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Original Paper

A Possibility to Overcome P-glycoprotein (PGP)-mediated Multidrug Resistance by Antibody-targeted Drugs Conjugated to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer carrier

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***N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers containing doxorubicin (DOX) and different targeting moieties were developed with the aim of specific chemotherapy. Two of them, HPMA-conjugated DOX and galactosamine-targeted DOX, are in phase II clinical trials in the U.K. We studied the effect of conjugates with different targeting moieties (anti-CD71, antithymocyte globulin, anti-CD4, transferrin) on human or mouse multidrug resistance (MDR) cell lines (CEM/VLB, P388-MDR). It was shown that targeting decreases the level of MDR for DOX and the level of MDR depends on the targeting moiety used. The combination of these conjugates with chemosensitisers (cyclosporin A, D, G) restored almost completely the sensitivity of MDR cell lines to that of parental sublines. These results suggest that different intracellular trafficking of these conjugates (in membrane-limited organelles) in contrast to free diffusion for low molecular weight compounds might partially overcome P-glycoprotein (Pgp)-mediated MDR. We also report here the development of biodegradable HPMA hydrogels suitable for prolonged release of the cytostatic drug and chemosensitiser as a potential approach to overcome MDR mediated by Pgp.**

Key words: HPMA, polymeric prodrugs, antibody targeting, doxorubicin, chemosensitisers, P-glycoprotein, multidrug resistance
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INTRODUCTION

THE DEVELOPMENT of multidrug resistance (MDR) to cytostatic drugs is a major factor limiting the response rate of cancer chemotherapy. The aetiology of this resistance is multifactorial and several mechanisms of such resistance have been reported, including the overexpression of the P-glycoprotein (Pgp) encoded by the *MDR1* gene [1]. Pgp which possesses ATPase activity and functions as an energy-dependent extrusion pump, is responsible for reduced intracellular drug retention, resulting in narrowing of the therapeutic window between tumour active anticancer dosage and the maximal tolerated dosage for bone marrow cytotoxicity [2, 3]. In mammals, high levels of Pgp are detected in several tissues, including colon, kidney, liver (bile duct) and the capillaries in the central nervous system [4].

The use of polymer-based drug delivery systems for chemotherapy has become an established approach in the development of new drugs designed for the treatment of cancer [5]. Copolymers based on *N*-(2-hydroxypropyl) methacrylamide (pHPMA) were developed as carriers of cytostatic and immunosuppressive drugs [6, 7]. Doxorubicin (DOX) conjugated to pHPMA (PK-1) has completed its phase I clinical study and is now under phase II clinical evaluation in the U.K. [8]. It is possible to increase the effectiveness and specificity of these conjugates by introducing a targeting moiety to the pHPMA carrier. Such conjugates, called HPMA polymeric prodrugs, have been studied as potential tumouristatics and immunomodulators [9]. Galactosamine-targeted pHPMA-conjugated DOX is now in phase I clinical trial in the U.K. [10]. Both the drug and the targeting moiety (carbohydrates,

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hormones or antibodies) are conjugated to oligopeptide side chains of the pHPMA polymeric carrier. The composition of these side chains ensures the stability of the conjugate in the bloodstream, its biodegradability by lysosomal enzymes and controllable and time-dependent release of the drug intracellularly. In contrast to the free drug, pHPMA conjugates are inactive during transportation in the bloodstream and become activated after cell internalisation [11]. Their higher *in vivo* therapeutic efficacy in different experimental models has been reported previously [12, 13] and may be partially due to prolonged systemic bioavailability and lower unspecific toxicity, including reduced myelotoxicity [14] and cardiotoxicity [15]. The specificity of binding to the particular cell target in mice has been shown previously with anti-CD4-targeted pHPMA-conjugated DOX [16].

A different mechanism of uptake and intracellular trafficking of the targeted pHPMA-conjugated drug (receptor-mediated endocytosis in membrane organelles in contrast to diffusion for the free drug) might overcome Pgp-mediated MDR by making the endosome/lysosome encapsulated drug inaccessible for Pgp. In this report we describe the effect of different targeted pHPMA conjugates of DOX in combination with different cyclosporins against the parental DOX-sensitive human cell line CEM, mouse P388 cell line and their DOX-resistant sublines (CEM/VLB and P388-MDR, respectively), overexpressing Pgp. We also report the possibility of using pHPMA-based hydrogel-incorporated drugs (cyclosporin A, DOX) for continuous release and prolonged systemic bioavailability of both the Pgp blocker and the cytostatic drug.

