophoresis. The RNA was treated with glyoxal before resolution by electrophoresis through a 1.5% agarose gel with 0.01 *M* sodium phosphate buffer (pH 8.0) [31]. The nucleic acids were transferred to nitrocellulose paper and baked for 2 h in vacuo at 80° C. Filters were prehybridized and hybridized to ^{32}P-labeled probes as described elsewhere [20]. The human *mdr1* DNA probe [24] was obtained from Dr. Igor Roninson, University of Illinois, Chicago, Illinois, and was labelled to a specific activity of 1.4×10^8 cpm/µg with [α-^{32}P]-deoxycytidine triphosphate (dCTP) using Amersham's multiprime labeling kit.

Western-blot analysis. Polyclonal antibody to P-glycoprotein was obtained through the courtesy of Dr. Marion Meyers, Memorial Sloan-Kettering Cancer Center, New York. Electrophoretic transfer blots were prepared as described by Towbin et al. [32]. Cells were lysed in 20 m*M* HEPES buffer (pH 7.2) containing 1% Triton X-100. Aliquots of 100,000-*g* supernatants containing 100 µg protein were subjected to electrophoresis in 7.5% gels and the proteins were transferred to nitrocellulose. After a blocking step in 5% powdered milk in 0.1 *M* TRIS buffer (pH 7.4), the filters were incubated in absorbed polyclonal antibody (1 : 50 dilution in the milk solution). Detection of antigens was completed with the use of peroxidase-conjugated goat anti-rabbit antiserum, with 4-chloro-2-naphthol and hydrogen peroxide serving as the substrate.

Preparation of nuclear extracts for assay of DNA topoisomerase II activity. Nuclear extracts were prepared as described by Sullivan et al. [27]. Protein content was determined as described by Lowry et al. [19]. Nuclear extracts obtained by this method also contain DNA topoisomerase I activity that relaxes supercoiled DNA dimers in the absence of adenosine triphosphate (ATP). However, since DNA topoisomerase I cannot efficiently catenate PBR322 [9] or unknot P4 DNA [14] and its effect on DNA is ATP-independent, the activities of DNA topoisomerase I and DNA topoisomerase II are easily distinguished.

Preparation of phage P4 DNA and assay of DNA topoisomerase II activity. Bacteriophage P4 virl del^{10} was provided by Dr. L. F. Liu, Johns Hopkins University School of Medicine, Baltimore, Maryland. Preparation of phage P4 DNA and assay of DNA topoisomerase II activity was accomplished as described by Liu et al. [14].

Results

The development and characterization of human colon and lung adenocarcinoma cell lines selected for resistance to etoposide has been described in detail elsewhere [15, 18]. In our laboratory, the colon and lung adenocarcinoma cell lines used in this study also did not exhibit gene amplifica-

Table 1. Relative resistance of proliferating cultured HCT116/VP48 cell populations to DNA topoisomerase II inhibitors

Agent	Mean LC_{50} (ng/ml) ±SE[a]		DOR[b]
	HCT116/VP48	HCT116	
Etoposide	11,800 ±225	210 ±29	56
Teniposide	1,800 ± 52	58 ± 1.7	31
Adriamycin	170 ± 5	18 ± 0.57	9.4
Daunomycin	48 ± 1.1	17 ± 0.57	2.8
m-AMSA	150 ± 5.1	52 ± 1.7	2.9
Actinomycin D	1.2± 0.04	0.19± 0.01	6.3

[a] LC_{50} is defined as the concentration that inhibits the growth of a test culture by 50% relative to the growth of a control culture. The mean LC_{50} value for each agent is based on three separate experiments. SE, Standard error of the mean. The difference in the means of the two cell populations was significant ($P < 0.01$)

[b] DOR, Degree of resistance = $\frac{\text{Mean } LC_{50} \text{ HCT116/VP48}}{\text{Mean } LC_{50} \text{ HCT116}}$

Table 2. Relative resistance of proliferating cultured A549B/VP29 cell populations to DNA topoisomerase II inhibitors

