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HPMA-hydrogels result in prolonged delivery of anticancer drugs and are a promising tool for the treatment of sensitive and multidrug resistant leukaemia

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Abstract

Treatment of an established BCL1 leukaemia in mice showed that the use of hydrogels is advantageous in comparison with free doxorubicin (DOX), partially due to the different pharmacokinetic profile of the drug release. Pharmacologically active concentrations ranging from 100 to 800 ng/ml were detectable in the bloodstream for more than 4 days when DOX-loaded hydrogels were implanted into mice. Animals treated with free DOX survived for 35 days, survival of hydrogel-DOX treated animals increased up to 60 days and long-term survivors were achieved, when the second hydrogel was implanted 2 weeks after the first one. Hydrogels containing vinblastine (VLB) were ineffective. *N*-(2-hydroxypropyl)methacrylamide (HPMA) hydrogels were also used in combined therapy against multidrug resistant leukaemia P388-MDR to achieve a synergistic effect of both the cytostatic drug and chemosensitising agent. It was shown that when 4 times the maximal tolerated dose (MTD) of free DOX was incorporated into HPMA-hydrogels, tumour volume was reduced by approximately 50% after implantation of the hydrogel containing DOX and cyclosporine A (CsA) and survival was slightly prolonged. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *N*-(2-Hydroxypropyl)methacrylamide; Biodegradable HPMA-hydrogels; Doxorubicin; Cyclosporines; P-Glycoprotein; Multidrug resistance

1. Introduction

It has been previously shown that polyalkylcyanoacrylate nanoparticles containing both cyclosporine A (CsA) and free doxorubicin (DOX) significantly inhibited the growth of various resistant cancer cell lines *in vitro* [1]. However, the efficacy of chemosensitisers plus cytostatics incorporated into formulations for prolonged release against multidrug resistance (MDR) *in vivo* has not been investigated thoroughly. Biodegradable hydrogels based on *N*-(2-hydroxypropyl) methacrylamide (HPMA) [2] offer an attractive possibility of incorporating various cytostatic drugs into their polymeric matrix. The biodegradation of such hydrogels allows the selective release of incorporated drugs from the polymer matrix for a defined time according to the level of cross-linking [3]. This would help to decrease the rate of drug elimination and to maintain higher drug concentrations in the blood and other tissues for a prolonged period. In addition, high peak concentrations would be eliminated, thus reducing adverse drug reactions, including acute toxicity.

The overexpression of P-glycoprotein (Pgp), encoded by the *MDR1* gene, has been associated with the MDR phenotype of tumour cells which are resistant to high levels of cytotoxic chemotherapeutic agents [4,5]. Pgp is an adenosine triphosphate (ATP)-dependent pleiotropic efflux pump for a variety of different compounds that enter cells by passive diffusion, including anthracyclines, vinca alkaloids, podophyllotoxins or rhodamine R123 [6,7]. Pgp expression in the plasma membrane results in a lowering of the intracellular concentration of MDR-type drugs and this Pgp-mediated transport of cytostatic drugs out of resistant tumour cells is thought to be the major cause of experimental and, probably also, clinical MDR [8]. Among the several strategies to overcome

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MDR, co-administration of chemosensitising agents, generally acting as Pgp inhibitors, has been widely investigated even in clinical practice [9,10]. Verapamil, CsA, its non-immunosuppressive analogue SDZ PSC 833 and GF 120918 belong amongst the best known MDR modulators [11,12]. This raised the hope that the addition of chemosensitisers to cytostatics might improve the chemotherapy success rate. Unfortunately, the results from clinical trials have not been satisfactory, often because of limited tolerance of the MDR-blockers by themselves, which precluded the achievement of pharmacologically active levels in the patients.

Pharmacokinetic characteristics of hydrogel-based drug delivery systems, as well as their efficacy in various cancer models, including sensitive and resistant leukaemia, were examined.

