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Review Article

Antibody-targeted HPMA copolymer-bound anthracycline antibiotics

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CONTENTS

Abstract

Conjugates based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) represent a new generation of polymeric anticancer drugs with improved therapeutic potential and considerably decreased nonspecific side effects. Antibody-targeted derivatives show specific active targeting and nonspecific passive accumulation in solid tumors due to the enhanced permeability and retention (EPR) effect, decreased immunogenicity of conjugated proteins, long-term circulation in the bloodstream, partial activity towards multidrug-resistant (MDR) cells, and immunoprotective and immunomobilizing activities *in vivo*. Conjugates of HPMA and doxorubicin containing intravenous human immunoglobulin (IVIg) were used as a palliative treatment in patients with advanced cancer.

Introduction

Classical cancer chemotherapy is limited by the lack of adequate selectivity for neoplastic over normal tissue. The majority of currently available cytostatic drugs exhibit limited efficacy, modest selectivity and considerable toxicity against normal intensively proliferating cells, which results in unwanted side effects. Among the most dangerous complications and limiting factors of antitumor therapy that prevent the use of the most effective cytotoxic substances in very high doses is a significant increase in the patient's sensitivity to bacterial and viral infections, *i.e.*, immunosuppression.

The dilemma, *i.e.*, the search for more effective cytotoxic drugs with weak general cytotoxic effects, could be solved by finding a targeting system which would ensure that the drug will reach and act only on the pathological process itself. The selectivity of treatment may be accomplished by the preferential action of a particular drug or by preferential localization, *i.e.*, targeting of a drug that acts nonspecifically. Such a new approach is called affinity therapy. The aim of affinity therapy is to accumulate the effective drug in the target tissue and eliminate, or at least minimize, its effect on healthy tissues and organs (1, 2).

Affinity therapy is being studied mainly for possible use in the therapy of cancer, but it may also be applied in other clinical situations. For instance, immunosuppressive drugs used for the treatment of severe autoimmune diseases, currently the most challenging scientific and clinical area in the field of immunology, or in transplantation, also exert nonspecific toxicity and can cause damage to healthy tissues (3, 4).

The first targeted drugs were immunotoxins where the toxin moiety, such as the α-chain of ricin, abrin or diphtheria toxin, was bound directly to targeting monoclonal antibodies (5-9) to promote the delivery of the cytotoxic agent specifically to a particular tumor. Several practical difficulties must be overcome for this technique to be successful. First, covalent coupling of the biologically active molecule to an antitumor antibody should be accomplished without loss of its toxicity or antibody specificity. Second, a sufficient amount of antibody must reach an appropriate tumor target before it is cleared from the blood by Fc receptor-bearing phagocytic cells. Third, an

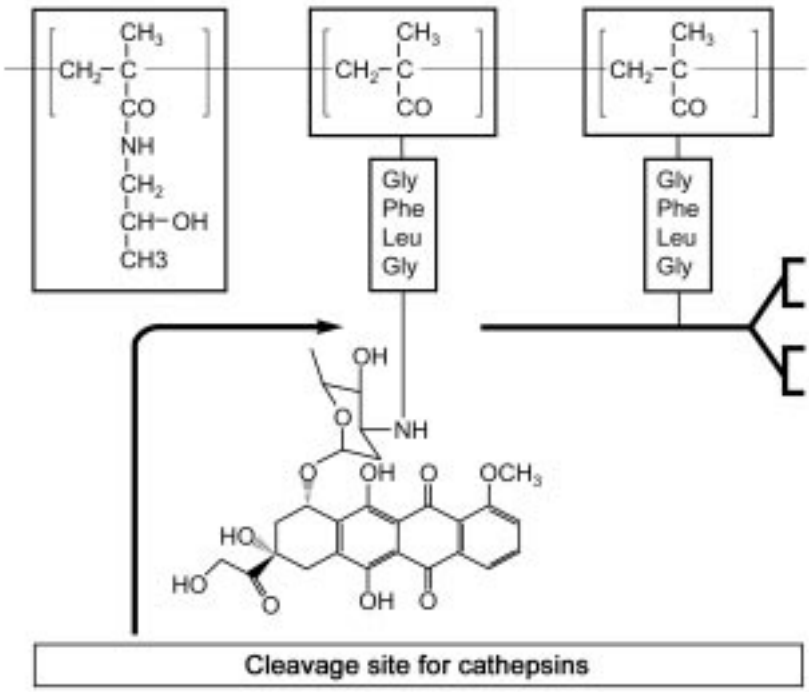

Fig. 1. Chemical structure of antibody-targeted HPMA copolymer–doxorubicin conjugate containing tetrapeptide (Gly-Phe-Leu-Gly) side-chains.

immune response against the toxin and the targeting antibody must be prevented. The second-generation immunotoxins, which have greatly improved stability, have been demonstrated to be effective not only *in vitro* but also *in vivo* in animal models and in patients. However, hepatotoxicity, vascular leak syndrome and the appearance of HAMA (human anti-mouse antibodies) directed against targeting monoclonal antibodies are common problems with immunotoxin therapy. Because of these practical difficulties, clinical trials of immunotoxins have had variable and modest success (10, 11).

The direct binding of a drug to monoclonal antibodies has a number of disadvantages. Foremost, the drug is not released from such a complex in a controlled manner. Controlled release means that the drug is inactive during transport in body fluids and is activated only at the site of the pathological process. To find a generally applicable delivery system which could be used in most cases where affinity therapy is indicated, a carrier has to be used (either natural or synthetic) as a bridge between the drug and the targeting moiety, which can be changed according to the nature of the pathological process. Synthetic polymers are advantageously used as drug carriers, since in contrast to natural macromolecules, they can be modified in a defined way.

In recent years, water-soluble synthetic polymers based on an *N*-(2-hydroxypropyl)methacrylamide copolymer (HPMA) backbone have been recognized as useful carriers in targeted drug delivery (12-15). Both the anticancer agent and the targeting moiety, *e.g.,* antibodies (16-39), carbohydrates (19, 23, 40-48), lectins (23, 49, 50), hormones (51) and oligopeptides (52), are conjugated by a well-defined chemical bond to the variable oligopeptide side-chains of an HPMA carrier, mostly Gly-Phe-Leu-Gly (53) (Fig. 1). The composition of the oligopeptide side-chains ensures the stability and inactivity of the prodrug during transport in the bloodstream, and its activation only after cell internalization by the action of thiol-dependent lysosomal proteases, which release the drug from its polymeric carrier (54, 55). In this way, enhanced antitumor activity is combined with decreased peripheral toxicity, leading to a substantial increase in the therapeutic index (47).

Experimental and clinical data obtained so far demonstrate the following advantages of HPMA copolymer-based macromolecular therapeutics: 1) targetability; 2) an enhanced accumulation in solid tumors due to the enhanced permeability and retention (EPR) effect; 3) decreased immunogenicity of conjugated proteins; 4) increased efficacy compared to free drug; 5) cytostatic activity *in vitro* and *in vivo*; 6) long-term circulation in the bloodstream; 7) reduced myelotoxicity, hepatotoxicity, cardiotoxicity, nephrotoxicity and toxicity to thymus; 8) increased maximum tolerated dose; 9) activity towards multidrug-resistant (MDR) cells; and 10) immunoprotective and immunomobilizing activities *in vivo*. These advantages are thought to be a consequence of changes in specific cell binding and uptake, intracellular processing, pharmacokinetics, biodistribution and bioavailability.

Immunogenicity of HPMA copolymers

Prior to any application of polymeric prodrugs in medicine, their immunogenicity must be determined with the aim of finding conjugates inducing the least possible defense reaction in the host organism. Immunogenicity is already known to be a problem associated with the application of murine antibodies in the clinic, for diagnostic purposes, radiotherapy or in the form of antibody conjugates for chemotherapy. The human immune system recognizes the rodent antibody as foreign and mounts an HAMA response, resulting in patient antibodies against the rodent monoclonal. An HAMA response and response against the toxin moiety develop in about 50% of patients after a single dose and in about 90% after a second treatment. Subsequent doses of antibody are then removed from the circulation before they can reach the disease target (56). Individuals with a functional immune system make anti-immunoglobulin antibodies even when humanized antibodies are used. Circulating anti-conjugate antibodies can inhibit the efficacy of antibody-targeted drugs by increasing their rate of clearance and/or by blocking the binding site on the targeting antibody.

There is no measurable antibody response to an HPMA homopolymer, *i.e.*, a polymer without the oligopeptide side-chains attached. The modification of the homopolymer with oligopeptidic side-chains results in a very weak, thymus-independent, IgM-restricted antibody response which is influenced by many factors, including the copolymer molar mass, dose and composition of oligopeptidic side-chains, and by the genetic background of the conjugate recipient (57-59). Only a very high concentration (20 g/l) of the homopolymer and copolymers differing in oligopeptidic side-chains activates complement pathways *in vitro*. Lower concentrations (2 or 0.2 g/l) do not activate either the classical or the alternative complement pathway (60). High-dose and chronic treatment of mice with an HPMA copolymer (total injected dose 2 g/kg during 2 months) did not disturb the phagocytic activity of peripheral blood leukocytes, did not damage the bone marrow stem cells, did not activate alternative or classical complement pathways, and did not influence the antibody-forming capacity of the injected mice to thymus-dependent and thymus-independent antigens (61).

HPMA copolymers not only fail to induce a significant immune response against themseleves, but they also have the capacity to dramatically reduce the antibody response against proteins, including immunoglobulins, bound to them as a targeting moiety (16, 26, 31, 62, 63; see also last section), permitting the use of multiple-dose protocols. The mechanism of reduction of protein immunogenicity after binding to HPMA copolymers is not known. The nonimmunogenic HPMA copolymer might simply mask the antigenic determinants of the targeting protein by sterically hindering their accessibility, a mechanism ascribed to polyethylene glycol (PEG) reduction of protein immunogenicity following conjugation (64). Alternatively, the up to 50% decrease observed in proteolytic degradation of antibody bound to HPMA copolymer compared to parent antibody (47) may interfere with normal pathways of intracellular protein degradation necessary for antigen processing and presentation, resulting in decreased immune response. Antigen processing is the conversion of native proteins into major histocompatibility class (MHC)-associated peptides. This process consists of the introduction of protein antigens into antigen-presenting cells (APCs), proteolytic degradation of these proteins into peptides, binding of the peptides to MHC molecules, and the display of the peptide–MHC complexes on the APC surface for recognition by T-cells. Antigen-processing pathways in APCs utilize basic cellular proteolytic mechanisms.

Passive accumulation and active targeting

Macromolecular prodrugs can be accumulated in solid tumors either by active targeting involving cell-surface receptors on target tumor cells, or by passive accumulation due to the EPR effect first described by Maeda *et al.* (65-67). It is supposed that the EPR effect is a consequence of an increased vasopermeability of solid tumors. The vasculature of tumor endothelium is often discontinuous and allows extravasation and accumulation of macromolecules in solid tumor tissue, which usually does not have an effective lymphatic drainage. Consequently, the clearance of tumor-accumulated macromolecules larger than 50 kDa is strictly limited (68).

HPMA copolymers are, under physiological conditions, uncharged and easily deformable since they lack any rigid tertiary structure. Both of these properties are known to enhance extravasation, as uncharged and flexible molecules easily pass through an endothelial layer (69). As a result of the EPR effect, even nontargeted polymeric conjugates based on HPMA show elevated therapeutic potential *in vivo* in a variety of experimental tumors (66, 70-72), and also in certain human tumors (73).

Essential in active drug delivery is the selection of appropriate cell-surface receptors for targeting. An ideal targeted drug conjugate should be both cytotoxic and selective in its action. Until now, a variety of ligands, such as asialoglycoproteins, transferrin, hormones, different growth factors, lectins, folic acid and carbohydrates, have been used as targeting moieties. Antibodies, both monoclonal and even polyclonal, are very efficient as they are able to distinguish specific antigenic determinants (epitopes) of the target tissue and react exclusively with them. Monoclonal antibodies are potentially the most selective and widely applicable of tumoritropic molecules. However, as free molecules, they have restricted extravasation, are immunogenic, susceptible to degradation by proteolytic enzymes and have a rather limited drug-carrying capacity. In theory, HPMA copolymer–antibody conjugates have advantages over the use of free antibodies in each of these four areas (14).

