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Cellular pharmacology of the partially non-cross-resistant anthracycline annamycin entrapped in liposomes in KB and KB-V1 cells

Roman Perez-Soler[1], Yi He Ling[1], Yiyu Zou[1], Waldemar Priebe[2]

[1] Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA
[2] Department of Clinical Investigation, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

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Abstract. The in vitro cytotoxicity, cellular pharmacology, and DNA lesions induced by the lipophilic anthracycline annamycin (Ann) were studied in KB and KB-V1 (multidrug-resistant) cells. Ann was tested in suspension in saline and 10% dimethylsulfoxide (DMSO: final concentration, 0.05%–0.5%) or entrapped in multilamellar liposomes (median size, 1.57 μm). Doxorubicin (Dox) was about twice as cytotoxic as Ann or liposome-entrapped Ann (L-Ann) against KB cells. Both Ann and L-Ann displayed a partial lack of cross-resistance with Dox (resistance indices: >60 for Dox, 4.7 for Ann, 4.0 for L-Ann). Accumulation of Ann in KB and KB-V1 cells was consistently about 2–3 and 10–20 times higher, respectively, than that of Dox. Cellular retention of Ann in KB and KB-V1 cells was about 2 and 30 times higher, respectively, than that of Dox as a result of the different efflux patterns of the two drugs: Dox was not effluxed from KB cells but was significantly effluxed from KB-V1 cells (66% at 1 h, whereas Ann efflux was similar in both cell lines (about 50% at 1 h). Dox retention in KB-V1 cells was increased by a factor of 2 in the presence of verapamil or cyclosporine A, but Ann retention was not. In addition, accumulation of Dox in KB-V1 cells was enhanced by the metabolic inhibitor deoxyglucose/azide and the membrane carboxylic ionophore monensin, whereas accumulation of Ann was not affected by either agent. All these findings indicate significant differences in the cellular transmembrane transport systems between Dox and Ann and suggest that Ann efflux is not mediated by P-glycoprotein. Liposome entrapment reduced by a factor of 1.3–2.0 the cellular accumulation of Ann without affecting its cytotoxicity. As compared with Dox, both Ann and L-Ann induced 3 times more DNA double- and single-strand breaks in KB cells. In KB-V1 cells, Dox did not induce DNA damage, whereas the extent of DNA breaks induced by both Ann and L-Ann was similar to that induced by Dox in KB cells. Our results indicate (1) that the lack of cross-resistance between Ann and Dox is associated with a markedly enhanced accumulation and retention of Ann in KB-V1 cells and (2) that the type of liposomes used does not significantly affect the cellular effects of Ann.

Introduction

Tumor drug resistance at maximum tolerated doses remains the most important obstacle to the successful treatment of disseminated human cancer with cytotoxic therapy. Acquired multidrug resistance (MDR) to different natural drugs is a phenomenon that is being extensively studied and is mediated in most cases by the overexpression of a membrane energy-dependent efflux pump, P-glycoprotein, which is capable of reducing the intracellular accumulation of different and structurally unrelated antitumor agents [1, 2]. The precise mechanism of drug efflux is not known, but direct drug binding to P-glycoprotein has been suggested. Recent studies suggest that overexpression of P-glycoprotein may be a clinically relevant mechanism of resistance in several human malignances such as leukemia, lymphoma, and breast carcinoma and an independent adverse prognostic factor in diseases such as childhood neuroblastoma or soft-tissue sarcoma [3, 4].

Different ways of overcoming MDR have been explored and have proved to be effective at least in in vitro systems, including the use of modulators that can inhibit drug efflux mediated by P-glycoprotein [5]. The preparation of structurally different analogs of MDR drugs with decreased interaction with P-glycoprotein or other drug-efflux mechanisms constitutes an alternative approach. In the case of anthracyclines, several analogs with a lack of cross-resistance properties have been reported during the last few years [6–14].

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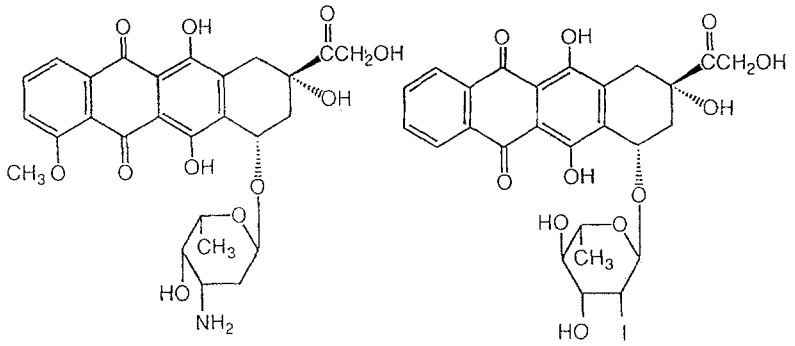
Correspondence to: Roman Perez-Soler, M. D., Box 80, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA



DOXORUBICIN (Dox) ANNAMYCIN (Ann)

Fig. 1. Chemical structures of Dox and Ann

Annamycin (Ann), a highly modified doxorubicin (Dox) analog (Fig. 1) with marked affinity for lipid membranes, has shown a significant lack of cross-resistance in P388/MDR cells that overexpress P-glycoprotein [15]. Ann accumulation in P388 and P388/MDR cells was about 3 and 6 times higher, respectively, than that of Dox. Ann cellular accumulation and level of induced DNA lesions in P388/MDR cells were similar to those of Dox in sensitive cells [15]. Ann was capable of inducing apoptosis in P388/MDR cells, whereas Dox was ineffective [16].

Because Ann is insoluble in water and has a high affinity for lipid membranes, we have developed liposomal formulations for its i.v. administration in preclinical animal experiments and future clinical studies [17]. Ann is easily amenable to formulation in a wide variety of liposomes with different physicochemical properties, thus offering the possibility of modifying the pharmacokinetics and organ-distribution profile of the drug in accordance with its intended use. The multilamellar liposomal formulation of Ann used in the current study is expected to enter clinical trials during 1994.