MATERIALS AND METHODS

Chemicals

1-Aminopropane-2-ol, methacryloylchloride, glycyl-L-phenylalanine, L-leucylglycine, glycylglycine, dimethylsulphoxide (DMSO), *N*,*N*′-dicyclohexylcarbodiimide (DCCI), daunomycin hydrochloride (DNM.HCl), 4-nitrophenol and ethylenediamine were obtained from Fluka AG, Buchs (Switzerland) and doxorubicin hydrochloride (DOX.HCl) was a kind gift from Pharmacia-Farmitalia Carlo Erba (Italy). All other chemicals and solvents were of analytical grade. The solvents were dried and purified by conventional procedures and distilled before use. Cyclosporin A and its derivatives (cyclosporin D, cyclosporin G, cyclosporin U) were a kind gift from M. Flieger (Institute of Microbiology, ASCR, Prague). Individual cyclosporins are cyclic undecapeptides differing in one or more amino acids in their chemical structure.

Antibodies and purification

Antithymocyte globulin was generated as described previously [17]. Monoclonal anti-CD4 (clone MEM-115, kindly provided by V. Hořejší, Institute of Molecular Genetics, ASCR, Prague, Czech Republic) and anti-CD71 (clone RL34, kindly provided by J. Kovář, Institute of Microbiology, ASCR, Prague, Czech Republic) antibodies were purified either from ascites of BALB/c mice or from hybridoma supernatants by protein-A Sepharose 4B (Pharmacia-LKB, Sweden) affinity chromatography in 0.1 M Tris–HCl buffer, pH 8.5, containing 3 M sodium chloride. The antibodies were eluted with 50 mM sodium phosphate buffer (pH 6.0) containing 150 mM sodium chloride, then subjected to ion exchange chromatography on Mono S HR5/5 (Pharmacia) and eluted with a 0–500 mM gradient of sodium chloride in a buffer of 50 mM sodium phosphate. Transferrin as a targeting moiety was obtained from Sigma (St Louis, Missouri, U.S.A). Commercial (fluorescein isothyocyanate) FITC-labelled anti-CD4 Monoclonal antibody was obtained from PharMingen (San Diego, California, U.S.A.) and secondary FITC-labelled swine antimouse IgG antibodies were purchased from the Institute of Sera and Vaccines, Prague, Czech Republic.

Cell lines and cell culture

The human acute lymphoblastic T-cell leukaemia cell line CEM and the MDR subline CEM/VLB were provided by L. Seymour (Cancer Research Campaign Group, Birmingham, U.K.).

The mouse leukaemia cell line P388 and its resistant subline P388-MDR were obtained from I. Lefkovits (Basel Institute for Immunology, Switzerland). Cells were propagated at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma). Stock cultures of CEM/VLB were grown in the presence of 50 ng/ml vinblastin (VLB), P388-MDR cell line in the presence of 250 ng/ml DOX to maintain the MDR phenotype. The cell lines were tested for mycoplasma infection.

Cellular drug sensitivity assays

To test the cytostatic effect of the drugs in the presence or absence of chemosensitisers, cell growth inhibition was determined using the ^{3}[H]-thymidine incorporation assay. A fresh vial of cells from the same stock was thawed for each assay to minimise phenotypic instability. The cells (2×10^{4} per well) were plated in 96-well FB tissue culture plates (NUNC, Naparville, Illinois, U.S.A.) and after 72 h of cultivation, each well was pulsed with 1 mCi (37 kBq) of [^{3}H]-thymidine for 6 h. The cells were then harvested on to glass-fibre filters. Scintillation mixture was added to each filter and counted in a scintillation counter (MicroBeta, Wallac, Finland). Each sample was determined in triplicate. Cell growth in the presence of different drug concentrations was calculated relative to the value obtained in the absence of the drug. In the same cases, the cytotoxic effect of the drugs was also measured using the MTT assay according to the protocol described previously [18].

Inhibition of Pgp-mediated efflux of rhodamine 123 (R123) *measured by flow cytometry*

The inhibition of Pgp function by cyclosporins was measured by flow cytometry according to methods published previously [19]. Briefly, the cells were incubated with Pgp substrate R123 (Sigma) for 1 h at 4°C. Then the cells were washed with cold phosphate buffered saline (PBS) and the individual Pgp blockers were added for 1 h. After further washing, the cells were analysed by flow cytometry and gated according to R123 fluorescence (FACSorter, Becton-Dickinson).