The effectiveness and selectivity of the antibody-targeted conjugates depend on the affinity and character of

the antibodies (polyclonal or monoclonal), their class (IgG or IgM) or subclass (IgG_1, IgG_2 or IgG_3), specificity and affinity. Not only targeting antibodies, but also the number and particular target epitopes of cell-surface receptors, as well as the rate of complex internalization, determine the final pharmacological activity of a conjugate (74). A nonspecific interaction of antibody-targeted conjugates includes cross-reactivity of the antibodies towards similar antigens on nontarget cells, absorption of conjugates due to interactions between hydrophobic cytotoxic agents and the cellular plasma membrane, and nonspecific endocytosis by fluid-phase uptake of the conjugate, which is proportional to its extracellular concentration. The intensity of such nonspecific binding depends again on the affinity and specificity of the antibodies used, on the composition of the cell surface, on the density and uniqueness of the cell-surface target molecule, on anatomical barriers, local concentration of the targeted drug and on the rate of nonspecific endocytosis. It was observed that following saturation of specific binding sites, a nonapeptide-targeted HPMA copolymer nonspecifically adsorbed to the cell surface. The amount of nonspecific binding was proportional to the concentration of the conjugate, but considerably lower than the specific binding (52). Thus, affinity therapy with antibody-targeted polymeric conjugates should ideally be based on a high selectivity and affinity of the antibodies for the target antigen and on a low nonspecific binding to normal cells and organs.

Another problem to be solved in antibody-targeted drug delivery is the outgrowth of "antigen loss variants" of the tumor cells that are common in rapidly growing tumors. Given the high mitotic rate of tumor cells and their genetic instability, mutations or deletions in genes encoding tumor antigens are common. If these antigens are not required for the growth of the tumors, the antigen-negative tumor cells have a growth advantage in the host. It was shown that the loss of antigens correlates with increased cancer growth and metastatic potential. Nonuniform expression of antigens on the surface of cancer cells is a phenomenon rather common to cancer cell populations and does not necessarily mean that tumor-targeted therapy will not work. One way to avoid this problem may be to use a mixture of targeting moieties recognizing different antigens/receptors expressed on the same tumor.

The specificity of the pharmacological effect of free and antibody-targeted HPMA copolymer-bound drug was tested *in vitro* and *in vivo*. Primary cultures of mouse splenocytes and human peripheral blood lymphocytes were stimulated *in vitro* with concanavalin A (Con A) or SpA (*Staphylococcus aureus* Cowan I protein). Con A is a T-cell mitogen with specificity for the T-cell receptor and which induces proliferation of T-lymphocytes only. These cells are the target for an anti-Thy 1.2-targeted HPMA copolymer containing daunorubicin (mouse model) or an anti-CD3-targeted HPMA copolymer containing doxorubicin (human model). SpA stimulates B-lymphocytes through their Ig receptor (BCR). B-cells are not a target in affinity therapy directed to surface receptors of T-lymphocytes (Thy 1.2 antigen or CD3 antigen). The results showed that the IC_{50} for lymphocytes stimulated with Con A (T-lymphocytes) and treated with anti-CD3- or anti-Thy 1.2-targeted HPMA-bound daunorubicin/doxorubicin was 2.26-5.3 μM, while the IC_{50} for lymphocytes stimulated with SpA (B-lymphocytes) was 520-890 μM. This demonstrated the high specificity of targeting, as proliferation of nontarget B-lymphocytes was inhibited only if they were cultivated in the presence of a very high concentration of an antibody-targeted polymer-bound cytotoxic drug. This nonspecific effect of anti-CD3- or anti-Thy 1.2-targeted drug on B-cells could be explained either by nonspecific fluid-phase pinocytosis of the antibody-targeted conjugate or by its binding to the Fc receptor on B-lymphocytes. If antibodies are bound to HPMA by aminolysis, the Fc part of the antibodies is not specifically blocked by binding to a polymeric carrier and B-cells may internalize conjugates by receptor-mediated endocytosis via their Fc receptor (75).

The specificity of targeting was tested also *in vivo* on the antibody response of immunodeprived athymic *nu/nu* BALB/c mice (a strain of mice that lack T-lymphocytes) and on the antibody response of normal BALB/c mice. Both strains were immunized with thymus-dependent (arsanilic acid-bovine gamma globulin, ARS-BGG) or thymus-independent (trinitrophenyl-*Escherichia coli,* TNP-*E. coli)* antigen. Theoretically, treatment with a cytotoxic drug targeted to the T-cell compartment should eliminate the T-cell help for B-cell activation and this should result in an impairment of the thymus-dependent response, while not affecting the thymus-independent response. The results have shown that a polymeric prodrug composed of anti-Thy 1.2-targeted HPMA copolymer-bound daunorubicin affects only the thymus-dependent antibody reaction *in vivo*. This proves the *in vivo* specificity of antibody-targeted drug application (75).

Another model used for the evaluation of specific active targeting was murine B-cell leukemia BCL_1 cells treated with HPMA copolymer-bound doxorubicin targeted with strictly leukemia cell-specific B1 monoclonal antibodies. FACS (fluorescence-activated cell sorter) analysis showed that both free B1 MAb and a B1 antibody-targeted HPMA conjugate bound with high affinity to BCL_1 cells, while no binding was observed to nonrelated mouse B-cell lymphoma 38C13 cells, used as negative control. Similarly, conjugate containing human nonspecific Ig instead of B1 monoclonals did not bind to BCL_1 cells (76). Nontargeted HPMA copolymer-bound doxorubicin exhibited an IC_{50} close to 1.5 μM doxorubicin equivalents. In contrast, HPMA copolymer-bound doxorubicin targeted with B1 MAb had an IC_{50} of about 35 nM. The conjugate containing human nonspecific IgG had an IC_{50} comparable to a nontargeted conjugate (76).

During experimentation with immunotoxins and HPMA copolymer carrier-bound drugs, a direct correlation was not consistently found between the ability of conjugates to bind to the target cell surface and their cytotoxicity (38, 77), nor did the rate of endocytosis always predict the efficacy of the immunoconjugates (78, 79). Thus,

it seems that the most important event occurs at later stages in delivery, *e.g.,* during intracellular routing of the drug–antibody complexes. It was reported that the intracellular fate of antibody-targeted conjugates is considerably influenced by an epitope on the target surface molecule to which the antibody molecule is directed (80), and by targeting antibodies themselves. Different monoclonal antibodies targeting the same surface antigen vary greatly not only in their rate of endocytosis but also in their subsequent intracellular routing (80-82). Those monoclonals, which are internalized but rapidly degraded inside the cell, perhaps in the lysosomal compartment, are usually not efficient enough in targeting. The performance of the various monoclonals appears to correlate very closely with their ability to transport the drug from the cell surface to the interior of a metabolically active target cell and accumulate the drug within the target cells (83).

One of the many approaches to increasing the efficacy of targeting is to increase the amount of selected surface receptors on target cells. To prove this theoretical assumption and compare the targeting efficacy of different monoclonal antibodies, we used a panel of anti-T-lymphocyte antibodies bound to an HPMA copolymer conjugated with doxorubicin. As model systems, we used spontaneously proliferating and Con A-stimulated mouse T-cells. Concanavalin A as a T-cell mitogen has the ability to increase the number of T-cell surface-associated activation markers that represent appropriate targets for antibody-driven drug delivery in hematological malignancies and in all situations where T-cells participate in antagonistic reactions, such as autoimmune diseases and rejection episodes after allogeneic transplantation. The tested antibodies were monoclonals directed against T-cell antigens (Thy 1.2 antigen, CD4 receptor, CD25 [IL-2R receptor] and TCR/CD3 complex) and against surface antigen expressed on APCs (class II MHC glycoprotein). (For results, see also the section: Affinity therapy with antibody-targeted HPMA copolymer-bound daunorubicin (doxorubicin) applied to the immune system.)

Flow cytometric analysis showed an intensive T-lymphocyte binding for HPMA copolymer carrier-conjugated doxorubicin targeted with specific antibody, whereas no binding was recorded with HPMA carrier-bound doxorubicin containing nonspecific mouse IgG. The binding was considerably increased in Con A-stimulated T-lymphocytes when compared with unstimulated cells, *i.e.,* spontaneously proliferating controls (84).

The accumulation in isolated mouse T-lymphocytes was most intensive with [^{125}I]-labeled anti-Thy 1.2-targeted and anti-CD4-targeted monoclonals, whereas no accumulation was seen after incubation of mouse T-cells with a nonspecific doxorubicin–HPMA–mouse IgG conjugate (84). Interestingly, the intracellular accumulation of the drug was influenced by the structure of the oligopeptide side-chains via which doxorubicin was attached to the polymeric carrier. A higher membrane association and intracellular accumulation into T-lymphocytes were seen with conjugates containing Gly-Phe-Leu-Gly sequences compared with conjugates containing Gly-Gly sequences. The effect of oligopeptidic side-chains was demonstrable only in T-cells exposed to a nontargeted HPMA copolymer-bound drug. The difference is probably due to the more strongly hydrophobic character of the Gly-Phe-Leu-Gly side-chains, which contributes to the increase in cell-surface binding and internalization of the nontargeted HPMA copolymer-bound drug in T-cells (85). Similarly, Duncan (86) found a higher uptake into the yolk sac tissue of HPMA copolymers containing higher amounts of hydrophobic comonomers.

[^{3}H]-Thymidine incorporation was measured as a marker of cytotoxicity *in vitro.* Generally, the binding of daunorubicin to the copolymer carrier always reduced its pharmacological activity. Free daunorubicin induced a 50% inhibition of [^{3}H]-thymidine incorporation by mouse T-lymphocytes at the lowest concentration of 0.1 mg/l. Five times more daunorubicin bound to a biodegradable HPMA copolymer with targeting anti-Thy 1.2 antibodies and 500 times more daunorubicin bound to a nontargeted biodegradable HPMA copolymer was necessary to achieve the same result (87). Inhibition of Con A-stimulated proliferation of T-lymphocytes was always greater compared to cultures of spontaneously proliferating T-cells and directly proportional to the intracellular accumulation of the conjugate. Anti-Thy 1.2-targeted HPMA copolymer-bound doxorubicin reduced the proliferation of unstimulated T-cells with an IC_{50} of 10 μg/ml, while proliferation of Con A-activated T-cells was reduced with an IC_{50} of 0.1 μg/ml, *i.e.,* at a 100-fold lower concentration. Similarly, anti-CD4-targeted HPMA copolymer-bound doxorubicin was effective in unstimulated T-cell cultures with an IC_{50} of 10 μg/ml and in Con A-stimulated T-cells with an IC_{50} of 1 μg/ml. The values for anti-MHC class II-targeted and anti-CD25-targeted HPMA copolymer-bound doxorubicin were 100 and 10 μg/ml, respectively. The nontargeted HPMA copolymer-bound doxorubicin was effective only at a concentration > 100 μg/ml and no substantial difference between unstimulated and stimulated T-cells was seen. The effect of unconjugated doxorubicin on mitogen-stimulated cell cultures was also considerably greater than in unstimulated cultures, confirming the higher sensitivity of rapidly proliferating cells to the toxic action of the cytostatic drug (84).

These results showed that the targets of the inhibitory effect of antibody-targeted HPMA-bound doxorubicin are predominantly rapidly proliferating cells, which are generally more susceptible to cytotoxic agents. Inhibitory efficacy is also positively influenced by a surface target antigen density, where a higher expression permitting a higher concentration of effector molecules on target cells (88, 89) results in stronger inhibition. Antibody-targeted chemotherapy might also be significantly influenced by the antigenic modulation, which is defined as the redistribution of surface antigen after binding of antibody and may involve internalization and degradation of surface antigen or shedding of the antibody–antigen complex from the cell surface. The factors regulating antigenic modulation are not clear. Some antigens modulate rapidly, some slowly or not at all, and still others modulate

when exposed to multiple antibodies directed against unrelated antigens.

An interesting observation is the finding that, in the case of only a 10-min contact *in vitro* between the polymeric prodrug based on HPMA with Gly-Phe-Leu-Gly side-chains and target T-cells, even a very high concentration (70 μg/ml) was without any detectable inhibitory effect. We attribute this to the shape of the conjugate molecule. It has been found (69) that HPMA copolymers containing hydrophobic side-chains associate in water, forming submicellar structures containing up to 5 macromolecules where the hydrophobic side-chains are enclosed in an outer hydrophilic coat of the polymer. The association increases with increasing temperature and results in a decrease in the rate of enzymatically catalyzed drug release (69). We hypothesize that the formation of associates results in a reduced interaction of the polymer with the cell surface, thus decreasing its uptake. To detect some effect, prolonged incubation or a lower concentration is needed (87).

