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Reversal of Adriamycin Resistance by Verapamil in Human Ovarian Cancer

Abstract. *The effectiveness of adriamycin in the treatment of ovarian cancer and other human tumors has been limited by the development of drug resistance. Verapamil, a calcium channel blocking agent, completely reversed adriamycin resistance in human ovarian cancer cells with moderate (three- to sixfold) degrees of resistance and partially reversed resistance in highly (150-fold) resistant cells. The potentiating effect of verapamil was due to inhibition of adriamycin efflux in the resistant cells. These results have led to a clinical trial of adriamycin and verapamil in refractory ovarian cancer patients.*

Adriamycin is a widely used anticancer agent whose effectiveness has been limited, in part, by the frequent development of drug resistance (*1*). The impact of drug resistance on the clinical usefulness of adriamycin is particularly evident in ovarian cancer, where adriamycin has a 40 percent response rate in previously untreated patients (*2*). The median duration of response is 3 months, and when the drug is administered in combination with alkylating agents the response duration is increased to only 7 to 9 months (*3*). Neither the nature of the intrinsic resistance of the patients who do not respond to treatment with adriamycin nor the acquired resistance that develops rapidly in patients who initially respond to the drug has been well characterized. Studies performed with P388 leukemia cells in vitro have shown that adriamycin-resistant cells have a decreased intracellular accumulation of adriamycin primarily because of an enhanced active efflux (*4, 5*). Verapamil, a calcium channel blocker, produces a seven- to tenfold enhancement of adriamycin cytotoxicity in such cells (*6*) and a twofold enhancement in human hematopoietic tumor cell lines (*7*). In studies of mice bearing daunomycin-resistant Ehrlich ascites tumor, the combination of verapamil plus daunomycin produced a doubling of survival rate compared to treatment with daunomycin alone (*8*). These observations, together with the availability of human tumor cell lines with varying degrees of resistance to adriamycin (*9, 10*), led us to examine the effect of verapamil on adriamycin resistance in ovarian cancer.

The ovarian cancer cell lines used in this study included (i) two cell lines sensitive in vitro to adriamycin [A1847 and A2780 (*11*)] and their adriamycin-resistant variants (1847^{AD} and 2780^{AD}) produced in vitro; (ii) cell lines NIH:OVCAR-2, -3, and -4 derived from ovarian cancer patients resistant to combination chemotherapy with and without adriamycin; and (iii) a fibroblast cell line established from the ovarian cancer patient from whom OVCAR-4 was established.

The concentration of verapamil used in cytotoxicity studies in the ovarian cancer cell lines encompassed the clinically achievable peak concentrations (250 to 1000 ng/ml) seen in patients who receive verapamil (Table 1) (*12, 13*). Verapamil completely reversed adriamycin resistance (Fig. 1A) and cross-resistance to vinblastine in 1847^{AD}, a line six times more resistant to adriamycin than its parent line. A similar dose-modifying factor (DMF) of verapamil on adriamycin cytotoxicity was observed with 2780^{AD}, a cell line 150 times more resistant to adriamycin than A2780. The DMF ranged from 3 to 12 as the verapamil dose was raised from 300 to 1000 ng/ml. However, in contrast to its effect on 1847^{AD}, verapamil did not restore sensitivity to adriamycin in 2780^{AD} to the corresponding sensitivity of the parent cell line.

Potentiation of adriamycin cytotoxicity by verapamil in the human ovarian cancer cell lines A2780 and A1847 established from previously untreated patients was also observed (Table 1). However, the degree of potentiation in these cell lines (DMF's of 1.5 and 3) was less than that observed in cell lines with resistance induced in vitro. Potentiation was greater in A1847 compared to A2780, even though A1847 is endogenously more resistant to adriamycin than is A2780. The DMF of verapamil was also smaller in the OVCAR cell lines (1.5 to 4) compared to that in the lines with resistance acquired in vitro. The optimal DMF was achieved in the clonogenic assay when the cells were exposed to verapamil for 24 hours.

In the absence of verapamil, the net

Table 1. Cytotoxicity of adriamycin alone and in combination with verapamil in human ovarian cancer cell lines.