Synthesis of HPMA–drug antibody conjugates

Polymer conjugates of DOX with antibodies or proteins (transferrin) was carried out in three reactions steps. In the first step, monomers, e.g. HPMA, methacryloylglycylglycine 4-nitrophenyl ester (MaGlyGlyONp) and methacryloylglycylphenylalynylleucylglycine 4-nitrophenyl ester (MaGlyPheLeuGlyONp), were prepared as described previously [11]. Methacryloylglycylphenylalanylleucylglycyl DOX (MaGlyPheLeu GlyDOX) was prepared by aminolysis of MaGly-

PheLeuGlyONp with DOX.HCl in dimethylformamide in the presence of an equivalent amount of triethylamine, according to the synthesis of its daunomycin analogue described previously [11, 20]. The monomer containing DOX was purified by gel filtration on Sephadex LH 20 in methanol and isolated by precipitation into diethylether. The final product was characterised by amino acid analysis, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The ratio of amino acids Gly:Phe:Leu was 2.02:1.0:1.0 (amino acid analyser LDC analytical with precolumn derivatisation, U.S.A.). TLC was acetone: tetrachlormethane 5:2, $R = 0.28$. HPLC was carried out in a reverse phase C18 column (Tessek), the solvent being methanol:water, gradient from 50 to 90 vol.% methanol in aqueous solution, with a flow rate of 1ml/min, and UV/VIS detection at 485 nm. Only a single peak was detected at 20.8 min.

In the second step, polymeric precursors bearing reactive ester groups at the end of their oligopeptide side chains were prepared by the radical precipitation copolymerisation of HPMA with the methacryloylated *p*-nitrophenyl ester (ONp) of the respective tetrapeptide in acetone as described previously [21]. The polymer precursor was dissolved in DMSO (15 wt%) and 50 mol% of DOX.HCl (relative to the ONp content in the precursor) was added. Under stirring, one equivalent of TEA (related to DOX.HCl) was added in four small portions. The reaction mixture was stirred at room temperature for 2 h, the solution filtered and the polymer precipitated into an acetone:diethyl ether mixture. The product was purified on Sephadex LH-20 in methanol and isolated by precipitation into acetone:diethyl ether. In the third step, targeting moieties were coupled to the precursor bearing DOX via consecutive aminolytic reactions as described previously [19, 22]. The polymer, dried in a vacuum, was dissolved in distilled water (10–15 wt%) and, under stirring and cooling at 10°C, added to a solution of the antibody (or respective protein) in phosphate buffer (15–25 mg/ml). The pH of the reaction mixture was adjusted to 8 under stirring at 10°C by adding a saturated solution of $Na_2B_4O_7$ (pH-stat Radiometer). The course of the reaction was monitored by the consumption of the borate solution. The reaction was completed at pH 8.5.

Characterisation of pHPMA conjugates

The polymer precursor was characterised by ultraviolet spectrophotometry (the content of oligopeptide side chains terminated in *p*-nitrophenoxy groups) and by GPC (weight- and number-average molecular weights, Mw and Mn) after aminolysis of reactive groups with 1-aminopropan-2-ol. All polymers, including copolymers containing both the antibody and the drug, were characterised and tested for the content of free polymer, drug or antibody by GPC using a Sepharose 4B/6B 100×1.6 cm column (ultraviolet and refraction index detection) or FPLC Pharmacia equipped with Superose 6 column and by electrophoresis (Pharmacia-LKB Phast System, Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) gels with gradient 4-15) [23]. The antibody content in the conjugates was estimated by amino acid analysis (LDC Analytical, precolumn OPA derivatisation) and the DOX content by ultraviolet spectrophotometry [24]. The conjugates with antibody or protein were purified using a Sephadex G-25 column eluted with phosphate buffer pH 7.4. Neither GPC nor electrophoresis showed significant amounts of free antibody or free drug in the conjugates. The samples were kept in the frozen state (−20°C) until biological evaluation. The characteristics of individual HPMA conjugates are shown in Table 1.

Synthesis of HPMA hydrogels

HPMA was prepared by the reaction of methacryloyl chloride with 1-amino-2-propanol in acetonitrile, as described previously [25]. The crosslinking agent, *N*,*O*-dimethacryloylhydroxylamine (DMHA), was prepared by the reaction of hydroxylamine hydrochloride with methacryloyl chloride in pyridine [26].

Hydrogels were prepared by radical solution copolymerisation of HPMA with DMHA in ethanol at 60°C using azo-*bis*-isobutyro-nitrile (AIBN) as the initiator of polymerisation [27]. Dry gels were loaded with the drug by swelling in a solution of the drug in ethanol (cyclosporin A) or in an ethanol–water mixture (DOX.HCl) followed by drying in air. The content of the drug in the hydrogel was estimated spectrophotometrically in a solution after hydrogel degradation (DOX) or using amino acid analysis of sample hydrolysate (cyclosporin A).

Hydrolytic degradation of hydrogels

The hydrolytic degradation of gel samples was studied at 37°C in 0.1 M phosphate buffer of pH 7.4 containing 0.15 M NaCl. The gel (rod 3×2 mm) was incubated under stirring in 20 ml of buffer and the time required for its total degradation into soluble products was measured.