The objectives of the work presented in this manuscript were to study in more depth the cellular pharmacology, membrane transport, and DNA damage induced by Ann and to study whether presenting the drug entrapped in multilamellar vesicles composed of dimyristoylphosphatidyl choline (DMPC) and dimyristoylphosphatidyl glycerol (DMPG) modulated its cytotoxicity and cellular pharmacology. We used KB and KB-V1 cells since they are adherent, thus allowing for removal of particulate liposomes from cell cultures as required by the different experiments. Our results indicate (1) that Ann is partially non-cross-resistant with Dox in this cell system, (2) that Ann retention in KB-V1 cells is 12–30 times higher than that of Dox, (3) that the lack of cross-resistance of Ann is associated with its ability to induce significant amounts of single- and double-strand DNA breaks in KB-V1 cells, and (4) that the entrapment of Ann in this type of multilamellar liposome reduces cellular accumulation of drug by a factor of 2 without significantly affecting its cytotoxicity.

Materials and methods

Drugs and chemicals. Dox was purchased from Ben Venue Laboratories, Inc. (Bradford, Ohio). Ann was prepared as previously reported [18], with slight modifications. DMPC and DMPG were purchased from Avanti Polar Lipids, Inc. (Alabaster, Ala). Multilamellar liposomal annamycin (L-Ann) was prepared as described previously [19]. In brief, appropriate amounts of Ann, DMPC, and DMPG in a weight ratio of 1:10.5:4.5 were dissolved in chloroform and dried under reduced pressure at 42° C in a rotary evaporator RE140 (Buchi, Switzerland). After the addition of normal saline (1 ml/mg of Ann), the liposome suspension was obtained by rotating the flask in the rotavapor at 30–40 rpm for 1.5 h at 30°–45° C. The entrapment efficiency was found to be >98% and the median size, 1.57 μm. The final Ann concentration in the preparation was 0.5 mg/ml. Free Ann was prepared as a stock suspension (1 mg/ml) in saline and 10% DMSO. The final DMSO concentration was 0.05%–0.5% for Ann concentrations of 5–50 μg/ml.

Dox and Ann concentrations of 5–10 μg/ml were used in all experiments because they are close to the Ann concentration resulting in the survival of 50% of treated KB cells (ID_{50}; see Results) and are easily achievable in different organs at tolerated doses after i.v. administration in mice [19].

Verapamil, quinidine, cytochalasin B, ouabain, 2-deoxyglucose, sodium azide, *N*-ethylmaleimide, chloroquine, and monensin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Cyclosporin A was a gift from the Fujian Institute of Microbiology (Fugian, China).

Minimum essential medium (MEM) and fetal calf serum were purchased from Gibco (Gaithersburg, Md.). [^{14}C]-Thymidine (2.11 GBq/nmol) was obtained from Amersham International Co. (Arlington Heights, Ill.). Other chemicals were purchased from either Sigma Chemical Co. (St. Louis, Mo.) or Fisher Scientific Co. (Springfield, N. J.).

Cell culture. Human KB and KB-V1 carcinoma cells were obtained from Dr. Michael Gottesman, National Cancer Institute, and maintained in MEM supplemented with 10% fetal calf serum, 100 units of penicillin/ml, and 2 m*M* glutamine at 37° C in a humidified incubator containing 5% CO_2/95% air. KB-V1 cells were developed by chronic exposure of KB cells to stepwise concentrations of vinblastine and were maintained in the same medium containing 0.5 μ*M* vinblastine. The resistance characteristics of these cell lines have been described by Willingham et al. [20].

Cytotoxicity assay. KB and KB-V1 cells (10^4/well) were seeded in 96-microwell plates for 12 h and then exposed to different concentrations of Dox, Ann, or L-Ann for 4 h at 37° C. Following the removal of drugs, the cells were washed twice with cold phosphate-buffered saline

Table 1. In vitro cytotoxicity of Ann and L-Ann[a]

Drug	ID_{50} (μg/ml)		Resistance index[b]
	KB	KB/V1	
Dox	3.20±0.51	202±66	63.0
Ann	7.85±0.91	37± 5	4.7
L-Ann	8.40±3.47	34± 3	4.0

[a] KB and KB-V1 cells were exposed to various concentrations of drugs at 37° C for 4 h, washed twice with cold PBS following the removal of drugs, and recultured in drug-free medium for 3 days. Cytotoxicity was measured by the MTT assay as described in Materials and methods

[b] Resistance index = ID_{50} for resistant cells : ID_{50} for sensitive cells

(PBS) and reincubated in drug-free medium for 72 h. Cell survival was measured using the 3-(4,5-dimethylthiazol-2-yl-)-2-5-diphenyltetrazolium bromide (MTT) reduction assay [21]. The ID_{50} was defined as the drug concentration resulting in 50% cell survival relative to the control value and was calculated by extrapolation of the percentage of cell-survival data obtained at each drug concentration tested.

Determination of intracellular drug accumulation and efflux. Intracellular drug accumulation and efflux were determined as previously reported [15]. In brief, both KB and KB-V1 cells ($0.25-0.5 \times 10^6$ cells/22-mm dish) were incubated with various concentrations of Dox, Ann, or L-Ann at 37° C. At the appropriate time points, the culture medium was removed and the cells were rinsed three times with cold PBS (pH 7.4) and detached with 1 ml of 0.5 m*M* ethylenediaminetetraacetic acid (EDTA)-PBS solution. Cell numbers were determined by the Coulter Counter Model ZM system (Coulter Electronics, Hialeah, Fla.). Drugs were extracted from the cells with chloroform : methanol (9 : 1, v/v) according to the method described by Speth et al. [22] and were quantified using a Perkin-Elmer MPF-44A spectrofluorometer (Perkin-Elmer, Norwalk, Conn.). The excitation and emission wavelengths were 490 and 590 nm for Dox and 500 and 560 nm for Ann, respectively. The intracellular drug content was calibrated with a standard curve and expressed in micrograms per 10^6 cells.