Cell line	Resistance produced		ICR_{50} adriamycin‡ (uM)	ICR_{50} adriamycin + verapamil (DMF)§
	In vivo*	In vitro†		
A1847			0.044	0.013 (3.3)
A2780			0.015	0.01 (1.5)
1847^{AD}		AD	0.18	0.03 (6)
2780^{AD}		AD	2.5	0.8 (3 to 6)
NIH:OVCAR-2	CP, AA		0.088	0.037 (2.4)‖
NIH:OVCAR-3	AA, AD, CP		0.03	0.02 (1.5)‖
NIH:OVCAR-4	AA, AD, CP		0.13	0.03 (4.3)‖

*Indicates cell line established from patient clinically refractory to indicated drugs: AA, alkylating agent; AD, adriamycin; CP, cisplatin. †Resistance was induced in vitro by stepwise incubation with adriamycin. ‡ICR_{50} refers to the drug concentration that results in a 50 percent reduction in colony formation (cell lines 2780, 2780^{AD}, 1847, 1847^{AD}, OVCAR −3 and −4) or a 50 percent reduction in cell count (OVCAR-2) after a 24-hour exposure to adriamycin plus verapamil. Colony formation was assessed 14 days after plating in agar, and cell count was assessed 7 days after exposure to drugs. §DMF (dose-modifying factor) represents the ratio of ICR_{50} (adriamycin) to ICR_{50} (adriamycin plus verapamil). Verapamil concentration was 250 to 500 ng/ml. ‖Maximum DMF achieved with verapamil at 3 μg/ml concentration.

cellular accumulation of adriamycin after a 2-hour incubation in the sensitive cell line, A1847, was three times that observed in the resistant variant 1847^{AD} (Fig. 1B). Incubation of 1847^{AD} cells with adriamycin plus verapamil increased the intracellular adriamycin to an amount similar to that found in A1847 cells incubated without verapamil. Verapamil had a smaller effect (30 percent increase) on the accumulation of adriamycin in the sensitive cell line. The steepness of the dose-response curve for this cell line, however, suggests that a moderate increase in cellular adriamycin can produce the DMF of 3 (Table 1).

To examine the effect of verapamil on the efflux of adriamycin from resistant cells, we first incubated 1847^{AD} cells with adriamycin plus verapamil and then changed to adriamycin-free medium with or without added verapamil. In the absence of verapamil there was a rapid depletion of cellular adriamycin in 1847^{AD} cells (15 percent of the peak cellular value) within 30 minutes (Fig. 1B). In contrast, cells incubated in the presence of verapamil for 30 minutes had cellular concentrations of adriamycin decreased by only 10 percent. These results are consistent with other studies that showed that adriamycin influx occurs by simple Fickian diffusion and that efflux is an active transport process (*14*). The data in Fig. 1, A and B, suggest that the increased concentration of adriamycin present in both resistant and sensitive cells after exposure to verapamil was associated with an increase in cytotoxicity. Results in cell lines OVCAR-3 and -4 derived from patients clinically resistant to adriamycin also showed less accumulation of adriamycin compared to the sensitive cell line A1847 (data not shown). Human ovarian cancer cells thus have the capacity to acquire resistance that is related to a net decrease in anthracycline accumulation, and this resistance can be reversed completely or partially by verapamil. The mechanism of the verapamil effect on adriamycin efflux and the role of calcium transport and calcium channels in this process remain to be determined.

The cytotoxicity studies in the highly resistant 2780^{AD} cell line and in the OVCAR cell lines in which verapamil did not completely restore sensitivity to adriamycin show that additional mechanisms for adriamycin resistance are likely to exist in some human tumors. Primary resistance and cross-resistance in the OVCAR cell lines may be complex phenomena that reflect simultaneous exposure in vivo to multiple drugs with different mechanisms of cytotoxicity.

The verapamil effect on chemotherapeutic agent cytotoxicity appears to be related to drugs with similar transport pathways. The cross-resistance to vinblastine [a drug with the same transport system as adriamycin (*15*)] observed in 1847^{AD} was overcome by verapamil, whereas resistance to melphalan in 1847^{ME} (a cell line with primary resistance to melphalan induced in vitro) was not affected by verapamil (data not shown).

The observation that adriamycin cytotoxicity was not potentiated by verapamil in a fibroblast cell line whereas a tumor cell line, OVCAR-4, from the same patient did show increased adriamycin cytotoxicity in the presence of verapamil suggests the possibility that a difference exists between normal and tumor cells that can be exploited to increase tumor cytotoxicity without increasing toxicity to normal tissue.

These observations have led to a clinical trial at the Medicine Branch, National Cancer Institute, of verapamil plus adriamycin in refractory ovarian cancer patients (*16*). Results have indicated that verapamil infusions are feasible and that verapamil blood concentrations of 1000 ng/ml and greater can be achieved at a time when adriamycin concentrations in plasma ranged from 0.1 to 0.13 μM. Thus, the adriamycin and verapamil concentrations required in vitro for potentiation of adriamycin cytotoxicity can be achieved in ovarian cancer patients. Enhancement of adriamycin cytotoxicity to normal tissues by verapamil seems not to occur, which is consistent with the results of the fibroblast studies in vitro.