2. Materials and methods

2.1. Chemicals

1-Aminopropane-2-ol, methacryloylchloride, dimethylsulphoxide (DMSO), *N*,*N*′-dicyclohexylcarbodiimide (DCCI), 4-nitrophenol, ethylenediamine and doxorubicin hydrochloride (DOX.HCl) were obtained from Fluka AG, Buchs (Switzerland), vinblastine sulphate was purchased from Sigma, USA. All other chemicals and solvents were of analytical grade. The solvents were dried and purified by conventional procedures and distilled before use. CsA was a kind gift of Dr M. Flieger (Inst. of Microbiology, ASCR, Prague). Sandimmune Neoral (Sandoz, Switzerland) was used for the oral administration of free CsA.

2.2. Cell lines and cell culture

The murine B cell leukaemia BCL1 is a well-described cell line that grows rapidly and consistently in immunocompetent Balb/c mice. This cancer cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Routine culture and all experiments with BCL1 cell line were conducted in Roswell Park Memorial Institute (RPMI) 1640 medium with extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), 2-mercaptoethanol (5×10^{-5} M), HEPES (4-(2-hydroxy-ethyl)-piporazine-1-ethane sulpone acid (10 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% (v/v) 0.1 μm filtered fetal bovine serum. Mouse leukaemia cell line P388 and its resistant sub-line P388-MDR were obtained from Prof. I. Lefkovits (Basel Institute for Immunology, Basel, Switzerland) and it was propagated in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma, USA). Stock cultures of the P388-MDR cell line in the presence of 250 ng/ml DOX were used to maintain the MDR-phenotype. Both cell lines were grown at 37 °C in a 5% CO_2 atmosphere. The cell lines were tested for mycoplasma infection.

2.3. 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 a ^{3}[H]-thymidine incorporation assay. A fresh vial of cells from the same stock was thawed for each assay to minimise phenotypic instability. 2×10^4 cells per well were plated in 96-well flat bottom (FB) tissue culture plates (NUNC, Denmark) 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 onto glass-fibre filters. Scintillation mixture was added to each filter and they were 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-assay according to the protocol described earlier in Ref. [13].

2.4. Mice

Mice of the inbred strain Balb/c were obtained from the breeding colony of the Institute of Physiology (Academy of Sciences of the Czech Republic) and kept in standard housing. DBA-2 mice were obtained from the breeding colony of Anlab Ltd. (Prague, Czech Republic). Animals aged 9–15 weeks and in the 19–22 g weight range were used for the *in vivo* experiments. The Animal Welfare Committee of the Institute of Microbiology Academy of Sciences of the Czech Republic approved all of the experiments.

2.5. In vivo *tumour therapy models*

Cells in exponential growth with viability greater than 95% were used for all of the experiments. Balb/c mice were intraperitoneally injected (i.p.) with 5×10^5 cells of BCL1 leukaemia in a total volume of 100 μl. Animals bearing day 11-established BCL1 leukaemia were treated with i.p. injections of three DOX doses (100 μg/mice on days 11, 13 and 15) or HPMA-hydrogels with a degradation time of 48 h containing 300 μg of DOX were subcutaneously (s.c.) implanted on day 11. Mice were weighed every other day to monitor the weight increase due to leukaemia (weight gain by more than 10% is considered to be a sign of leukaemia growth or relapse). The therapeutic effect of the drugs inhibits the weight gain (histological studies, M. Kovář, Inst. of Microbiology).

5×10^5 cells of MDR P388-MDR were s.c. injected in the right flank of DBA-2 mice and the treatment was initiated when the tumour was palpable (on days 6 and 7). CsA was given orally (p.o.) in a total dose of 400 μg 1–2 h before i.p. administration of DOX (100 μg/mice) on days 6, 8 and 10). HPMA-hydrogels containing various drug combinations were implanted on the other side of the body. The mean tumour size was monitored three times per week. Control animals received empty hydrogel. All experiments were performed two times using individual treatment groups of 6–8 mice.

2.6. Reticulocyte assay

Retic-COUNT contains thiazole orange dye with an affinity for DNA and RNA. Reticulocytes are immature erythrocytes released from the bone marrow containing remains of DNA and RNA and they are therefore detectable by fluorescent activated cell sorting (FACS) (Becton-Dickinson) after incubation with Retic-COUNT. 5 μl of the mouse blood from the tail vein was taken and either added to a tube containing 0.5 ml of PBS with 0.1% NaN_3 (to set up a control marker) or to a tube with 0.5 ml of Retic-COUNT solution (Becton-Dickinson). Tubes were left in the dark for 1 h at laboratory temperature and 50 000 erythrocytes were analysed using flow cytometry.