To measure drug efflux, KB and KB-V1 cells were loaded with a 10-μg/ml concentration of Dox, Ann, or L-Ann at 37° C for 1 h. After the removal of drugs, cells were washed three times with cold PBS and immediately reincubated in prewarmed fresh medium at 37° C. Following incubation at 37° C for the indicated intervals, cell aliquots were collected, the cells were counted, and the cellular drug retention was determined using the extraction and quantification methods described above.

To assess the effects of different types of MDR odulators on drug uptake, KB and KB-V1 cells ($0.25-0.5 \times 10^6$ cells/ml) were incubated at 37° C for 1 h with a 10-μg/ml concentration of Dox, Ann, or L-Ann in the absence or presence of verapamil, cyclosporin A, or quinidine at the same concentration. To determine the effects of metabolic inhibitors on drug uptake, cells were preincubated at 37° C with either 3 m*M* sodium azide plus 25 m*M* 2-deoxyglucose, 2 m*M* ouabain (membrane Na+/K+ ATPase inhibitor), 20 μ*M* *N*-ethylmaleimide (enzyme inhibitor), 50 μ*M* chloroquine (lysosomatropic agent), 7 μ*M* monensin (carboxylic ionophore), or 20 μ*M* cytochalasin B (inhibitor of microtubule or microfilament polymerization) prior to the addition of anthracyclines at 10 μg/ml [23]. After a 60-min incubation period, KB and KB-V1 cells were washed three times with cold PBS solution and detached with 0.5 m*M* EDTA-PBS solution. The intracellular drug content was extracted and measured as described above.

Fluorescence microscopy of drug accumulation. KB and KB-V1 cells were plated in tissue-culture chamber/slides (Lab-Tek, Naperville, Ill.) for 12 h and then exposed to Dox, Ann, or L-Ann at 5 μg/ml for different periods at 37° C. Drugs were removed and the slides were prepared and immediately photographed using a Nikon T-104 fluorescence microscope.

Assessment of DNA damage. Single- and double-strand DNA breaks were determined by alkaline elution as previously described [24]. In brief, exponentially growing KB and KB-V1 cells (0.25×10^6 cells) were labeled with [^{14}C]-thymidine for 24 h at 37° C in MEM supplemented with 10% fetal calf serum. The labeled cells were washed and chased for another 3 h before drug treatment. Following a 1-h period of incubation with Dox, Ann, or L-Ann at 10 μg/ml, cells were washed with PBS, lysed in 2% sodium dodecyl sulfate/25 m*M* Na_4 EDTA (pH 9.6), and eluted under alkaline conditions. The amounts of DNA strand breaks were calculated as rad equivalents as described previously [15].

Results

Cytotoxicity

Table 1 shows the cytotoxicity of Dox, Ann, and L-Ann against KB and KB-V1 cells as determined by MTT assay using a 4-h drug exposure. Results represent the mean values ± SD for at least three experiments. Against KB cells, Dox was about 2 times more cytotoxic than Ann or L-Ann (ID_{50}: 3.20±0.51, 7.85±0.91, and 8.40±3.47 μg/ml, respectively). Against KB-V1 cells, the ID_{50} of Dox was 202±66 μg/ml, whereas those of Ann and L-Ann were 37±5 and 34±3 μg/ml, respectively. As a result, the resistance indices were 63 for Dox, 4.7 for Ann, and 4.0 for L-Ann. Ann and L-Ann are therefore partially non-cross-resistant with Dox. Dox preparations containing the same concentrations of DMSO as the Ann preparations did not display an enhanced cytotoxic effect against KB or KB-V1 cells at the concentration range tested (data not shown).

Drug accumulation and efflux

Figure 2 shows the concentration-course accumulation of Dox, Ann, and L-Ann in KB and KB-V1 cells. Accumulation was determined at 1 h of drug exposure. For all drugs, drug accumulation was concentration-dependent but not linear, the extent of accumulation showing a tendency to plateau with increasing drug concentration.

Dox accumulation of KB-V1 cells was reduced by about 90% as compared with that in KB cells, and the difference tended to increase with increasing Dox concentrations. Accumulation of Ann was about 3-fold that of Dox in KB cells. Ann accumulation was lower in KB-V1 cells than in KB cells, and the difference increased with increasing drug concentrations, as in the case of Dox. However, the reduction in cellular accumulation in KB-V1 cells was markedly lower in the case of Ann (30% for Ann vs 90% for Dox). As a result, accumulation of Ann in KB-V1 cells was about 20 times higher than that of Dox. Accumulation of Ann was consistently about 2-fold that of L-Ann in both cell lines.

Figure 3 shows the time-course accumulation of Dox, Ann, and L-Ann. The patterns of drug accumulation were similar for all drugs, consisting of an initial phase of rapid drug uptake (10 min) followed by a phase of slower drug uptake (10–60 min). Dox accumulation in KB-V1 cells was reduced by 75% as compared with that in KB cells. In the case of Ann and L-Ann, accumulation in KB-V1 cells

