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LIPOSOME ENCAPSULATION ENHANCEMENT OF METHOTREXATE SENSITIVITY IN A TRANSPORT RESISTANT HUMAN LEUKEMIC CELL LINE*

JOHN A. TODD†, EDWARD J. MODEST‡, PETER W. ROSSOW‡ and ZOLTÁN A. TÖKÉS†§

†Department of Biochemistry, USC School of Medicine and the USC Comprehensive Cancer Center, Los Angeles, CA 90033, U.S.A. and ‡Division of Pharmacology, Sidney Farber Cancer Institute, Boston, MA 02115, U.S.A.

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Abstract—A stable mutant of human leukemia CCRF/CEM cells has recently been isolated which is transport resistant for methotrexate (MTX). Encapsulation of MTX in cationic unilamellar liposomes increased the association of the drug 5-fold with the sensitive, and 50-fold with the resistant, cells as compared to the uptake of free drug. The liposome-mediated associations of MTX with sensitive and transport deficient cell lines were similar. Cytostatic studies demonstrated that liposome encapsulation increased MTX activity 4-fold towards the transport resistant cell line. The addition of cholesterol to the vesicles decreased their effectiveness. A 4-fold increase in drug sensitivity due to encapsulation may allow such transport resistant tumor cells to become responsive to chemotherapeutic doses of MTX which are currently feasible in human clinical protocols.

The utility of antifolates in controlling both malignant and nonmalignant diseases is complicated by the problem that some tumors either possess intrinsic resistance or acquire resistance to these compounds. At least three major types of mechanisms by which cells can acquire resistance to antifolates have been described. These mechanisms include: (i) an altered or different form of dihydrofolate reductase, the target enzyme [1–4], (ii) overproduction of this enzyme [1, 3–7], and (iii) a defect in the transport of antifolates into the cell [3, 8–11]. Acquisition of resistance may involve the simultaneous development of one or more of these alterations [3].

Recently, a clone of human CCRF-CEM cells has been isolated which is resistant to methotrexate (MTX)‖ [11]. These cells possess a diminished ability to transport MTX. This phenomena is due in part to the loss of the low affinity plasma membrane component for MTX transport [12]. The high affinity transport component is slightly altered though still present. However, due to competitive inhibition by a serum component, 5-methyltetrahydrofolate, MTX is prevented from freely entering the cell by this route.

In our earlier studies with human chronic lymphocytic leukemia cells, we demonstrated that the liposome encapsulation enhanced up to two hundred times the quantity of MTX that could associate with the cells [13]. Although the encapsulated drug was not as efficient as the entrapped MTX in inhibiting dihydrofolate reductase, significant enzyme inhibition was still observed. This observation suggests the possibility of using liposome entrapped MTX to overcome transport resistance. Such an approach would deliver the antifolate by a mechanism independent of the normally available high and low affinity carriers.

We report here a series of experiments designed to investigate the potential for entrapping antifolates in liposomes to overcome drug transport resistance in human CCRF-CEM cells. Our observations demonstrate that when MTX is entrapped in liposomes it associates fifty and five times more readily with drug resistant and parent CCRF-CEM cells respectively. Furthermore, the increased association leads to a 4-fold increase in cytostatic activity towards the MTX resistant cell line.

MATERIALS AND METHODS

Chemicals. Phospholipids were obtained from the Sigma Chemical Co. (St. Louis, MO) and contained less than 2% contaminants as determined by silica gel thin-layer chromatography in chloroform–methanol–water (75:22:3), developed with I_2 vapor. Stearylamine was obtained from the Eastman Kodak Co. (Rochester, NY). MTX was obtained from the Sigma Chemical Co. [3′,5′,7-^{3}H]MTX (20 Ci/mmole) was obtained from Moravek Biochemicals (City of Industry, CA) and was judged to be greater

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§ Address all correspondence to: Zoltán A. Tökés, Cancer Research Laboratory, Comprehensive Cancer Center, Kenneth Morris Jr. Cancer Research Institute, University of Southern California, 1303 North Mission Rd., Los Angeles, CA 90033, U.S.A.

