PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 164, 51–56 (1980)

Partial Cross-Resistance of Cultured Murine Leukemia Vincristine-Resistant P388 Cells to 4′-Demethylepipodophyllotoxin Ethylidene-β-D-Glucoside (40823)[1]

LEE J. WILKOFF AND ELIZABETH A. DULMADGE

Cell Biology Division, Kettering-Meyer Laboratory, Southern Research Institute, 2000 Ninth Avenue South, Birmingham, Alabama 35205

The semisynthetic derivative of podophyllotoxin, 4′-demethylepipodophyllotoxin ethylidene-β-D-glucoside (VP 16-213),[2] demonstrates significant cytotoxicity *in vitro*, is active against experimental animal tumors, and is undergoing clinical trials for the treatment of human malignancies (1–9). In contrast to podophyllotoxin, colchicine, or the vinca alkaloids, inhibition of microtubule assembly and metaphase arrest does not occur in cells treated with this agent (10–12).

Drug-induced resistance of tumor cell populations to a large heterocyclic antitumor compound may result in cross-resistance to other large heterocyclic antitumor compounds (13, 14). For instance, Ehrlich ascites tumor selected for resistance to vincristine is also cross-resistant to vinblastine, adriamycin, and daunomycin (15–17). A recent report has indicated that murine leukemia P388 resistant to vincristine has a limited degree of cross-resistance to adriamycin and actinomycin D (18).

We have recently reported that cultured P388 cells resistant to vincristine (P388/VCR) derived from resistant cell populations developed *in vivo* are cross-resistant to actinomycin D, adriamycin, daunomycin, and *N*-trifluoroacetyladriamycin-14-valerate but not to cinerubin A (19). Since VP 16-213 is also a relatively large heterocyclic antitumor compound of potential clinical utility, this study was conducted to determine if any cross-resistance of P388/VCR cells to VP 16-213 could be demonstrated, independent of host factors, by using cultured P388/VCR cells as target cells. Preliminary results of this study have been reported (20).

Materials and methods. The P388/VCR tumor was developed by treatment of (C57BL × DBA/2)F_1 (BDF_1) mice bearing the P388 ascites tumor with vincristine, 1 mg/kg body weight on Days 1, 5, and 9 post ip implantation of 10^6 or 10^7 P388 cells as previously described (19). Proliferating cultured leukemia P388/0 cells were developed from sensitive cell populations passaged *in vivo* in BDF_1 mice and cultured P388/VCR cells were established from resistant cell populations developed after the 16th *in vivo* passage (19).

P388/0 and P388/VCR cells were propagated in D-EMEM (21) (Flow Laboratories, Rockville, Md.) supplemented with 10% horse serum (Flow Laboratories) and 2-mercaptoethanol (22) (5 μmole/liter of medium; Sigma Chemical Co., St. Louis, Mo.) using 75-cm^2 plastic tissue culture flasks (Corning, Corning, N.Y.). The cultures were incubated at 37°C in air. Under these conditions the leukemia cell populations exponentially proliferated with doubling times (mean ± SD) of 14.2 ± 2.0 hr (P388/0) and 16.5 ± 1.9 hr (P388/VCR) between cell densities of 5.0×10^4 and 1.0×10^6 cells/ml. At the time of these studies, the P388/VCR cell line had completed approximately 1500 doublings of the population in culture.

To determine the effect of VP 16-213 or vincristine on the viability of proliferating cell populations, cultures were initiated at a density of 5.0×10^4 cells/ml in a total volume of 100 ml medium in spinner flasks (Bellco Glass Co., Vineland, N.J.) and allowed to proliferate to a population density of 5.0×10^5 cells/ml. The spinner cultures were incubated at 37°C on a magnetic stir-

[1] Supported by Public Health Service Contract NO1-CM-97309 from the Division of Cancer Treatment, National Cancer Institute.

