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ScienceDirect
Cancer Letters 257 (2007) 165–171
CANCER Letters
www.elsevier.com/locate/canlet

Immunization with liposome-anchored pegylated peptides modulates doxorubicin sensitivity in P-glycoprotein-expressing P388 cells

Grégory Gatouillat [a], Johann Odot [a], Emilie Balasse [a], Claude Nicolau [b], Pierre-François Tosi [a], David T. Hickman [b], María Pilar López-Deber [b], Claudie Madoulet [a,*]

[a] *Laboratory of Biochemistry, IFR53, Faculty of Pharmacy, 51 rue Cognacq-Jay, 51096 Reims cedex, France*
[b] *AC Immune SA, PSE-B EPFL, 1015 Lausanne, Switzerland*

Received 19 February 2007; received in revised form 4 April 2007; accepted 4 April 2007

Abstract

The clinical use of chemotherapy in cancer treatment is limited by the occurrence of multidrug resistance (MDR) associated with the overexpression of membrane transporters, one of the best known is P-glycoprotein (Pgp), that actively expels drugs out of tumor cells. To overcome Pgp-mediated MDR, synthetic peptides corresponding to fragments from extracellular loops 1, 2 and 4 of the murine Pgp were coupled to polyethylene glycol–distearoylphosphatidylethanolamine and inserted into empty or monophosphoryl lipid A-containing liposomes. This formulation elicited specific antibodies which blocked Pgp-mediated efflux of doxorubicin, resulting in increased intracellular drug accumulation and subsequent potentiation of the cytotoxic effect of doxorubicin on multidrug-resistant P388 (P388R) cells. Previous immunizations with MDR1 peptides improved the efficiency of chemotherapy against P388R cells *in vivo*, with an increase of 83% of mice survival time. Overall, these results suggest that this approach can modulate Pgp activity by blocking drug efflux and may have clinical relevance as an alternative strategy to toxic chemosensitizers in drug-resistant cancer therapy.

Keywords: Multidrug resistance; P-Glycoprotein; Immunization; Pegylated peptides; Liposomes

1. Introduction

Failure of chemotherapy often occurs in cancer treatment because most tumors become resistant to a broad range of structurally and functionally unrelated anticancer drugs. There is now a great amount of evidence indicating that one of the major causes of this phenomenon, termed multidrug resistance (MDR), is the overexpression in tumor cells of the *mdr1* gene which codes for the 170 kDa P-glycoprotein (Pgp) [1]. This membrane-bound glycoprotein acts as an active efflux pump towards many anticancer drugs, extruding them out of the cell. Therefore, numbers of exogenous substrates are actively transported out of tumor cells by Pgp, resulting in reduced intracellular concentration,

* Corresponding author. Tel.: +33 3 26 91 37 32; fax: +33 3 26 91 37 30.
E-mail address: claudie.madoulet@univ-reims.fr (C. Madoulet).

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doi:10.1016/j.canlet.2007.04.001

tumor cell survival and resistance to anti-neoplastic drugs. Clinically, Pgp was shown to be expressed in acute myelocytic leukaemia cells in approximately 30% of patients when diagnosed and over 50% at relapse [2,3]. To overcome MDR and improve the efficacy of chemotherapy, several strategies attempting to reverse the MDR phenotype have been developed. A variety of agents are able to inhibit Pgp-mediated drug efflux, including calcium channel blockers such as verapamil [4], the immunosuppressor cyclosporine and its non-immunosupressive analog PSC-833 [5], or monoclonal antibodies (mAbs) such as MRK16 [6] or UIC2, recognizing specific epitopes to the MDR1 protein [7].

The murine *mdr1* cDNA encodes a 1276-amino acid protein with a structure similar to its human homologue and shows 80% homology with the human Pgp [8].

Previous studies from our laboratory reported the *in vitro* and *in vivo* reversal of multidrug resistance by immunization with palmitoylated peptides derived from the extracellular loops of the murine Pgp in L1210- and P388-resistant cells [9,10].