Effect of different composition of oligopeptidic spacer on pharmacological efficacy

If the cytotoxic drug bound to the polymer acts upon the cell surface, the degradation of the bond between the spacer and the drug is not the main problem. On the other hand, this assumes greater importance if the drug to be bound acts only intracellularly. Such a drug must be accumulated in the target cell by endocytosis and must be released there from the polymeric carrier to become active (12).

The release of the drug depends on the character of the oligopeptidic side-chains of the HPMA copolymer carrier. A drug bound to a nondegradable sequence such as Gly-Gly is not released from the polymer either during its transport in the bloodstream or in the target tissue. On the other hand, if the bond between the drug and an oligopeptidic sequence such as Gly-Phe-Leu-Gly is degradable by lysosomal enzymes, the conjugate is stable in plasma and serum (54), and pharmacologically active drug is released only intracellularly in target cells (55, 90).

The sensitivity to cleavage by lysosomal enzymes is correlated with the pharmacological activity of the preparation. Daunorubicin bound to a noncleavable Gly-Gly sequence is practically inactive both *in vitro* and *in vivo*, while the same drug bound to the cleavable sequence Gly-Phe-Leu-Gly is highly cytotoxic *in vitro* (16, 17, 43), and effectively suppresses the antibody response *in vivo* (16, 18), as well as the growth of experimental tumors (44).

The toxicity of the conjugates with degradable sequences considerably exceeds that of the conjugate with nondegradable sequences. The HPMA copolymer containing doxorubicin bound via Gly-Phe-Leu-Gly oligopeptide side-chains and targeted with anti-Thy 1.2 antibodies possesses *in vitro* cytotoxicity even at a concentration of 1.8 μg/ml (IC_{50} cytotoxic index) daunorubicin equivalents, while more than 100 times more daunorubicin is needed for the same toxicity for the polymer with a nondegradable Gly-Gly sequence (IC_{50} cytotoxic index = 250 μg/ml) (16).

Similar results were also obtained *in vivo*. HPMA copolymer conjugates with Gly-Phe-Leu-Gly side-chains decreased the antibody response by 60-80%, while no effect was seen using an HPMA copolymer conjugate where daunorubicin was bound via Gly-Gly side-chains. This is discussed further below. Daunorubicin bound to the copolymer via a degradable peptide spacer and used for the treatment of DBA2 mice bearing L1210 leukemia caused a marked delay in the onset of tumor appearance and greatly increased the mean survival time (from 22 days to 31 days) compared with daunorubicin bound to the copolymer via a nondegradable spacer (mean survival time of 22 days, the same as recorded for the controls) (91).

There is no doubt about the influence of the composition of the HPMA copolymer side-chains on the final pharmacological activity of polymeric compound. The results support the assumption that the drug has to be bound to a sequence from which it could be released in a controlled way at the site of the assumed effect. A random or insufficiently defined bond between the drug and its carrier may easily lead to a loss of activity of the whole complex. However, the rate of drug release is not the only factor responsible for conjugate efficacy, as we did not confirm a direct correlation between the rate of *in vitro* drug release and the *in vitro* cytotoxicity against human peripheral blood T-lymphocytes and mouse T-cell lymphoma EL4 cells.

We have selected HPMA conjugates with various tripeptide and tetrapeptide spacers, and the rate of release of doxorubicin *in vitro* by cathepsin B was as follows: Gly-Leu-Phe-Gly > Gly-Phe-Leu-Gly > Gly-Leu-Gly > Gly-Phe-Gly > Gly-Gly (92). Even when the cytotoxic activity of the conjugates releasing doxorubicin at the highest rate was most pronounced (IC_{50} = 1 μg/ml), a direct correlation was not observed. The conjugate with Gly-Leu-Gly tripeptide had an IC_{50} of 10 μg/ml, the conjugate with Gly-Phe-Gly tripeptide an IC_{50} of 1-10 μg/ml, and the conjugate with the nondegradable dipeptide Gly-Gly an IC_{50} of 100 μg/ml, which was unsuitable for targeting.

Similar results were obtained with anti-Thy 1.2-targeted conjugates tested against mouse T-cell lymphoma EL4 (84). As, in addition to rapid drug release, conjugates containing Gly-Phe-Leu-Gly sequences show a higher membrane association compared to conjugates with Gly-Gly side-chains (85), the pharmacological efficacy of conjugates with "biodegradable" side-chains seems to confirm the hypothesis of Hovorka *et al.* (93) that the toxicity of HPMA-based conjugates is a combination of the toxic effect of released doxorubicin directed against the nucleus and inducing apoptosis, and the toxic effect of doxorubicin in polymer-bound form directed against cell membranes and causing necrosis.

We have also compared the efficacy of conjugates with a tetrapeptidic spacer containing only D-phenylalanine or L-phenylalanine. In the presence of cathepsin B and after 24 h of incubation, the Gly-Phe(L)-Leu-Gly spacer releases 35% of bound doxorubicin, the Gly-Phe(D)-Leu-Gly spacer releases 12% and the Gly-Phe(D,L)-Leu-Gly spacer releases 22% of bound doxorubicin. Surprisingly, the use of a spacer containing both isomers of phenylalanine gives the best cytostatic activity *in vitro* and antitumor efficacy *in vivo* (38). This confirms that the rate of drug release is not the only decisive factor for the final pharmacological effect of antibody-targeted HPMA copolymer-bound drug.

Recently, we have also introduced HPMA copolymer-bound doxorubicin conjugates with a pH-sensitive bond between the drug and the spacer. In this type of conjugate, the doxorubicin is attached to the spacer through a hydrazone bond. The hydrazone bond is relatively stable at physiological pH 7.4 but is quickly hydrolyzed at pH 5.0. Thus, unlike aminolytically bound doxorubicin, the drug can be released already in endosomes as the pH in this cell compartment is favorable (94). It was shown that HPMA copolymer conjugates with doxorubicin conjugated via a hydrazone bond exhibit very high cytostatic activity *in vitro* against a variety of tumor cell lines, including those with a limited lysosomal content (95), and antitumor effect *in vivo* (96).

Influence of the method of synthesis on the binding affinity of antibody-targeted HPMA conjugates

The efficacy and specificity of antibody targeting depends primarily on the availability of a functional antibody binding site. The attachment of antibodies to a polymeric carrier usually decreases their binding activity, although we have never seen a decrease so great as to prevent efficient targeting. Conformation, and thus the targeting activity, of a coupled antibody could be influenced by the synthetic method selected for the attachment of the antibody molecule to the synthetic carrier, and by the physicochemical characteristics of the carrier molecule itself. In fact, antibodies can be bound to an HPMA copolymer either randomly by aminolysis (random binding), *i.e.*, via the N^{ε}-amino groups of lysine residues (approximately 75 lysine residues may be involved) and reactive 4-nitrophenyl ester (ONp) groups at the end of oligopeptide side-chains of the HPMA copolymer–doxorubicin conjugates, or via oxidized carbohydrate moieties of the Fc part of the IgG molecule (oriented binding). Aminolysis could involve amino acid residues that are a part of, or close to, the antibody binding site, which might result in a decrease of the targeting capacity of the construct. Oriented binding through the Fc part of the immunoglobulin molecule considerably increases the targeting capacity, reduces the nonspecific interaction of the antibody-targeted conjugate with nontarget somatic cells, and consequently increases the pharmacological activity of the antibody-targeted polymeric prodrug (20, 34, 46).

Three methods of covalently binding the monoclonal antibody OV-TL16, recognizing the OA-3 antigen expressed on the surface of OVCAR-3 ovarian carcinoma cells, and its Fab' fragments to an HPMA carrier containing doxorubicin were used to compare the effect of the mode of binding on the targeting ability of the final product. The affinity constant estimated by radioassay on cell monolayers revealed antigen-binding heterogeneity of the conjugate when the antibodies were bound by aminolysis. Binding via hydrazone bonds formed by the reaction of aldehyde groups on the oxidized antibody with hydrazide groups on the HPMA copolymer conjugate resulted in conjugates with a more homogeneous distribution of affinity constants. However, both methods resulted in up to an 8-fold decrease in the affinity constant compared to the parent antibody, probably as a consequence of conformational changes in the antibody structure (97). A third method involves thioether bonds formed by the reaction of sulfydryl groups of Fab' fragments with maleimido groups on the side-chain termini of the HPMA copolymer–drug conjugate. In such conjugates, the affinity constant is also reduced, but the distribution was more homogeneous than in either conjugate prepared with the whole antibody (97).

Seymour *et al.* (47) examined the ability of the B72.3 monoclonal antibody, its fragments and the various HPMA copolymer conjugates to localize on human colorectal carcinoma LS 174T xenografted to nude mice. Aminolysis was selected for the attachment of parent antibody and Fab' and $F(ab')_2$ fragments to the HPMA carrier. An HPMA conjugate with nonspecific mouse IgG was used as a control. The intact unmodified antibody showed the greatest quantitative targeting, localizing up to 25% of recovered dose per g of tumor. The unmodified $F(ab')_2$ fragment also showed a good quantitative localization (over 10% of recovered dose per g of tumor), wheras the unmodified Fab' fragment and all of the copolymer conjugates (HPMA–B72.3, HPMA–$F(ab')_2$ and HPMA–Fab') showed levels of targeting that were no greater than those achieved by the nonspecific mouse IgG and its HPMA conjugate, respectively. This is almost certainly due to masking of the antigen recognition site of the antibody by polymer chains, either folded across it or even covalently linked to it (31). This masking of the antigen recognition site is not entirely unpredictable, since aminolysis involves binding the copolymer chains to any available primary amine residues of the protein, some of which are present in the antigen recognition site itself. Results *in vitro* using membrane preparations isolated from explanted LS 174T tumors revealed a complete lack of antigen binding by the B72.3 antibody-targeted HPMA conjugate, despite extensive binding by the parent antibody (47). When targeting was expressed as tumor-to-blood ratio, the Fab' and $F(ab')_2$ fragments of B72.3 antibody performed better than the intact monoclonal even though the absolute quantity of material delivered to the tumor was less (47). More precisely controlled chemistry of derivatization, and probably also the selected class, subclass, polyclonal or monoclonal character of

antibodies, could affect the targeting efficacy of antibody-targeted polymeric conjugates.

We have directly compared random and oriented binding in conjugates targeted with anti-Thy 1.2 antibodies and containing chlorin e6 (34, 46) or conjugates targeted with anti-thymocyte globulin and containing ciclosporin (20). In the conjugate prepared by aminolysis, antibodies were bound randomly via N^{ε}-amino groups of lysine residues. In the conjugate with an oriented binding, a saccharide moiety in the Fc portion of the IgG molecule was oxidized and bound to HPMA copolymer side-chains terminating in aliphatic amino groups. Random and oriented binding confers a different targeting and also effectiveness of polymeric conjugates when tested in primary cultures of mouse splenocytes and isolated T-lymphocytes. The targeting capacity of the HPMA copolymer conjugate where antibodies were bound through oxidized saccharide (oriented binding in which the antibody binding site is protected) was enhanced by up to 2 orders of magnitude compared to the conjugate where antibodies were bound randomly, and the pharmacological efficacy of such a conjugate even exceeded the activity of the free drug (19, 46).

In conjugates prepared by an aminolytic reaction, part of the multiple ONp groups of the HPMA copolymer react with the amino groups of daunosamine in the doxorubicin molecule, followed by the reaction of the rest of the ONp groups of the HPMA copolymer with amino groups of lysine residues in the antibody molecule. Thus, the branching cannot be avoided in the reaction where a multivalent polymer precursor reacts with a multivalent antibody. The effect of branching can only be minimized by careful performance and control of pH, ionic strength and temperature of the reaction. Nevertheless, the reaction product is still a mixture of molecules consisting of a single antibody modified with polymer chains, and molecules containing 2 or more antibody molecules connected to many polymer chains into a branched structure. Consequently, precise characterization of such a "classic" conjugate is rather difficult. Its molecular weight is high and its polydispersity fairly broad, as the targeting and drug moieties are bound via linkages randomly distributed along the polymer chain (53). An advantage of aminolytic conjugation is the relative ease of performance on a large scale (38).