[2] Abbreviations used: VP 16-23, 4′-demethylepipodophyllotoxin ethylidene-β-D-glucoside; D-EMEM, Dulbecco's modified Eagle's minimum essential medium; S_f, surviving fraction(s); TMC, theoretical maximum concentration(s).

0037-9727/80/050051-06$01.00/0

rer. Proliferating cell populations were exposed to selected drug concentrations for 1, 2, 4, 6, or 24 hr, and the number of surviving cells was determined by cloning in semisolid medium.

The semisolid medium used was D-EMEM supplemented with 20% horse serum, 5 μM 2-mercaptoethanol, and a final concentration of 0.15% agarose type II (Sigma Chemical Co., St. Louis, Mo.). Cells from the drug-treated P388 cultures were cloned at 10,000, 1000, and 100 cells/tube in quadruplicate, and the cells from the control cultures were cloned at 100 cells/tube in a series of 12 tubes. The tubes were incubated for 10–12 days at 37°C in 5% CO_2. Only clones with 50 or more cells were counted (23), and cloning efficiencies of control cultures ranged from 50 to 65%.

Vincristine was dissolved in sterile distilled and deionized water and diluted with cell culture medium to predetermined concentrations. VP 16-213 was dissolved in a minimum volume of *N,N*-dimethylformamide (DMF) and then diluted with cell culture medium to the desired concentration. The final DMF concentration was 0.025% in the medium; when this solvent was used, the control cultures also contained 0.025% DMF.

The sensitivities of cultured leukemia P388/VCR and P388/0 cell populations were compared by χ^2 analysis. The standard errors of the ratios of surviving fractions of P388/VCR and P388/0 cell populations were calculated as described by Kendall and Stuart (24).

The *in vitro* concentrations of the agents used in this study can be compared with the estimated maximum concentration that can be attained in the body fluids of mice, if one assumes rapid equal distribution in all fluids following the administration of a drug dose lethal to 10% of the animals (25). The TMC for vincristine is 4 μg/ml and for VP 16-213 it is 35 to 49 μg/ml.

Results and discussion. Cultured P388/VCR cells were significantly resistant to 6.1×10^{-6} M (5.0 μg/ml) vincristine for periods of exposure up to 24 hr (Table I). For instance, at 24 hr the S_f of the P388/VCR cell population was about 15-fold greater than that of the P388/0 cell population. However, the data indicate that the S_f of the P388/VCR cell population decreased as a function of time. The P388/VCR cells were significantly resistant to the high concentration of 6.1×10^{-5} M (50 μg/ml) vincristine. The P388/VCR cells are resistant to relatively high concentrations of vincristine *in vitro,* since the estimated maximum concentration that can be attained in the body fluids of mice is 4 μg/ml.

Cultured P388/VCR cells treated with VP 16-213 exhibited a degree of cross-resistance to this agent (Table II). For example, the S_f of the P388/VCR cell population was about fourfold greater than the S_f of the P388/0 cell population after exposure for 6 hr to 6.1×10^{-6} M VP 16-213. However, after exposure for 24 hr to this concentration of VP 16-213 or after exposure for 2 hr to 6.0×10^{-5} M VP 16-213 there was no significant difference in the surviving fractions of P388/VCR and P388/0 cell populations.

In comparing the sensitivity of P388/VCR cells to vincristine (Table I) with their sensitivity to VP 16-213 (Table II) as a function of time, it is evident that at each time interval the S_f of the P388/VCR cells treated with vincristine was significantly greater than the S_f of the P388/VCR cells treated with VP 16-213. Thus, even though there is a degree of cross-resistance of P388/VCR cells to VP 16-213, the P388/VCR cells are significantly sensitive to VP 16-213.

