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Antitumor effect of mitoxantrone-containing liposomes

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Abstract

The antitumor activity of mitoxantrone-containing liposomes was compared in vitro and in vivo with that of free mitoxantrone using L1210 leukemia cells. The in vitro inhibition of the tumor cells was tested after treatment for 24 and 72 h. It was found that mitoxantrone-containing liposomes showed a slower antitumor reaction and higher antitumor activity after treatment of the cells for 72 h. This was attributed to the liposomes sustained and enhanced the action of mitoxantrone. The in vivo antitumor effect of L1210 bearing mice treated with either mitoxantrone-containing liposomes or free mitoxantrone demonstrated no significant difference in survival time. However, an increase in survival rate for mitoxantrone-containing liposomes was shown. After a high dose treatment of the tumor bearing mice with mitoxantrone-containing liposomes, the survival rate was increased. This was due to the increase of antitumor activity and reduction of toxicity of mitoxantrone by liposomes.

Keywords: Mitoxantrone; Liposome; L1210 cell; CDF_1 mouse; Antitumor effect

1. Introduction

In previous works, the entrapment of mitoxantrone in liposomes was studied to investigate the loading efficiency, in vitro release characteristics and chemical stability (Law et al., 1991; Law et al., 1994; Law et al., 1995). It was found that negatively charged liposomes demonstrated a high loading efficiency depending on the ionic strength and pH of the medium. Neutral liposomes showed a profile of loading efficiency similar to that of the negatively charged liposomes but with a lower magnitude of loading efficiency. With positively charged liposomes, the loading efficiency increased with the added concentration of mitoxantrone. The effect of galactocerebroside on the loading efficiency increased with added concentration of mitoxantrone. The loading efficiency was dependent on neither the phospholipid concentration of liposomal membrane nor the methods for preparing and separating the liposomes. The release characteristics of mitox-

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antrone-containing liposomes showed that the effect of lipid composition on release rate resulted in an order of positively charged liposome > neutral liposome > negatively charged liposome. The release of mitoxantrone from liposomes after the incorporation of galactocerebroside was not dependent on the concentration of added galactocerebroside. The increase in temperature brought about an increase of release rate. At 4, 25 and 37°C, the release profiles fitted a linear concentration-square root of time plot which indicated the diffusion-controlled release of entrapped mitoxantrone from the membrane matrix. At high pH, the partitioning of mitoxantrone into the membrane matrix was considerable and the mitoxantrone was released rapidly. The result of chemical stability of mitoxantrone entrapped in liposomes showed that the effect of silanization on glass containers, charge characteristics of the liposomes, pH of the medium, addition of ascorbic acid in the medium, and temperature for storage on the degradation of mitoxantrone followed a pseudo-first-order reaction. The loss of concentration for mitoxantrone was found at zero time of storage in plain glass vials at pH 5.8 and 7.4. At pH 3.6 and 4°C, the mitoxantrone entrapped in negatively charged liposomes in silanized glass vials resulted in an optimum half-life. The degradation of mitoxantrone in solution and in liposomes increased with increasing pH of the medium. The degradation of mitoxantrone in negatively charged, positively charged and neutral liposomes showed no significant difference. The aqueous and organic solvent methods of silanization on the degradation of mitoxantrone in liposomes resulted in the similar rate constants and half-lives. The addition of ascorbic acid to mitoxantrone-containing liposomes exhibited no effect on the increase of stability for the entrapped mitoxantrone.

In the present study, an attempt was made to investigate the antitumor effect of mitoxantrone-containing liposomes, using the L1210 leukemia cells. The results of in vitro and in vivo antitumor activity were compared with those obtained from free mitoxantrone.

2. Materials and methods

2.1. Materials

Mitoxantrone was obtained from Kingdom Pharmaceutical Co. (ROC). Dioleoylphosphatidylcholine and cholesterol were purchased from Sigma (USA). Dicetylphosphate was obtained from Pharmacia P-L Chemicals (Sweden). RPMI 1640 culture medium was purchased from Grand Island Biological Co. (USA).

2.2. Animals

CDF_1 female mice (Tri-Service General Hospital Animal Breeding Center, ROC) weighing about 18-22 gm (8-10-week-old) were used for in vivo study.

2.3. Tumor cells

L1210 cells were grown in vivo in the peritoneal cavity of CDF_1 mice and transplanted weekly.

2.4. Methods

2.4.1. Preparation of mitoxantrone-containing liposomes

Liposomes were prepared by a method described previously (Law et al., 1991). Phospholipids were dissolved in chloroform in a 50-ml round-bottom flask and dried in rotary evaporator under reduced pressure at 37°C to form a thin film on the flask. The desired concentration of mitoxantrone in 0.9% sodium chloride solution was added to the film. Multilamellar liposomes were formed by constant vortex and sonication. Dioleoylphosphatidylcholine was the main lipid component for the construction of liposomes and a molar ratio of 1.6 was used. Cholesterol was added to the liposomes at a molar ratio of 1.0. Dicetylphosphate was added at a molar ratio of 0.15 to form negatively charged liposomes.