2.7. Synthesis of HPMA hydrogels

HPMA was prepared by the reaction of methacryloyl chloride with 1-amino-2-propanol in acetonitrile, as described earlier in Ref. [14]. The crosslinking agent, *N*,*O*-dimethacryloylhydroxylamine (DMHA), was prepared by the reaction of hydroxylamine hydrochloride with methacryloyl chloride in pyridine [2].

Hydrogels were prepared by radical solution copolymerisation of HPMA with DMHA in ethanol at 60 °C using azo-bis-isobutyro-nitrile (AIBN) as an initiator of polymerisation [15]. Dry gels were loaded with the drug by swelling in a solution of the drug in ethanol (CsA), ethanol–water mixture (DOX.HCl) or physiological solution (VLB) followed by drying in the air. After the drying, 30 μl of pure ethanol was added to the microwell with the hydrogel (to achieve the sterility and to retract part of the drug, which could get stuck on upper part of the gel, into its lumen). Hydrogels were then kept in sterile vials in the dark until their immediate use.

2.8. Hydrolytic degradation of hydrogels in vitro and kinetics of the drug release

The time dependence of the drug amount released from the hydrogels incubated in 0.1 M phosphate buffer, pH 7.4 containing 0.15 M NaCl at 37 °C was determined using ultraviolet (UV) spectrophotometry (DOX, VLB) and amino acid analysis (CsA). Content of DOX was determined after its extraction into chloroform at $\lambda = 488$ nm, $\epsilon = 1.15\times10^4$ l mol^{-1} cm^{-1} using a procedure described for the determination of DOX in the blood. Content of CsA was determined by amino acid analysis after drug extraction into chloroform and its hydrolysis in 6 N HCl (115 °C, 16 h). Alanine, valine and α-amino butyric acid were used for the calibration and drug content calculation. Content of VLB released into the incubation media was analysed directly in the supernatant at $\lambda = 269$ nm using molar extinction coefficient $\epsilon = 1.5\times10^4$ l mol^{-1} cm^{-1}. Supernatant from the hydrolysis of the unloaded hydrogel was used as a reference. The total amount of the drug absorbed in the hydrogel was determined in the incubation media after complete hydrolysis of the gel. Drug release was expressed as the relative amount of drug (relative to the total dose) released in a given time period.

2.9. Hydrogel implantation

Mice were i.p. injected using chloralium hydrate (0.2 ml of 8% solution, Inst. of Clinical and Experimental Medicine, Prague, Czech Republic) to achieve anaesthesia. Skin of mice was shaved, sterilised and cut (2 mm in diameter) by scissors. Hydrogel rods were washed in ethanol and sterile distilled water and they were immediately implanted s.c. Wound edges were held by tweezers and stuck together using the tissue adhesive Histoacryl (B. Braun Surgical, Germany). The wound was covered with antiseptics and mice were placed into clean aquaria. All instruments used for the surgery were sterilised prior to use.

2.10. Determination of DOX content in the blood

Samples of blood and organs (homogenised) were tested for the content of total DOX in solution. The amount of free DOX was determined after its extraction from the incubation media into chloroform. A mixture of 0.1 ml of sample solution and 0.3 ml of buffer (0.2 M Na_2CO_3/$NaHCO_3$, pH 9.8) was extracted with 0.6 ml of chloroform. DOX was determined by high performance liquid chromatography (HPLC) (Tessek gel C18 column, methanol–water gradient 10–90% methanol, fluorescent detector with excitation at 488 nm and emission at 560 nm). The calibration (yield of extraction) was carried out using DOX.HCl.

2.11. Statistical analysis

The Wilcoxon's statistics (multiple comparison method) and Student's *t*-test were used to evaluate the differences among experimental groups. P values <0.05 were taken as statistically significant.