In another experiment we compared the sensitivities of P388/VCR and P388/0 cells to different concentrations of vincristine and VP 16-213 using a 6 hr exposure period (Table III). Over the concentration range 6.1×10^{-8} to 6.1×10^{-5} M, resistance to vincristine was demonstrated when comparing the S_f of the P388/VCR cell populations to the S_f of the P388/0 cell populations. Cross-resistance to VP 16-213 was detected at concentrations between 6.1×10^{-8} and 6.1×10^{-6} M. It should be noted that a VP 16-213 concentration of 6.1×10^{-5} M (36 μg/ml) was quite effective in reducing the number of viable cells in both the P388/VCR and P388/0 cell populations (S_f 13/40,000; 3.5 log reduction in viability).

TABLE I. SENSITIVITY OF CULTURED LEUKEMIA P388/VCR AND P388/0 CELLS TO 6.1×10^{-6} OR 6.1×10^{-5} *M* VINCRISTINE SULFATE[a]

M[b]	Exposure period (hr)	S_f[c] P388/VCR	S_f[c] P388/0	Ratio ± SE[d] S_f P388/VCR / S_f P388/0	*P*[e]
6.1×10^{-6}	1	400/400	17/40,000	2353 ± 571	<0.001
	2	400/400	17/40,000	2353 ± 571	<0.001
	4	400/400	17/40,000	2353 ± 571	<0.001
	6	140/400	17/40,000	824 ± 207	<0.001
	24	132/40,000	9/40,000	14.7 ± 5.1	<0.001
6.1×10^{-5}	1	400/400	17/40,000	2353 ± 571	<0.001
	2	400/400	17/40,000	2353 ± 571	<0.001
	4	60/400	17/40,000	354 ± 95	<0.001
	6	40/400	17/40,000	235 ± 67	<0.001

[a] Proliferating cultured P388/VCR or P388/0 cells were exposed to 6.1×10^{-6} *M* or 6.1×10^{-5} *M* vincristine sulfate tor the indicated exposure periods and the number of cells surviving drug treatment was estimated by assay in semisolid medium as described under Materials and methods.
[b] *M* is molarity.
[c] S_f is surviving fraction.
[d] Standard error of the ratio was estimated as described in Ref. (24).
[e] S_f's for each exposure period were compared by use of χ^2 analysis.

This concentration is a physiologically attainable concentration since the estimated maximum concentration of VP 16-213 that can be attained in the body fluids of mice is approximately 42 μg/ml.

Cultured P388/0 cells may be more sensitive to VP 16-213 than to vincristine. For example, a 6-hr exposure of P388/0 cells to 6.1×10^{-7} *M* VP 16-213 results in a 2.8-log reduction in viability (S_f = 64/40,000) whereas exposure to 6.1×10^{-7} *M* vincristine results in a 0.5-log reduction in viability (S_f = 144/4,000; Table III).

It is interesting to compare the sensitivity of cultured P388/0 cells to VP 16-213 with the reported sensitivities of other cultured

TABLE II. SENSITIVITY OF CULTURED LEUKEMIA P388/VCR AND P388/0 CELLS TO 6.1×10^{-6} OR 6.5×10^{-5} *M* VP 16-213[a]

	Exposure period (hr)	S_f[c] P388/VCR	S_f[c] P388/0	Ratio ± SE[d] S_f P388/VCR / S_f P388/0	*P*[e]
6.1×10^{-6}	1	152/400	12/40,000	1267 ± 374	<0.001
	2	240/40,000	12/40,000	20 ± 5.9	<0.001
	4	200/40,000	12/40,000	16.7 ± 5.0	<0.001
	6	52/40,000	12/40,000	4.3 ± 1.4	<0.001
	24	7/40,000	7/40,000	1.0 ± 0.5	NS[f]
6.1×10^{-5}	1	68/4,000	12/40,000	56.7 ± 17.7	<0.001
	2	12/40,000	12/40,000	1.0 ± 0.4	NS
	4	12/40,000	12/40,000	1.0 ± 0.4	NS
	6	12/40,000	12/40,000	1.0 ± 0.4	NS

[a] Proliferating cultured P388/VCR or P388/0 cells were exposed to 6.1×10^{-6} *M* or 6.1×10^{-5} *M* VP 16-213 for the indicated exposure periods and the number of cells surviving drug treatment was estimated by assay in semisolid medium as described under Materials and methods.
[b] *M* is molarity.
[c] S_f is surviving fraction.
[d] Standard error (SE) of the ratio was estimated as described in Ref. (24).
[e] S_f's for each exposure period were compared by use of χ^2 analysis.
[f] NS is not significant.