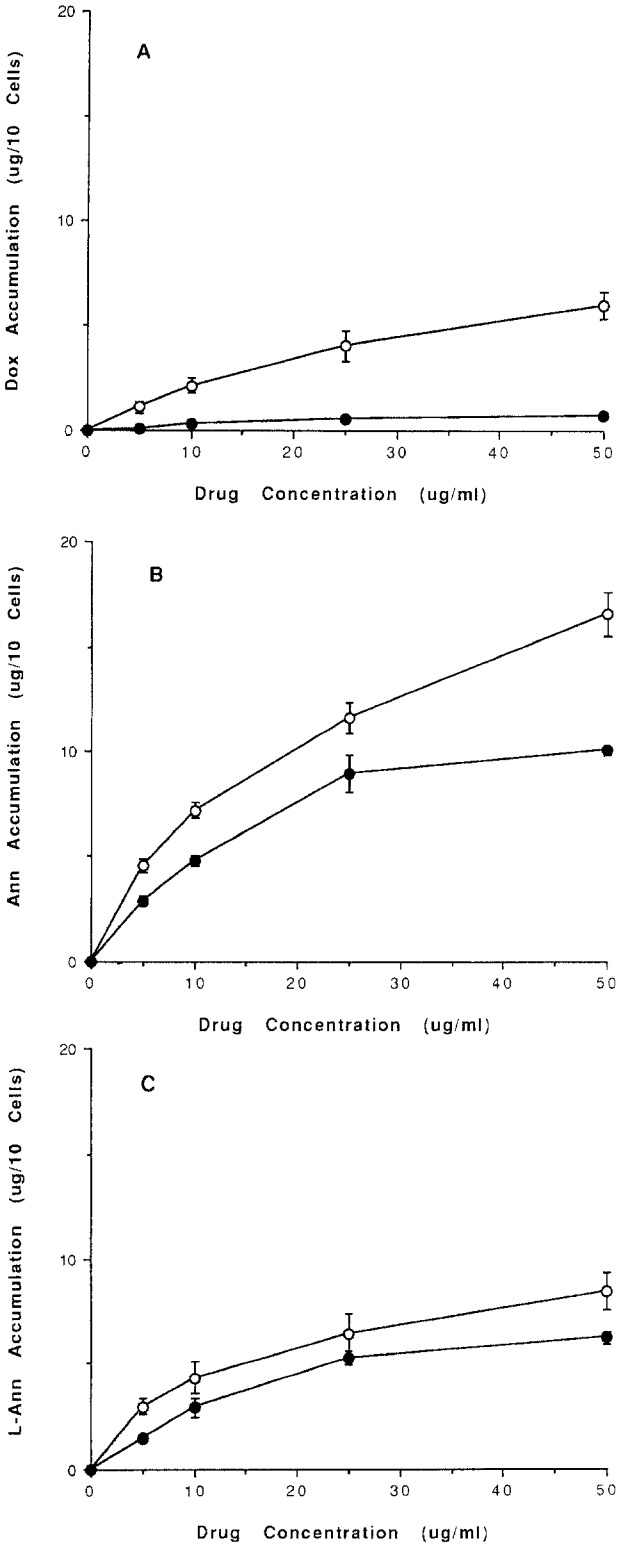


Fig. 2A–C. Cellular accumulation (concentration course) of Dox (**A**), Ann (**B**), and L-Ann (**C**) in KB (○) and KB-V1 (●) cells. Time of drug exposure, 1 h

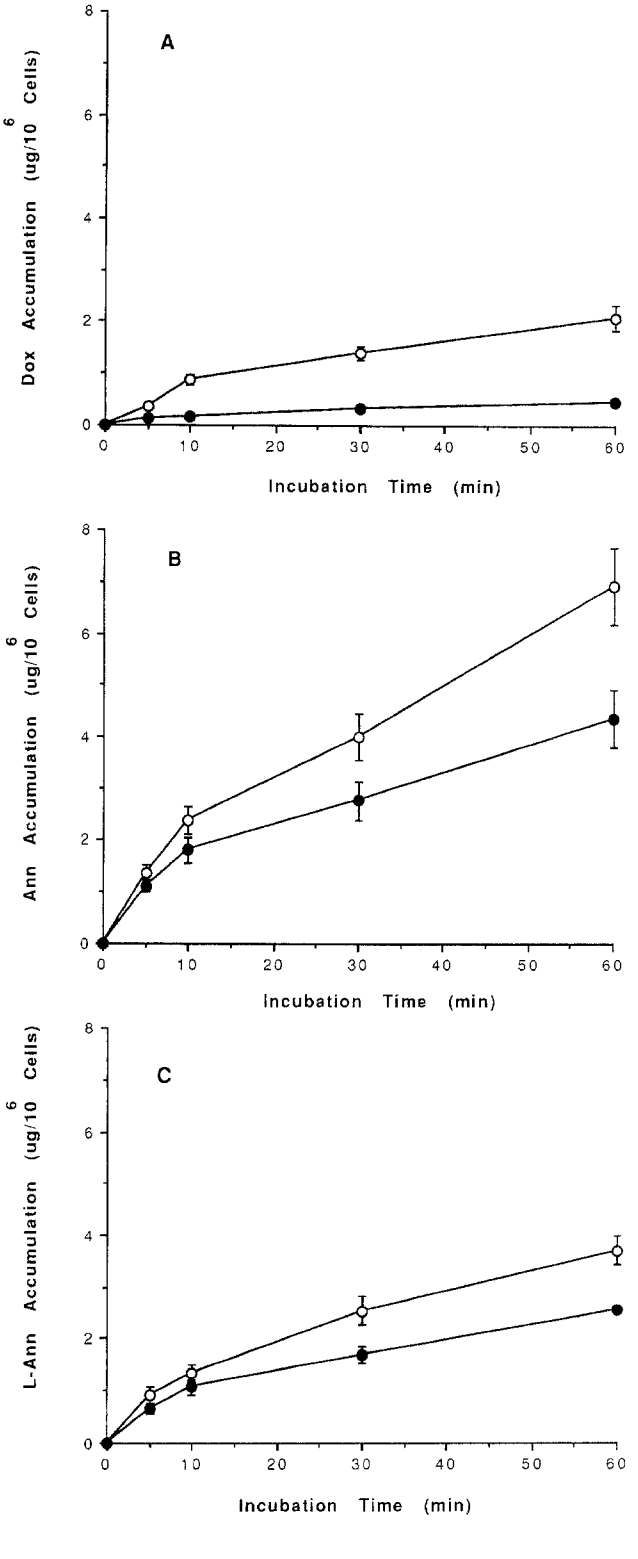


Fig. 3A–C. Cellular accumulation (time course) of Dox (**A**), Ann (**B**), and L-Ann (**C**) in KB (○) and KB-V1 (●) cells. Drug concentration, 10 μg/ml

was also reduced, but only by about 30%. Accumulation of Ann in KB cells was about 3-fold that of Dox and 2-fold that of L-Ann. In KB-V1 cells, accumulation of Ann was about 8-fold that of Dox and 30%–40% higher than that of L-Ann. Cellular accumulation was minimal as measured in experiments performed at 4° C for all drugs, thus suggesting that binding to the cellular membrane is a minor component of the measured cellular accumulation (data not shown).

