Targeted liposomal genetic drug carriers for leukemia treatment.

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INTRODUCTION AND OBJECTIVE

Very promising strategy of treatment of the neoplastic diseases is antisense gene therapy. Molecules such as antisense oligodeoxynucleotides (asODN), siRNA or miRNA can be used to inhibit particular gene expression. It could be combined with other treatments to enhance therapeutic effect. In acute leukemias overexpression of the antiapoptotic gene *Bcl-2* is observed in more than 70% cases. Reduction of Bcl-2 protein level that could, itself, prevent the development of cancer or more probably could help sensitize cancer cells to apoptosis inducers. The main objective of our work is to obtain carriers characterized by high transfection efficiency, stability in the presence of serum, as well as toxicity and specificity for targeted (leukemic) cells. Proposed here liposomal carriers consist of a core composed of antisense oligonucleotides complexed with synthetic polycation, polyethyleneimine (PEI) (Guillem, 2002) encapsulated within negatively charged liposomes containing polyethylenoglycol (PEG). Carriers are enriched with covalently-bound antibodies recognizing well characterized marker expressed on the surface of leukemia cells.

MATERIALS AND METHODS

Cell cultures Human B cell lines Daudi (CCL-213), Raji (CCL-86) and primary cells isolated from peripheral blood were used as a model of target cells. Cells were cultured at 37^{o}C in a humidified atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 μg/ml penicillin/ streptomycin.

Antibodies Terapeutic antibodies MabThera were purchased from Roche. Rabbit anti-Bcl-2 primary antibodies and anti-human b-actin primary antibodies were purchased from Santa Cruz Biotechnology. Anti-rabbit HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories, INC.

Liposome Preparation Targeted liposome preparation containing PEI-asODN complex was obtained by the method which is a subject of patenting procedure.

Stability tests Protective capability of liposomes towards oligonucleotides was tested by incubation with commercial DNase and with human serum. Carriers after incubation were disrupted with Triton X-100 followed by gel electrophoresis.

Western Blotting Cell samples after incubation with liposomes were collected at the indicated time points and resolved in 12% SDS-PAGE gels, than blotted onto nitrocellulose membrane and developed with proper primary and secondary antibodies.

Cytotoxicity assay Proliferation of investigated cells cultured in medium containing immunoliposomes was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays according to the manufacturer's protocol.

Confocal microscopy Cells were incubated with DiD-stained immunoliposomes and than washed with PBS, fixed with 4% paraformaldehyde and centrifuged onto glass slides. Additionaly cells were permeabilized in cold acetone:methanol (9:1) and stained with DAPI. The images were aquired on a Zeiss LSM 510 confocal microscope (Carl Zeiss; Inc.) with 60x objective lenses, using laser excitation at 405 nm and 633 nm.

RESULTS AND DISCUSSION

The chemical nature of genetic drug requires suitable carrier system that protects it from enzymatic digestion. Incubation with human serum indicates that our carrier protects encapsulated DNA from degradation by nucleases (Fig. 1).

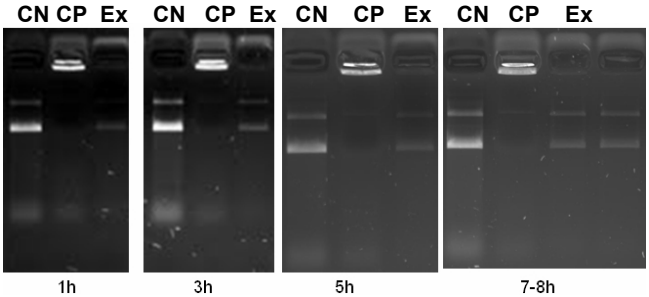
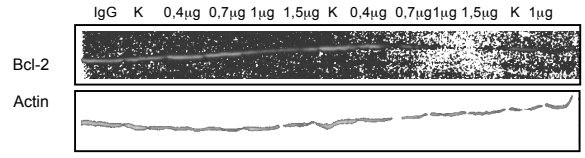