TABLE III. SENSITIVITY OF CULTURED P388/VCR AND P388/0 CELLS TO 6.1×10^{-8} TO 6.1×10^{-5} *M* VINCRISTINE SULFATE OR VP 16-213 FOR A 6-HR EXPOSURE PERIOD

	Vincristine sulfate[a]				VP 16-213[a]			
	S_f[c]		Ratio ± SE[d] S_f P388/VCR		S_f[c]		Ratio ± SE[d] S_f P388/VCR	
M[b]	P388/VCR	P388/0	S_f P388/0	P[e]	P388/VCR	P388/0	S_f P388/0	P[e]
6.1×10^{-8}	400/400	60/400	6.7 ± 0.8	<0.001	400/400	48/400	8.3 ± 1.0	<0.001
6.1×10^{-7}	400/400	144/4,000	27.8 ± 2.3	<0.001	116/400	64/40,000	181 ± 27	<0.001
6.1×10^{-6}	140/400	17/40,000	824 ± 207	<0.001	52/40,000	13/40,000	4.0 ± 1.2	<0.001
6.1×10^{-5}	40/400	17/40,000	235 ± 67	<0.001	13/40,000	13/40,000	1.0 ± 0.4	NS[f]

[a] Proliferating cultured P388/VCR or P388/0 cells were exposed to 6.1×10^{-8} to 6.1×10^{-5} vincristine sulfate or VP 16-213 for 6 hr and the number of cells surviving drug treatment was estimated by assay in semisolid medium as described under Materials and methods.

[b] *M* is molarity.

[c] S_f is surviving fraction.

[d] Standard error (SE) of the ratio was estimated as described in Ref. (24).

[e] S_f's for each exposure period were compared by use of χ^2 analysis.

[f] NS is not significant.

cells to this agent. Greider *et al.* (26) have reported that cell proliferation was inhibited in cultures of P815 cells exposed to either 1 or 10 μg/ml of VP 16-213 and after 1.5 hr; the cell number decreased with the higher concentration below the initial cell density. The decrease in cell number was attributed to the increasing number of dead cells observed by dye exclusion after 6 hr of treatment. Stähelin (27) has reported that the number of surviving cells of cultured P815 cell populations exposed to VP 16-213 depended on drug concentration and time of exposure. The data indicated that when P815 cells were treated with 1 μg/ml for one-third hr there was a 0.3-log reduction in viability of the cell population and when the cell populations were treated with 3.2 μg/ml for 3 hr, the reduction in viability of the cell population was 1.0 log. Drewinko and Barlogie (28) have reported that the 1 hr-D_0 dose (mean lethal dose equal to the concentration required to reduce the number of surviving cells by 63%) for cultured human lymphoid T_1 cells was 3 μg/ml. In the present study treatment of P388/0 cell populations with 6.1×10^{-6} *M* VP 16-213 for 1 hr resulted in a 3.5-log reduction in viability of the cell population (S_f = 12/40,000; Table II). Furthermore, the decrease in the number of viable cells when P388/0 cells were exposed to 6.1×10^{-7} *M* (0.36 μg/ml) VP 16-213 for 6 hr was 2.8 log (S_f = 64/40,000; Table III). The results in Tables II and III indicate that cultured P388/0 cells are more sensitive to VP 16-213 than the P815 or T_1 cells.