Table 2. Drug accumulation and retention at 1 h in KB and KB-V1 cells

Drug	KB			KB-V1			KB:KB-V1
	μg/10^6 cells	Ann: Dox	Ann: L-Ann	μg/10^6 cells	Ann: Dox	Ann: L-Ann	
Accumulation							
Dox	1.60±0.22	–	–	0.26±0.08	–	–	6.15
Ann	8.07±1.27	5.04	2.14	6.38±1.67	24.5	2.05	1.26
L-Ann	3.76±0.89	2.35	–	3.11±0.33	12.0	–	1.20
Retention (% of accumulation)							
Dox	1.87±0.17 (117)	–	–	0.09±0.02 (35)	–	–	20.7
Ann	4.11±0.03 (51)	2.19	2.25	2.81±0.95 (44)	31.2	1.39	1.46
L-Ann	1.82±0.23 (48)	0.97	–	2.01±0.11 (65)	22.3	–	0.90

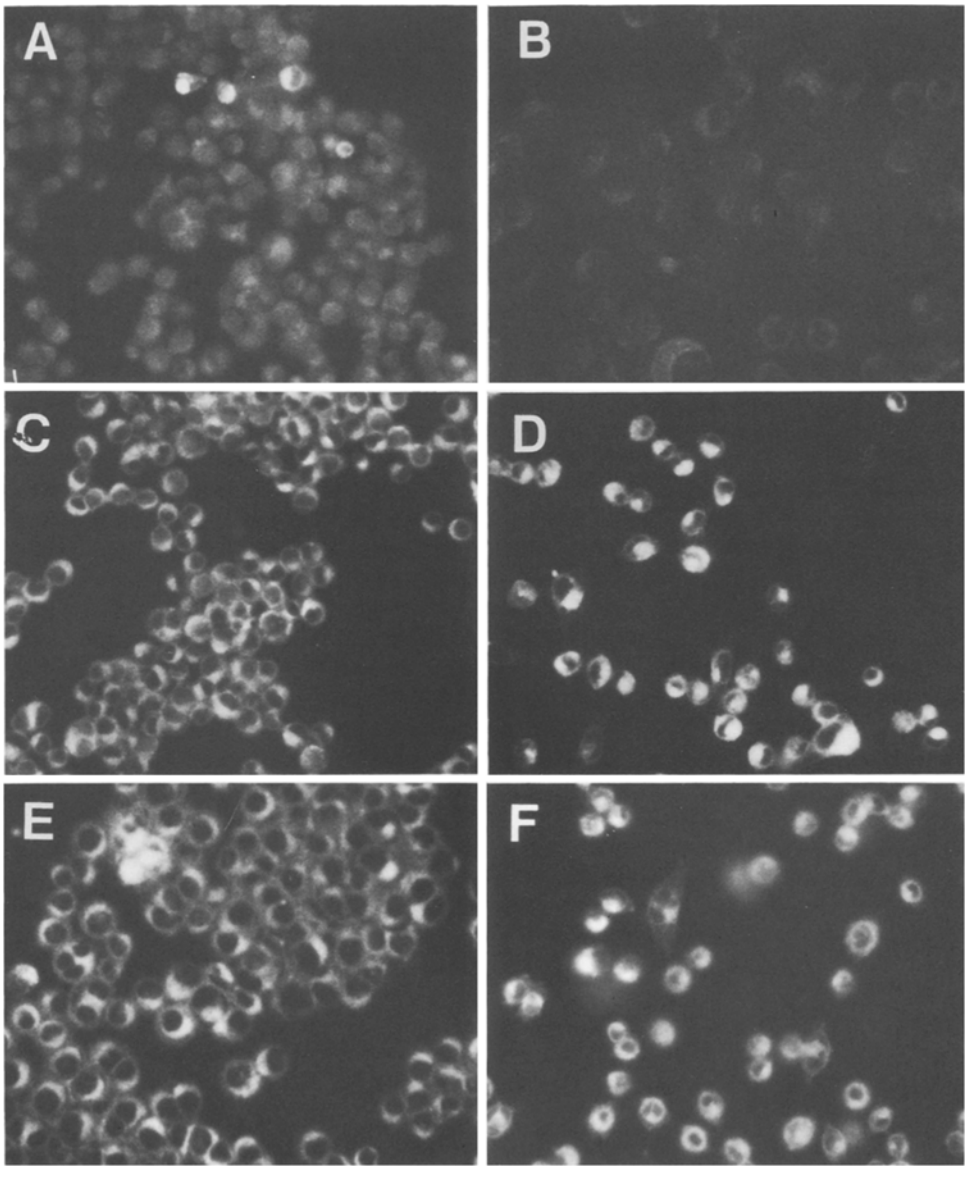


Fig. 4 A–F. Fluorescence microscopy observation of KB (**A, C, E**) and KB-V1 cells (**B, D, F**) exposed to Dox (**A, B**), Ann (**C, D**), and L-Ann (**E, F**) at 5 μg/ml for 5 min

Table 3. Drug accumulation in the presence or absence of resistance modifiers

Treatment	Cellular accumulation (μg/10^6 cells)	
	KB	KB-V1
	(% of drug alone)	
Dox	1.60±0.21 (100)	0.26±0.08 (100)
+ Verapamil	1.82±0.26 (114)	0.50±0.09 (192)*
+ Cyclosporin A	1.86±0.28 (116)	0.51±0.14 (196)*
+ Quinidine	1.93±0.26 (120)	0.38±0.14 (146)
Ann	8.07±1.27 (100)	6.38±1.67 (100)
+ Verapamil	9.39±1.73 (116)	8.77±1.52 (138)
+ Cyclosporin A	7.96±1.38 (99)	4.83±1.08 (75)
+ Quinidine	9.88±1.90 (122)	10.81±2.59 (169)
L-Ann	3.76±0.89 (100)	3.11±0.33 (100)
+ Verapamil	3.40±0.65 (91)	3.77±0.81 (121)
+ Cyclosporin A	3.59±0.74 (96)	3.00±0.72 (96)
+ Quinidine	3.69±0.86 (98)	4.22±0.83 (136)