Mitoxantrone-containing liposomes were separated from the unentrapped mitoxantrone by ultracentrifugation and washed three times with 0.9% sodium chloride solution. The concentration of entrapped mitoxantrone was determined by spectrophotometry at 242 nm.

2.4.2. In vitro antitumor effect

Freshly washed L1210 leukemia cells with a concentration of 1×10^5 viable cells/ml were incubated in a 24-well plate at 37°C in RPMI 1640 medium in the presence of mitoxantrone or mitoxantrone-containing liposomes. The drug concentration range of free mitoxantrone and mitoxantrone-containing liposomes used in this assay was from 3×10^{-1} to 3×10^2 ng/ml. Empty liposomes were used as control. After treatment for 24 and 72 h, cells were removed and measured for cell death by the MTT method: 250 ml of (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide solution (5 mg/ml phosphate-buffered saline) was added to the well without prior removal of the culture medium. The mixture was incubated for 2 h at 37°C and 1 ml of the extraction buffer (12.5% sodium dodecyl sulfate, 45% dimethylformamide and with 6.0 M HCl to adjust pH to 4.7) was added to dissolve the formazan-protein complex and mixed well. The resultant solution was measured at 570 nm. The positive well with the live cells demonstrated a purple to brown color; whereas, the negative well of the dead cells showed a yellow color.

2.4.3. In vivo antitumor activity

The antitumor effect of free mitoxantrone and mitoxantrone-containing liposomes was studied with L1210 leukemia in CDF_1 mice. Eighty CDF_1 mice, divided into five groups (16 mice per group), were inoculated intraperitoneally with 5×10^5 L1210 cells/mouse. One day after inoculation the groups of mice were treated intraperitoneally with drugs. The mitoxantrone doses were 3 and 10 mg/kg body weight, and for mitoxantrone-containing liposomes were equivalent to 3 and 10 mg mitoxantrone/kg body weight.

3. Results and discussion

The results of in vitro antitumor activity of empty liposomes, free mitoxantrone and mitoxantrone-containing liposomes after incubation with L1210 cells for 24 h are shown in Fig. 1. The empty liposomes demonstrated no effect on the viability of the L1210 cells. The mitoxantrone

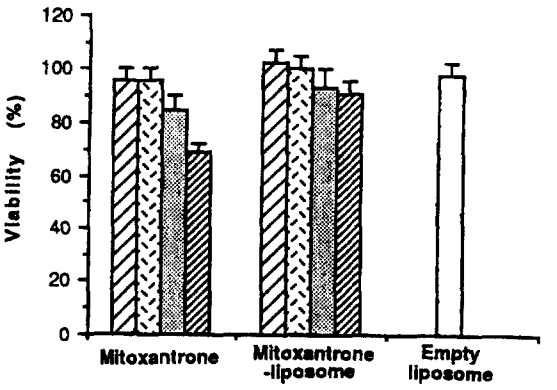

Fig. 1. Viability of L1210 cells after treatment with mitoxantrone, mitoxantrone-containing liposomes and empty liposomes for 24 h: (▨) 0.3, (▩) 3, (■) 30 and (▨) 300 ng/ml of mitoxantrone.

entrapped in liposomes also showed no significant antitumor effect in the concentration range from 3×10^{-1} to 3×10^2 ng/ml. The free mitoxantrone showed an inhibition on cell activity as drug concentration increased. At a mitoxantrone concentration of 3×10^2 ng/ml, a 68% viability of the cells was observed.

Fig. 2 shows the results of in vitro treatment of L1210 cells with empty liposomes, free mitoxantrone and mitoxantrone-containing liposomes for 72 h. It appears that the empty liposomes exerted no effect on the growth of cells. Whereas, the free mitoxantrone and mitoxantrone-contain-

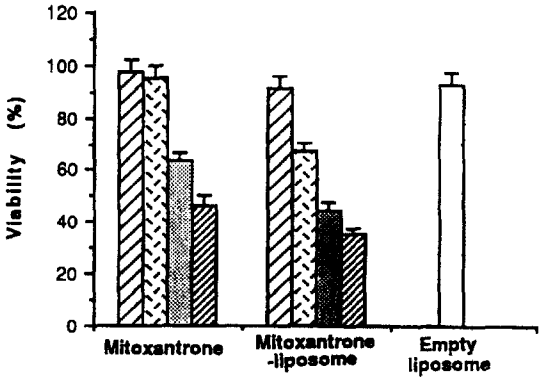

Fig. 2. Viability of L1210 cells after treatment with mitoxantrone, mitoxantrone-containing liposomes and empty liposomes for 72 h: (▨) 0.3, (▩) 3, (■) 30 and (▨) 300 ng/ml of mitoxantrone.