Since the degree of cross-resistance to VP 16-213 appears to be a function of concentration and time, partial barriers to the biological action of VP 16-213 must exist at some level. Although VP 16-213 has a different mechanism of action than vincristine (10–12), the partial cross-resistance of P388/VCR cells to VP 16-213 (Tables II and III) was not totally unexpected since cross-resistance to antitumor agents that are relatively large heterocyclic molecules does occur (13, 14). However, the basis for resistance and cross-resistance to these relatively large heterocyclic molecules has not been delineated. Several factors may be involved: decreased drug transport across cell membranes (18, 29–33), decreased drug retention by cells (15, 18, 32–37), decreased binding affinities to target macromolecules (33, 38), altered intracellular distribution (15, 19, 33, 38), and changes in cell membrane structure (4, 18, 33, 39, 40).

In this study, cultured P388/VCR cells, although sensitive to VP 16-213, exhibit partial cross-resistance to this agent. The degree of cross-resistance appears to be a function of concentration and time. The partial cross-resistance indicates that the P388/VCR cells are not as sensitive to VP 16-213 as the P388/0 cells. The results using cultured P388/0 and P388/VCR cells correlate with the results of Schabel *et al.* (4) in a study of *in vivo* passaged P388/0 and P388/VCR tumors. These authors presented data that demonstrated that the P388/0 tumor may be more sensitive to VP 16-213 than to vincristine and that the P388/0 tumor is approximately 10-fold more sensitive to treatment with VP 16-213 than is the P388/VCR tumor. Since vincristine and VP 16-213 are agents of clinical interest, this cell culture system can provide an experimental model for the investigation of the basis for resistance to vincristine and the partial cross-resistance to VP 16-213 at the cell level.

Summary. Cultured P388 cells derived from vincristine-resistant cell populations developed *in vivo* (P388/VCR) retain their resistance to vincristine, although the degree of resistance of the P388/VCR cell populations appears to be a function of concentration and time of exposure. The P388/VCR cell population is sensitive to 4′-demethylepipodophyllotoxin ethylidene-β-D-glucoside (VP 16-213) but a degree of cross-resistance to VP 16-213 can be demonstrated. The degree of cross-resistance to VP 16-213 also appears to be a function of concentration and time of exposure.

We thank Dr. H. E. Skipper, Dr. F. M. Schabel, Jr., and Dr. R. W. Brockman for their helpful discussions and encouragement; Mr. John A. Burdeshaw for statistical analysis of the data; Dr. W. R. Laster and Mrs. Mary Trader for providing the leukemia P388 and vincristine-resistant P388 tumors; and Mrs. Carol S. Eldridge for excellent technical assistance.