In order to optimize this MDR-reversing strategy, we investigated in this study whether immunizations with synthetic Pgp-derived peptides covalently conjugated to polyethylene glycol (PEG) chains and inserted into liposomes, with or without addition of the adjuvant monophosphoryl lipid A (MPLA), could break the immune tolerance towards the MDR1 protein. We showed that this approach elicited antibodies specific to the extracellular epitopes and that these antibodies were efficient in blocking *in vitro* and *in vivo* Pgp activity.

2. Materials and methods

2.1. Mice and cell lines

Female B6D2F1 mice, 6–8 weeks old, obtained from Charles River (Iffa Credo, L'Arbresle, France), were held under specified pathogen-free conditions at the animal facility of the Faculty of Pharmacy of Reims according to standard guidelines. All experiments were conducted according to the animal care and use of the European Community.

A resistant murine leukemia P388 cell subline (P388R) was established by exposure of the sensitive parental P388 cells to 1 μM doxorubicin (DXR). P388R cells were cultured in RPMI 1640 medium (Gibco-Invitrogen, Cergy-Pontoise, France) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Invitrogen) at 37 °C in a humidified atmosphere of 5% carbon dioxide/95% air.

2.2. Synthetic PEG-peptides

The pegylated peptides corresponding to fragments from the extracellular loops 1, 2 and 4 of the murine Pgp were obtained from AC Immune (Lausanne, Switzerland): mPEGp1: K*GNMTDSFTKAEASILPSIK*G-OH; mPEGp2: K*KVLTSFTNKELQAYAKK*G-OH; mPEGp4: K*SRDDDHETKRQNCNK*G-OH. Immunogenic peptides were predicted by three-dimensional analysis of the extracellular loops. Each peptide was coupled to polyethylene glycol–distearoylphosphatidylethanolamine (PEG–DSPE) on the Lys residue noted K*. For the three loops, the lyophilized peptides consisted in a mixture of peptides that were either monopegylated or dipegylated with DiPEG/monoPEG ratio 70/30. Mean molecular weight was 5735 ± 213 Da for monoPEG peptides and 9324 ± 227 Da for diPEG peptides.

2.3. Western blot analysis

After washing with phosphate-buffered saline (PBS pH 7.2) (Gibco), the cells were lysed in ice-cold buffer (100 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100, 0.1% SDS, 0.1% NP-40) for 45 min and centrifuged at 4 °C for 20 min at 20,000*g*. The protein content of the supernatants was determined by using the Bradford assay with bovine serum albumin as standard (Sigma, St. Quentin Fallavier, France). The samples were subjected to a 4–12% SDS–PAGE and then transferred into a nitrocellulose membrane. Immunodetection of Pgp was performed by using the Western Breeze chemiluminescent immunodetection kit (Invitrogen) and the mAb JSB-1 (1:750 dilution, Proteogenix, Oberhausbergen, France).

2.4. Immune formulations and immunization protocol

Liposomes were prepared by mixing dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG) and cholesterol (Avanti Polar Lipids, Alabaster, AL, USA) in the molar ratios of 0.9:0.1:0.7, respectively. MPLA (List Biologicals Labs, CA, USA) was added at a concentration of 40 μg per μmol of phospholipids [11]. Three suspensions of liposomes were prepared: control: DMPC, DMPG, cholesterol; Lp1: DMPC, DMPG, cholesterol, PEG-peptides; Lp2: Lp1 plus MPLA. The three DSPE–PEG-peptides (mPEGp1, mPEGp2, mPEGp4) were added together (10 μg of each peptide) to the liposome preparation. Solvents were evaporated and the resultant film was hydrated with sterile PBS and adjusted to a final phospolipid concentration of 4 mM. For the second and third immunizations, each batch of liposomes was kept frozen. Before injection, the liposome suspension (750 μl) was mixed with 200 μl of sterile 1.3% aluminium hydroxide (alum) (Brenntag, Frederikssund, Denmark).