A new HPMA conjugate with a "star-like" structure represents a better characterized material (Fig. 2). The differences reside in the methods of synthesis, attachment of targeting antibodies and resultant physicochemical characteristics. In "star-like" conjugates, HPMA copolymer chains containing only one reactive *N*-hydroxysuccinimide group at the end of the molecule react with the lysine ε-amino groups of the antibody molecule. Thus, the antibody molecule is surrounded by approximately 20-30 HPMA copolymer chains exposing the drug (doxorubicin) bound through a Gly-Phe-Leu-Gly tetrapeptide on the surface (98, 99). No cross-linking or branching of polymeric chains was found and the distribution of molecular weight was narrow. Thus, the star structure of HPMA copolymer-bound doxorubicin targeted with antibody provides a more homogeneous and better characterized conjugate compared to the classic structure (38, 98, 99). FACS analysis repeatedly showed that the attachment of antibodies to the HPMA copolymer carrier always decreases their targeting capacity. Comparison of two HPMA conjugates containing doxorubicin and targeted with B1 monoclonal antibodies specifically recognizing the BCR receptor of BCL_1 mouse B-cell leukemia revealed that the binding of "star-like" conjugates is impaired by conjugation to the polymeric carrier more than the binding of "classic" conjugates, but the "star-like" conjugate showed 3 times higher cytostatic activity *in vitro* (IC_{50} = 10.5 nM doxorubicin equivalents) than the "classic" conjugate (IC_{50} = 32.6 nM doxorubicin equivalents). Both targeted conjugates exhibited much greater cytostatic activity than nontargeted HPMA copolymer-bound doxorubicin (IC_{50} = 1450 nM doxorubicin equivalents), which is not surprising as this nontargeted conjugate has no specific receptor on target cells.

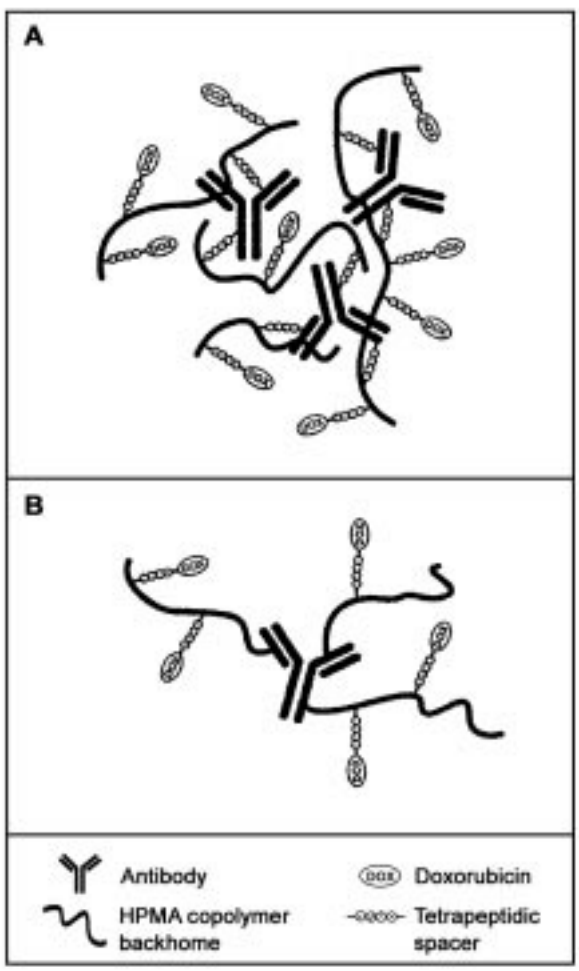

Fig. 2. Schematic drawing of the classical structure of antibody-targeted HPMA copolymer-bound doxorubicin conjugate (A) and the star structure of antibody-targeted HPMA copolymer-bound doxorubicin conjugate (B).

Receptor-mediated endocytosis via the Fc receptor is probably not involved, as "targeting" using irrelevant antibody (anti-Thy 1.2) bound to a polymeric carrier was not seen. Nontargeted conjugate is taken up only by fluid-phase pinocytosis, which is a slow process and requires a high concentration of the conjugate to produce cytostatic activity (86).

Similar results were obtained with the mouse T-cell lymphoma EL4 and human colorectal carcinoma SW620. While the binding of "classic" conjugates to the targets was always superior to the binding of "star-like" conjugates, their cytotoxicity was lower. The "star-like" anti-Thy 1.2-targeted HPMA-bound doxorubicin was 10-fold more cytotoxic (IC_{50} = 1.14 μM doxorubicin equivalents) compared to the "classic" conjugate (IC_{50} = 12.73 μM doxorubicin equivalents). When SW620 cells were incubated with a "star-like" conjugate targeted by anti-CD71 monoclonals, the IC_{50} (0.25 μM doxorubicin equivalents) was 10-fold lower than with the "classic" conjugate targeted by an identical antibody (IC_{50} = 2.76 μM doxorubicin equivalents) (77).

Both the "classic" and the "star-like" conjugates of HPMA copolymer-bound doxorubicin targeted with B1 MAb signficantly prolonged the survival of mice. One dose of free doxorubicin (50 μg) given on day 11 after the inoculation of cancer cells prolonged the survival of mice to 114% relative to control mice. The "classic" conjugate prolonged the survival to 138% relative to control mice, but did not produce any cures. The "star-like" conjugate, on the other hand, showed an extraordinary effect, completely curing 55% of mice and extending the average lifespan of the remaining mice to 258% of that found in the controls (38). Similarly, the "star-like" conjugates containing anti-CD71 monoclonals as targeting structures were more effective against human colorectal cancer SW620 xenografts than the "classic" conjugates. Biodistribution studies revealed that the "star-like" conjugate remained in a relatively high concentration in the blood for a few days. Nineteen percent of the injected dose remained after 48 h, compared to only 3.8% of the "classic" conjugate and 2.9% of nontargeted HPMA-bound doxorubicin (PK1) (77). In an experimental model of mouse T-cell lymphoma EL4 tumors, both anti-Thy 1.2-targeted conjugates ("classic" and "star-like") were effective and cured all tumor-bearing mice. Data obtained with "star-like" conjugates confirmed that the intensity of binding is only one of many factors responsible for the pharmacological activity of targeted polymeric drugs.

As already mentioned, the binding of antibodies to HPMA copolymer-bound doxorubicin can involve the antibody binding site, and hence decrease the antibody-binding potential. Thus, in "classic" conjugates we have tried to protect the antigen binding site by dimethylmalein anhydride. However, no significant difference was seen in the cytotoxicity of conjugates with protected and unprotected antibodies (98, 99), and this rather laborious method of protection was not used further for routine synthesis of conjugates. Among other methods used to prevent inactivation of the antibody binding site of the immunoglobulin molecule are the already-mentioned "oriented" binding of oxidized antibody to a polymer containing hydrazide groups (19, 20, 34, 46, 97, 98), or binding of polymerized Fab' antibody fragments to an HPMA copolymer via a thioether group formed by a reaction of Fab' SH group with a maleimide group in a methacryloylated unit (97, 100).

Affinity therapy with antibody-targeted HPMA copolymer-bound daunorubicin (doxorubicin) applied to the immune system

One of the main problems of anticancer affinity therapy is the selection of the most specific targeting structure. The same holds for intervention on the immune system, where physiological (*e.g.,* rejection of transplanted graft) or pathological (autoimmune disorders) reactions depend on the cooperation of different cellular subsets characterized by specific surface structures. For this reason, the effect of affinity therapy on lymphatic tissue depends mainly on the specificity of the antibody used as a targeting structure.

We have chosen a well-defined model which allows study of the pharmacological efficacy of the antibody–polymer–drug conjugates *in vitro* and *in vivo*. By testing targeting to lymphatic tissue, we have compared the targeting abilities of polyclonal anti-thymocyte globulin (ATG) and monoclonal anti-Thy 1.2, anti-CD4, anti-CD25, anti-CD3 or anti-class II MHC glycoprotein antibodies, all of which are directed to well-defined surface molecules on cells involved in immune reactions. Once bound to its membrane receptor, anti-Thy 1.2 antibody loss from the cell surface is low and this retention of surface-associated antibody is maintained *in vitro,* as well as *in vivo. In vivo,* 1 min is sufficient for reproducible binding of the polymeric conjugate to peripheral blood leukocytes (32). Thy 1.2 (CD90), CD4, CD25 and CD3 molecules are expressed on T-lymphocytes and class II MHC molecules on specialized APCs, such as dendritic cells, macrophages, B-lymphocytes, certain endothelial cells and thymic epithelial cells. Anti-thymocyte globulin is a polyclonal antiserum directed against a number of different surface molecules of thymocytes and T-cells. T-lymphocytes, cells important for all types of immune response, recognize and respond to peptide antigens attached to class I or II MHC glycoproteins expressed on APCs. FACS analysis showed that all selected antibodies, including ATG, bind strongly and specifically to normal T-lymphocytes and to mouse EL4 T-cell lymphoma (84).

Loading with a selected antibody-targeted polymer-bound drug leads to the killing, inactivation or inhibition of cells involved in the immune reaction. As a result of elimination of APCs and/or T-lymphocytes, the cooperation in the immune system is disturbed and consequently, the cellular and humoral (antibody) response is decreased. The intensity of the antibody reaction *in vivo* can be tested either by serum antibody levels (ELISA, RIA), or more precisely by direct estimation of antibody-releasing

B-cells by the so-called plaque-forming cell (PFC) technique. It was observed that ATG-, anti-Thy 1.2-, anti-CD4-, anti-CD25-, anti-CD3- and anti-class II MHC glycoprotein-targeted daunorubicin and doxorubicin bound to an HPMA copolymer carrier via a Gly-Phe-Leu-Gly sequence are cytotoxic to mouse splenocytes and human peripheral blood lymphocytes *in vitro* (16-18, 87; see also section on "Passive accumulation and active targeting"). *In vivo,* such conjugates effectively suppress both primary and secondary antibody responses directed against sheep red blood cells (SRBCs) by 60-85% depending on the concentration of the injected drug equivalent (16-18). Daunorubicin bound to the copolymer containing a degradable Gly-Phe-Leu-Gly sequence is active, but compared with free daunorubicin, a higher quantity of conjugated drug is needed for the same suppression. Daunorubicin bound to the copolymer with a nondegradable sequence appears to be inactive, similar to the situation *in vitro*.

The degree of depletion of T-lymphocytes and/or APCs reflected in a decrease of the immune response *in vivo* is directly related to the pharmacological activity of the antibody–HPMA copolymer carrier–drug conjugates. The most effective suppression of anti-SRBC responses *in vivo* was induced by injection of a conjugate with Gly-Phe-Leu-Gly side-chains targeted with an antibody against class II MHC molecules. A total of 260 μg of daunorubicin injected in 3 consecutive doses decreased the number of plaque-forming (antibody-producing) cells (PFCs) by 65%, while a similar conjugate targeted with polyclonal ATG was less effective and decreased the number of PFCs by only 50%. Surprisingly, the least effective was the conjugate containing monoclonal anti-Thy 1.2 antibody, where only 35% suppression of PFCs was recorded. Nontargeted HPMA copolymer carrier-bound daunorubicin had rather limited activity, as the number of PFCs was suppressed by only 17%. Daunorubicin and ATG bound to HPMA copolymer carrier via nonbiodegradable Gly-Gly sequences were inactive (16, 18). By eliminating the APCs that carry class II MHC molecules on their surface, the immune response is blocked at the beginning. Anti-class II MHC antiserum has been successfully used for the treatment of experimental autoimmune diseases (101) and affinity therapy using the same antibodies as a targeting structure might be even more effective. Class II MHC molecules are expressed in large quantities, mostly on activated cells, while resting cells carry on their surface only a limited number of class II MHC glycoproteins.

The optimal time for loading of antibody-targeted conjugate into isolated T-lymphocytes was 3 h (85). With a shorter incubation time (10 min), it was necessary to considerably increase the dose to achieve the same inhibitory effect. Cultivation of immunocompetent cells (mouse splenocytes, human peripheral blood leukocytes) with antibody–HPMA–daunorubicin conjugates at 4 °C instead of 37 °C had only a limited effect on the intensity of antibody response, confirming that an active metabolic process is important for intracellular accumulation of such an antibody-targeted conjugate (87).