‖ Abbreviations: MTX, methotrexate; CEM/O, CCRF/CEM human lymphoblastoid cell line; CEM/MTX, methotrexate resistant subline of the CCRF/CEM cell line; PC, phosphatidyl choline; SA, stearylamine; CH, cholesterol; PS, phosphatidyl serine; SUV, neutral small unilamellar vesicles; SUV^+, cationic small unilamellar vesicles; SUV$^-$, anionic small unilamellar vesicles; and MLV^+, cationic multilamellar vesicles.

than 95% pure by paper chromatography in butanol–pyridine–water (1:1:1). All other chemicals were reagent grade. Tissue culture supplies were obtained from Flow Laboratories (Rockville, MD).

Liposome preparation. The composition and molar ratios of the various liposome preparations were as follows: PC:SA (4:1); PC:CH:SA (4:3:1); PC:PS (9:1); and PC. Small unilamellar vesicles (SUV) containing MTX were prepared by evaporating 30 mg of lipid under N_2 to form a shell. Fifteen mg of MTX in 0.75 ml of 0.9% saline was added, and the mixture was vortexed to disperse the lipid and sonicated for 12 min at 125 W under an N_2 atmosphere with a microprobe sonicator (Biosonik IV; VWR Scientific Division, Univar, Los Angeles, CA). The transparent liposome preparation was separated from non encapsulated drug by gel filtration on a 1 × 35 cm Sephadex G-50 column equilibrated and eluted with 0.9% NaCl. Non-encapsulated MTX, which was in the included volume, was saved for experiments in which the free form of drug was required. Vesicles containing [^{3}H]MTX were prepared by adding 30 μCi of [^{3}H]MTX to the unlabelled MTX prior to sonication. Empty liposomes were prepared analogously, but without the addition of MTX. SUV containing MTX, empty SUV, or non-entrapped MTX were made sterile by passage through 0.2 μm millipore filters. The concentrations of non-entrapped or liposome-entrapped MTX were determined spectrophotometrically at 303 nm in methanol. Vesicle size ranged from 500 to 1500 Å as determined by negative stain electron microscopy [14]. The efficiency of MTX entrapment averaged between 1 and 2%. In the remainder of the text, the various small unilamellar vesicle preparations will be referred to as: (i) SUV^+, cationic vesicles, (ii) SUV^-, anionic vesicles, and (iii) SUV, neutral vesicles.

Large multilamellar vesicles (MLV) containing MTX were prepared similarly as SUV except that the sonication time was limited to 2 min. Since these vesicles were larger than 0.2 μm, they could not be millipore filtered. Instead, the liposomes were prepared using sterile reagents and equipment in a laminar flow hood. The efficiency of MTX entrapment in these liposomes averaged 5%.

Liposome stability. Five mg of the liposome preparations containing MTX and a trace of [^{3}H]MTX was added to 4 ml of medium RPMI-1640 (Flow Laboratories) containing 10% newborn calf serum and gentamycin. The suspension was placed in a shaking incubator at 37° and 125 μl samples were withdrawn at 1 min, 1, 18, 24, and 48 hr. These aliquots were applied to Sephadex G-200 colums that had been equilibrated and developed with 0.9% NaCl. Serum protein–[^{3}H]MTX complexes were partially included, and free [^{3}H]MTX was eluted in the included volume. Liposome entrapped [^{3}H]MTX eluted in the excluded volume and was assumed to represent intact vesicles. The time required to reach 50% disruption of the vesicles was determined.

Cells. The CCRF/CEM human lymphoblastoid cell line CEM/O [15] and a MTX-resistant subline (CEM/MTX) [11, 12] were grown in 25 cm^2 flasks with medium RPMI-1640 supplemented with gentamycin, 8% newborn calf serum, and 2% fetal calf serum. The resistant cells possess a diminished ability to transport MTX [11, 12].