Groups of four mice, weighing 19–22 g, were immunized by three intraperitoneal (i.p.) inoculations at 2-week intervals with 200 μl of the different immune preparations. One group of mice immunized with empty liposomes was used as control. Blood samples were collected from the tail vein 12 days before each immunization and after the last immunization. Each sample was processed and serum was collected and stored at 4 °C less than 4 days until use.

2.5. Analysis of immune response

The analysis of immune response was performed using a dot blot assay. After blocking and washing the nitrocellulose papers, each serum sample (dilution 1:800) was incubated for 2 h in the presence of nitrocellulose-adsorbed pegylated peptides (100 ng) as previously described [9]. The membranes were probed with a peroxidase-conjugated secondary antibody (1:3000 dilution, Interchim, Uptima, Montluçon, France) for 1 h at room temperature, then washed and revealed using enhanced chemoluminescence (Interchim). Films were scanned using a densitometer and antibody titres were estimated from standard dilutions of purified IgG (Interchim).

2.6. In vitro activity of the elicited antibodies

The effects of anti-Pgp antibodies on the growth inhibitory effects of doxorubicin were assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. P388R cells (5×10^3) were seeded in each well of a 96-well plate, incubated with 1.5% serum (control or antiserum) in growth medium for 1 h and then grown for 48 h with 0.5, 1 or 2.5 μM DXR (Teva Classics, Paris, France). Subsequently, 20 μl of MTT (2.5 mg/ml) in PBS were added to each well, followed by incubation for 3 h at 37 °C. Formazan crystals were dissolved in dimethylsulfoxide (DMSO). Absorbance was determined with a microplate reader (Multiskan Ascent, Labsystems) at 540 nm. Results were expressed as percentages of cell viability compared to untreated cells. To evaluate the effects of the elicited antibodies, verapamil (VPL) (Sigma) was used as a reference modulator of resistance and cell viability was analysed under the same conditions.

2.7. Flow cytometric assays

Indirect immunofluorescent labelling was performed to determine the reactivity of each serum with P388R cells. Cells were incubated for 1 h in presence of 10% serum from control or immunized mice. After washing the cells, the supernatant was removed and the cells were stained with FITC-conjugated goat $(Fab')_2$ anti-mouse secondary antibody (Beckman–Coulter, dilution 1:100) for 30 min on ice. Cells were washed and resuspended in PBS for flow cytometry analysis. The excitation and emission wavelengths were respectively 490 and 523 nm.

For intracellular accumulation of DXR, cells (1×10^5) were grown for 1 h in culture medium containing 10% of each serum or 3 μM VPL. Doxorubicin (1 μM) was added to the cells, gently mixed and incubated at 37 °C. Cells were then centrifuged, suspended in PBS and fluorescence emitted by DXR was analyzed using a FACSAria flow cytometer (Becton–Dickinson) with excitation and emission wavelengths of 470 and 585 nm.

2.8. In vivo evaluation of the immune formulations

Groups of four mice immunized with the different liposomal preparations received by i.p. injection 10^6 P388R cells on day 0. Mice were injected with 5.5 mg/kg DXR and 2.5 mg/kg vinblastine (VBL) (Sigma) on days 1, 9, 17 and on days 5, 13, 21, respectively. During that period, eating, drinking, alertness and weight were noted and survival time of each mouse was recorded.

3. Results

3.1. Expression of Pgp in DXR-resistant P388 cells

Western blot analysis was performed to detect Pgp expression in P388 and P388R cells. Using the mAb JSB-1, Pgp was detected in P388R cell line while it was not detected in its DXR-sensitive counterpart (Fig. 1). The DXR IC_{50} for the P388R cell subline was 3 μM compared to 6 nM for the drug-sensitive parental cells (data not shown).