Affinity therapy with antibody-targeted HPMA copolymer-bound doxorubicin in experimental cancer models

Several experimental models of cancer were used to determine the efficacy of antibody-targeted HPMA copolymer carrier-bound doxorubicin.

The mouse EL4 T-cell lymphoma was selected because of its low sensitivity to treatment with free doxorubicin (102). The *in vivo* therapeutic efficacy of HPMA copolymer-bound doxorubicin targeted with polyclonal anti-EL4 antibody, polyclonal ATG, monoclonal anti-Thy 1.2 antibody or its $F(ab')_2$ fragment, which roughly corresponds to 2 disulfide-linked Fab' fragments and has divalent antigen-binding capacity, was compared with the efficacy of doxorubicin conjugated to HPMA copolymer carrier containing nonspecific mouse IgG or BSA. It was suggested that using smaller fragments which maintained the desirable tumor-targeting characteristics might be advantageous because their accumulation and targeting in solid tumors are more rapid (103, 104).

The polyclonal anti-EL4 antibody-targeted conjugate caused a significant delay in tumor growth and an extension of the lifespan of treated mice to 184% compared to control. The effect was comparable with that of HPMA copolymer-bound doxorubicin targeted with polyclonal ATG, while conjugates targeted with monoclonal anti-Thy 1.2 antibody or its $F(ab')_2$ fragment were much more effective. However, a considerable antitumor effect was also seen for conjugates targeted with syngeneic nonspecific IgG, human IVIg and BSA. In mice treated with doxorubicin–HPMA–mouse IgG, survival was extended by up to 133% compared to controls, and in those treated with doxorubicin–HPMA–BSA, it was prolonged by up to 130% compared to controls. The delay in tumor growth was comparable in mice treated with IgG- and BSA-containing HPMA–doxorubicin, but the death rate was lower in the former group (24). We reasoned that the therapeutic efficacy seen after the administration of HPMA copolymer carrier-bound doxorubicin containing nonspecific IgG or BSA is due to the EPR effect described by Maeda *et al.* (65-67), as in biodistribution studies performed with [^{125}I]-labeled conjugates, rather high accumulation of IgG- and BSA-containing HPMA bound-doxorubicin conjugates has been reported (Jelínková, unpublished results). A modest effect was seen with nontargeted HPMA copolymer carrier-bound doxorubicin (PK1), which might be explained by its lower molecular weight compared to protein-containing conjugates (24).

In this model, the difference in the therapeutic potential of the conjugates targeted with whole anti-Thy 1.2 antibody or its $F(ab')_2$ fragment was not significant. This is in agreement with Waldman (105), who reported that intact IgG antibodies are retained better by the tumor, which appears to be better for therapy. Similarly, it was

reported by Juweid *et al*. (106) that the $F(ab')_2$ fragment of a murine monoclonal antibody against carcinoembryonic antigen (CEA) exhibits a similar targeting sensitivity and tumor dose as reported for the IgG form. EL4 T-cell lymphoma is associated with a rapid spread of micrometastatic tumors invading all lymphatic tissues, including the spleen, which might be even more life-threatening than the primary solid tumor. Thus, it is most likely that the EPR effect is only partially responsible for the final pharmacological efficacy of the conjugates and the difference between samples with different molecular weight cannot be proven. The decision to use intact IgG or $F(ab')_2$ or Fab' fragments for targeting will therefore depend on the characteristics and microanatomy of a particular tumor (24).

The transferrin receptor (CD71) has already been used as a target for immunotoxins (107) and targeted gene therapy (108). CD71 represents a marker of activated and dividing cells that is quickly internalized after ligand binding. As a well-defined antigen that is strongly expressed on cancer cells, it is suitable for targeted cancer chemotherapy (30). The number of receptors per cell is thought to be dependent upon the cell type and growth. We have compared the targeting efficacy of transferrin as a natural ligand and anti-CD71 monoclonals in affinity therapy against mouse B-cell lymphoma 38C13.

Conjugation of an anti-CD71 antibody to the HPMA copolymer carrier had little effect on its binding to the CD71 receptor expressed on B-cell lymphoma. On the other hand, binding of transferrin to an HPMA copolymer carrier resulted in a significantly reduced binding capacity to target cells.

The cytotoxic activity of doxorubicin attached to the HPMA copolymer carrier was decreased more than 1,000-fold when tested *in vitro* by [^{3}H]-thymidine uptake. Targeting with anti-CD71 antibodies increased cytotoxicity approximately 9-fold. As a targeting moiety, transferrin was less effective, as the cytotoxic effect of the transferrin-targeted conjugate was 4-fold lower compared to that obtained with the anti-CD71-targeted conjugate. The therapeutic effect of anti-CD71- or transferrin-targeted HPMA copolymer-bound doxorubicin was evaluated on cancer growth and survival in C3H/HeN mice with established 38C13 B-cell lymphoma. Similar to the *in vitro* studies, HPMA copolymer-bound doxorubicin targeted with anti-CD71 monoclonals had a much stronger therapeutic effect, significantly delaying tumor growth, significantly prolonging survival and completely curing 30% of tumor-bearing mice, while the transferrin-targeted conjugate cured only 15% of 38C13 tumor-bearing mice. Free or nontargeted HPMA copolymer-bound doxorubicin showed only a slight antitumor effect (37).

Mouse BCL_1 B-cell leukemia represents a B-cell-derived malignancy with BCL_1 cells possessing a surface IgM B-cell receptor (BCR). As IgM BCR is highly expressed on B-cells and rapidly internalized after ligand binding (109), it should be advantageous to use a B1 monoclonal antibody recognizing the BCL_1 BCR idiotype as a targeting moiety. *In vitro,* a B1 monoclonal-targeted conjugate demonstrated 40-fold higher cytotoxicity than nontargeted or human nonspecific Ig containing HPMA copolymer-bound doxorubicin. The IC_{50} for the nontargeted conjugate was 1360 nM, a concentration similar to that obtained with nonspecific human Ig (IC_{50} = 1170 nM). The IC_{50} of a B1-targeted conjugate was 34 nM and that of free doxorubicin was 1.5 nM. Intravenous administration of 5 mg/kg doxorubicin equivalents in a B1 MAb-targeted conjugate on days 11 and 14 completely cured 70% of mice and extended the average lifespan of the remaining mice to 246% of that found in the controls. The conjugate containing human nonspecific Ig prolonged the survival of mice to only 136% of the control group and did not produce cures. It was found that an essential factor in the efficacy of the therapy was the time interval between tumor cell inoculation and the beginning of treatment. While 2 doses given on days 11 and 14 were able to cure 70% of experimental mice, 3 doses given on days 15, 18 and 21 completely cured only 10% of mice (76). In the late stages of BCL_1 leukemia, mice have detectable serum levels of BCL_1-specific IgM antibodies which are probably released from dying BCL_1 cells. These serum antibodies with the BCL_1 idiotype can compete or even block the binding of B1 MAb-targeted conjugates to target BCL_1 cells, thereby decreasing the efficacy of the therapy (76).

As previously mentioned, the OV-TL16 antibody recognizes the OA-3 antigen expressed on the surface of OVCAR-3 ovarian carcinoma cells (35, 36, 39, 110, 111). Binding of doxorubicin to a nontargeted HPMA copolymer carrier drastically decreased its cytotoxicity *in vitro* towards OVCAR-3 cells. The IC_{50}, determined by the MTT assay, increased from 2 μM for free doxorubicin to 150 μM for the doxorubicin–HPMA conjugate PK1 (35). It was suggested that this increase in IC_{50}, regularly seen after the attachment of different drugs to an HPMA copolymer carrier, might reflect the different mechanisms of cell uptake, resulting in altered intracellular drug concentration. The increased molecular weight of the conjugate prevents its free diffusion through the plasma membrane and limits its uptake by pinocytosis, which seems to be a rather inefficient process when compared to the rapid transport of free drug through the plasma membrane (35, 112). According to our observations, the accumulation of nontargeted conjugate (doxorubicin–HPMA [PK1]) is a rapid event occurring within minutes if a cell is exposed to a very high extracellular concentration. However, drug effect is seen only after 12-16 h (Kovář, Hovorka unpublished observation). It should also be stressed that measurement of [^{3}H]-thymidine uptake, which we routinely use in our laboratory, is at least 10 times more sensitive for inhibition of proliferation compared to the MTT assay, and consequently the IC_{50} obtained by these two methods can differ greatly.

On the other hand, a doxorubicin–HPMA conjugate targeted with an OV-TL16 antibody, which enters the cells by receptor-mediated endocytosis, was substantially more toxic *in vitro* and *in vivo* than a nontargeted conjugate. *In vitro*, the low IC_{50} value for doxorubicin was

practically restored by antibody targeting. The IC_{50} for doxorubicin-HPMA–OV–TL16 antibody was found to be 4.4 μM doxorubicin equivalents, which is 34-fold less than that for the nontargeted conjugate. The internalization of the conjugate was seen within 5 min. *In vivo,* a single i.v. dose of doxorubicin–HPMA–OV-TL16-targeted conjugate (2.2 mg/kg doxorubicin equivalents) was able to suppress the growth of OVCAR-3 carcinoma xenografts in nude mice. Three i.v. injections of the same conjugate demonstrated the best efficacy in inhibiting xenograft growth, although no tumor cures were found (39).

Biodistribution and pharmacokinetics

To be effective, HPMA-based polymeric prodrugs must have an increased plasma half-life and must accumulate in target tissue with long-term retention. Daunorubicin injected in the free form disappears from the bloodstream very quickly in a biphasic pattern (44, 85, 113, 114), and less than 30% of the dose remains after 30 min. Compared to blood, the decline of free iodinated drug or its metabolites in the liver, spleen and thymus is considerably slower (85), which is probably due to the fact that daunorubicin is metabolized and excreted mainly via the hepatobiliary route (115), and the spleen and thymus contain rapidly proliferating cell compartments. It was reported that the concentration of daunorubicin found in animal tissues after i.v. administration correlates with the number of nuclei present in a particular tissue, as daunorubicin binds with high affinity to nucleic acid, predominantly by intercalation into the DNA double helix (116).

The elimination of an HPMA copolymer carrier after i.v. or i.p. injection is directly related to its molecular weight. Copolymers with a molecular weight of < 45 kDa are quantitatively eliminated from the organism within hours by urinary excretion and do not accumulate in any tissue. Preparations exceeding this threshold are lost from the bloodstream only slowly by extravasation. Following subcutaneous administration, the largest HPMA copolymer fraction (molecular weight of 778 kDa, diameter of approximately 30 nm) showed increased retention at the site of injection, approximately 20% of the dose remaining there after 21 days (117).

Coupling of the drug to the synthetic polymeric carrier considerably alters its body distribution (118). [^{125}I]-Daunorubicin bound to an HPMA copolymer is detectable in the blood and other tissues in a higher concentration for a longer time compared with free drug. An interesting finding is that after injection of the nontargeted conjugate with biodegradable Gly-Phe-Leu-Gly side-chains, the level of radioactivity, particularly in the blood and liver, is higher than after injection of copolymer with nonbiodegradable Gly-Gly sequences (85). This is in agreement with the observation *in vitro* that higher membrane association and intracellular accumulation into T-lymphocytes were seen with conjugates containing Gly-Phe-Leu-Gly sequences compared with conjugates containing Gly-Gly sequences (85). The differences in the fates of these two copolymers are probably due to the highly hydrophobic character of Gly-Phe-Leu-Gly side-chains, which contributes to the increase in binding and internalization by some cell types (93). Similar results, *i.e.*, a higher accumulation of HPMA copolymers containing hydrophobic comonomers, were reported by Duncan *et al.* (118) for yolk sac.

