Controlled Delivery of Methotrexate from Channel-Protein Containing Liposomes

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Published in:
Journal of Controlled Release

Publication date:
2005

CONTROLLED DELIVERY OF METHOTREXATE FROM CHANNEL-PROTEIN CONTAINING LIPOSOMES

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Summary

The goal of this study is to develop a controlled methotrexate delivery system that releases its content by a target specific stimulus. To accomplish this, both methotrexate and an engineered channel protein (MscL) were incorporated into DOPC/CH/DSPE-PEG liposomes, providing a controlled drug delivery system. Reconstitution and encapsulation methods were optimized in order to ensure optimal channel gating activity and high drug:lipid ratio. At the optimal conditions, a 95% release of MTX could be reached.

Introduction

In tumor therapy, many potential drugs cannot be used because they are toxic to several vital organs. For these drugs, encapsulation in liposomes can be a solution. Further improvements in the design of liposomes resulting in specific release of drug at the tumor site will make the utilization of liposomes more feasible. To accomplish this, we are developing a liposomal system containing a pH-sensitive proteinacious valve that opens as a response to lowered pH values, such as these reported, e.g., for solid tumors [1]. This proteinacious valve is the mechanosensitive channel of large conductance (MscL) from *E.coli*. The glycine at the 22nd position of the MscL is mutated to a cysteine, which is used to target either pH-sensitive chemical groups or [2-(trimethylamonium)ethyl] methanethiosulfonate bromide (MTSET) to the protein. Binding of MTSET introduces a positive charge into the hydrophobic channel pore leading to opening of the channel. MscL-G22C was reconstituted in therapeutically optimized liposomes composed of DOPC/Cholesterol/DSPE-PEG2000 (70:20:10), which was shown to be compatible with the channel protein activity. The encapsulation method for MTX has been optimized.

Experimental methods

The preparation of MscL containing liposomes with encapsulated MTX combines the detergent-mediated reconstitution of MscL in liposomes by using BioBeads and the encapsulation of MTX by one of the following methods: (a) lipid film rehydration in a drug-containing solution, (b) reverse phase evaporation and (c) addition of the drug in the presence of detergent during protein reconstitution. For the analysis of drug:lipid ratio, MTX-proteoliposomes were separated from the free drug on a Sephadex G50 column. Total lipid content was determined from a sensitive fluorescence assay, using diphenylhexatriene as the probe (excitation at 355 nm and emission at 440 nm). MTX content was measured by HPLC (detection at 294/356 nm [2]). Drug:lipid ratio was calculated as μg drug per mg total lipid.

Release of MTX after activation with MTSET (pH nonsensitive) was determined after separation of liposomes and free drug on a desalting PD10 column followed by the measurement of total lipid and drug concentrations.

Results and discussion

In order to see which MTX encapsulation method gives the highest drug:lipid ratio in combination with high drug release after activation, three methods were compared. The results can be seen in Table 1. All three encapsulation methods tested gave similar drug:lipid ratios (between 5 and 7 μg/mg) and also similar release (about 95%) upon the activation of the channel. For practical reasons, the method in which the drug was encapsulated during protein reconstitution was used in further experiments.

Table 1
Drug:lipid ratios and drug release after different MTX encapsulation methods

Encapsulation method	Drug:lipid (μg/mg)	Released drug after activation (%)
Reverse phase evaporation	6.99	95
During protein reconstitution	6.39	95
Lipid film rehydration	5.13	94

Conclusion

A proteoliposomal delivery system for MTX has been developed, which can release almost all encapsulated drug upon the activation of the channel protein by chemical labeling. We are currently busy with the development of pH-sensitive proteoliposomes.

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TARGETED NORFLOXACIN IS ACTIVE IN VIVO AGAINST PERSISTENT *MYCOBACTERIUM BOVIS* BCG

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Summary

Tuberculosis is difficult to treat because *Mycobacterium tuberculosis* persistent forms resist the usual antituberculous agents. This results in 2–3 million deaths each year. In order to improve tuberculosis treatment, we established a short-term in vivo model of persistent mycobacteria. Also, we synthetized a macromolecular prodrug targeted toward macrophages and demonstrated its in vivo efficacy against persistent mycobacteria. To our knowledge, this is the first report of a drug active in vivo against persistent mycobacteria.

Introduction

Tuberculosis infects 2 billion people worldwide. Even if most of them present no symptoms and will never develop the disease, the eventual development of an overt tuberculosis, up to decades after contamination [1,2], results in 2–3 million deaths each year.

The possible late development of tuberculosis results from the induction of *Mycobacterium tuberculosis* persistence by different conditions, such as nutrient starvation [3] or hypoxia [4]. This means that *M. tuberculosis* keeps its infectious capacity intact while it no longer multiplies. Persistent bacilli resist antituberculous agents, such as isoniazid, although the same antibiotics rapidly eliminate actively multiplying *M. tuberculosis*. Consequently, tuberculosis is very difficult to treat: the usual regimen requires four antibiotics for two months, then two antibiotics for the next four months, watching out for the return of persistent bacteria to normal metabolism.

In patients, persistent bacilli survive in macrophages after phagocytosis where they are rather well protected from immune response and antibiotic treatment. It is expected that antibiotics targeted toward macrophages improve treatment efficacy by concentrating drugs close to bacteria. The aim of this work was to design a conjugate able to deliver an antibiotic into macrophages phagosomal vacuole in close contact with the intracellular bacteria.

We synthesized targeted macromolecular prodrugs composed of a macromolecular carrier, of a targeting device and of an antibiotic active in vitro against *M. tuberculosis*. The carrier, dextran, bears both mannose, the homing device intended to target the macrophage, and norfloxacin, to be delivered into the phagosomal vacuole [5]. Norfloxacin was linked to the macromolecular carrier through two different peptide arms.

M. bovis BCG was used as a mycobacterial model because this bacterium has a physiology closely related to the physiology of *M. tuberculosis*, while being at the same time much less pathogenic than the latter, and because the immune response of the mice rapidly controlled extracellular infection and left only intracellular bacilli, i.e., the goal of the targeted norfloxacin.

This model takes advantage of different pO_2 levels in different mouse organs, hence of different persistence statuses of bacteria in these organs, and it therefore allowed us to test the in vivo antibiotic activity of our macromolecular prodrugs against persistent *M. bovis* BCG in mice. In this paper, we report the measurement of pO_2 in liver and spleen of mice. We study the therapeutic activity of our macromolecular prodrugs versus isoniazid and ofloxacin used as controls against well oxygenated and hypoxic, hence persistent, bacilli by counting them in lungs, spleen and liver. These organs are rich in macrophages and possess different oxygenation statuses.