3. Results

3.1. The in vivo efficacy of free and hydrogel-entrapped drugs against BCL1 leukaemia

Survival and onset of disease (weight gain) were observed over a period of more than 100 days (Fig. 1). The efficacy study revealed that the maximal tolerated dose (MTD) of free DOX (3×5 mg/kg) slightly increased the mean survival of the mice from 30 to 35 days. However, the survival of mice treated with s.c. implanted HPMA-hydrogel containing 15 mg/kg of DOX (H-DOX) was prolonged significantly (60 days). H-VLB in a dose of 1.7 mg/kg was almost ineffective (mean survival of mice 37 days), while the same VLB dose injected i.p. was too high for mice and they died within 15 days due to toxicity (Fig. 1). Implantation of the empty hydrogel (without the drug) had no effect on the survival of the mice.

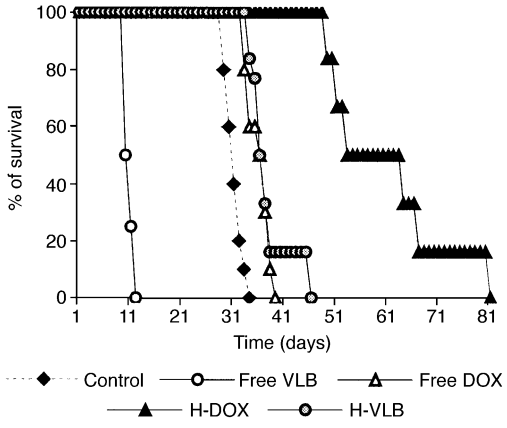

Fig. 1. The treatment of BCL1 leukaemia (5×10^5 intraperitoneally (i.p.)) was initiated on day 11. Free doxorubicin (DOX)—5 mg/kg i.p. on days 11, 12, 13. Hydrogel-entrapped DOX (H-DOX)—15 mg/kg. Free vinblastine (VLB) and hydrogel-entrapped VLB—1.7 mg/kg i.p. or subcutaneously (s.c.), respectively. Control mice were implanted with hydrogel without the drug, 6–10 mice (controls) per group were used.

Fig. 2 shows that only H-DOX, which continuously released the drug over a period of 48 h, was able to control leukaemia onset until day 40. In contrast, there was almost no difference between the time of weight gain in the group of mice treated either with free DOX or hydrogel containing VLB (H-VLB) in comparison with the control mice.

These results led us to test whether the implantation of a second drug-loaded hydrogel would protect the mice from leukaemia relapse and keep the progression of the disease under the control. In order to determine the side-effects of chemotherapy on the immune system, we tested Balb/c mice for restoration of bone marrow (BM) activity after treatment with the MTD dose of free DOX. We have used a reticulocyte assay, which reflects the state of the bone marrow *in vivo*. When the proliferation of bone marrow progenitors is affected by chemotherapy, the number of reticulocytes is reduced, while their increase reflects the period of increased BM activity, which compensates for the bone marrow damage by the anticancer agents. It was shown that the haemopoietic activity of the bone marrow was restored approximately 2 weeks after chemotherapy (data not shown). Implantation of H-DOX caused weight loss during 1 week and it took another week for the weight to return to baseline levels. Based on these findings, the second hydrogel was implanted 2 weeks after the first one, on day 25, thus avoiding damage to the host immune system. We observed almost no difference when a second hydrogel containing VLB was implanted (increase of mean survival from 37 to 40 days). In contrast, when the second H-DOX was implanted s.c., the mean survival increased from 60 (one H-DOX) up to 88 days (Fig. 3). In addition, the onset of the disease was delayed, although the overall survival was only slightly

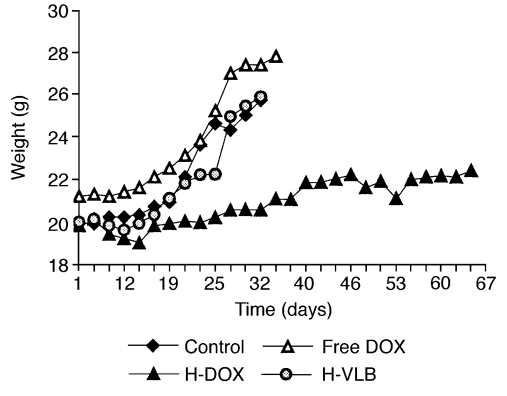

Fig. 2. Balb/c mice bearing BCL1 leukaemia were weighed every other day to monitor the therapeutic effect and leukaemia onset (weight gain by more than 10%). While free doxorubicin (DOX) had almost no effect on leukaemia onset, hydrogel-entrapped DOX was able to control it until day 40. Free vinblastine (VLB) was too toxic (data not shown) and hydrogel-entrapped VLB was ineffective.