Agent	Mean LC_{50} (ng/ml) ±SE[a]		DOR[b]
	A549B/VP29	A549B	
Etoposide	2,600 ±90	540 ±19	4.8
Teniposide	96 ± 2.9	58 ± 1.7	1.6
Adriamycin	34 ± 0.98	17 ± 0.51	2.0
Daunomycin	38 ± 1.1[c]	34 ± 0.98[c]	1.1
m-AMSA	110 ± 3.8	33 ± 1.2	3.3
Actinomycin D	1.8± 0.05	3.1± 0.09	0.6

[a] LC_{50} is defined as the concentration that inhibits the growth of a test culture by 50% relative to the growth of a control culture. The mean LC_{50} value for each agent is based on three separate experiments. SE, Standard error of the mean. The difference in the means of the two cell populations was significant ($P < 0.01$)

[b] DOR, Degree of resistance = $\frac{\text{Mean } LC_{50} \text{ A549/VP29}}{\text{Mean } LC_{50} \text{ A549B}}$

[c] The difference in the means was not significant ($P > 0.05$)

tion of the human *mdr1* sequence, the 4.3-kb P-glycoprotein transcript, or the membrane P-glycoprotein (data not shown) as reported by Long et al. [18].

The HCT 116/VP48 cell line was approximately 50-fold resistant to the selecting agent etoposide and 30-fold resistant to teniposide (Table 1). The degree of resistance to other DNA topoisomerase II inhibitors was of a lower magnitude: Adriamycin, 9-fold; daunomycin, 3-fold; m-AMSA, 3-fold; and actinomycin D, 6-fold. On the other hand, the A549B/VP-29 cell line exhibited a lower degree of resistance to the selecting agent etoposide (5-fold) and approximately a 2-fold resistance to teniposide (Table 2). The cells exhibited a 2-fold resistance to Adriamycin but were sensitive to daunomycin and showed a 3-fold resistance to m-AMSA. These cells were sensitive to actinomycin D. The HCT 116/VP48 cell line showed a 7-fold resistance to vincristine and a 2-fold resistance to vinblastine but was sensitive to homoharringtonine (Table 3). Similarly, the A549B/VP29 cell line was also resistant to vinblastine (2-fold) and sensitive to homoharringtonine (Table 3).

Discussion

Two categories of cellular resistance to DNA topoisomerase II-reactive antitumor drugs have been described [38]. The first involves the multiple-drug-resistance (MDR) phenotype associated with an overexpression of P-glycoprotein [6]. The second category of resistance involves quantitative or qualitative differences between DNA topoisomerase II in drug-sensitive and drug-resistant cells [13, 14, 38].

The status of the expression of the *mdr1* gene has been evaluated at both the DNA and the RNA level in human colon and lung adenocarcinoma cell lines selected for resistance to etoposide [18] and was confirmed in the HCT116/VP48 and A549/VP29 cell populations employed in the current study. There was no amplification of the human *mdr1* DNA sequence, the 4.3-kb P-glycoprotein transcript, or the P-glycoprotein.

Table 3. Sensitivity of cultured HCT116/VP48 and A549B/VP29 cell populations to vincristine, vinblastine, and homoharringtonine

Agent	Mean LC_{50} (ng/ml) ±SE[a]		DOR[b]			DOR[b]
	HCT116/VP48	HCT116		A549B/VP29	A549B	
VCR	8.0±0.23[c]	1.1±0.03[c]	7.3			
VBL	9.6±0.34[c]	5.0±0.17[c]	1.9	15±0.44[c]	9.0±0.31[c]	1.7
HHT	13 ±0.46[d]	15 ±0.51[d]	0.9	14±0.40[d]	13 ±0.37[d]	1.1

[a] LC_{50} is defined as the concentration that inhibits the growth of a test culture by 50% relative to the growth of a control culture. The mean LC_{50} value for each agent is based on three separate experiments. SE, Standard error of the mean

[b] DOR, Degree of resistance = $\frac{\text{Mean } LC_{50} \text{ HCT116/VP48 or A549B/VP29}}{\text{Mean } LC_{50} \text{ HCT116 or A549B}}$

[c] The difference in the means of the two cell populations was significant ($P < 0.01$)

[d] The difference in the means of the two cell populations was not significant ($P > 0.05$)