RESULTS

The ability of targeted pHPMA-conjugated DOX to overcome Pgp-mediated MDR

As shown in Figure 1a, the CEM-VLB cell line was 50 times more resistant to free DOX than the CEM line. The IC_{50} for DOX was calculated to be 2.5 μM in comparison with 0.05 μM for the sensitive CEM cell line. The IC_{50} for antithymocyte globulin-targeted pHPMA-conjugated DOX (sample no.1) was determined to be 10 μM for CEM and 250 μM for the MDR subline CEM-VLB (Figure 1b). Similar results were shown for an anti-CD71-targeted pHPMA conjugate of DOX (sample no.2) with the IC_{50} for CEM and

Table 1. *Characterisation of N-(2-hydroxypropyl) methacrylamide (HPMA) conjugates of doxorubicin (DOX)*

Sample no.	Type of HPMA–DOX conjugate	Content of targeting moiety (wt%)	Content of the drug (wt%)
1	ATG–HPMA–DOX	25	6.25
2	Anti-CD71–HPMA-DOX	25	5.02
3	Anti-CD4–HPMA–DOX	25	5.50
4	HPMA–DOX	–	8.50
5	Transferrin–HPMA–DOX	25	4.14

ATG, antithymocyte globulin.

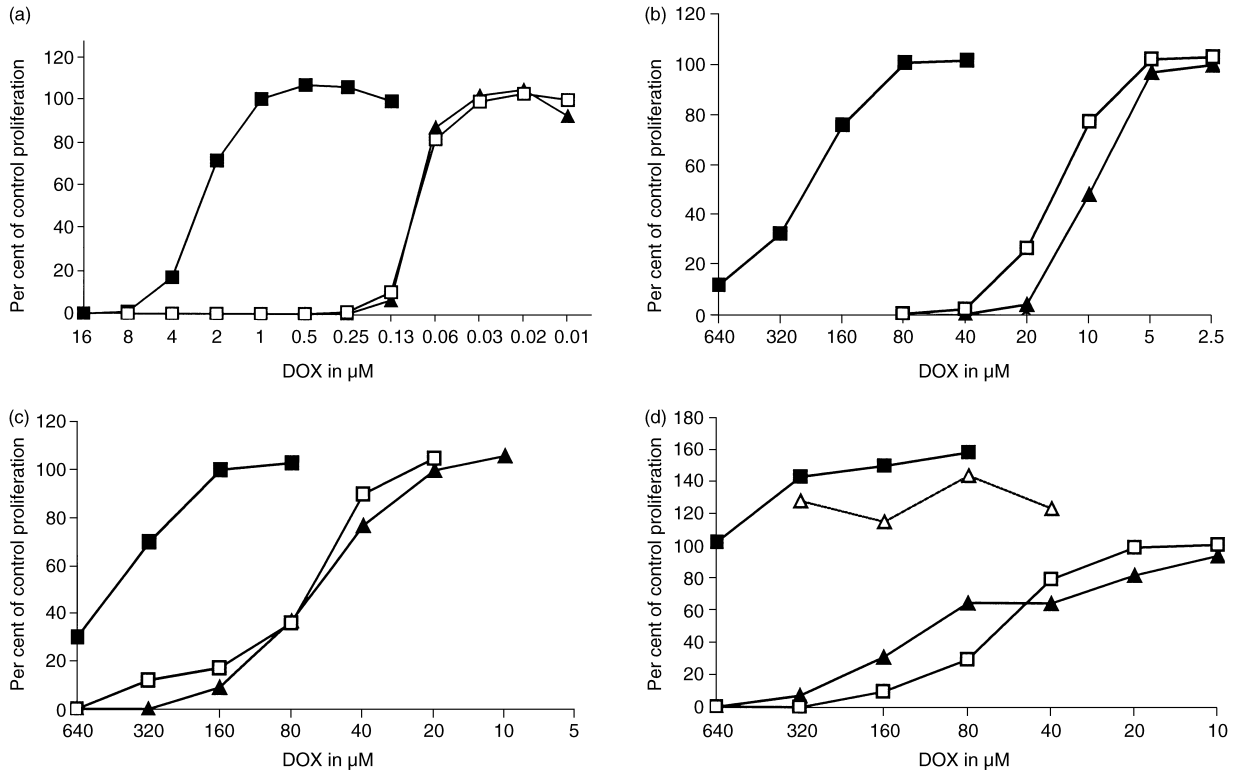

Figure 1. Effect of free DOX (a), HPMA-conjugated DOX targeted by anti-thymocyte globulin (b), anti-CD71 (c) and anti-CD4 (d) and their combination with cyclosporin A on proliferation of sensitive CEM and resistant CEM-VLB cell lines. Each sample was determined in triplicate and the data represent the average of three independent experiments. The number of impulses in the control wells was calculated to be 100%. —■—, resistant CEM-VLB; —□—, CEM-VLB + cyclosporin A 1.6 μM; —▲—, sensitive CEM; —△—, CEM incubated with anti-CD4 conjugated to N-(2-hydroxypropyl) methacrylamide (without doxorubicin (DOX)).