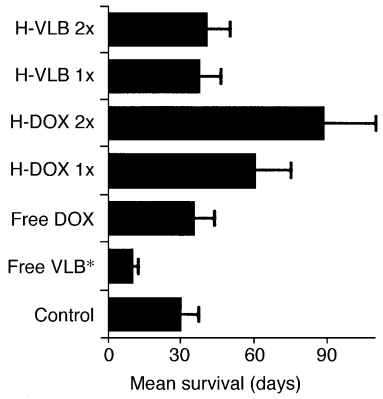

Fig. 3. Mice bearing BCL1 leukaemia treated either intraperitoneally (i.p.) with free drugs or drugs incorporated in *N*-(2-hydroxypropyl)methacrylamide (HPMA)-hydrogels. Free vinblastine (VLB) (*) was too toxic and mice died within 15 days. The doses were the same as in Fig. 1.

increased (two mice from the 12 were long-term survivors). No mice survived after the implantation of one H-DOX.

3.2. Degradation of HPMA-hydrogels and comparison of the drug release in vitro and in vivo

In vitro experiments showed that DOX is released from HPMA-hydrogel in a biphasic mode (Fig. 4). Approximately 40% of the drug is released within the first 10 h and it probably corresponds to free diffusion of the drug from hydrogel. Then a plateau-phase of approximately 30 h was observed during which another 10–20% of the drug was released. The remaining part of DOX (40–50%) was released during the last 10 h, the phase being characterised by complete degradation of the polymer network. DOX concentration measured *in vivo* after hydrogel implantation actually copies the *in vitro* release. However, we were able to detect DOX at pharmacologically active concentrations (100–800 ng/ml) even 96 h after the implantation.

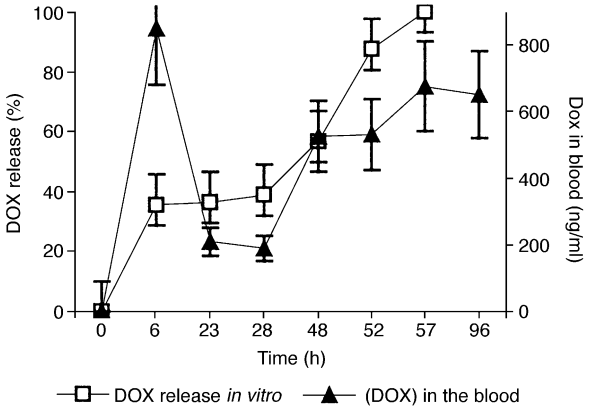

Fig. 4. Kinetics of doxorubicin (DOX) release from the *N*-(2-hydroxypropyl)methacrylamide (HPMA) hydrogel (degradation 48 h) *in vitro* and *in vivo*. Free DOX (100 μg intraperitoneally (i.p.)) was not detected in the blood 3 h after injection (data not shown), while 600 ng/ml of DOX were detected 96 h after implantation.

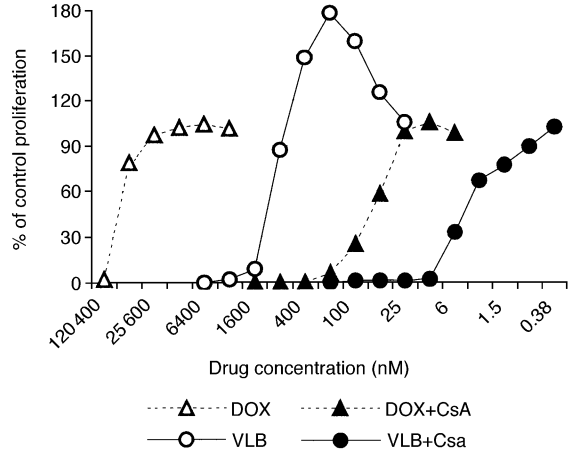

Fig. 5. P388-MDR cells were plated into tissue culture plates with appropriate drug concentrations and after 72 h the proliferation was measured using ^{3}[H]-thymidine uptake. Each sample was determined in triplicate. The control samples were calculated to be 100%.