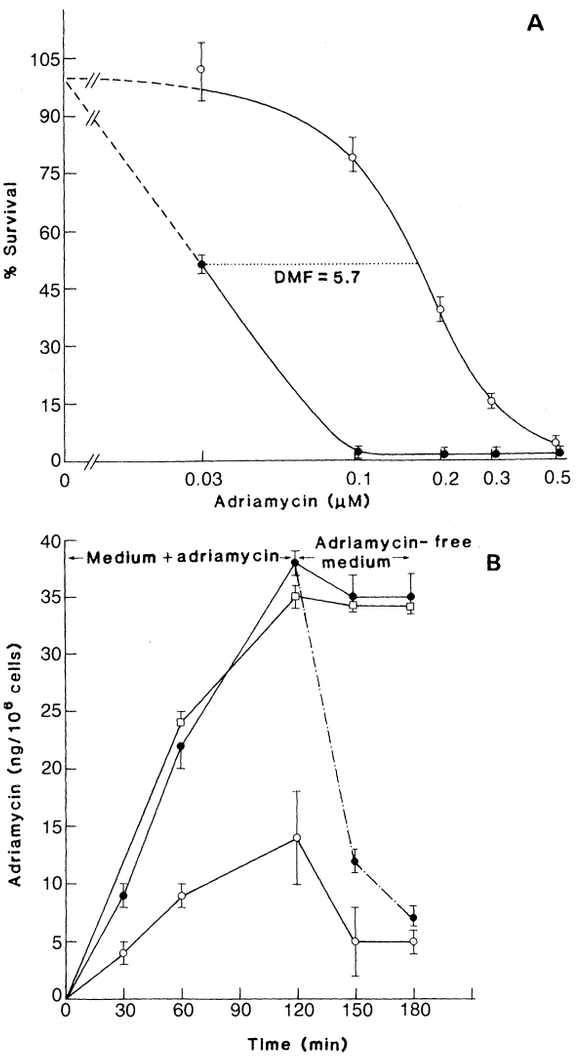

Fig. 1. (A) Enhancement of adriamycin cytotoxicity by verapamil. Cells of 1847^{AD} were exposed (24 hours) in a monolayer culture system to adriamycin alone (○) or in combination with verapamil (250 ng/ml) (●), then harvested with trypsin and cloned in a double-layer agar system (*17*). Tumor cell colonies (> 50 cells) were enumerated on an Omnicon FAS II Image Analysis System 14 days after plating. Each point is the mean of triplicate determinations. Percent survival was calculated by $(C_p/C_a) \times 100$, where C_p is colonies formed in the presence of drug and C_a is colonies formed in the absence of drug. A standard two-sample t-test yielded $P < 0.01$ for each comparison between the two curves at each dose. (B) Cellular accumulation and retention of adriamycin in the absence and presence of verapamil in A1847 and 1847^{AD} ovarian cancer cells. For studies of adriamycin accumulation, cells in monolayer were incubated in growth medium (37°C, 5 percent CO_2 by volume) with verapamil (6.6 μM) or adriamycin (0.16 μM) or both. At the points shown, cells were harvested quantitatively with trypsin and phosphate-buffered saline. For dissociation studies, cells were first incubated for 2 hours with adriamycin in the presence or absence of verapamil, changed to adriamycin-free medium with or without verapamil, and harvested at the subsequent time points. After addition of internal standard (daunomycin), cells and harvest medium were lyophilized and the residue extracted with methanol. The extract was analyzed by high-performance liquid chromatography with detection by fluorescence (*18*). A1847 (□) and 1847^{AD} (○) influx and efflux of adriamycin in the absence of verapamil; 1847^{AD} (●) influx of adriamycin in the presence of verapamil and efflux in the absence (—·—) or presence (——) of verapamil.

Although establishment of the true therapeutic effect of verapamil in combination with adriamycin awaits the completion of this trial and others, the in vitro effects suggest that reversal, or at least modification, of adriamycin resistance is a clinical possibility.