CEM-VLB being 70 and 480 μM, respectively (Figure 1c). Anti-CD4-targeted pHPMA-bound DOX (sample no. 3), which has previously been tested for its binding specificity on peripheral blood leucocytes using two-colour flow cytometry, was effective only on parental CEM with an IC_{50} of approximately 120 μM (Figure 1d). In contrast, proliferation of CEM-VLB was not inhibited when the highest tested concentration (640 μM) of anti-CD4-targeted pHPMA-conjugated DOX was used. The IC_{50} for non-targeted pHPMA-conjugated DOX is shown in Table 2.

The IC_{50} for DOX against the P388 cell line (macrophage-like origin) was estimated to be 0.24 μM for sensitive P388 and 72 μM for its resistant subline P388-MDR (300 times more resistant). We also tested non-targeted pHPMA-bound DOX (sample no. 4) and transferrin-targeted pHPMA-conjugated DOX (sample no. 5). The IC_{50} of free or pHPMA-conjugated drug against different cell lines are shown in Table 2. Figure 2 shows the level of DOX resistance (ratio IC_{50} for resistant subline to IC_{50} for parental cell line) for different targeted conjugates of DOX in CEM/VLB (Figure 2a) and P388-MDR (Figure 2b).

Sensitisation of the MDR cell line CEM-VLB to free and antibody-targeted pHPMA-coupled DOX

In order to determine whether the sensitivity of the CEM cell line can be restored for MDR-subline CEM-VLB by Pgp blockers, cytotoxic curves for various forms of free and antibody-targeted pHPMA-coupled DOX were constructed in the presence or absence of cyclosporin A and its derivatives (cyclosporin D, cyclosporin G). It was shown that the

Table 2. IC_{50} of different cytostatic drugs and N-(2-hydroxpropyl) methacrylamide (HPMA) conjugates against different multidrug resistant cell lines

		IC_{50} (μm) for sensitive cell lines (CEM, P388) and their resistant counterparts (CEM-VLB, P388-MDR, respectively)			
Type of drug	Sample no.	CEM	CEM-VLB	P388	P388-MDR
Free DOX	–	0.05	2.5	0.24	72
Free VLB	–	0.0013	0.052	0.016	1.6
ATG–HPMA–DOX	1	10	250	–	–
Anti-CD71–HPMA–DOX	2	70	480	–	–
Anti-CD4–HPMA–DOX	3	120	NA	–	–
HPMA–DOX	4	100	NA	32	NA
Transferrin–HPMA–DOX	5	–	–	25	625

NA, not active at maximally tested concentration (640 μM); –, not tested; DOX, doxorubicin; VLB, vinblastin; ATG, antithymocyte globulin.

sensitivity of MDR cells is restored by cyclosporins not only towards free DOX but also antibody-targeted conjugates of DOX. Using 0.4–1.6 μM concentrations of various cyclosporins, the parental sensitivity of CEM-VLB was almost completely restored (Figure 1), both for free and targeted pHPMA-conjugated DOX. Similar results were obtained when HPMA-conjugated DOX or transferrin-targeted DOX were tested in combination with 1.6 μM cyclosporin A against mouse P388-MDR (data not shown).

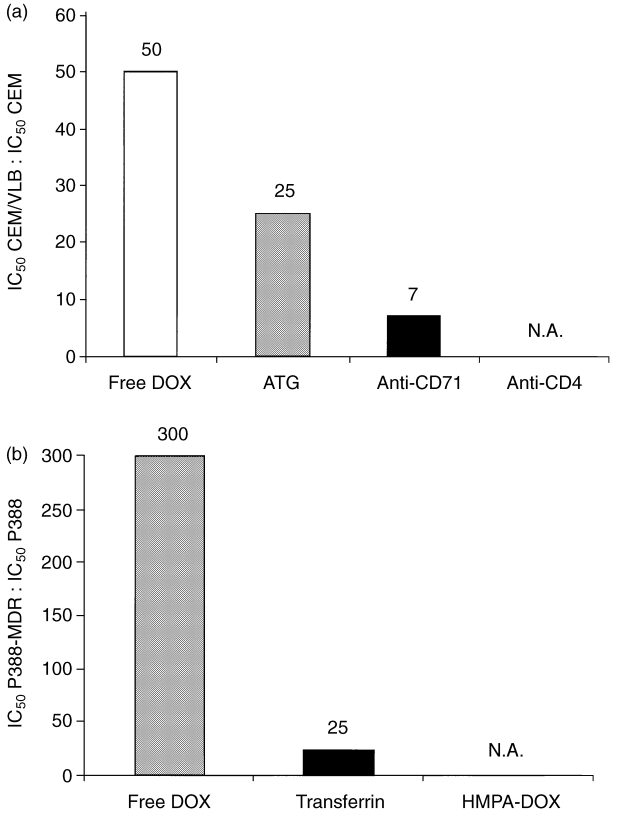

Figure 2. The level of resistance for free DOX and different targeted HPMA-conjugated DOX in CEM/VLB (a) and P388-MDR cell lines (b). N.A., not active.