3.3. Efficacy of hydrogels containing cytostatic drug and chemosensitiser to inhibit MDR

In comparison with its sensitive subline P388, mouse MDR leukaemia cell line P388-MDR is several hundred times less sensitive to various anticancer agents, including DOX, VLB and etoposide due to the overexpression of Pgp. *In vitro* studies showed that Pgp function could be completely blocked by CsA (Fig. 5), as well as by its derivatives.

Therefore, HPMA hydrogels containing various combinations of cytostatic drug with chemosensitiser were synthesised and their potential to inhibit Pgp-mediated MDR was confirmed *in vitro* [22].

Therapy was initiated when the tumour was palpable (3–4 mm in diameter, approximately day 6). Hydrogels were implanted on the other side of the back, while free drugs were applied either i.p. (DOX) or orally (p.o.) (CsA). The results of the first hydrogel generation are shown in Fig. 6. Although the tumour volume was reduced by half with free CsA

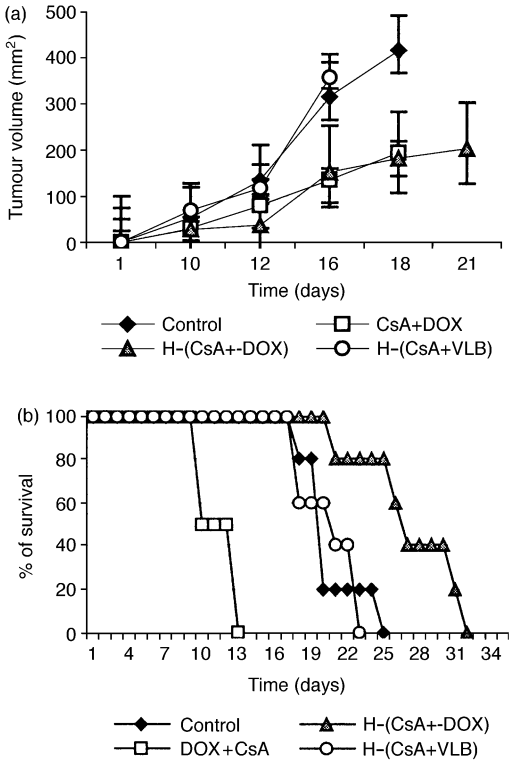

Fig. 6. P388-MDR cells (5×10^5) injected subcutaneously (s.c.), the treatment started on day 6, when the tumour was palpable (6–8 mice per group). Free CsA (3×15 mg/kg orally (p.o.) on days 6, 7, 8) + doxorubicin (DOX) (3×5 mg/kg intraperitoneally (i.p.), 2 h after CsA). Hydrogels containing CsA and DOX: total CsA and DOX doses were 45 and 15 mg/kg, respectively.

Table 1
Release of various drugs from the HPMA-hydrogels *in vitro* PBS, pH 7.4

Hydrogel containing	Drug release (%)				
	1 h	8 h	16 h	30 h	42 h
DOX	55[a]	70	74	80	95
DOX/CsA	55/10	74/20	80/27	87/36	100/100
VLB	76	90	94	100	
VLB/CsA	53/7	95/17	100/25	100/36	100/100
CsA	1	8	20	32	60

HPMA, *N*-(2-hydroxypropyl)methacrylamide; PBS, phosphate-buffered solution; DOX, doxorubicin; CsA, cyclosporine A; VLB, vinblastine.

[a] Numbers represent the percentage of the drug released from HPMA hydrogel (relative to total drug amount; average value from three independent measurements).

(15 mg/kg) + DOX (5 mg/kg) (Fig. 6a), this combination using the MTD dose of CsA resulted in the deaths of mice due to toxicity. Hydrogels containing CsA and DOX also reduced the tumour growth and overall survival was prolonged in comparison with the control animals (Fig. 6b). Hydrogel containing the combination of VLB + CsA had no effect on tumour volume and survival, although the previous *in vitro* studies documented that the cytostatic effect of VLB against resistant P388-MDR is better than that of DOX (Fig. 5). Data concerning the release profile of the various drugs from the HPMA hydrogels are shown in Table 1.