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Loss of Adhesion of Murine Erythroleukemia Cells to Fibronectin During Erythroid Differentiation

Abstract. *Uninduced murine erythroleukemia cells specifically attached to fibronectin-coated dishes but not to dishes coated with laminin or type I or IV collagen. Dimethyl sulfoxide–induced differentiation of these cells caused a dramatic decrease in adhesion to fibronectin that was correlated with synthesis of the erythrocyte glycoprotein "band III," a membrane marker of the differentiated erythrocyte. Loss or modification of fibronectin binding sites on the cell surface during erythroid differentiation may cause the release of reticulocytes from the interstitial matrix of bone marrow into the blood.*

Differentiation of the mammalian erythrocyte from its nucleated erythroblast precursor takes 4 to 6 days and involves extensive remodeling of the cell surface membrane (*1*). At least two cell surface glycoproteins, "band III" (the anion-exchange protein) and glycophorin, are unique to erythrocytes (*2*) and are synthesized preferentially during erythropoiesis (*3, 4*). Spectrin, the principal structural protein of the submembrane cytoskeleton in erythrocytes (*5*), is also synthesized during erythropoiesis (*3, 6*). Erythroid differentiation also involves loss of glycoproteins characteristic of the erythroblast, such as the transferrin receptor (*7*).

We report here that erythroid differentiation by murine erythroleukemia (MEL) cells in vitro is also accompanied by loss of their ability to attach to the glycoprotein fibronectin. Fibronectin is a major fibrous component of the extracellular matrix in bone marrow (*8*) and is found on the surface of many types of cells and in the blood plasma. It and other extracellular matrix–associated proteins, like collagens and laminin, promote attachment and subsequent spreading of many cell types in vitro (*9*). Fibronectin also appears to be involved in the regulation of several differentiation pathways, including myogenesis, chondrogenesis, and adrenergic differentiation in explanted neural crest cells (*9*). We speculate that loss of fibronectin binding sites during erythroid differentiation may be involved in the release of erythrocytes from the interstitial matrix of bone marrow into the blood.

Murine erythroleukemia cells provide a valuable in vitro model for studying many aspects of erythroid differentiation (*10*). Treatment of these cells with dimethyl sulfoxide (DMSO) results in the initiation of a differentiation process in which they synthesize hemoglobin, spectrin (*6*), and band III protein (*11*). Although MEL cells normally grow in suspension culture, attachment to the surface of culture flasks can be mediated by serum fibronectin (*12*).

Exponentially growing MEL cells attached to fibronectin-coated dishes but not to dishes coated with bovine serum albumin (BSA) (Table 1). Attachment was dependent on the amount of fibronectin applied to the dishes: 50 percent adhesion was observed at 5 μg per 10-cm^2 dish and > 90 percent adhesion at 12 μg. Most of the added cells became firmly attached within 60 minutes of plating and most of the adhering cells retained their spherical shape; only 10 to 15 percent had spread by 60 minutes (Fig. 1a). Attachment of MEL cells was not promoted by collagen type I (50 to 500 μg per dish), collagen type IV (50 to 500 μg), or laminin (1 to 25 μg) (Table 1). This negative result cannot be attributed to possible inactivation of these proteins during the preparation of dishes, because dishes coated with collagens promoted attachment and spreading of Chinese hamster ovary (CHO) fibroblasts (Table 1) (*13*). Furthermore, CHO cells attached to dishes coated with fibronectin or laminin. We also examined the adhe-

Table 1. Adhesion of MEL, CHO, and HepG2 cells (*18*) to plasma fibronectin, collagen, and laminin. Attachment of cells to 35-mm petri dishes coated with proteins (*19*) in the indicated amounts was measured by incubating 4×10^5 cells at 37°C in a CO_2 incubator. After 90 minutes the medium was removed and unattached cells were counted in a Coulter counter; the dishes containing the attached cells were washed three times with the incubation medium and once with Hanks buffered salt solution before trypsinization (0.2 percent trypsin and 0.02 percent EDTA) to detach the cells. Detached cells were quickly resuspended in fresh incubation medium containing soybean trypsin inhibitor (25 μg/ml) and counted as above. Data are means ± standard errors for four replicate dishes.

Addition to dish	Attached cells (percent)		
	MEL	CHO	HepG2
None	5 ± 2.7	10 ± 4.9	7 ± 3.0
Fibronectin (12 μg)	90 ± 5.9	79 ± 5.0	25 ± 3.6
Laminin (12 μg)	10 ± 6.0	72 ± 4.0	85 ± 4.5
Collagen type 1 (200 μg)	4 ± 2.6	83 ± 5.6	20 ± 4.0
Collagen type IV (150 μg)	6 ± 3.7	90 ± 4.0	18 ± 5.6