Uptake studies. Both cell lines were harvested by centrifugation and washed twice with 0.9% saline. Usually 1×10^8 cells were resuspended in 10 ml RPMI-1640 containing 10% newborn calf serum and were incubated for 2 hr at 37°. Either SUV^+ entrapped [^{3}H]MTX, non-entrapped [^{3}H]MTX, or empty SUV^+ plus non-entrapped [^{3}H]MTX was added to a final concentration of 0.5 μM. At 5-, 15-, 30-, 60- and 120-min intervals, 0.5 ml of the suspension was removed and placed in 2 ml of ice-cold 0.9% saline. The samples were then centrifuged at 200 *g* and washed four times with 5 ml of cold saline to remove non-cell associated [^{3}H]MTX. Cell viability was greater that 95% at the beginning and end of each assay as determined by trypan blue exclusion. The final pellet was suspended in 0.5 ml water and measured for ^{3}H-label in PCS II scintillation fluid (New England Nuclear Corp., Boston, MA) using a Beckman LS8000 liquid scintillation counter.

Cytostatic assay. One ml of the cell suspension containing 8% newborn and 2% fetal calf serum and gentamycin in medium RPMI-1640 was placed in each well of 2 ml multiwell plates (Falcon, Oxnard, CA) at an initial density of 5×10^4 cells/ml. After allowing the cells to grow for 24 hr, the plates were centrifuged at 200 *g* and the medium was removed. One ml of fresh medium containing 10% newborn calf serum, antibiotics, and either liposome entrapped MTX, non-entrapped MTX, or empty liposomes plus non-entrapped MTX was added to each well. In the first type of cytostatic assay, these plates were allowed to incubate for 4 days at 37° under a 5% CO_2 atmosphere. On day 4, the final cell density in each well of the plate was determined with a Coulter Counter Model Zb equipped with a channelyzer. In the second type of cytostatic assay, the cells in the plates were centrifuged at 200 *g* and the medium was removed. The new medium containing the drug was added to each appropriate well. This procedure was repeated every 24 hr for 3 consecutive days. Twenty-four hr after the last change of medium, the final cell density was determined. Cellular sensitivity to the drug was defined as the mean IC_{50}, which is the concentration of MTX required to inhibit cell growth to 50% of the untreated cell population.

RESULTS

The stability of liposome preparations at 37° in medium RPMI-1640 with 10% newborn calf serum is a function of composition and vesicle size (Table 1). The addition of cholesterol to SUV^+ during preparation approximately doubled vesicle stability. Multilamellar vesicles containing cholesterol were substantially more stable than unilamellar vesicles of an equivalent phospholipid composition. Their half-times of degradation were 24, 52 and 113 hr for SUV^+, cholesterol-containing SUV^+, and cholesterol-containing MLV^+ respectively.

Since the CEM/MTX cell line has been described previously as possessing a decreased ability to transport MTX [11], experiments were performed to test

Table 1. Percentage of intact liposomes after incubation for various lengths of time*

Incubation time	Percentage of intact liposomes: Small unilamellar vesicles [PC:SA]	Small unilamellar vesicles [PC:CH:SA]	Multilamellar vesicles [PC:CH:SA]
1 min	91%	99%	99%
1 hr	79%	83%	95%
18 hr	52%	60%	89%
24 hr	50%	58%	85%
48 hr	46%	51%	76%

* Incubations contained 5 mg of liposome lipid in 4 ml of RPMI-1640 with 10% newborn calf serum; the conditions were as described in Materials and Methods. Intact liposomes containing [^{3}H]MTX were quantitated by passing the incubation medium through a Sephadex G-200 column. The results are the average of two separate experiments.

whether liposome encapsulation could increase MTX association with these cells. Figure 1A shows that encapsulation of [^{3}H]MTX in SUV$^+$ increased its association 5-fold with the drug-sensitive cell line.