3.2. Induction of a humoral immune response against the extracellular loop fragments of the murine Pgp

It was then determined whether the synthetic peptides derived from the murine Pgp inserted in liposomes induced an antibody response. Three different immune formulations were prepared as described in Section 2. Sera were assessed for antibody levels by a dot blot assay. As shown in Fig. 2a, immunization with mPEGp1, mPEGp2

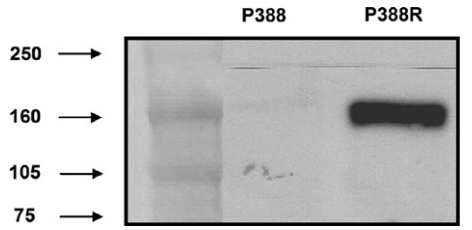

Fig. 1. Western blot analysis of Pgp expression in P388R cells. Proteins were subjected to a 4–12% polyacrylamide gel elctrophoresis and then transferred into a nitrocellulose membrane. The membrane was probed with the JSB-1 primary antibody (1:750) for 1 h and revealed using the Western Breeze chemiluminescent immunodetection kit according to the manufacturer's instructions.

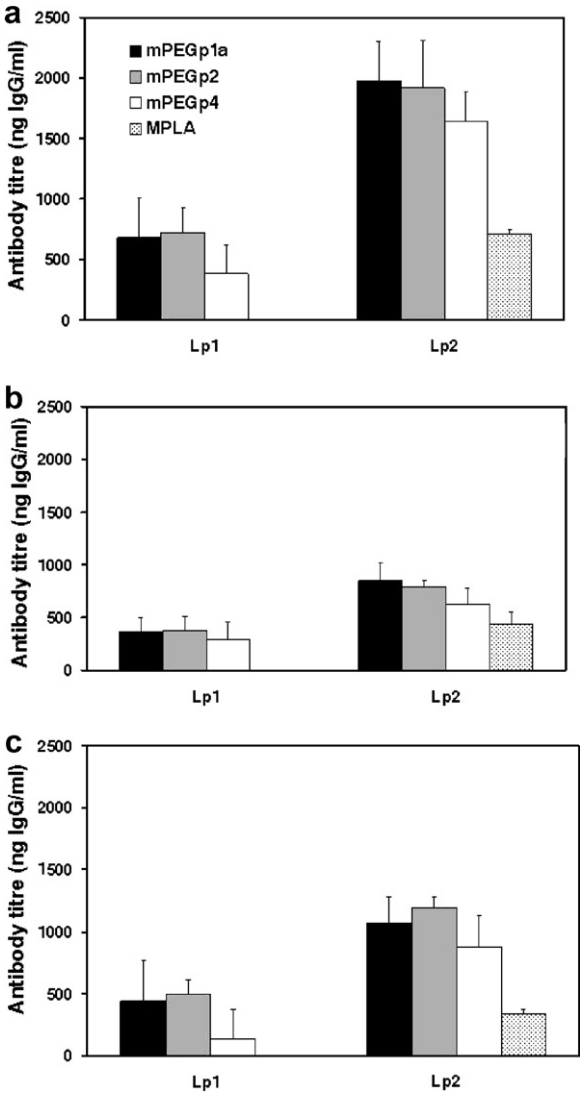

Fig. 2. Measurement of IgG levels in sera collected 15 (a), 30 (b) and 45 (c) days after the last immunization with Pgp-derived peptides. PEG-peptides were adsorbed in nitrocellulose and then incubated with the different sera. IgG-epitopes complexes were detected with a peroxidase-conjugated secondary antibody and revealed using chemiluminescence. Means ± standard deviations from four sera of each group are shown.

and mPEGp4 together (Lp1) elicited IgG against the three peptides 15 days after the third challenge. The addition of the adjuvant MPLA to this formulation (Lp2) increased the antibody levels with 2.9-, 2.7- and 4.2-fold increases, respectively, against mPEGp1, mPEGp2 and mPEGp4 compared to Lp1. The immune response in mice either immunized with Lp1 or Lp2 decreased 30 days after the last immunization (Fig. 2b) and became stable 45 days after the last challenge (Fig. 2c). High antibody levels were still detectable 80 days after the third immunization with average values of total antibody titres of 1.1 and