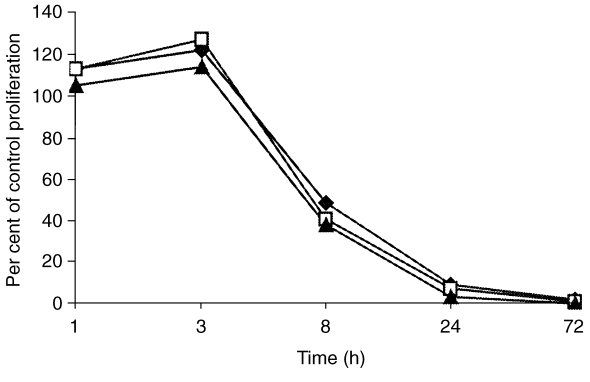

Figure 3. Effect of time on efficacy of DOX in combination with different cyclosporin analogues (cyclosporin A, cyclosporin D, cyclosporin G) against multidrug resistant CEM/VLB cell line. The MTT assay was performed according to protocol described previously [28]. Each sample was determined in triplicate. The cell growth of the control was calculated to be 100%. —◆—, cyclosporin A; —□—, cyclosporin D; —▲—, cyclosporin G.

The effect of time on the efficacy of the cyclosporin + DOX combination against CEM/VLB

In general, chemosensitisers and cytostatic drugs have very short plasma half-lives, with the majority of the drug being excreted within the first 1–8 h. When the MDR cell line CEM/VLB was exposed to non-toxic concentrations of both drugs for 1, 3, or 8 h, their growth was only partially inhibited (40–50% of control proliferation). A 24 h exposure of CEM/VLB cells to chemosensitiser + DOX combination was long enough for complete inhibition of proliferation (Figure 3). This is consistent with previous observations that DOX causes apoptosis after 12–24 h [28].

The inhibition of proliferation of the CEM/VLB cell line after exposure to a non-toxic concentration of DOX (1 μM) in combination with different concentrations of cyclosporin A, cyclosporin D, cyclosporin G and cyclosporin U is shown in Figure 4a. It was observed that cyclosporin D and cyclosporin G are better than cyclosporin A and cyclosporin A is better than cyclosporin U in their ability to block Pgp-mediated MDR cell lines. Similar results were obtained by flow cytometry experiments measuring the ability of cyclosporins to inhibit Pgp-mediated efflux of R123 from CEM/VLB (Figure 4b).

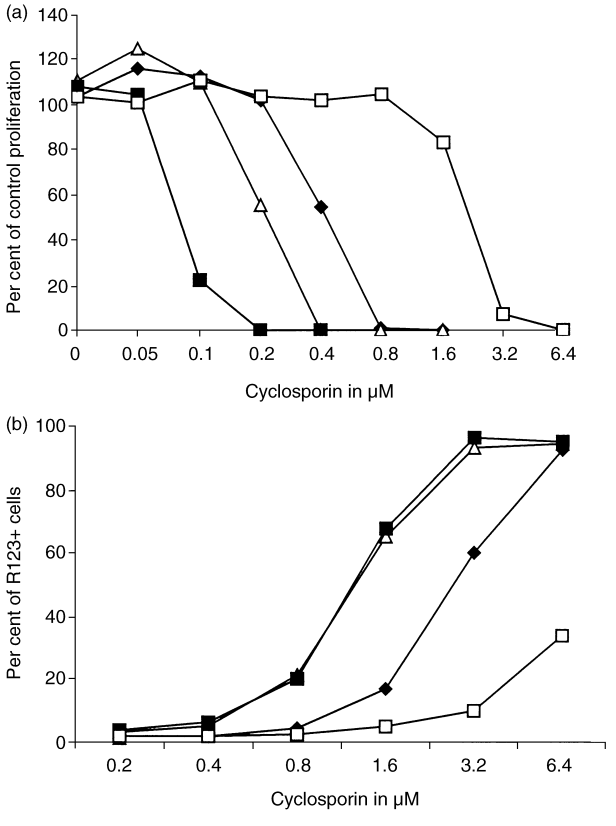

Figure 4. The efficacy of different cyclosporins to block Pgp measured by (a) inhibition of proliferation of CEM/VLB cell line in combination with DOX, (b) R123 efflux from Pgp-positive cell line CEM/VLB. The efficacy if individual cyclosporins to inhibit P-glycoprotein (Pgp)-mediated efflux of rhodamine 123 (R123) from CEM/VLB was measured by flow cytometry. R123 is transported from Pgp+cells and these cells are negative for fluorescence unless the function of Pgp is blocked by chemosensitisers, when R123 remains inside and the cells become positive for fluorescence. —◆—, cyclosporin A; —△—, cyclosporin D; —■—, cyclosporin G; —□—, cyclosporin U.