Conjugation of antibodies and their fragments to HPMA copolymers results in extended circulation times and most probably contributes to passive accumulation in solid tumors by the EPR effect. Covalent binding of [^{125}I]-daunorubicin to an HPMA copolymer containing targeting anti-Thy 1.2 antibodies increased its level in the blood 5-20-fold and considerably decreased its rate of elimination compared to the free drug. The greatest accumulation of anti-Thy 1.2-targeted conjugate was detected in the spleen, thymus and liver 2 h after i.v. administration, while only liver accumulation was observed if [^{125}I]-daunorubicin–HPMA copolymer containing nonspecific rabbit gamma globulin (RGG) instead of specific antibodies was injected (85). This is consistent with *in vitro* experiments, where the highest accumulation of radioactivity in isolated T-lymphocytes was found 2 h after incubation with [^{125}I]-daunorubicin–HPMA–anti-Thy 1.2-targeted conjugate (85). The same results were obtained after i.p. administration, suggesting that the conjugate was rapidly drained from the peritoneal cavity either through lymphatic and blood capillary walls or through the diaphragma lymphatic system. Seymour *et al.* (117) also reported that molecular weight does not influence the movement of copolymers from the peritoneal compartment to the bloodstream after i.p. injection.

A very important observation is that HPMA copolymers with and without immunoglobulin show a comparable distribution between the plasma and blood cells (85). Similarly, Kovář *et al.* (76) did not detect any considerable binding of conjugate containing nonspecific Ig to BCL_1 B-cell leukemia cells, indicating that massive uptake of polymer-bound doxorubicin through Fc receptors on nontarget cells is not expected.

It is necessary to keep in mind that the body distribution of an antibody-targeted conjugate will always be strongly influenced by the specificity of targeting antibodies, and thus data obtained for anti-Thy-1.2-targeted conjugates might not necessarily fit the data obtained with other antibody-targeted conjugates.

Following i.v. administration to normal mice, [^{125}I]-labeled whole B72.3 monoclonal antibody (murine IgG_1 that recognizes the TAG72 antigen expressed on LS 174T human colorectal carcinoma) showed slow clearance from the bloodstream, probably due to both extravasation and metabolism. A decrease to 50% of the initial value took about 10 h, and gradual decline continued, reaching 70% after 48 h. The rate of clearance appeared to be independent of the administered dose. Parent antibody and its fragments showed molecular weight-dependent blood clearance following i.v. injection. While the intact molecule remained in the circulation for an

extended period, the Fab' fragment was cleared rapidly, predominantly via the kidney into the urine. The larger $F(ab')_2$ fragment demonstrated intermediate blood clearance kinetics, with a half-life of about 3 h (47).

The blood clearance of the B72.3-targeted HPMA conjugate was similar to the parent B72.3 antibody, while the clearance of both fragment conjugates was substantially slower. Most striking was the HPMA–Fab' conjugate, which displayed a half-life of about 5 h compared with a few minutes for the unmodified fragment, over 20% remaining in the circulation after 24 h (47).

Biodistribution and pharmacokinetics are among the factors that substantially influence the pharmacological activity of macromolecular therapeutics (74). Our data show that the "star-like" conjugates of HPMA copolymer-bound doxorubicin targeted with anti-Thy 1.2 antibody, with considerably greater antitumor activity compared to the conjugate with a "classic" structure, have a prolonged blood elimination profile (77).

Subcellular trafficking and processing

It has been repeatedly reported that attachment of a drug to a nontargeted HPMA copolymer carrier substantially decreases its cytotoxicity. It is generally supposed that such a decrease in pharmacological activity reflects the change in the mechanisms of cell entry. Free drugs enter the cell via transmembrane transport or adsorptive pinocytosis, while fluid-phase endocytosis of nontargeted drugs and receptor-mediated endocytosis of antibody-targeted drugs are responsible for intracellular accumulation of macromolecular therapeutics. Targeted conjugates were found to be much more toxic (86).

When transported by diffusion, the drug will reach the cytoplasm of the cell, whereas all endocytic processes are lysosomotropic (119). The interaction and processes that determine the ultimate fate of a targeted macromolecular therapeutic depend on both the receptor and its ligand. Among factors that decisively influence the efficacy of antibody-targeted polymeric conjugates are: 1) the affinity of the antibodies; 2) the nature and density of the target antigen; 3) the type of cell target; 4) the rate of endocytosis; and 5) the intracellular pathways of the conjugate (74).

Intrinsic fluorescence of doxorubicin was used to follow the uptake and subcellular localization of OV-TL16 monoclonal antibody-targeted doxorubicin bound to an HPMA copolymer carrier in OVCAR-3 ovarian cancer cells. After 24 h of incubation, doxorubicin fluorescence was detectable by confocal fluorescence microscopy in round-shaped acidic organelles, most probably lysosomes, predominantly clustered around nuclei. Later on, the doxorubicin fluorescence was detected in nuclei of OVCAR-3 cells incubated with a degradable spacer Gly-Phe-Leu-Gly-containing HPMA conjugate. A similar accumulation in nuclei was seen after incubation of cancer cells with free drug. On the other hand, cells exposed for the same time to an HPMA conjugate containing doxorubicin attached via a nondegradable Gly-Gly spacer did not demonstrate staining of nuclei (36).

It was seen that the OV-TL16-targeted HPMA–doxorubicin conjugate binds and is taken up by OVCAR-3 cells relatively quickly. Both free antibodies and the antibody-targeted drug-containing HPMA conjugate were localized inside the cells as early as 5 min after the onset of incubation. The total amount of cell-associated material gradually increased as a function of time, but only 30% of the total amount of cell-associated material was internalized over the 90-min interval (35). A similar result was reported for an anti-Thy 1.2-targeted conjugate. After 90 min of incubation, the conjugate was still localized mainly on the cell surface. After 48 h, the majority of the targeted complex was already internalized and doxorubicin accumulated in cytoplasmic structures, but antibodies were still detectable on the surface of target cells. In contrast to the results with OV-TL16 antibodies, after 3 days of incubation, cells exposed to an anti-Thy 1.2-targeted HPMA conjugate containing biodegradable Gly-Phe-Leu-Gly showed detectable doxorubicin fluorescence only in the cytoplasmic structures (93).

Low nonspecific toxicity of antibody-targeted HPMA conjugates

Many free drugs, and also immunotoxins, are hepatotoxic, myelotoxic, nephrotoxic and cardiotoxic, and also induce other undesirable side effects (120). The aim of affinity therapy is not only to accumulate the drug at the site of the pathological process, but also to eliminate its toxic effects on normal tissue.

We and others reported that conjugation of cytotoxic or immunosuppressive drugs to a water-soluble copolymer carrier based on HPMA significantly reduces their nonspecific toxicity, including myelotoxicity (18, 121), hepatotoxicity (18), cardiotoxicity (73, 122, 123), nephrotoxicity (4, 20), toxicity to thymus (3, 4) and immunotoxicity (124).

Bone marrow contains a heterogeneous population of hematopoietic and lymphopoietic precursors which are highly sensitive to the action of cytotoxic drugs. The myelotoxicity of macromolecular therapeutics can be experimentally measured by counting so-called spleen cell colony-forming units (CFU-s). First, experimental mice are injected with free drug or polymeric conjugates, and after a selected time, their bone marrow cells are transplanted to sublethally irradiated recipients. A part of these transplanted cells, especially the hematopoietic precursors, form colonies in different tissues and organs, which in the spleen are known as as CFU-s. The decrease in the number of CFU-s compared to controls is directly related to the myelotoxicity of the tested material.

For instance, free daunorubicin is strongly myelotoxic and doses of 6-24 mg/kg injected i.v. in 3 consecutive doses led to an almost total depletion of hematopoietic precursors in the bone marrow, whereas the same dose applied i.p. left 30% of precursors alive (18).

Administration of the homopolymer poly(HPMA), *i.e.,* a polymer without the oligopeptidic side-chains, did not affect the number of hematopoietic precursors in bone marrow (125). An antibody-targeted HPMA conjugate without the drug and a drug-containing HPMA copolymer without the targeting moiety were also nontoxic. Repeated injection of anti-Thy 1.2-targeted daunorubicin conjugated to an HPMA copolymer reduced the number of bone marrow stem cells only slightly. The myelotoxicity of such antibody-targeted HPMA copolymer carrier-bound drug was reduced by up to 80-fold compared to the free drug (18, 121).

The side effects of an anti-Thy 1.2-targeted HPMA copolymer–drug conjugate were also assessed by histological examination of the thymus, liver, spleen, heart and kidneys. Morphological findings corroborated the results obtained while testing the direct effect of antibody-targeted HPMA copolymer-bound drug on stem cells from the bone marrow. Hyperplasia and pronounced irritation of Kupffer cells in liver, as well as cardiotoxicity, were observed only after injection of free daunorubicin, whereas the antibody-targeted conjugate had no such pathological effect (18).

Possibility to overcome P-glycoprotein-mediated multidrug resistance (MDR)

Cancer is characterized by intra- and intertumor heterogeneity. Intertumor heterogeneity is responsible for the different toxicity of similar conjugates compared in different tumor models. Intratumor heterogeneity arises during cancer progression from originally homogeneous cell populations representing mostly drug-sensitive cells. Depending on the type of cancer, and sometimes also as a consequence of chemotherapy, MDR mutants appear in originally homogeneous and sensitive cell populations. This development of MDR is a major factor limiting the response rate of cancer chemotherapy. Several mechanisms of MDR have been reported. The MDR phenotype is brought about by members of the ATP-binding cassette (ABC) family of transporters, P-glycoprotein (Pgp) and proteins of the MDR family (MDRP). P-glycoprotein functions as an energy-dependent extrusion pump, causing reduced intracellular drug retention and resulting in narrowing of the therapeutic window between tumor-active anticancer doses and the maximum tolerated dose (126-128). The success of treatment of patients suffering from chemoresistant cancer depends above all on the elimination of such MDR cancer cells.

It has been proposed (36, 129) that the different mechanisms of uptake and intracellular trafficking of antibody-targeted HPMA-conjugated drugs (receptor-mediated endocytosis in membrane-limited organelles) in contrast to diffusion of the free drug might overcome, at least partially, Pgp-mediated MDR by making the endosome/lysosome-encapsulated drug inaccessible for Pgp. Thus, we have tested three different antibody-targeted HPMA conjugates of doxorubicin against the sensitive human lymphoblastic T-cell line CEM and against its MDR subline CEM-VLB overexpressing Pgp. It was shown that the ability of the conjugates to decrease doxorubicin resistance depends primarily on the selected targeting moiety. An anti-CD71-targeted conjugate was effective in both the sensitive and the resistant cell line, with an IC_{50} of 70 and 480 μM, respectively. The most potent was the ATG-targeted conjugate of doxorubicin, with an IC_{50} for CEM of 10 μM and for CEM-VLB of 250 μM.

CEM-VLB cells were 50 times more resistant to free doxorubicin than parental CEM cells. The level of resistance (IC_{50} MDR cells/IC_{50} sensitive cells) decreased to 25 and 7 for ATG- and anti-CD-71-targeted conjugates of doxorubicin, respectively. An anti-CD4-targeted conjugate was unable to overcome MDR of selected human leukemia. Inhibition of proliferation by an anti-CD4-targeted conjugate was recorded only in the sensitive cell line, while the proliferation of the MDR subline CEM-VLB was not affected even when the highest doxorubicin concentration (640 μM) was used (33). This might be due to poor internalization of antigen*, i.e.,* the CD4 molecule (130), or its ligand, *i.e.*, the monoclonal anti-CD4 antibody, as it has been reported that the IgG_{2a} subclass is much less internalized compared with IgG_1 (131). The inability of an anti-CD4-targeted conjugate to overcome MDR was not a consequence of its low binding to target leukemia cells, as specific binding of the conjugate to CD4-positive human peripheral blood lymphocytes, CEM and CEM-VLB cells was confirmed by flow cytometry (33). In contrast to the results obtained with sensitive A2780 and doxorubicin-resistant A2780/AD ovarian carcinoma (129), the nontargeted doxorubicin–HPMA conjugate (PK1) and a mixture of a free drug and free HPMA copolymer were not effective in the CEM-VLB line at the highest tested concentration (640 μM) (33). This confirms that the circumvention of MDR is not an intrinsic ability of HPMA and that HPMA does not block Pgp, as has been shown for some other synthetic polymers (132-135).

Omelyanenko *et al.* (36) directly demonstrated increased intracellular concentrations of HPMA copolymer-bound doxorubicin in the MDR A2780/AD line relative to the free drug, and confirmed the hypothesis of a change in the intracellular gradient of the drug bound to an HPMA copolymer carrier. In the case of the free drug, the gradient is directed from the plasma membrane to the perinuclear membrane. In the case of HPMA copolymer-bound drug, where the drug is released in lysosomes, the gradient is from the perinuclear region to the plasma membrane. Thus, the probability of the drug interacting with nuclear DNA is higher than the probability of its recognition by the Pgp pump (36, 129).

