

Received 15 May 2002
Accepted 25 July 2002

Short Communication

USE OF THE POST-INSERTION METHOD FOR THE FORMATION OF LIGAND-COUPLED LIPOSOMES

THERESA M. ALLEN, PUJA SAPRA and ELAINE MOASE
Department of Pharmacology, University of Alberta, Edmonton, Alberta,
Canada T6G 2H7

Abstract: A new technique is described for the formation of ligand-targeted liposomes that can be used with whole antibodies, antibody fragments, peptides or other ligands. The ligands are coupled to polyethylene glycol micelles and then transferred in a simple incubation step from the micelles into the outer monolayer of pre-formed, drug-loaded liposomes. This versatile method allows a combinatorial approach to the design of targeted liposomes that minimises manufacturing complexities, allowing a variety of ligands to be inserted into a variety of pre-formed liposomes containing a variety of drugs. This allows the ligand-targeted therapeutics to be tailored to the needs of individual patients.

Key Words: Immunoliposomes, Antibodies, Ligand-Targeted Therapeutics, Polyethylene Glycol, Doxorubicin, Vincristine

INTRODUCTION

The use of targeted liposomes is recognized as a promising strategy for improving the selective targeting of drugs to diseased tissues *in vivo*, leading to reductions in drug toxicity and improvements in therapeutic outcomes. A large variety of targeting molecules have been attached to the surface of liposomes to date, using a variety of coupling methods [1-5]. If targeted liposomes are to be useful in clinical applications, simple and flexible preparation methods are required so they can be tailored to the patient's disease profile without the need for separate manufacturing procedures for each ligand and drug combination. In addition, the coupling chemistries for attaching antibodies to liposomes are sometimes incompatible with the optimal conditions for loading drugs into

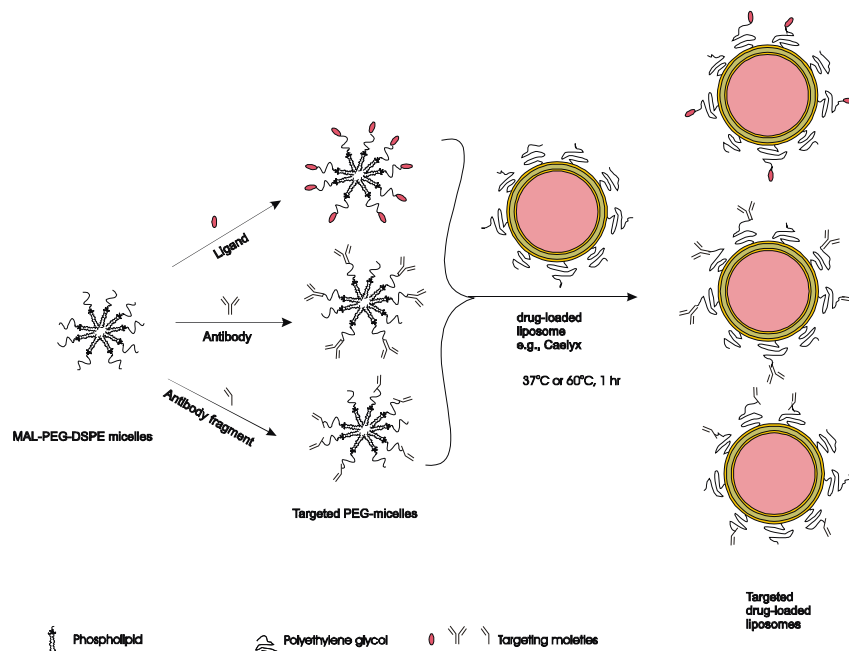


Fig. 1. Cartoon depicting the post-insertion method for preparation of immunoliposomes. The ligands of choice are coupled to micelles composed of Mal-PEG-DSPE or mixtures of Mal-PEG-DSPE and mPEG-DSPE, then incubated with the preformed drug-loaded liposomes of choice under conditions that allow the transfer of ligand-PEG-DSPE into the liposomes. The method simplifies the preparation of immunoliposomes, allowing flexibility in the choice of target and drug.

liposomes, e.g. the requirement for a particular pH for loading and a different pH for coupling. In our laboratory we have developed an approach that allows us to insert the desired ligands, covalently attached to polyethylene glycol (mPEG)-lipid micelles, into preformed drug-loaded liposomes. A schematic diagram depicting this approach to the formation of ligand-targeted liposomes is given in Figure 1. The preparation and characterization of these ligand-targeted liposomes, termed post-insertion liposomes (PIL) are described below.

MATERIALS AND METHODS

Liposomes were prepared as described previously [6] and were normally composed of either hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CHOL) in a 2:1 molar ratio or sphingomyelin:cholesterol (SM:CHOL) liposomes in an 55:45 molar ratio and contained various percentages of mPEG₂₀₀₀-distearoylphosphatidylethanolamine (mPEG-DSPE). Sheep IgG, anti-CD19 whole monoclonal antibodies or Fab' fragments, or peptide (antagonist G), were coupled to micelles composed of maleimide-terminated PEG-DSPE

(Mal-PEG-DSPE) with or without additional mPEG-DSPE, and then the ligand-coupled mPEG-DSPE was transferred to preformed liposomes containing either doxorubicin or vincristine during a simple incubation step at 60 - 65°C for 0.5-1 hour, as described [6-8 and Sapra, P., unpublished results]. Doxorubicin or vincristine was loaded into liposomes using published techniques [9, 10]. Binding studies with drug-free PIL were performed to evaluate targeting of the PIL to cell lines according to previously described procedures [7].