HPMA hydrogels containing DOX or cyclosporin A

When it was shown that both the chemosensitiser (cyclosporin A) and the cytostatic drug (DOX) have to be present in the appropriate concentration ratios for at least 12–24 h, biodegradable hydrogels based on HPMA were synthesised. The biodegradation of such hydrogels allows the selective release of the entrapped drugs from the gel matrix for a defined time. This would help to decrease the rate of drug elimination and to maintain the drug concentration in the bloodstream and tissues higher for a longer time. HPMA hydrogels loaded with DOX or cyclosporin A were synthesised (degradation time 12 h—data not shown). We tried to test the effect of hydrogel-entrapped cyclosporin A (H-CsA) in combination with free DOX or hydrogel-entrapped DOX (H-DOX) on the growth and viability of P388-MDR *in vitro*. Both combinations were effective, while H-CsA or H-DOX alone were not able to inhibit cell growth and viability (Figure 5).

DISCUSSION

We and others [29] proposed that antibody-targeted pHPMA-conjugated drugs might overcome PgP-mediated MDR. Lysosomotropic delivery of these conjugates to the cells by receptor-mediated endocytosis could at least partially overcome PgP-mediated drug efflux. In addition, it has recently been published that *in vitro* chronic exposure of cancer cells to pHPMA-conjugated DOX (PK1)—in contrast to free DOX—did not induce Pgp-mediated MDR [30].

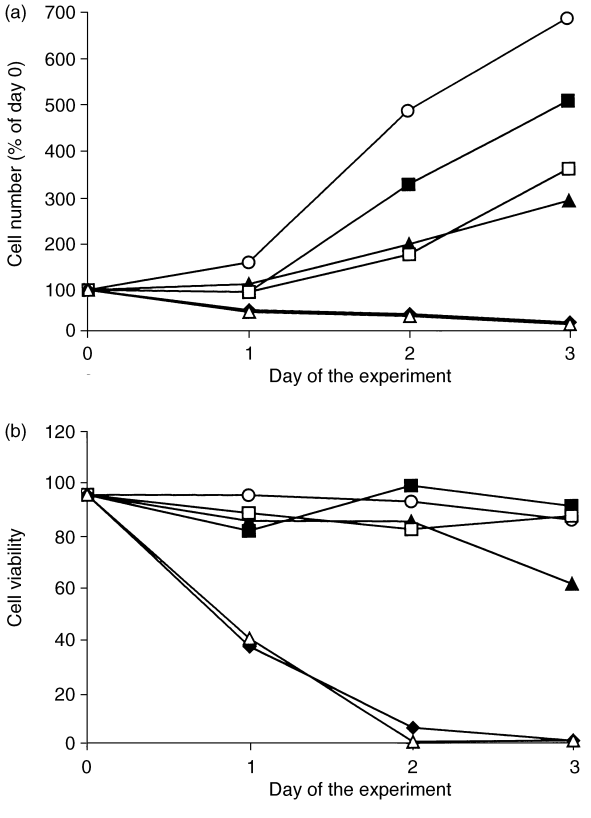

Figure 5. The *in vitro* effect of HPMA-hydrogel entrapped cyclosporin A (H-CsA) in combination with free or HPMA-hydrogel entrapped DOX (H-DOX) on cell number (a) and viability (b) of P388-MDR cell line. —■—, control; —○—, hydrogel-entrapped cyclosporin A (H-CsA); —▲—, doxorubicin (DOX); —□—, hydrogel-entrapped DOX (H-DOX); —◆—, H-CsA+DOX; —△—, H-CsA+H-DOX.

We tested three different antibody-targeted pHPMA conjugates of DOX against the human lymphoblastic T-cell line CEM and its MDR subline CEM-VLB overexpressing Pgp. It was shown that the ability of the conjugates to decrease DOX resistence depends very much on the targeting moiety. Anti-CD71-targeted conjugate was effective in both sensitive and resistant cell lines, with an IC_{50} of 70 and 480 μM, respectively. The most potent was the antithymocyte globulin-targeted conjugate of DOX with an IC_{50} for CEM of 10 μM and 250 μM for the CEM-VLB cell line. However, anti-CD4-targeted conjugate was effective only when the sensitive cell line CEM was used in the experiment. The proliferation of the MDR subline CEM-VLB was not inhibited even when the highest DOX concentration was used (640 μM). This might be due to a poor internalisation of the CD4 molecule [31] and/or the fact that the isotype of anti-CD4 antibody was IgG_{2a}, i.e. a much less internalised isotype if compared with IgG_1 [32]. It was shown that these resistant cell lines were in fact stimulated by anti-CD4-targeted pHPMA conjugates. It has previously been published that CD4 crosslinking may activate T-cells [33]. A similar stimulatory effect was shown for the control pHPMA conjugate containing only anti-CD4 antibody (Figure 1d). This stimulation is not due to the low binding activity of anti-CD4 antibodies conjugated to pHPMA copolymer carrier, as we have confirmed the specific binding of these conjugates to CD4 positive human peripheral blood lymphocytes by flow cytometry. Binding of these conjugates to the surface of CEM and CEM-VLB cells was also detected (data not shown).