1. Grieder, A., Mauer, R., and Stähelin, H., Cancer Res. **34**, 1788 (1974).
2. Stähelin, H., Eur. J. Cancer **9**, 215 (1973).
3. Nissen, N. I., Larsen, V., Pederson, H., Thomsen, K., Cancer Chemother. Rep. **56**, 769 (1972).
4. Schabel, F. M., Jr., Skipper, H. E., Trader, M. W., Laster, R., Jr., Corbett, T. H., Griswold, D. P., Jr., Eur. J. Cancer, in press.
5. Mathe, G., Schwarzenberg, L., Pouillart, P. *et al.*, Cancer **34**, 985 (1974).
6. European Organization for Research on the Treatment of Cancer, Clinical Screening Group, Brit. Med. J. **3**, 199 (1973).
7. Cohen, M. H., Broder, L. E., Fossick, B. E. *et al.*, Cancer Treat. Rep. **61**, 489 (1977).
8. Eagen, R. T., Ingle, J. N., Creagen, E. T., *et al.*, Cancer Treat. Rep. **62**, 843 (1978).
9. Cecil, J. W., Quagliana, J. M., Coltman, C. A., *et al.*, Cancer Treat. Rep. **62**, 801 (1978).
10. Stähelin, H., Eur. J. Cancer **6**, 303 (1970).
11. Loike, J. D., Horowitz, S. B., Biochem. **15**, 5435 (1976).
12. Krishan, A., Paika, K., Frei, E. III, J. Cell Biol. **66**, 521 (1975).
13. Hall, T. C., Cancer Chemother. Rep. **6** (Part 3) 227, (1975).
14. Johnson, R. K., Chitnis, M. P., Embrey, W. M., Gregory, E. B., Cancer Treatment Rep. **62**, 1535 (1978).
15. Danø, K., Acta. Pathol. Microbiol. Scand. [A] (Suppl. N) **256**, 11 (1976).
16. Danø, K., Cancer Chemother. Rep. **56**, 701 (1972).
17. Danø, K., *in* "Adriamycin" (S. K. Carter, A. Dimarco, M. Ghione, *et al.*, eds), p. 90. Springer-Verlag, Berlin (1972).
18. Brockman, R. W., Yagosaira, U., Ling, V., et al., *in* "Current Chemotherapy" (W. Siegenthaler, R. Liithy, eds.), Vol. 1, p. 97. Amer. Soc. Microbiol., Washington, D.C. (1978).
19. Wilkoff, L. J., Dulmadge, E. A., J. Nat. Cancer Inst. **61**, 1521 (1978).
20. Wilkoff, L. J., Dulmadge, E. A., Proc. Amer. Assoc. Cancer Res. **19**, 37 (1978).
21. Morton, H. J., In Vitro **6**, 89 (1970).
22. Broome, J. D., Jeng, M. W., J. Nat. Cancer Inst. **49**, 579 (1972).
23. Courtenay, V. D., Br. J. Cancer **34**, 39 (1976).
24. Kendall, M. G., Stuart, A., *in* "The Advanced Theory of Statistics" Vol. 1, p. 232. Griffin, London (1963).
25. Schabel, F. M., Jr., Skipper, H. E., Trader, M. W., *et al.*, Cancer Chemother. Rep. **48**, 17 (1965).
26. Grieder, A., Mayer, R., Stähelin, H., Cancer Res. **34**, 1788 (1974).
27. Stähelin, H., *in* "Progress in Chemotherapy" (G. K. Daikos, ed.), Vol. 3, p. 819. Hellenic Society for Chemotherapy, Athens (1974).
28. Drewinko, B., Barlogie, B., Cancer Treatment Rep. **60**, 1295 (1976).
29. Biedler, L. J., Riehm, H., Cancer Res. **30**, 1174 (1970).
30. Riehm, H., Biedler, J. L., Cancer Res. **31**, 409 (1971).
31. Golstein, M. N., Hamm, H., Amrod, E., Science **151**, 1555 (1966).
32. Kessel, D., Wodinsky, I., Biochem. Pharmacol. **17**, 161 (1968).
33. DiMarco, A., *in* "Fundamentals in Cancer Chemotherapy" (F. M. Schabel, Jr., ed.), Vol. 23, p. 216. Karger, Basil (1978).
34. Kessel, D., Botterill, V., Wodinsky, I., Cancer Res. **28**, 938 (1968).
35. Skoosgaard, T., Cancer Res. **38**, 4722 (1978).
36. Inaba, M., Johnson, R. K., Cancer Res. **37**, 4629 (1977).
37. Inaba, M., Kobayashi, H., Sakurai, Y., Johnson, R. K. Cancer Res. **39**, 2200 (1979).
38. Schwartz, H. S., Kanter, P. M., Cancer Chemother. Rep. **6** (Part 3), 107 (1975).
39. Bosmann, B. H., Kessel, D., Mol. Pharmacol. **6**, 345 (1970).
40. Bosmann, B. H., Nature (London) **233**, 566 (1971).

Received October 26, 1979. P.S.E.B.M. 1980, Vol. 164.