VCR, Vincristine; VBL, vinblastine; HHT, homoharringtonine

In general, we observed the same patterns of resistance to DNA topoisomerase II inhibitors previously reported by Long et al. [18], although quantitatively the resistant patterns seen for the HCT116/VP48 cells as compared with the HCT116/VP35 cells used by Long et al. [18] were marginally different. For instance, Long et al. [18] reported that HCT116/V35 cells were only 9-fold resistant to the selecting agent etoposide and 7-fold resistant to teniposide. Their resistance to Adriamycin was 6-fold and these authors reported no resistance to actinomycin D. The HCT116/VP35 cells were not cross-resistant to colchicine [18], whereas the HCT116/VP48 cells were cross-resistant to vincristine and vinblastine (Table 3). The two cell populations are different in that the HCT116/VP-35 cells were selected with 35 cycles of etoposide selection [18], whereas the cell population used in the current study (HCT116/VP48) was selected with 48 cycles of etoposide selection. This suggests that increased exposure to etoposide in the selection process has resulted in cross-resistance to natural-product microtubule inhibitors even though there is no overexpression of P-glycoprotein.

The resistance patterns shown by the A549B/VP29 cell population that we used and the A549/VP28 cells used by Long et al. [18] to DNA topoisomerase II inhibitors are quite similar. Both cell lines were resistant to etoposide, teniposide, and Adriamycin and sensitive to actinomycin D. Long et al. [18] have reported that the potential mechanism(s) of resistance to DNA topoisomerase II inhibitors in the HCT116/VP-35 and A549/VP28 cell populations may be due to decreased levels of DNA topoisomerase II m-RNA and DNA topoisomerase II enzymatic activity. Preliminary data obtained in our laboratory indicate that in the absence of etoposide, no difference in DNA topoisomerase II catalytic activity could be detected between the HCT116/VP48 and HCT116 cell populations. In another preliminary experiment, the HCT116/VP48 cells required an increased concentration of etoposide (approximately 100-fold) to inhibit DNA topoisomerase II catalytic activity as compared with that required by the HCT116 cells. Additional experiments will be required to investigate in detail the potential basis for resistance to DNA topoisomerase II inhibitors in the HCT116/VP48 cell populations.

The results of the present study indicate that human colon HCT116/VP48 and human lung A549B/VP29 cell populations that exhibit resistance to DNA topoisomerase II inhibitors are sensitive to homoharringtonine. On the other hand, these cell lines did show a degree of resistance to the vinca alkaloids. DNA topoisomerase II mutations would not per se account for vinca alkaloid resistance. Due to the method of original selection used to derive the drug-resistant sublines (i.e., selection as a population), it is probable that more than one mechanism may be contributing to the cross-resistance pattern seen in these cell populations. This problem is difficult to investigate further in these cell lines without first obtaining clonal cell lines.

Combinations of vinca alkaloids and epipodophyllotoxins are effective against certain malignancies [37]. Jackson et al. [11, 12] demonstrated therapeutic synergism in L1210-bearing mice when vincristine was combined with etoposide. In children with acute lymphocytic leukemia who have relapsed after the end of therapy, etoposide and teniposide have been used in combination with vincristine to overcome clinical resistance to vincristine [1, 23].

This study shows that during the process of selecting human colon and lung adenocarcinoma cell lines for resistance to etoposide using etoposide as the selecting agent, cross-resistance to the vinca alkaloids developed. These cell lines did not exhibit gene amplification of the human *mdr1* gene or the 4.3-kb P-glycoprotein transcript and there was no amplification of the 170-kDa P-glycoprotein. This development of cross-resistance to the vinca alkaloids raises the question of whether this could occur in clinical situations in which patients undergo treatment with epipodophyllotoxins and vinca alkaloids. Many tumor samples derived from clinically refractory patients do not exhibit amplified P-glycoprotein expression [4]. Since chemotherapy is routinely given at or near the maximum tolerated dose, it is usually not possible to increase the dose of a chemotherapeutic agent by even a small, e.g., 2-fold, increment [25]. Therefore, the degree of drug resistance necessary for a tumor to become clinically refractory to chemotherapy is probably not large. The sensitivity of cells exhibiting a non-P-glycoprotein-mediated MDR phenotype to homoharringtonine suggests a potential use for this agent in treating tumors with this type of antitumor drug resistance.

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