Cells were exposed for 1 h to Dox, Ann, or L-Ann at 10 μg/ml in the absence or presence of different modifiers at the same concentration
* $P < 0.05$ as compared with Dox alone

Table 4. Drug retention in the presence or absence of resistance modifiers

Treatment	Cellular retention (μg/10^6 cells)	
	KB	KB-V1
	(% of drug alone)	
Dox	1.87±0.17 (100)	0.09±0.02 (100)
+ Verapamil	1.94±0.23 (104)	0.17±0.06 (192)*
+ Cyclosporin A	1.93±0.25 (103)	0.18±0.06 (200)*
+ Quinidine	2.06±0.13 (110)	0.09±0.01 (100)
Ann	4.11±0.03 (100)	2.81±0.95 (100)
+ Verapamil	4.31±0.54 (105)	3.13±0.46 (111)
+ Cyclosporin A	3.87±0.23 (94)	1.79±0.15 (64)
+ Quinidine	4.55±0.41 (110)	3.35±0.23 (119)
L-Ann	1.82±0.23 (100)	2.01±0.11 (100)
+ Verapamil	1.72±0.08 (95)	1.62±0.26 (81)
+ Cyclosporin A	1.59±0.06 (87)	1.15±0.23 (56)
+ Quinidine	1.75±0.09 (96)	2.05±0.16 (102)

Cells were exposed for 1 h to Dox, Ann, or L-Ann at 10 μg/ml in the absence or presence of different modifiers at the same concentration. Cells were washed and cellular drug retention was measured 1 h thereafter
* $P < 0.05$ as compared with Dox alone

Table 2 shows the results of separate cellular accumulation (1-h drug incubation) and retention (1 h after drug removal) studies with the different drugs (10 μg/ml) in KB and KB-V1 cells. The drug accumulation and retention ratios between the different drugs and cell lines are shown. Dox accumulation in KB-V1 cells was about 15% of that in KB cells, whereas that of L-Ann or Ann was about 75%–80%. As compared with that of Dox, accumulation of Ann was 5 and 24 times higher in KB and KB-V1 cells, respectively. Because accumulation of Ann was about 2-fold that of L-Ann, the differences in accumulation noted between L-Ann and Dox in KB and KB-V1 cells corresponded to factors of 2.5 and 12, respectively.

Retention of Dox in KB-V1 cells was about 5% of that in KB cells, whereas that of Ann was about 50% and that of L-Ann was not significantly different. As compared with that of Dox, retention of Ann was only 2 times higher in KB cells but was 31 times higher in KB-V1 cells. As compared with that of L-Ann, retention of Ann was again 2 times higher in KB cells and 40% higher in KB-V1 cells. As a result, retention of L-Ann was similar to that of Dox in KB cells and about 20 times higher than that of Dox in KB-V1 cells.

Dox showed a different drug-efflux pattern as compared with Ann or L-Ann since it was not effluxed from KB cells (drug retention at 1 h, 117%) and was markedly effluxed from KB-V1 cells (drug retention at 1 h, 34.7%). Ann and L-Ann showed similar drug-efflux patterns. They were both similarly effluxed from KB and KB-V1 cells (Ann retention at 1 h, 51% in KB cells vs 44% in KB-V1 cells; L-Ann retention at 1 h, 48% in KB cells vs 65% in KB-V1 cells).

Observation under fluorescence microscopy

Figure 4 shows pictures of KB (left panels A, C, E) and KB-V1 (right panels B, D, F) cells exposed to a 5-μg/ml concentration of Dox (top panels A, B), Ann (middle panels C, D), or L-Ann (bottom panels E, F) at 5 min as observed under fluorescence microscopy. The pictures were taken on the same day using fresh cell preparations and the same photographic technique. Drugs were removed before microscopic observation. When the fluorescence intensity in the different cell cultures was compared, it was obvious that the cellular accumulation of Dox in KB-V1 cells was markedly reduced as compared with that in KB cells, whereas the cellular accumulation of both Ann and L-Ann was similar in the two cell lines. These findings confirm the results of the quantitative cellular accumulation studies described in the previous section. Observation of cells exposed to drugs for up to 20 min gave similar results (pictures not shown). Interestingly, most cellular uptake of Ann appears to occur in the plasma membrane and cytoplasm, whereas the subcellular distribution pattern of Dox is more homogeneous.

Modulation of drug accumulation and efflux

Tables 3 and 4 show the effect of the modulators verapamil, cyclosporin A, and quinidine on the cellular accumulation and retention of Dox, Ann, and L-Ann. None of the modulators increased the accumulation or retention of any of the drugs in KB cells. In KB-V1 cells, the presence of verapamil or cyclosporin A increased the cellular accumulation and retention of Dox by a factor of about 2. Changes in Ann or L-Ann accumulation or retention as a result of the presence of modulators were minimal.