4. Discussion

Despite the fact that the concept of drug delivery systems (DDS) is not new, great progress has recently been made in the treatment of a variety of diseases. Research areas cover novel properties that have been developed, increased efficacy of the drug delivery, improved release profiles and also drug targeting (reviewed in Ref. [16]). Several strategies to improve drug bioavailability were developed including nanoparticles [17], microspheres [18], as well as hydrogel-based DDS [19]. To convey a sufficient dose of drug to the diseased tissue, suitable carriers of drugs are needed. In this study, the HPMA copolymer was used for the synthesis of biodegradable hydrogels for prolonged, time-dependent drug release [20,21].

The *in vivo* data from the treatment of BCL1 leukaemia suggest that the use of hydrogels is advantageous in comparison with the application of free drugs. Histological study showed that the number of liver and lung metastases was significantly reduced after the implantation of the DOX-loaded hydrogel. The reason for the increased efficacy of the hydrogel-entrapped DOX was apparent from the comparison of drug bioavailability after the injection of free drug (i.v. or s.c.) and after the s.c. implantation of the hydrogel containing the drug. Pharmacokinetic study revealed sustained DOX release from the hydrogel over a prolonged period of time *in vivo*—DOX concentration in the blood 96 h after hydrogel implantation was approximately 600 ng/ml, a level well above the IC_{50} of DOX for BCL1 leukaemia *in vivo* (1–2 ng/ml). Moreover, no signs of systemic toxicity or local necrosis were present after the implantation of hydrogel containing 400 μg of DOX (i.e. 20 mg of DOX/kg—this dose is approximately 4 times higher than the MTD of the free drug in mice).

These encouraging results led us to test combined drug delivery using HPMA hydrogels against multidrug resistant mouse leukaemia cells P388-MDR. Hydrogels were loaded with cytostatic drug (DOX or VLB) and with a chemosensitising agent (CsA) to block Pgp function. While the application of free CsA and DOX was toxic (mean survival 12 days), the same drug dose in hydrogel form was non-toxic and resulted in a slightly prolonged survival of mice bearing s.c. growing P388-MDR from 21 to 28 days, but no long-term survivors were obtained. There may be several explanations for these results. First of all, previous *in vitro* studies have shown that CsA has to be present in the cell culture for at least 8–12 h together with DOX to inhibit the proliferation of P388-MDR [22]. In addition, marked differences concerning the drug release from the HPMA hydrogels were observed for hydrophilic (DOX, VLB) and hydrophobic drugs (CsA). While the majority of DOX is released from this type of hydrogel within the first 10 h by diffusion, release of hydrophobic CsA actually copies the hydrogel degradation, with most of the drug being released during the last phase of hydrogel degradation (Table 1). Therefore the synergistic effect of chemosensitiser and cytostatic drug may be affected by these differences. Finally, while BCL1 leukaemia is a relatively sensitive disseminated cancer, P388-MDR is a highly resistant model growing s.c. as a solid tumour. Although *in vitro* studies indicate that proliferation of P388-MDR is inhibited by a combination of CsA (1 μM) with DOX (1 μM) and we were able to detect these concentrations in the bloodstream under physiological conditions, these levels may not be achievable at the tumour site due to the unique physiology of solid tumours, which makes them more resistant to anticancer therapy [23]. It is interesting to note here that although VLB was shown to be more effective than DOX in the inhibition of BCL1 and P388-MDR proliferation *in vitro*, its efficacy *in vivo* was very weak. It confirms the clinical data, that *in vitro* sensitivity of cancer cells from human biopsies exposed to various cytostatics does not necessarily reflects the *in vivo* situation [24]. Moreover, it was recently published that in addition to drug efflux activity, Pgp may possess also an anti-apoptotic function that protects Pgp-expressing cancer cells from the caspase-dependent pathway leading to apoptosis [25].

There is an effort to develop the second generation of hydrogels, which would allow the release of the cytostatic

agent and chemosensitiser at the same time to achieve their synergistic effect. This might be achieved by the combination of a more easily degradable outer part of hydrogel containing the hydrophobic drug (CsA or its more potent derivatives), and an inner part with a longer degradation time, where DOX release would depend on diffusion and hydrogel degradation.

Acknowledgements

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