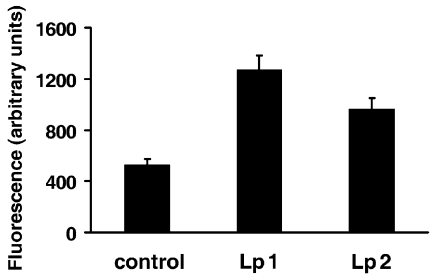

Fig. 3. Fluorescence emitted from P388R cells incubated with sera from control mice or from Lp1 and Lp2 mice. P388R cells were incubated with the different sera and subsequently stained with the FITC-conjugated secondary antibody. After washing the cells, fluorescence was measured by flow cytometry. Results are mean ± SD from three independent experiments.

1.7 μg/ml in Lp1 and Lp2 sera, respectively (data not shown). During treatment, there was no loss of weight in treated mice.

The binding capacity of the elicited antibodies was then analyzed by incubating sera harvested 15 days after the last immunization with Pgp-expressing P388 cells. Fluorescence was analyzed by flow cytometry. As seen in Fig. 3, fluorescence was 2.4 and 1.8 greater when cells were incubated with sera from Lp1 and Lp2 mice compared to control serum. These results show that there is no correlation between antibodies levels and their affinity to Pgp-expressing cells.

3.3. Effect of the elicited antibodies on doxorubicin sensitivity in P388R cells

The ability of anti-Pgp antibodies to reverse DXR resistance was investigated. P388R cells were incubated with the different sera and DXR. The elicited antibodies potentiated the cytotoxic effect of DXR. The IC_{50} was 2.1 μM in cells treated with DXR plus control serum and decreased to 0.65 μM in the presence of Lp1 and Lp2 sera (Fig. 4). At 2.5 μM DXR, Lp1 serum increased

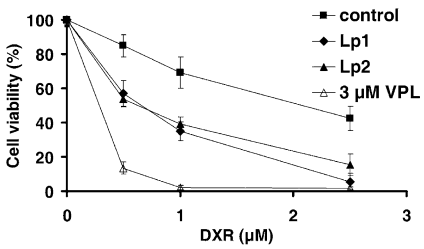

Fig. 4. Cytotoxicity induced by DXR in the presence of serum from naive, Lp1 and Lp2 mice or 3 μM VPL. P388R cells were incubated with each reversal agent (VPL or serum) for 1 h before addition of 0.5, 1 or 2.5 μM DXR. After incubating for 48 h, cell viability was determined by the MTT assay. Each point represents mean ± SD of triplicates experiments.

cell death of 37% compared to control and was as efficient as 3 μM VPL. Lp1 and Lp2 sera alone had no effect on cell viability in the absence of DXR treatment. Similar results were obtained when the sera used were warmed at 56 °C to inactivate complement (data not shown). This indicates that incubation with the induced antibodies increases cell cytotoxicity by a mechanism that does not imply the complement system. These data suggest that the elicited antibodies can restore DXR sensitivity in Pgp-expressing cells. Although the addition of the adjuvant MPLA to the immune formulation (Lp2) increased the immune response against P-gp-derived peptides compared to the Lp1 formulation, it did not improve the reversal activity of the antibodies. Thus, the immune formulation containing the three peptides is more efficient than the same formulation plus MPLA.

3.4. Effect of the elicited antibodies on doxorubicin uptake in P388R cells

To explore the effect of the antibodies on intracellular accumulation of DXR, P388R cells were incubated with different sera in the presence of 1 μM DXR, Lp1 serum was the most efficient in increasing DXR uptake (47% compared to serum from control mice, see Fig. 5), and was as efficient as 3 μM VPL. Although a strong antibody response was observed in mice immunized with Lp2 (the same formulation than Lp1 with MPLA), these antibodies failed to block the efflux pump at the same level than Lp1 antibodies, with only a 13% increase in DXR uptake compared to control. This supports our findings that the formulation containing the three peptides is the most efficient and that the serum levels of the elicited antibodies is not correlated to their activity.