Xenogeneic targeting

Spontaneous human cancers often do not express surface antigens suitable for targeting. Recent progress in gene therapy-based approaches offers the possibility of overcoming the problem of the absence of specific

tumor markers, at least in some tumors. This approach is based on transfer of selected genes into tumor cells and subsequent utilization of these gene products as targets for monoclonal antibodies (136), or for a monoclonal antibody-targeted polymeric drug. Thus, the question was addressed whether the human colorectal cancer cell line SW620 transfected with the mouse *Thy 1.2* gene would result in a xenogeneic gene-marked human cancer suitable for effective site-specific drug targeting. A monoclonal anti-Thy 1.2 antibody as targeting moiety and doxorubicin as drug were conjugated to HPMA acting as a carrier responsible for controlled intracellular drug release. FACS analysis showed that the transfectant, the SW620/T subline, binds the anti-Thy-1.2 monoclonal antibody and anti-Thy 1.2-targeted polymeric conjugate to the same extent as do Thy-1.2$^+$ mouse T-lymphocytes and Thy-1.2$^+$ mouse T-cell lymphoma EL4. No such binding was detected with the Thy-1.2$^-$ parent SW620 cancer cell line (23).

The conjugates of HPMA copolymer carrier-bound doxorubicin targeted with an anti-Thy 1.2 monoclonal antibody or its F(ab')$_2$ fragment specifically inhibited the proliferation of only the transfectant SW620/T in a concentration-dependent manner *in vitro*. Nonspecific inhibition of the proliferation of the parental SW620 cell line was seen only at very high concentrations of targeted doxorubicin, probably reflecting a nonspecific accumulation of the compound by fluid-phase pinocytosis (23).

These results represent the first indication of the suitability of transfection of human cancer cells with a mouse gene controlling the synthesis of a selected surface marker for site-specific affinity therapy of malignancies.

Dual cytostatic and immunomobilizing activity of antibody-targeted HPMA-bound drugs

Efficient affinity therapy can eradicate cancer cells with a sufficiently high density of cell-surface target antigens. Cells with a low density or without target antigens, which eventually prevail during cancer progression, represent minimal residual disease (MRD), which has to be eliminated by the defense system of the cancer host. Although there is still no direct evidence that immunosurveillance actually protects individuals from tumors, numerous observations support the concept that cytolytic (cytotoxic) T-lymphocytes (Tc lymphocytes, CTLs), natural killer (NK) cells, lymphokine-activated killer (LAK) cells and macrophages provide antitumor immunity *in vivo*. The recognition machinery used by these cells is different, while the mechanisms of target cell destruction are similar. Two possible cytocidal mechanisms have been proposed: 1) the apoptotic mechanism, in which the effector cell triggers an autolytic cascade in the target cell characterized by prelytic fragmentation of the genomic DNA; and 2) the lytic mechanism, in which lytic molecules, notably perforin, are secreted by the effector cell into the intercellular space and polymerize to form lytic pores in the target cell membrane.

Such antitumor defense reactions of the tumor-bearing host are often depressed either primarily as a consequence of extensive growth of the cancer, or secondarily as a result of intensive treatment with classical cytostatic drugs, which are generally immunotoxic. To achieve better results in cancer therapy, a new generation of anticancer drugs has to be developed to effectively kill intensively proliferating cancer cells while simultaneously sparing intensively proliferating cells of the immune system, among others.

A series of experiments peformed in our laboratory showed that the lysis of target EL4 tumor cells is decreased if effector killer cells (CTLs and NK cells) are isolated from C57BL/10 mice bearing mouse T-cell lymphoma EL4 and repeatedly treated with free doxorubicin. If instead of free drug the mice were injected with the drug conjugated to an HPMA copolymer carrier targeted with anti-Thy 1.2 antibody, an efficient tumoricidal activity of CTL and NK cells was observed. In fact, this was the first report indicating that the targeted form of an HPMA copolymer-bound cytostatic drug, in contrast to free drug, protects the immune effector functions in tumor-bearing animals (24, 124). Similarly, we have detected an impairment of NK activity in *nu/nu* mice bearing human colorectal cancer SW620 xenografts and treated with free doxorubicin, while a significant increase in NK activity was observed in cancer-bearing mice treated with doxorubicin-bound to an HPMA copolymer carrier (Šírová, to be published).

There are indications that HPMA copolymer carrier-bound drugs not only spare, but in some way mobilize, the defensive antitumor response of the tumor-bearing host. HPMA-bound doxorubicin targeted with selected monoclonal antibodies, or even with a human polyclonal IVIg, represents a very efficient treatment of experimental cancer. Up to 40% of mice cured from mouse BCL_1 cell leukemia by doxorubicin–HPMA–B1 monoclonals and retransplanted with a lethal dose of the same malignant cells survived without any other treatment. Similarly, up to 30% of experimental mice transplanted with mouse B-cell lymphoma 38C13 and cured with doxorubicin–HPMA–anti-CD71 monoclonals survived retransplantation with a lethal dose of the same malignant cells. The same applies to mouse T-cell lymphoma EL4, where up to 90% of mice cured by doxorubicin–HPMA–anti-Thy 1.2 monoclonals survived retransplantation. If the mice developed a tumor, its growth was considerably slower compared to control mice (25, 124; Kovář, to be published). Antitumor efficacy and systemic anticancer immunity were also demonstrated in mice bearing murine EL4 T-cell lymphoma and in nude mice bearing human colorectal carcinoma SW620 treated with a doxorubicin–HPMA–IVIg conjugate (Šírová *et al.,* to be published). According to our preliminary results, the establishment of systemic immunity during treatment with antibody-targeted polymeric drugs requires a sufficient amount of antigen (cancer cells) available for a certain period of time when the immune system of the host is not yet exhausted (Kovář, to be published).

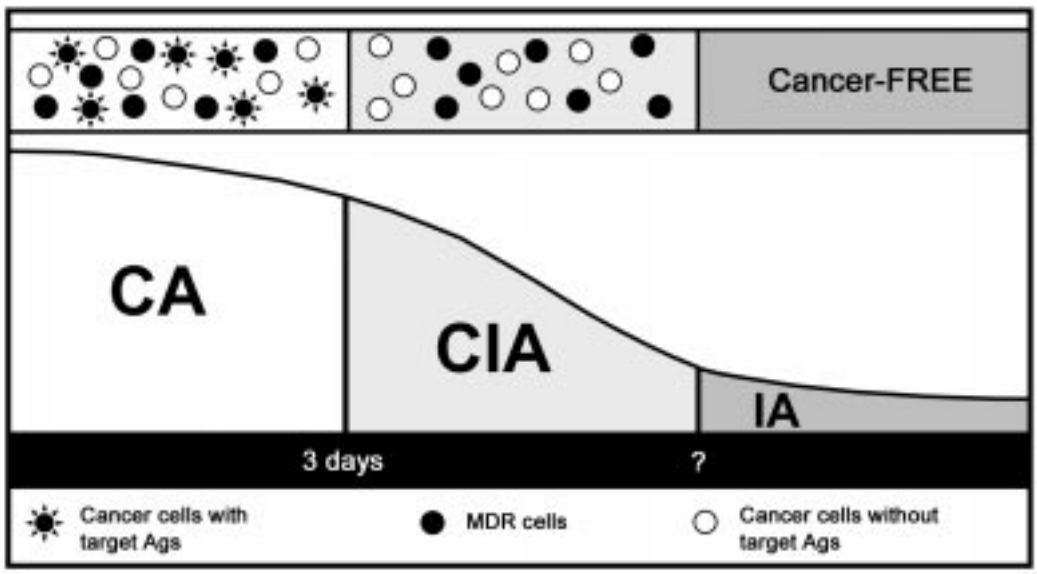

Fig. 3. Hypothetical cytotoxic and immunomobilizing activity of antibody-targeted HPMA copolymer–doxorubicin conjugate.

These results strongly support our hypothesis that, in addition to the proven cytostatic and cytotoxic activity, the impressive antitumor effect of antibody-targeted HPMA copolymer carrier-bound doxorubicin is also due to its remarkable immunoprotective and immunomobilizing activity. It is thought that, immediately after injection, due to the high levels of the drug, the primary activity of the polymeric conjugate is cytotoxic and cytostatic. Subsequently, long-term circulating HPMA-bound drug, at concentrations lower than the minimal inhibitory levels, mobilizes the defense mechanisms of the host (Fig. 3).

It was recently observed that stress proteins such as HSP70 stimulate immature dendritic cells (DC). Dendritic cells, which often infiltrate tumors, are potent APCs with the unique capacity to initiate primary immune responses. After binding antigen in the cancer tissue, they move to lymph nodes and activate T-cells. Activated effector T-lymphocytes by themselves or with the help of other cells of the defense system (macrophages, granulocytes, NK cells) kill cancer cells. It was reported that stress proteins are not released and thus the dendritic cells are not activated during apoptosis (137-139). This might explain why free drugs regularly induce only apoptosis and do not stimulate the immune response *in vivo*. As cancer cells exposed to antibody-targeted HPMA-bound drug die mostly by necrosis (93, 140), stress proteins released during necrotic death in large amounts might be involved in the immunostimulatory and immunomobilizing activity of antibody-targeted HPMA-bound drugs *in vivo*.

Other available data might also explain an immunoprotective effect of antibody-targeted HPMA-bound doxorubicin. One of the mechanisms by which malignant cells escape immune surveillance is Fas counterattack. The Fas receptor (Fas, FasR, APO-1, CD95) and its ligand (FasL, CD95L) are transmembrane proteins which, in complex, trigger a caspase-dependent apoptotic signal that culminates in programmed cell death, or apoptosis (141). It was initially thought that Fas/FasL-mediated apoptosis is only involved in physiological thymocyte clonal deletion, tolerance acquisition, immune response termination and elimination of transformed and infected cells. Recently, nonphysiological FasL expression has been demonstrated in various cancer cell lines and in several human malignancies *in vivo*. Fas counterattack induced by $FasL^+$ cancer cells in tumor-infiltrating Fas^+ effector cells of the immune system might eliminate, or at least minimize, any local antitumor immune response.

It has also been reported that the treatment with anticancer drugs such as doxorubicin, methotrexate, cytarabine, etoposide, cisplatin and others at therapeutic concentrations leads to the induction of FasL on cancer cells (142, 143). As free drugs and polymer-bound drugs differ in their intracellular accumulation and in their ability to induce drug-dependent apoptosis (93, 140), we addressed the question of whether surface expression of FasL could be reduced if malignant cells are exposed to a nontargeted or antibody-targeted HPMA copolymer-bound drug instead of free drug. Human colorectal cancer SW620 cells exposed to free doxorubicin or nontargeted or anti-CD71-targeted HPMA-bound drug were tested for surface FasL expression by FACS. Significant expression of FasL was observed only on the surface of SW620 cancer cells exposed to free doxorubicin, while on the cells exposed to nontargeted or an anti-CD71-targeted polymeric conjugate, FasL expression was not seen (124). This suggests that the signals involved in the increase of the surface expression of FasL are different in malignant cells exposed to the free or polymer-bound drug and that the administration of antibody-targeted HPMA polymer-bound doxorubicin, which does not

increase cancer cell-surface FasL expression, could spare the effector antitumor mechanisms of the host immune system.

Effect of doxorubicin bound to HPMA and containing human IVIg in patients with advanced cancer

Autologous immunoglobulin (Ig) and/or commercial IVIg were used as actively/passively targeting moieties in HPMA conjugates for the treatment of patients with disseminated cancer. One patient with generalized angiosarcoma and another with generalized breast carcinoma were treated with conjugates containing autologous Ig, while other patients were injected with HPMA-bound doxorubicin containing commercial IVIg (Intraglobin F; Biotest Pharma).

Intravenous immunoglobulin (IVIg, HuIg) is human serum Ig that is mainly composed of IgG prepared from plasma pools of thousands of healthy blood donors. It was demonstrated that administration of IVIg to mice inoculated with melanoma or sarcoma cells induced a statistically significant inhibition of metastatic lung foci and prolongation of survival time (144). *In vitro* studies revealed that IVIg stimulates the production of IL-12, a cytokine with a significant effect on the tumor-specific immune response, activates NK cells and contains natural antibodies directed against cell-membrane antigens of some tumors.