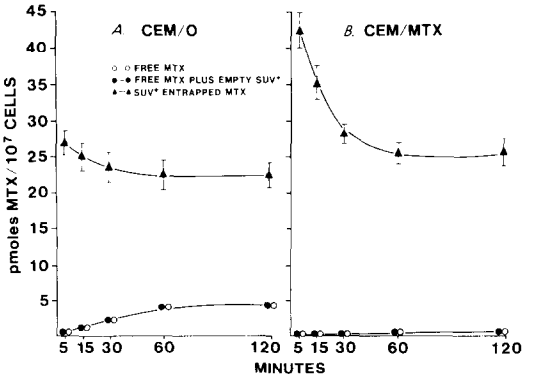

Fig. 1. Time course of MTX uptake by either sensitive CEM/O (A) or drug-resistant CEM/MTX (B) human leukemic cells. Cells in 10 ml of RPMI-1640 with 10% newborn calf serum were presented with 0.5 μM MTX either non-entrapped or entrapped in 0.85 mg of lipid in cationic unilamellar vesicles (ULV$^+$). These values represent the mean ± S.D. for pmoles of MTX associated with 10^7 cells after incubation at 37° and five consecutive washings with PBS.

Empty vesicles had no effect on the uptake of non-entrapped drug, indicating that they do not modify the membrane transport mechanism for MTX. The uptake of [^{3}H]MTX by the drug-resistant cell line in Fig. 1B was approximately 10-fold less than for the sensitive cells. This finding is in agreement with previous reports [11]. Encapsulation of [^{3}H]MTX in SUV$^+$ increased the association of the drug with the resistant cells by 50-fold. Empty SUV$^+$ had no effect on the uptake of [^{3}H]MTX by CEM/MTX. Association of encapsulated drug with both cell lines was rapid and reached maximum levels within 5 min. This rapid binding can be explained by an initial adsorption of liposomes to the surface of the cells [16]. By 60 min, the quantity of cell-associated drug decreased to constant levels of approximately 25 pmoles/10^7 cells for both cell lines.

Experiments were performed to assess the cytostatic activity of liposome-entrapped MTX towards CEM/O and CEM/MTX cells. The cells were added to culture medium containing either non-entrapped or liposome-entrapped drug, and the final cell density was determined after 86 hr of incubation. The results are presented in Table 2. When MTX was presented to CEM/O cells either free, free plus empty SUV$^+$, entrapped in SUV$^+$, or entrapped in MLV$^+$, the IC_{50} values were not significantly different. Under these culture conditions, liposome-entrapped MTX was only equally effective as non-entrapped MTX in inhibiting the growth of both these cell lines.

Table 2. Cytostatic activity of liposome entrapped MTX towards CEM/O and CEM/MTX cells using a single addition of drug preparations*

Form of MTX added	IC_{50} (μM): CEM/O	CEM/MTX
Non-entrapped	0.048 ± 0.02	1.6 ± 0.3
Non-entrapped + empty SUV$^+$[PC:SA]	0.059 ± 0.01	1.4 ± 0.4
SUV$^+$[PC:SA]	0.056 ± 0.02	1.5 ± 0.1
MLV$^+$[PC:CH:SA]	0.059 ± 0.02	2.1 ± 0.4

* Cells were added to media containing the different forms of MTX and allowed to grow for 86 hr as described in Materials and Methods. The amount of liposome lipid added was 0.2 mg/ml at MTX concentrations of 1 μM. The results are the mean values from three separate experiments ± S.D. for the concentration of MTX required to inhibit cell growth by 50%.

Table 3. Cytostatic activity of liposome entrapped MTX towards CEM/MTX cells when exposed to fresh liposomes every 24 hr*

Form of MTX added	IC_{50} (μM) CEM/MTX
Non-entrapped	1.8 ± 0.8
Non-entrapped + empty	1.5 ± 0.1
SUV [PC:PS]	2.5 ± 0.3
SUV⁻[PC:CH:SA]	1.4 ± 0.5
SUV[PC]	0.7 ± 0.4
SUV⁻[PC:SA]	0.39 ± 0.20

* Cells were centrifuged and fresh media containing the different forms of MTX were added as described in Materials and Methods. The amount of liposome lipid added was 0.2 mg/ml at MTX concentrations of 1 μM. The results are the mean values from four separate experiments ± S.D. for the concentration of MTX required to inhibit cell growth by 50%.