Table 5 shows the effect of 2-deoxyglucose plus sodium azide and monensin on the cellular accumulation of Dox, Ann, and L-Ann. Again, no effect was observed on drug accumulation in KB cells. In KB-V1 cells, the presence of the metabolic inhibitor 2-deoxyglucose plus sodium azide increased the accumulation of Dox by a factor of 2, as did

Table 5. Effect of different modifiers on drug accumulation in KB and KB-V1 cells

Treatment	Intracellular accumulation (μg/10^6 cells)	
	KB	KB-V1
	(% of drug alone)	
Control:		
Dox	2.11±0.14 (100)	0.21±0.02 (100)
Ann	7.01±0.87 (100)	3.76±0.96 (100)
L-Ann	3.56±0.14 (100)	1.64±0.61 (100)
Deoxyglucose (NaN_3 (25 m*M*/3 m*M*):		
Dox	2.15±0.54 (102)	0.41±0.05 (205)*
Ann	6.27±2.18 (90)	3.75±0.77 (100)
L-Ann	3.08±0.57 (86)	1.55 (100)
Monensin (7 μ*M*):		
Dox	2.18±0.30 (103)	0.49±0.05 (234)*
Ann	7.11±0.79 (101)	3.54±0.81 (95)
L-Ann	3.63±0.11 (102)	1.64±0.39 (100)

Cells were exposed for 1 h to Dox, Ann, or L-Ann at 10 μg/ml in the presence or absence of inhibitors at the indicated concentrations. No effect was observed with ouabain (2 m*M*), *N*-ethylmalemide (20 μ*M*), chloroquine (50 μ*M*), or cytochalasin B (20 μ*M*)
* $P < 0.01$ as compared with control

the presence of the carboxylic ionophore monensin, whereas the accumulation of Ann and L-Ann remained unchanged. The presence of the other inhibitors tested (2 m*M* ouabain, 20 μ*M* *N*-ethylmaleimide, 50 μ*M* chloroquine, 20 μ*M* cytochalasin) did not affect the cellular accumulation of the drugs in either KB or KB-V1 cells (data not shown).

DNA damage

Figure 5 shows the effects of Dox, Ann, and L-Ann in inducing double- and single-strand DNA breaks in KB and KB-V1 cells. All experiments were performed using a drug concentration of 10 μg/ml for 1 h. In KB cells, both Ann and L-Ann induced about 3 times more double- and single-strand DNA breaks than did Dox. Ann was slightly more effective than L-Ann in inducing both types of lesions. In KB-V1 cells, no DNA lesion was detected in cells exposed to Dox. Although both Ann and L-Ann were less effective in inducing double- and single-strand DNA breaks in KB-V1 cells as compared with KB cells, the level of DNA damage was similar to that caused by Dox in KB cells.

Discussion

The main objective of this study was to ascertain the mechanism of the lack of cross-resistance of the new anthracycline Ann, which is about to enter clinical trials, and to assess the consequences of liposome entrapment on its cellular effects. Our results indicate (1) that the lack of cross-resistance of Ann is associated with a markedly enhanced accumulation and retention of the drug in resistant cells as compared with Dox, thus resulting in induction of

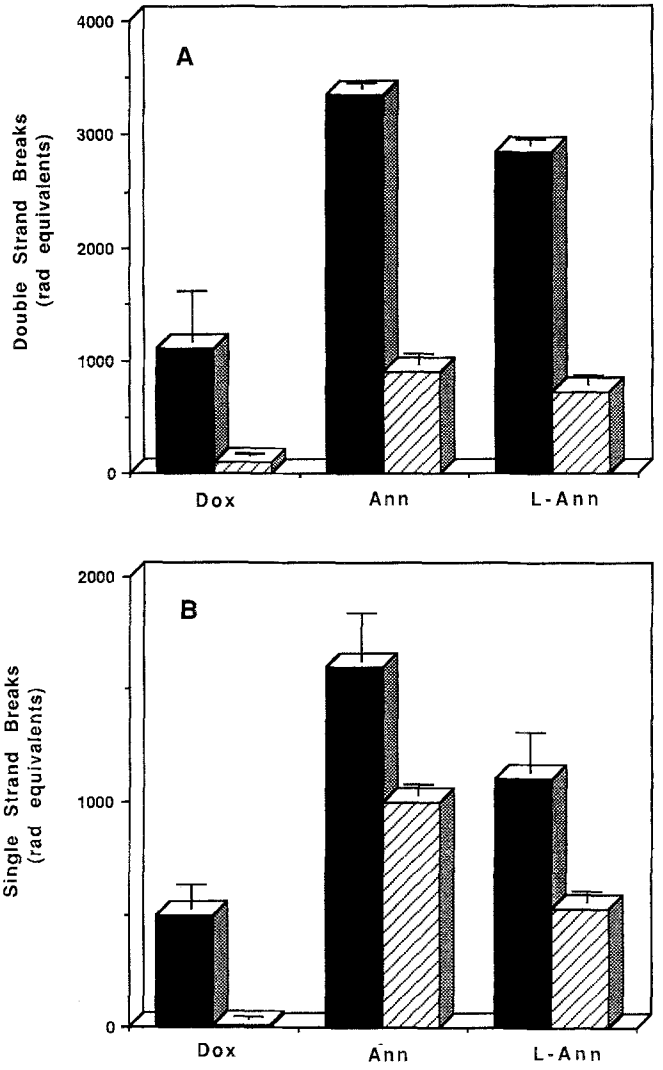


Fig. 5A, B. Double-strand (**A**) and single-strand DNA breaks (**B**) induced by exposure to Dox, Ann, and L-Ann at 10 μg/ml for 1 h in KB (*dark bars*) and KB-V1 cells (*striped bars*)

DNA lesions in resistant cells, and (2) that the liposomal carrier used has little effect on the cytotoxicity of Ann, although it tends to decrease drug accumulation in both cell lines and to enhance drug retention in resistant cells.

As compared with those of Dox, the cellular accumulation and retention of Ann were 2–3 times higher in KB cells and 10–30 times higher in KB-V1 cells as a result of differences in the uptake and efflux patterns of the two drugs. Ann displayed similar efflux patterns in KB and KB-V1 cells, whereas Dox was not effluxed from KB cells but was markedly effluxed from KB-V1 cells during the 1-h efflux period used in our studies. This observation together with the inability of verapamil and other modulators to increase the cellular accumulation of Ann in KB-V1 cells provides indirect evidence that Ann is not a good substrate for P-glycoprotein or does not interact with P-glycoprotein as a result of its high affinity for the lipidic portion of the cell membrane or the absence of a basic amino group in the sugar portion of the molecule as described for hydroxyrubicin [14]. In spite of the remarkable ability of Ann to be retained in KB-V1 cells, the cellular accumulation and

retention of Ann in KB-V1 cells were about 30% lower than those in KB cells, as was the extent of DNA breaks. This observation and the finding that the resistance in KB-V1 cells is multifactorial may explain why the resistance index was decreased by 20 times but not reduced to 1. In recently completed studies, both Ann and L-Ann have shown significant activity in delaying the growth of KB-V1 xenografts in nude mice [25].