Fig. 1 Drug protection abilities during 8 hours incubation with human serum. (Negative Control (CN)-untreated liposomes; Positive Control (CP) – liposomes digested by DNase, Ex- testes probes after 1- 8 hours incubation with human serum)

We have also shown that Bcl-2 antisense oligonucleotides encapsulated in prepared lipid vectors caused *Bcl-2* gene silencing after 48 h post

addition to cell culture. (Fig. 2a). Additionally, cells derived from patients diagnosed with B cell leukemia were treated with different amount of carriers to check the transfection efficiency (Fig.2b).

a.



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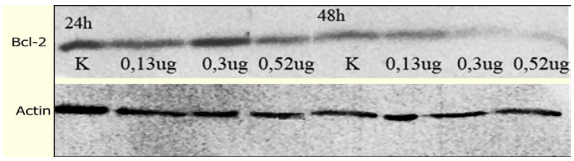

Fig. 2 Silencing of *Bcl-2* gene expression in a. Daudi cell line culture and b. in human B leukemia cell culture. Western blot obtained from both cell line cultures after different time point in the presence of indicated amounts of asODNs.

Further, we investigated cytotoxicity of our immunoliposomes versus model and control cell lines. Experiments confirmed the selectivity of liposomes coated with antibodies against targeted cells. Table 1 presents concentrations of liposomes needed for being effective against 10 (EC10), 50 (IC50) and 90 (IC90) % of treated population.

Table 1 Doses of liposomes which cause cytotoxicity towards different cell lines.

Cell line	Daudi	Raji	Jurkat	L1210
EC10[µg/ml]	41,3	28,2	51,4	62,2
IC50[µg/ml]	134,1	119,3	311,2	387,1
IC90[µg/ml]	330,8	264,3	-	-

Using confocal microscopy, we have shown that our carrier is specific towards human B cells. Model and control cells were incubated with DiD-labeled liposomes. Mixture of target and control cells was treated with anti-CD20 liposomes. Based on the different morphology (Fig 3a) and size (Fig 3b) it could be concluded that only target cells bound liposomes.

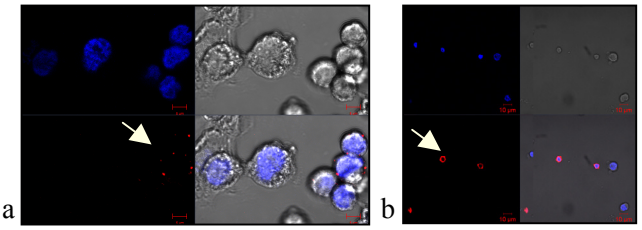

Fig. 3 Selectivity of immunoliposomes towards cells expressing targeted antigen. Cells showing red fluoresecence coming from liposomes interacting with plasma membrane marked by arrows. (a. suspension Daudi (CD20+) and adherent HeLa (CD20-) cells and b. PBLs (CD20+) cells and HEL (CD20-) cells.

CONCLUSIONS

- Immunoliposomes effectively reduce the expression of *Bcl-2* in cancer cells.
- Our data indicate that proposed carrier is selective towards target cells.
- Developed lipid carrier based on polyplex backbone additionally equipped with antibodies, was shown to be reasonable non-viral vector for specific oligonucleotide transfer into human cells.
- Invented carriers are even more attractive as human cells are known to be a very difficult object for standard transfection methods (Smisterova 2001).

REFERENCES

- Smisterova et al. (2001) *Molecular shape of cationic lipid controls the structure of cationic lipid/DOPE-DNA complexes and efficiency of gene delivery*. JBC vol. 276 pages: 47615-47622.
- Guillem et al. (2002) *Targeted oligonucleotide delivery in human lymphoma cell lines using polyethyleneimine based immunopolyplex*. Journal of Controlled release 83 pages:133-146.