Table 1
Effect of mitoxantrone and mitoxantrone-containing liposomes on the survial time and fraction of survival of CDFI mice with L1210

Drug	Mitoxantrone dose (mg/kg)	Survival fraction	Mean survival time (days)
Phosphate-buffered saline	-	5/16-	27.8 ± 3.5
Empty liposome	-	4/16	27.5 ± 5.5
Mitoxantrone	3	9/16	53.0 ± 13.3
Mitoxantrone-liposome	10	12/16	39.5 ± 5.3
Mitoxantrone	10	2/16	41.1 ± 13.1
Mitoxantrone-liposome	10	13/16	45.6 ± 9.2

ing liposomes suppressed the cell activity with increasing drug concentration.

The above results revealed that both the free mitoxantrone and mitoxantrone-containing liposomes demonstrated an antitumor effect. However, mitoxantrone-containing liposomes showed slower antitumor reaction and higher antitumor activity. This may be due to the carrier effect of liposomes which sustained and enhanced the action of mitoxantrone on tumor cells. In a previous study (Law et al., 1994), it was found that the mitoxantrone-containing liposomes released approximately 5% of the total content after incubation for 24 h. This corresponds to about 15 ng/ml of mitoxantrone released from the mitoxantrone-containing liposomes at the increased dose of 300 ng/ml in the case studied here. It is possible that at this mitoxantrone concentration, the cells would not be inhibited significantly (Fig. 1). Also, from the cell viability maintained at an average of about 100% after treatment with the liposome-encapsulated mitoxantrone for 24 h, it seems that the uptake of the drug from liposomes was too small and slow to kill the tumor cells (Fig. 1). However, after 72 h treatment with the mitoxantrone containing-liposomes, an inhibition of cells was found at concentrations of 3, 30 and 300 ng/ml of mitoxantrone (Fig. 2). At low mitoxantrone concentration (0.3 ng/ml), no inhibition was shown. According to previous results on mitoxantrone release from liposomes (Law et al., 1994), 7% of the entrapped drug was found after incubation for 72 h. Therefore, a liposome with entrapped mitoxantrone concentrations of 3, 30 or 300 ng/ml can release mitoxantrone directly in the medium at concentrations of 0.21, 2.1 and 21 ng/ml, respectively, after 72 h. At the release concentrations of 0.21, 2.1 and 21 ng/ml of mitoxantrone, the cells showed 67, 45 and 36% viabilities, respectively. However, from the results of the antitumor effect of free mitoxantrone after treatment for 72 h, at inhibition concentrations of 0.3 and 3 ng/ml, the cells demonstrated 100% of viability (Fig. 2). That is to say, at release concentrations of 0.21 and 2.1 ng/ml of mitoxantrone, it would have no effect on the viability of the cells. Hence, the inhibition of tumor cells at these concentrations may be due to the uptake of the mitoxantrone-containing liposomes by the cells, and then the mitoxantrone is released in the tumor cells to kill the cells effectively (Gregoriadis et al., 1974; Patel et al., 1982; Gabizon et al., 1983). At a release concentration of 21 ng/ml of mitoxantrone, or increased dose of 300 ng/ml, the inhibition of the cells may result from a direct release of mitoxantrone from liposomes to the medium as well as an uptake of mitoxantrone via the liposome carrier effect.

Table 1 shows the results of the in vivo antitumor activity obtained after administration with a single dose of free mitoxantrone and mitoxantrone-containing liposomes. The 3-mg/kg dose was selected as the optimum therapeutic dosage for intraperitoneal injection (Wallace et al., 1979; Batra et al., 1986). The 10-mg/kg dose was tested to investigate the high dose response. The injection of phosphate-buffered saline or empty liposomes into the L1210 tumor-bearing CDF_1 mice resulted in survival times of 27.8 ± 3.5 or 27.5 ± 5.5 days, respectively. Injection of such mice with free mitoxantrone or mitoxantrone-containing liposomes demonstrated a significant increase