3.5. In vivo activity of the elicited antibodies in combination with chemotherapy against lymphoid neoplasm

Previously immunized mice were injected with P388R cells 45 days after the last immunization and DXR/VBL chemotherapy was administered. Mice immunized with

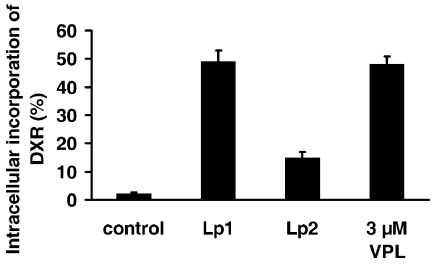

Fig. 5. Effects of the elicited antibodies on DXR accumulation in P388R cells. 1×10^5 cells were incubated with 3 μM VPL, control, Lp1 or Lp2 sera for 1 h before addition of 1 μM DXR. After washing the cells, the intrinsic fluorescence of DXR was analysed by flow cytometry.

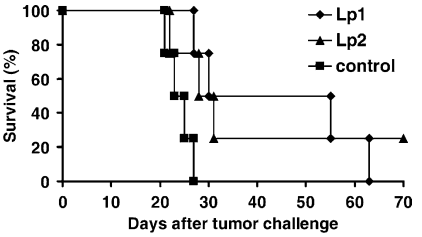

Fig. 6. Survival time of mice immunized by Lp1 or Lp2 formulation. At day zero, 10^6 P388 cells were inoculated intraperitoneally. At days 1, 9, 17, DXR (5.5 mg/kg) and at days 5, 13, 21, VBL (2.5 mg/kg) were injected. The numbers of survivors in each group was monitored. The experience was reproduced twice.

empty liposomes (control) had a mean survival time of 24 days (Fig. 6). The immunization with Lp1 induced an increase of mean survival time of 20 days compared to control, which corresponds to an improvement of 83%. In this group, only one mouse did not respond to the immunizations and died on day 27 after injections of tumor cells. The other mice, which developed an immune response against the peptides, died between days 30 and 63 after tumor inoculation. In the group immunized with Lp2, mean survival was 27 days. Mice died between 22 and 31 days, except one mouse which survived and was still alive 80 days after tumor injection. This mouse may be in cancer remission after chemotherapy treatment, which could be attributed to variations in individual responses to treatment.

4. Discussion

Several strategies for overcoming drug resistance have been reported. Among them, the development of reversal agents which target Pgp have been extensively studied. However, their clinical use has been limited because toxic side effects appeared with the use of efficient doses for systemic chemotherapy [12]. Therefore, the search for alternative approaches is needed.

Several mAbs directed against Pgp such as MRK16 and UIC2 were used to inhibit its activity. These antibodies recognize a single epitope which is not determined. On the contrary, the use of polyclonal antibodies, obtained by immunization with defined immunogenic peptide sequences, allows targeting Pgp at multiple sites.

The mAb UIC2 recognizes a conformational epitope which involves the first extracellular loop, because a deletion of about 20 amino acids in the extracellular loop 1 induces the loss of UIC2 binding to Pgp [13]. A recombinant antibody fragment

targeted to the same extracellular loop also inhibited Pgp-mediated anticancer drug efflux [14]. This justifies our choice to use a fragment from the extracellular loop 1 as immunogen.

A previous study from our laboratory described for the first time an anti-Pgp immunization that was efficient in reversing MDR *in vivo* [10]. Synthetic peptides coupled to palmitoyl chains and inserted in liposome bilayers, mimicking the extracellular loops of the murine Pgp, elicited anti-Pgp antibodies capable of improving the survival time of immunized mice in combination with chemotherapy. The manipulation of the immune system to break tolerance towards self-proteins was also used in a vaccination strategy that prevented formation or reduced development of β-amyloid plaque that occurs in Alzheimer's disease [15].