The use of anthracycline analogs to overcome MDR has been explored before. At least five different subfamilies of anthracyclines have been reported to be capable of overcoming MDR mostly in vitro: the morpholinyl anthracyclines [6–9]; the *N*-benzyl derivatives [10–12]; 4′-iododoxorubicin [13]; the 3′-deaminated anthracyclines, such as Ann [14]; and the 4-demethoxylated analogs, such as idarubicin and Ann itself [26]. All these compounds have in common a high lipophilicity, a markedly increased cellular accumulation in both sensitive and resistant cells as compared with that of Dox, and similar efflux patterns in sensitive and resistant cells, whereas Dox efflux is markedly increased in resistant cells as compared with sensitive cells. However, important differences are noteworthy. For example, approximately 75% of MX2, one of the best-studied morpholinyl anthracyclines, was found to be effluxed from both sensitive and resistant cells [17], whereas the efflux of Ann was only about 50% in our study. In addition, in contrast to Dox, the morpholinyl anthracyclines produce DNA interstrand cross-linking [27] and are not topoisomerase II inhibitors [28]. Therefore, a different mechanism of cytotoxicity may explain, at least in part, the cytotoxicity of the morpholinyl derivatives against MDR cells. Also, whereas MX2 and Dox are similarly effective in inducing double-strand DNA breaks in sensitive cells, Ann is 2–3 times more effective than Dox while being 2 times less potent in vitro.

Because of their marked lipophilicity, the development of an appropriate drug-delivery system is an important aspect of the preclinical development of most non-cross-resistant anthracyclines, including Ann. To facilitate our task, we specifically selected Ann from more than 30 lipophilic analogs because it had a very high affinity for artificial lipid membranes in addition to its remarkable activity, thus allowing for the use of liposomes as a natural drug-delivery system. As previously reported, most lipophilic anthracyclines, including some of the morpholinyl derivatives, tend to precipitate in the aqueous milieu rather than associating with phospholipid bilayers, which makes them poor candidates for liposomal delivery [17].

The multilamellar vesicles used to deliver Ann are easy to prepare and have been extensively used in humans without resulting in toxicity [29]. In our in vitro experiments, they did not significantly alter the cytotoxic properties of Ann. This finding implies that Ann does not need to reach the tumor cells associated with the liposomes to exert its cytotoxic effect. Liposomal incorporation tended to decrease cellular accumulation of Ann by a factor of 2, but this difference did not have an impact on its cytotoxicity. There are several possible explanations for this observation: (1) Ann tends to remain bound to the liposome membranes under in vitro conditions due to its high affinity for the liposome membranes; (2) the DMSO present in the free Ann preparation enhances its cellular accumulation; and (3) fluorescence quenching by the liposomes. The third possibility appears unlikely in view of the similar fluorescence intensity of free and L-Ann in ex vivo studies.

Because liposomes, including small unilamellar vesicles, are larger than the endothelial gaps, intact vesicles can certainly reach organs with fenestrated capillaries such as the liver, spleen, and bone marrow but cannot reach other organs. Recently, liposomes containing cardiolipin or phosphatidyl serine have been found to be capable of overcoming MDR when used as carriers of Dox in vitro [30–34] and in vivo in bone marrow cells [34]. Therefore, these studies indicate that liposomes of certain composition may favorably alter the cellular pharmacology of drugs. However, it appears unlikely that these formulations would be effective for the treatment of human solid tumors expressing the MDR phenotype, since the vascular barrier may prevent them from reaching the tumor cells intact and their ability to overcome resistance appears to require both the presence of specific lipids in the liposome membrane and direct contact of the intact vesicles with the cells. Because of their intrinsic lack of cross-resistance, this potential limitation does not apply to lipophilic anthracyclines such as Ann. We have explored the use of cardiolipin liposomes as carriers of Ann (data not shown) and found that they are capable of significantly increasing the cellular accumulation of Ann in both sensitive and resistant cells without changing the resistance index. Therefore, they do not appear to offer any additional advantage over those of Ann itself or Ann delivered in multilamellar liposomes of the composition used in our study.

We have described elsewhere the organ distribution of Ann and L-Ann in mice bearing s.c. B16 melanoma tumors and unexpectedly found that liposomal delivery actually enhances the tumor drug uptake by 2–3-fold without altering the heart levels [19]. Therefore, apart from being an appropriate vehicle for the administration of Ann, the multilamellar liposomes used can actually increase the delivery of Ann to the tumor site. This observation contrasts with the in vitro studies, in which liposomal incorporation tended to decrease Ann accumulation in tumor cells. It appears unlikely that this enhanced in vivo tumor targeting of the drug is mediated by a preferential localization of intact liposomes to the tumor tissue as discussed above; rather, it could to be the result of drug transfer to a second carrier such as serum lipoproteins.

In summary, our study shows that the cellular effects of the lipophilic anthracyclin Ann are little affected by presenting the drug either in a suspension containing DMSO or entrapped in large liposomes suspended in saline. These findings suggest that any advantage that L-Ann may display in future clinical studies will probably be due either to the drug's properties or to changes in its pharmacokinetics and organ distribution secondary to liposome entrapment. Because changes in liposome composition and size offer an opportunity to alter the pharmacokinetics and further improve the tumor-targeting properties of drugs entrapped in liposomal and because Ann is easily amenable to entrapment in a wide variety of liposomal carriers with negligible technical difficulties, there is room for further enhancement of its tumor-targeting capacity and secondary improvement

of its therapeutic index, specifically by using small liposomes with a prolonged serum half-life as extensively reported by numerous investigators [35–38].

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