Pharmacokinetics and biodistribution of liposomes was determined in female BALB/c mice according to previously described procedures [7]. The therapeutic effectiveness of doxorubicin- or vincristine-containing liposomes targeted with anti-CD19 whole antibody or Fab' fragments was evaluated in a murine model of human B-cell lymphoma [11].

RESULTS AND DISCUSSION

Ligands coupled to the terminus of polyethylene glycol (PEG) could be inserted into the outer monolayer of preformed, drug-loaded liposomes in a time and temperature-dependant manner [6]. Efficient transfer of sheep IgG-PEG-DSPE from micelles was achieved above the phase transition temperature of the lipids (around 60°C). The amount of IgG and mPEG-DSPE transferred into the liposomes was dependant upon the concentration of the IgG-PEG-DSPE micelles added (Table 1).

In our studies we achieved up to 0.67 nmol Ab/ μ mol PL and 3 mol % mPEG-DSPE could be transferred from micelles into liposomes. As the amount of mPEG-DSPE pre-existing in the recipient liposomes increased, the amount of IgG-PEG-DSPE that could be transferred into the liposomes decreased. We observed that, except for Caelyx/Doxil (9 mol % mPEG-DSPE in the outer monolayer of the liposome), we could achieve adequate transfer of IgG-PEG-DSPE micelles onto the preformed liposomes, so as to achieve good target binding. This suggests there is an upper limit to the amount of mPEG-DSPE in the recipient liposomes that will allow transfer from the micelles. The resulting formulations were found to have good stability in human plasma; after 48 h at 37°C no transferred IgG or mPEG-DSPE appeared to have dissociated from the liposomes. In addition, incubation of antibody-micelles with the preformed liposomes did not seem to accelerate the release of doxorubicin from the liposomes [6].

HSPC:CHOL:PEG-DSPE, 2:1:0.1 liposomes coupled to anti-CD19 (PCPIL[anti-CD19] and immunoliposomes formed by the conventional Mal-PEG-DSPE coupling procedure (PCSIL[anti-CD19]) had similar levels of binding to CD19+ B-lymphoma cell line (Namalwa) at similar levels of coupled antibody [7]. The cytotoxicity of DXR-loaded PCPIL[anti-CD19] against Namalwa cells, was similar to that of PCSIL[anti-CD19], and both were higher than non-targeted formulations. PCPIL[anti-CD19] and PCSIL[anti-CD19] had similar pharmacokinetic profiles and therapeutic efficacy in a murine model of human

B-cell lymphoma [7]. Both of the targeted formulations were cleared significantly more rapidly than the non-targeted liposomes in BALB/c mice, likely though binding to normal B cells (via CD19 epitopes) and to mononuclear phagocyte cells (via Fc receptors) in these immune-competent mice. Further, *in vivo* survival studies demonstrated comparable mean survival times for SCID mice injected with Namalwa cells and treated with doxorubicin-loaded PCPIL[anti-CD19] or PCSIL[anti-CD19] [7].

Tab. 1. Efficiency of transfer of various ligands conjugated to PEG-DSPE micelles to preformed liposomes.

Ligand	Preformed liposomes (molar ratio)	Molar ratio of mPEG-DSPE:MalPEG-DSPE in micelles	Molar ratio of micellar PEG-DSPE to liposomal phospholipid	PIL nmol ligand/ μ mol PL	Ref.
Sheep IgG	unloaded HSPC/Chol (67:33)	4:1	4	0.67	[6]
Sheep IgG	unloaded HSPC/Chol/mPEG-DSPE (65:33:2)	4:1	4	0.67	[6]
Sheep IgG	unloaded HSPC/Chol/mPEG-DSPE (64:32:4)	4:1	4	0.47	[6]
Sheep IgG	doxorubicin-loaded HSPC/Chol/mPEG-DSPE (61:30:9, Caelyx)	4:1	4	0.13	[6]
anti-CD19	doxorubicin-loaded HSPC/Chol/mPEG-DSPE (64:32:4)	4:1	5	0.17-0.27	[7]
anti-CD19	vincristine-loaded in SM/Chol (55:45, mol:mol)	4:1	5	0.3	Sapra, P., unpublished results
anti-CD19 Fab' fragment	vincristine-loaded SM/Chol (55:45, mol:mol)	4:1	5	0.45	Sapra, P., unpublished results
Antagonist G	doxorubicin-loaded HSPC/Chol/mPEG-DSPE (64:32:4)	0:4	4	0.36	[8]

We have also used the post-insertion method for inserting anti-CD19 as either whole Ab or Fab' fragments into SM:CHOL (55:45 mol/mol) liposomes loaded with vincristine (Vinc-SMPIL[anti-CD19]) (12). The rate of leakage of vincristine from Vinc-SMPIL[anti-CD19] was found to be similar to that from Vinc-SMSIL[anti-CD19] (Mal-PEG-DSPE coupling procedure). In addition, *in vitro* cytotoxicity of these immunoliposomes against Namalwa cells was found to be comparable to Vinc-SMSIL[anti-CD19]. Vinc-SMPIL[anti-CD19] had significantly improved therapeutic efficacies compared to vincristine-loaded non-targeted liposomes or free vincristine in the Namalwa model of human B-cell lymphoma.