Pegylation consists in the covalent attachment of PEG to polypeptides or other candidate molecules and is used to increase drug circulating half-life. The high affinity of PEG to water molecules leads to a larger conjugate with reduced clearance. PEG-proteins are used as therapeutic drugs, such as pegylated interferon in the treatment of chronic hepatitis C [16]. However, pegylation reduces peptide immunogenicity. Immunization with a synthetic peptide from the acetylcholine receptor bound to PEG reduced antibody response, leading to the tolerance of the unconjugated peptide [17]. This lack of immunogenicity was overcome by the stabilization of the PEG-peptides in liposomes [18].

Interestingly, our approach which consists in stabilizing PEG-peptides in liposomes induced a strong immune response against the extracellular loop fragments 1, 2 and 4 of the murine Pgp. The addition of synthetic MPLA enhanced antibody production, as it has previously been observed [19]. It is generally accepted that lipid A has greater Th1 than Th2-stimulating potential, but in some cases, MPLA has been shown to enhance both humoral and cellular immune responses [20]. However, when sera were incubated with P388R cells, antibody binding was greater with Lp1 serum, i.e. serum from mice that had not received MPLA. The addition of MPLA in the immune preparation increased antibody titre but it may have elicited antibodies with reduced affinity to Pgp.

The anti-Pgp antibodies from Lp1-immunized mice improved sensitivity to doxorubicin in P388R cells, and were as efficient as 3 μM VPL. The binding of antibodies to their specific epitopes, i.e. extracellular loops, may have blocked the pore formed by Pgp. It seems also possible that antibody binding may have blocked the conformational changes required to Pgp activity, leading to an inhibition of drug efflux and the subsequent DXR accumulation in cells, as demonstrated by flow cytometry analysis of DXR uptake. In other studies, passive immunization with mAbs also led to an increase in drug uptake [7,21]. Lp2 sera exhibited reduced ability to enhance drug uptake compared to Lp1 antibodies. The hypothesis that Lp2 antibodies have lower affinity towards Pgp could explain these results, confirming that the Lp1 immunization (without MPLA) is more efficient than the same preparation with MPLA.

The *in vivo* experiments showed that Lp1-immunized mice inoculated with P388R cells had an increased survival time up to 83%. Wang et al. [22] reported an increase in survival time of 25% with the combination therapy vinblastine plus bisbenzyllisoquinoline alkaloids as chemosensitizer. The combined use of chemotherapy and compound S9788 improved survival time of 49% [23]. The elicited antibodies did not act on Pgp-expressing normal cells. No auto-immune symptoms were detected in mice immunized. This selectivity could be attributed to the differential expression of Pgp in tumor cells and in normal secretory tissues. Indeed, Pgp is rather distributed on the luminal surface in the latter [24] and so, it may not be accessible to antibodies. However, complete eradication of tumor did not occur, since immunized mice died. The fact that UIC2 reactivity toward Pgp was increased by the addition of Pgp substrates led to the hypothesis that Pgp function involves conformational changes [25]. Therefore, the incomplete neutralization of Pgp may be due to the existence of different conformational states of Pgp expressed on tumor cells. A certain conformation, may allow binding of antibodies to their epitopes while these epitopes may not be accessible to antibodies in another conformational state. This could explain why complete neutralization of tumors did not happen. Alternatively, the level of induced antibodies may not be sufficient to block all the pumps. To achieve that, re-boosting the immune response against Pgp in association with chemotherapy could be performed several weeks after tumor challenge.

This approach, consisting in breaking tolerance to specific self-proteins by immunization with synthetic peptides reconstituted in liposomes, could be applied to other cell surface proteins involved in cancer chemoresistance and could lead to efficient treatments against chemoresistant cancers.

Acknowledgements

We thank Lydie Ventéo for histopathological studies. This work was supported by AC Immune SA, Lausanne, Switzerland.

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