From our stability studies, it was apparent that the various vesicle preparations would not remain intact during the time required for growth inhibition experiments. Therefore, our assays were redesigned to increase the time interval in which the cells would be exposed to intact vesicles. CEM/MTX cells were added to tissue culture media containing the different forms of drugs and were allowed to incubate for 24 hr. The cells were then centrifuged and resuspended in fresh media containing the different forms of drug every 24 hrs for 3 days. Results from these experiments are presented in Table 3. An example of a dose–response curve from a representative experiment is presented in Fig. 2. Entrapment in SUV⁺[PC-SA] or SUV[PC] increased the cytostatic activity of MTX approximately 4- and 2-fold, respectively, and partially overcame the drug resistance displayed by this cell line. Addition of empty vesicles did not effect the IC_{50} of the free drug. When vesicles

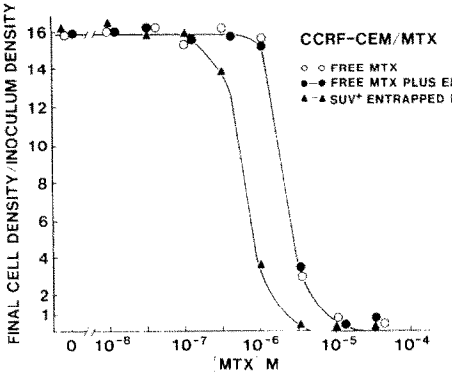

Fig. 2. Inhibition growth of drug-resistant CEM/MTX cells. Non-entrapped or cationic unilamellar liposome (ULV⁺) entrapped MTX was added to the cells in medium RPMI-1640 with 10% newborn calf serum. The amount of liposome lipid added was 0.2 mg/ml at MTX concentrations of 1 μM. Every 24 hr, the cells were centrifuged and resuspended in fresh media containing either form of drug. Final cell density was determined 84 hr later and plotted as a ratio of the inoculation cell density. Each symbol represents the mean density of two cultures.

containing cholesterol were used, they were only as effective as non-entrapped drug. Anionic vesicles containing MTX were less efficient at inhibiting cell growth.

Uptake experiments with fresh unilamellar vesicles were performed with CEM/MTX cells that had been incubated previously with encapsulated MTX for 24 hr. we found that the freshly added liposome–drug complexes did not bind to the cells or further increase the amount of MTX that was previously cell associated. These results indicated that the vesicle uptake sites in the surface of the cells remained saturated for up to 24 hr.

DISCUSSION

During the past few years, significant effort has been devoted to evaluating liposome–drug complexes for their anti-tumor potential. Many drugs have been shown suitable for entrapment and have displayed increased cytotoxicity towards established cell lines *in vitro* [17, 18] and experimental tumors *in vivo* [18–26]. Since liposomes can enter cells by mechanisms that are not available to anti-cancer drugs [27], it is conceivable that, by encapsulation, the normal route of drug entry could be bypassed. However, only limited reports exist on the ability of liposomes to overcome drug transport resistance [17, 28]. Recently, a stable mutant of human CCRF/CEM leukemic cells has been isolated which is transport resistant for MTX [11, 12]. The development of this type of resistance is postulated to be one of the mechanisms by which malignant cells cease responding to MTX [3, 8–11]. We felt, therefore, that liposome encapsulated MTX should be evaluated for its ability to overcome the resistance.

Non-entrapped MTX attained 10 fold higher levels of drug in sensitive leukemia cells than in resistant ones. In contrast, cationic unilamellar vesicles associated drug with either cell line much faster than when non entrapped. The rates of uptake and the plateau levels reached were similar for both cell lines, indicating that a common mechanism may exist to accumulate vesicle–drug complexes. Liposome-entrapped MTX accumulated rapidly with the cells and may have represented binding of vesicles to their surfaces. The initial quantity of cell-associated drug decreased to constant levels by 60 min. This decrease may be due to serum protein disruption of vesicles that were bound to the surface of the cells. Encapsulation increased MTX association 50-fold with the resistant cell line by 60 min. This cellular association does not necessarily mean that all of the drug got to its pharmacological site of action. However, if even a small proportion of MTX is available to dihydrofolate reductase, then the resistance might be partially overcome.