of survival time. A survival time of 53.0 ± 13.3 days in mice was obtained after injection with 3 mg/kg of free mitoxantrone. When the same dose of mitoxantrone-containing liposomes was administered, the survival time of the L1210-bearing mice was 39.5 ± 5.3 days. This indicated no significant difference from the result obtained with free mitoxantrone due to the large fluctuation of the survival time. However, from the fraction of survival, the free mitoxantrone resulted in 9/16 but the mitoxantrone-containing liposomes demonstrated 12/16. That is to say, the mitoxantrone-containing liposomes increased the survival rate of the L1210 tumor-bearing mice. Treatment of the tumor-bearing mice with 10 mg/kg of free mitoxantrone showed no improvement in the survival time and a worse survival fraction as compared with the results obtained with 3 mg/kg. A large fluctuation of the survival time was also observed. The decrease of the survival fraction with high dose mitoxantrone may be due to the toxicity of the drug. This can be confirmed by a significant decrease of body weight in mice after treatment with high dose mitoxantrone (Fig. 3). No apparent tumor growth was also found for these mice during the experiment. On the other hand, the high dose treatment with mitoxantrone-containing liposomes for tumor-bearing mice demonstrated a survival time of 45.6 ± 9.2 days and a survival fraction of 13/16.

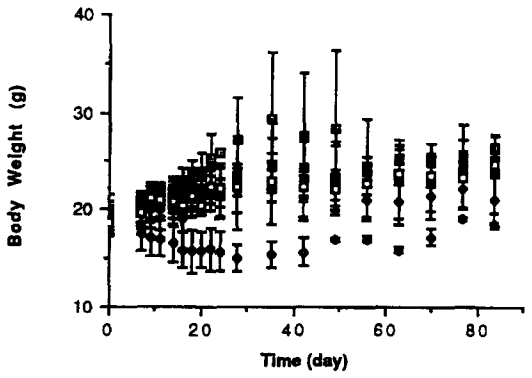

Fig. 3. Change of body weight of L1210 tumor-bearing mice after treatment with mitoxantrone, mitoxantrone-containing liposomes and empty liposomes. (□) empty liposome; (◆) 3 mg/ml mitoxantrone; (□) mitoxantrone-containing liposome with 3 mg/ml mitoxantrone; (◇) 10 mg/ml mitoxantrone; (■) mitoxantrone-containing liposome with 10 mg/ml mitoxantrone; (□) normal mice without treatment.

This was not significantly different from the survival time compared with the results obtained from high dose of free mitoxantrone but showed an increase in survival fraction. It appears that the presence of liposomes reduces mitoxantrone toxicity. This result is in good agreement with the recent report on mitoxantrone encapsulated into negatively charged liposomes of phosphatidic acid which demonstrated a reduction of drug toxicity and increase of cytostatic efficiency in L1210 leukemia (Schwendener et al., 1991).

Acknowledgements

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References

Batra, V.K., Morrison, J.A., Woodward, D.L., Siverd, N.S. and Yacobi, A., Pharmacokinetics of mitoxantrone in man and laboratory animals. *Drug Metab. Rev.*, 17 (1986) 311-329.

Gabizon, A., Goren, D., Fuks, Z., Barenholz, Y, Dagan, A. and Meshorer, A., Enhancement of adriamycin delivery to liver metastatic cells with increased tumoricidal effect using liposomes as drug carriers. *Cancer Res.*, 43 (1983) 4730-4735.

Gregoriadis, G., Swain, C.P., Wills, E.J. and Tavill, A.S., Drug-carrier potential of liposomes in cancer chemotherapy. *Lancet*, (1974) 1313-1316.

Law, S.L., Chang, P. and Lin, C.H., Characteristics of mitoxantrone loading on liposomes. *Int. J. Pharm.*, 70 (1991) 1-7.

Law, S.L., Jang, T.F., Chang, P. and Lin, C.H., Release characteristics of mitoxantrone-containing liposomes. *Int. J. Pharm.*, 103 (1994) 81-85.

Law, S.L., Jang, T.F., Chang, P. and Lin, F.M., Stability of mitoxantrone-containing liposomes. *Int. J. Pharm.*, 116 (1995) 87-93.

Patel, K.R., Jonah, M.M. and Rahman, Y.E., In vitro uptake and therapeutic application of liposome-encapsulated methotrexate in mouse hepatoma 129. *Eur. J. Cancer Clin. Oncol.*, 18 (1982) 833-843.

Schwendener, R.A., Fiebig, H.H., Berger, M.R. and Berger, D.P., Evaluation of incorporation characteristics of mitoxantrone into unilamellar liposomes and analysis of their pharmacokinetic properties, acute toxicity, and antitumor efficacy. *Cancer Chemother. Pharmacol.*, 27 (1991) 429-439.

Wallace, R.E., Murdock, K.C., Angier, R.B. and Durr, F.E., Activity of a novel anthracenedione, 1,4-dihydroxy-5,8-bis{{{2-[(2-hydroxyethyl)amino]ethyl}amino}}-9,10-anthracenedione dihydrochloride, against experi-mental tumors in mice. *Cancer Res.*, 39 (1979) 1570-1574.