CEM-VLB cells were 50 times more resistant to free DOX than parental CEM cells. The level of resistance decreased to 25 and 7 for anti thymocyte globulin- and anti-CD71-targeted conjugates of DOX, respectively. pHPMA conjugates of DOX (transferrin-targeted and non-targeted) were also tested against the mouse MDR cell line P388-MDR. It was shown that the level of DOX resistance decreased from 300 for free DOX to 25 for transferrin-targeted conjugate. The non-targeted pHPMA–DOX conjugate was not effective at the highest concentration tested (640 μM). DOX had to be conjugated to HPMA because the level of MDR was unchanged when the cells were incubated with free DOX and free HPMA. The circumvention of MDR is not then an intrinsic ability of HPMA (HPMA does not block Pgp) as it was shown for other copolymers [34, 35].

It seems that the level of internalisation of different conjugates is the factor responsible for the partial circumvention of MDR. We hypothesised that the receptor-mediated endocytosis and subcellular trafficking in membrane organelles make the DOX released from polymeric conjugate only partially inaccessible for Pgp. The conjugates, after being internalised, are exposed in the acidic lysosomal compartment to cathepsin proteases which degrade the bond between the oligopeptidic spacer GlyPheLeuGly (designed to be cleaved intracellularly by acidic proteases) and the drug. After its release from the pHPMA carrier, DOX enters the cytoplasm and eventually the nucleus. Cytoplasmic DOX might become a substrate for Pgp expressed in the vesicles travelling from endoplasmic reticulum towards the cell membrane. Pgp can pump DOX into the vesicles where the drug remains or it is released outside the cell compartment after the fusion of the vesicle with the cell membrane. The data support the concept that MDR is only partially overcome by these conjugates. When MDR cells were exposed to both targeted conjugate

and one of the cyclosporins (cyclosporin A, cyclosporin D, cyclosporin G) in concentrations ranging between 0.4 and 1.6 μM, the sensitivity of MDR cells almost equalled that of the parental cell lines.

Recently, we developed biodegradable HPMA hydrogels containing cyclosporin A as a chemosensitiser and/or cytostatic drug (DOX, vinblastin). These hydrogels—according to the level of crosslinking—have a degradation time of 12–56 h. We propose that these hydrogels could maintain the level of both drugs stable and high enough *in vivo* to reach the desired pharmacological effect. Their *in vitro* effectivity in combination with free DOX or H-DOX was shown (Figure 5).

However, even if Pgp-associated MDR proves to be a relevant and reversible cause of clinical drug resistance, numerous problems remain to be solved. The identification of more specific, potent, and less clinically toxic chemosensitiser/cytostatic drug combinations, as well as their most efficacious modulator regimens for clinical use, remains critical to the possible success of this approach. An important limiting factor for chemosensitiser/cytostatic drug combinations is their toxicity for normal tissues expressing Pgp. This side-effect might be decreased or even overcome when pHPMA polymer-bound drugs are used. It has been published previously that owing to their higher molecular weight (non-targeted conjugates 20–30 kDa, targeted conjugates approximately 200–300 kDa) they cannot cross the blood-thymus barrier [36]. The same might be true for the blood–brain barrier expressing Pgp, thus contributing to a lower toxicity of polymer-based drugs in comparison with free drugs. In addition, it is now well established that biocompatible macromolecules, including pHPMA copolymer-based prodrugs, may exhibit a substantial tumour accumulation due to the phenomenon of enhanced permeability and retention (EPR effect) [37]. Macromolecular conjugates can extravasate due to the leaky endothelium of the tumour tissue, but remain there for a longer time due to the lack of effective lymphatic drainage in the tumour. It is tempting to speculate that passive targeting (or antibody targeting) of macromolecular prodrugs could limit the efficacy of chemosensitiser/cytostatic drug combinations only to cancer tissue at least in those solid tumours where the EPR effect was reported [38]. The *in vivo* efficacy of HPMA copolymer conjugates with DOX in combination with free cyclosporin A or biodegradable H-CsA for the treatment of subcutaneous P388-MDR in mice is under evaluation.

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