Experiments were performed to evaluate the potential usefulness of encapsulation in overcoming transport resistance. When liposomes containing MTX were added to cultures of drug sensitive or resistant CEM/MTX cells and allowed to incubate for 86 hr, no increase in cytostatic activity was observed for the encapsulated drug. In both cell types, values for MLV⁺ or SUV⁺ entrapped MTX were almost identical to those obtained for free MTX

with or without empty liposomes. Since several serum proteins are known to disrupt the integrity of lipid bilayer vesicles [29–31], this finding most probably reflected the instability of the lipid vesicle–drug complexes in the incubation medium. Indeed, when tested for stability their degradation half-times were substantially less than the time required for the growth inhibition assay. Small unilamellar vesicles were less stable than large multilamellar vesicles. When cholesterol was added to unilamellar vesicles during preparation, their integrity increased, most probably reflecting a decrease in bilayer fluidity [32–34]. Due to the relative instability of the liposomes, it was necessary to design growth inhibition assays that reflected intact vesicle interactions with leukemic cells. When the cell culture medium was changed during the cytostatic assay and freshly prepared vesicles were added every 24 hr, a marked change in the IC_{50} values was observed. Unilamellar vesicles containing MTX and composed of PC and SA were approximately four times more effective than the free drug in inhibiting the drug-resistant cell growth. Using this technique we have not found an increase in cytostatic activity against the sensitive cell line. Empty vesicles did not affect the IC_{50} for free MTX, indicating that the liposomes were not exerting a cytostatic effect at this concentration. Furthermore, quantitation of empty vesicle toxicity revealed that the amount of lipid used in these experiments was eight times less than that required to exert 10% cytostatic activity. If SA was omitted from the vesicles containing MTX, they became less effective in inhibiting cell growth but were still twice as effective as the non-entrapped drug. The increase in cytostatic activity by the daily addition of vesicles did not reflect additive binding over the assay period. This is because the adsorption sites for liposomes on the cells surface remained saturated for at least 24 hr. Instead, this increase was probably mediated through a direct delivery of vesicle content to the pharmacological site of action of the drug. Further supporting this postulation is the observation that when cholesterol was added to PC:SA vesicles, their effectiveness decreased to that of the free drug. This probably reflects the mechanisms of vesicle-cell interaction. The cellular uptake of liposomes has been postulated to proceed by (i) cell surface adsorption, (ii) endocytosis, a process by which the liposome contents are delivered to endocytotic vesicles that may later fuse with lysosomes, and (iii) fusion with the plasma membrane in which the liposome contents are emptied directly into the cytoplasm [27]. Fusion is favored for vesicles that have a fluid membrane and endocytosis is favored for more rigid vesicles. Since cholesterol decreases the fluidity of phospholipid bilayers, it is possible that the cholesterol-containing vesicles did not fuse with the cells as readily as the more fluid liposomes. Consequently they were not as efficient in delivering MTX to its cytoplasmic site of action. Vesicles containing the anionic phospholipid PS were slightly less effective than non-entrapped MTX in inhibiting CEM/MTX growth. Since these vesicles were negatively charged, they may have been repelled by the overall negative charge on the surface of the cell. Although we have demonstrated only a 4-fold increase in cytotoxicity, it is probable that other vesicles can be designed to further increase MTX delivery to its site of action.

Our observations demonstrate that an alternative route of drug delivery, mediated by liposomes, renders a line in human leukemic cells that are transport resistant to MTX more sensitive to the drug. This type of increase in drug sensitivity may decrease the levels of MTX required to overcome such transport resistance clinically, and in turn decrease toxicity to normal tissues [35, 36]. Future experiments will require an assessment of MTX-induced toxicity when encapsulated.

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