The post-insertion method is also suitable for the insertion of peptide ligands into preformed Stealth immunoliposomes. Antagonist G-targeted DXR-loaded liposomes were prepared by the post-insertion method and tested in a human small cell lung cancer (H69) cell line. A maximum of 0.36 nmoles antagonist G/ μ mol PL could be inserted into HSPC:CHOL liposomes (PLG) (Table 1) and these PLG demonstrated similar binding to liposomes prepared by the conventional Mal-PEG-DSPE method. The cytotoxicities of the DXR-loaded PLG (DXR-PLG) were higher than those of conventional liposomes prepared by the Mal-PEG-DSPE method (DXR-SLG), likely because this hydrophobic peptide could non-specifically associate with the conventional liposomes through hydrophobic interactions [8]. This points out another advantage of the post-insertion method; it can decrease non-specific interactions of ligands with liposomes.

CONCLUSIONS

We have demonstrated that the post-insertion approach is suitable for the preparation of targeted liposomes using antibodies, antibody fragments or peptides. Since the conditions for the insertion of the ligand is now decoupled from the conditions for the preparation of and loading of drug into liposomes, conditions can be optimized for both drug loading and ligand insertion. These liposomes have *in vitro* drug leakage rates, cell association and cytotoxicity profiles comparable to liposomes made by conventional coupling procedures like the Mal-PEG-DSPE coupling method. Further, these liposomes have therapeutic efficacies similar to liposomes made by conventional coupling procedures and superior efficacies to non-targeted liposomes. In conclusion, the post-insertion approach to preparing targeted liposomal formulations seems to be simple, rapid and flexible, all desirable properties from both a research and a manufacturing viewpoint. The method allows a combinatorial approach to the design of targeted liposomes, where a variety of ligands can be inserted into a variety of pre-formed liposomes containing a variety of drugs, allowing the therapy to be tailored to the needs of individual patients.

REFERENCES

1. Hansen, C.B., Kao, G.Y., Moase, E.H., Zalipsky, S., Allen and T.M. Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures. **Biochim. Biophys. Acta** 1239 (1995) 133-144.
2. Allen, T.M., Hansen, C.B. and Stuart, D.D. Targeted sterically stabilized liposomal drug delivery, in **Medical Applications of Liposomes** (Lasic, D.D. and Papahadjopoulos, D. Eds.) 1998, Elsevier Science Publishers: Amsterdam 297-323.
3. Klibanov, A.L., Serbina, N., Torchilin, V.P. and Huang, L. Attachment of ligands to liposomes via PEG spacer for prolonged liposome circulation and targeting. **J. Liposome Res.** 6 (1996) 195-196.
4. Kirpotin, D., Park, J.W., Hong, K., Keller, G., Benz, C. and Papahadjopoulos, D. Binding and endocytosis of sterically stabilized anti-HER2 immunoliposomes by human breast cancer cells. **Proc. Am. Assoc. Cancer Res.** 37 (1996) 3186.
5. Zalipsky, S., Puntambekar, B., Bolikas, P., Engbers, C.M., Woodle and M.C. Peptide attachment to extremities of liposomal surface grafted PEG chains: Preparation of the long-circulating form of laminin pentapeptide, YIGSR. **Bioconj. Chem.** 6 (1995) 705-708.
6. Ishida, T., Iden, D.L. and Allen, T.M. A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs. **FEBS Lett.** 460 (1999) 129-133.
7. Iden, D.L. and Allen, T.M. In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion technique. **Biochim. Biophys. Acta** 1513 (2001) 207-216.
8. Moreira, J.N., Ishida, T., Gaspar, R. and Allen, T.M. Use of the post-insertion technique to insert peptide ligands into pre-formed Stealth liposomes with retention of binding activity and cytotoxicity. **Pharm. Res.** 19 (2002) 265-269.
9. Bolotin, E.M., Cohen, R., Bar, L.K., Emanuel, S.N., Lasic, D.D. and Barenholz, Y. Ammonium sulphate gradients for efficient and stable remote loading of amphipathic weak bases into liposomes and ligandosomes. **J. Liposome Res.** 4 (1994) 455-479.
10. Boman, N.L., Masin, D., Mayer, L.D., Cullis, P.R., Bally and M.B. Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors. **Cancer Res.** 54 (1994) 2830-2833.
11. Lopes de Menezes, D.E., Pilarski, L.M. and Allen, T.M. *In vitro and in vivo* targeting of immunoliposomal doxorubicin to human B-cell lymphoma. **Cancer Res.** 58 (1998) 3320-3330.