The patient with angiosarcoma underwent orthotopic heart transplantation and was kept on conventional combination adjuvant chemotherapy. In 1 year, she developed secondary tumors in the lung and was unsuccessfully treated with epirubicin dactinomycin and cyclophosphamide. Multiple secondaries in lungs and in soft tissues led to the initiation of palliative experimental therapy with HPMA copolymer-bound epirubicin targeted with autologous IgG, where specific antibodies against biopsy-proven soft tissue metastases were determined by ELISA. Administration of macromolecular therapeutics (the total dose of epirubicin was 265 mg/m^2) proceeded without any signs of intolerance. Antitumor efficacy was observed, with the disappearance of 95% of secondary lung tumors, as well as soft tissue metatases, after treatment. In fact, at this progressive stage of the disease, epirubicin–HPMA–IVIg was the only cytostatic with an obvious antitumor effect. Moreover, in contrast to free epirubicin, the polymeric conjugate did not show significant myelotoxicity, demonstrating its immunoprotective character (25, 27).

Seven patients with generalized breast carcinoma unsuccessfully treated by classical chemotherapy were injected with doxorubicin bound to an HPMA copolymer carrier containing commercial IVIg. The total dose was 495-741 mg doxorubicin equivalents/m^2. Analysis of plasma samples by gradient-based HPLC confirmed the persistence of macromolecular therapeutics in peripheral blood. Twenty-four hours after the first application of doxorubicin–HPMA–IVIg, the amount of doxorubicin in the blood of the first patient represented 70% of the injected dose, after 72 h it was 22% of the injected dose, and after 21 days about 1-2% of the injected dose remained (25, 28). In agreement with the data from clinical trials reported for nontargeted HPMA copolymer carrier-bound doxorubicin (PK1) (73, 145) and with the data obtained in experimental animals injected with nontargeted and antibody-targeted HPMA copolymer-bound daunorubicin (121), it was observed that the conjugate is stable and doxorubicin remains in the peripheral blood and urine, mostly in the polymer-bound form (27-29).

More than 116 biochemical and immunological parameters were tested in blood samples taken from the patients at different time intervals. A decrease of some parameters with pathological values before treatment was seen, and physiological values were even occasionally reached, including some tumor markers. Myelosuppression and cardiotoxicity were not observed. Doxorubicin–HPMA–IVIg does not induce an antibody response in the recipient organism, as up to 7 months after the first treatment of the first patient and more than 5 months after the first treatment of the second patient, ELISA did not reveal the presence of serum anti-human Ig antibodies (27-29).

The absolute number of $CD16^+56^+$ and $CD4^+$ cells was increased in the peripheral blood and activation of NK and LAK cells was seen in patients 72 h after the treatment, supporting data previously obtained in experimental animals pointing to a dual cytostatic and immunomobilizing character for doxorubicin–HPMA conjugates containing a targeting immunoglobulin moiety (26-29, 124).

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References

1. Vitetta, E.S., Uhr, J.W. *The potential use of immunotoxins in transplantation, cancer therapy, and immunoregulation.* Transplantation 1984, 37: 535-48.

2. Vitetta, E.S., Thorpe, P.E., Uhr, J.W. *Immunotoxins: Magic bullets or misquided missiles?* Immunol Today 1993, 14: 252-64.

3. Šťastný, M., Ulbrich, K., Strohalm, J., Rossmann, P., Říhová, B. *Abnormal differentiation of thymocytes induced by free cyclosporine is avoided when cyclosporine bound to N-(2-hydroxypropyl)methacrylamide copolymer carrier is used.* Transplantation 1997, 63: 1818-27.

4. Rossmann, P., Říhová, B., Strohalm, J., Ulbrich,K. *Morphology of rat kidney and thymus after native and antibody-coupled cyclosporin A application (reduced toxicity of targeted drug).* Folia Microbiol 1997, 42: 277-87.

5. Jansen, F.K., Blythman, H.E., Cariére, D. et al. *Immunotoxins: Hybrid molecules combining high specificity and potent cytotoxicity.* Immunol Rev 1982, 62: 185-216.

6. Arnon, R., Sela, M. *In vitro and in vivo efficacy of conjugates of daunomycin with anti-tumor antibodies.* Immunol Rev 1982, 62: 5-27.

7. Raso, V. *Antibody mediated delivery of toxic molecules to antigen bearing target cells.* Immunol Rev 1982, 62: 93-117.

8. Neville, D.M., Youle, R.J. *Monoclonal antibody-ricin or ricin A chain hybrids: Kinetic analysis of cell killing for tumor therapy.* Immunol Rev 1982, 62: 75-91.

9. Vitetta, E.S., Krolick, K.A., Uhr, J.W. *Neoplastic B cells as targets for antibody-ricin A chain immunotoxins.* Immunol Rev 1982, 62, 159-83.

10. Press, O.W., Howell-Clark, S., Anderson, I., Bernstein, I. *Retention of B-cell specific monoclonal antibodies by human lymphoma cells.* Blood 1994, 83: 1390-7.

11. Gottstein, C., Winkler, U., Bohlen, H., Diehl, V., Engert, A. *Immunotoxins: Is there a clinical value?* Ann Oncol 1994, 5: S97-S103.

12. Kopeček, J. *Controlled degradability of polymers - A key to drug delivery systems.* Biomaterials 1984, 5: 19-25.

13. Kopeček, J. *The potential of water-soluble polymeric carriers in targeted and site-specific drug delivery.* J Control Release 1990, 11: 279-90.

14. Kopeček, J., Kopečková, P., Minko, T., Lu, Z.R. *HPMA copolymer-anticancer drug conjugates: Design, activity, and mechanism of action.* Eur J Pharmaceut Biopharm 2000, 50: 61-81.

15. Kopeček, J., Kopečková, P., Minko, T., Lu, Z.-R., Peterson, C.M. *Water soluble polymers in tumor targeted delivery.* J Control Release 2001, 74: 147-58.

16. Říhová, B., Kopeček, J. *Biological properties of targetable poly[N-(2-hydroxypropyl) methacrylamide]-antibody conjugates.* J Control Release 1985, 2: 289-310.

17. Říhová, B., Kopeček, J., Kopečková-Rejmanová, P., Strohalm, J., Plocová, D., Semorádová, H. *Bioaffinity therapy with antibodies and drugs bound to soluble synthetic polymers.* J Chromatogr Biomed Appl 1986, 376: 221-33.

18. Říhová, B., Kopečková, P., Strohalm, J., Rossmann, P., Vetvička, V., Kopeček, J. *Antibody-directed affinity therapy applied to the immune system: In vivo effectiveness and limited toxicity of daunomycin conjugated to HPMA copolymers and targeting antibody.* Clin Immunol Immunopathol 1988, 46: 100-14.

19. Říhová, B., Krinick, N. L., Kopeček, J. *Targetable photoactivatable drugs.* J Mater Sci: Mater Med 1991, 2: 238-42.

20. Říhová, B., Jegorov, A., Strohalm, J., Mat'ha, V., Rossmann, P., Fornůsek, L., Ulbrich, K *Antibody-targeted cyclosporin A.* J Control Release 1992, 19: 25-39.

21. Říhová, B. *Antibody-targeted polymer-bound drugs.* Folia Microbiol 1995, 40: 367-84.

22. Říhová, B., Strohalm, J., Plocová, D. et al. *Cytotoxic and cytostatic effects of anti-Thy 1.2 targeted doxorubicin and cyclosporin A.* J Control Release 1996, 40: 303-19.

23. Říhová, B., Jelínková, M., Strohalm, J. et al. *The anti-proliferative effect of a lectin- and anti-Thy-1.2 antibody-targeted HPMA copolymer-bound doxorubicin on primary and metastatic human colorectal carcinoma and on human colorectal carcinoma transfected with mouse Thy-1.2 gene.* Bioconj Chem 2000, 11: 664-73.

24. Říhová, B., Jelínková, M., Strohalm, J. et al. *Polymeric drugs based on conjugates of synthetic and natural macromolecules. II. Anti-cancer activity of antibody or $(Fab')_2$-targeted conjugates and combined therapy with immunomodulators.* J Control Release 2000, 64: 241-61.

25. Říhová, B., Strohalm, J., Kubáčková, K. et al. *Acquired and specific immunological mechanisms co-responsible for efficacy of polymer-bound drugs.* J Control Release 2002, 78: 97-114.

26. Říhová, B. *Immunomodulating activities of soluble synthetic polymer-bound drugs.* Adv Drug Del Rev 2002, 54: 653-74.

27. Říhová, B., Strohalm, J., Kubáčková, K. et al. *Drug-HPMA-HuIg conjugates effective against human solid cancer.* In: Polymer Drugs in the Clinical Stage. Maeda et al. (Eds.). Kluwer Academic/Plenum Publishers: New York 2003, 125-43.

28. Říhová, B., Strohalm, J., Prausová, J. et al. *Cytostatic and immunomobilizing activities of polymer-bound drugs; experimental and first clinical data.* J Control Release 2003, 91: 1-16.

29. Říhová, B., Kubáčková, K. *Clinical implications of N-(2-hydroxypropyl)methacrylamide copolymers.* Curr Pharmaceut Biotechnol 2003, 4: 311-22.

30. Flanagan, P.A., Kopečková, P., Kopeček, J., Duncan, R. *Evaluation of protein-N-(2-hydroxypropyl)methacrylamide copolymer conjugates as targetable drug carriers. 1. Binding, pinocytic uptake and intracellular distribution of transferrin and anti-transferrin receptor antibody conjugates.* Biochim Biophys Acta 1989, 993: 83-91.

31. Flanagan, P.A., Duncan, R., Říhová, B., Šubr, V., Kopeček, J. *Immunogenicity of protein-N-(2-hydroxypropyl)methacrylamide copolymer conjugates in A/J and B10 mice.* J Bioact Comp Polym 1990, 5: 151-66.

32. Šťastný, M., Říhová, B., Strohalm, J., Ulbrich, K. *Comparison of the binding kinetics of antibody-targeted N-(2-hydroxypropyl)methacrylamide (HPMA)-bound doxorubicin in vitro and in vivo.* J Control Release 1996, 42: 229-36.

33. Šťastný, M., Strohalm, J., Plocová, D., Ulbrich, K., Říhová, B. *A possibility to overcome P-glycoprotein (PGP)-mediated multidrug resistance by antibody-targeted drugs conjugated to N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer carrier.* Eur J Cancer 1999, 35: 459-66.

34. Krinick, N.L., Říhová, B., Ulbrich, K., Strohalm, J., Kopeček, J. *Targetable photoactivatable drugs, 2. Synthesis of N-(2-hydroxypropyl)methacrylamide copolymer-anti-Thy 1.2 antibody-chlorin e_6 conjugates and a preliminary study of their photodynamic effect on mouse splenocytes in vitro.* Makromol Chem 1990, 191: 839-56.

35. Omelyanenko, V., Gentry, C., Kopečková, P., Kopeček, J. *HPMA copolymer-anticancer drug-OV-TL16 antibody conjugates. II. Processing in epithelial ovarian carcinoma cells in vitro.* Intl J Cancer 1998, 75: 600-8.

36. Omelyanenko, V., Kopečková, P., Gentry, C., Kopeček, J. *Targetable HPMA copolymer-adriamycin conjugates. Recognition, internalization, and subcellular fate.* J Control Release 1998, 53: 25-37.

37. Kovář, M., Strohalm, J., Ulbrich, K., Říhová, B. *In vitro and in vivo effect of HPMA copolymer-bound doxorubicin targeted to transferrin receptor of B-cell lymphoma 38C13.* J Drug Targ 2002, 10: 23-30.

38. Kovář, M., Strohalm, J., Etrych, T., Ulbrich, K., Říhová, B. *Star structure of antibody-targeted HPMA copolymer-bound doxorubicin: A novel type of polymeric conjugate for targeted drug delivery with potent antitumor effect.* Bioconj Chem 2002, 13: 206-15.

39. Shiah, J.-G., Sun, Y., Kopečková, P., Peterson, C.M., Straight, R.C., Kopeček, J. *Combination chemotherapy and photodynamic therapy of targetable N-(2-hydroxypropyl)methacrylamide copolymer-doxorubicin/mesochlorin e_6-OV-TL 16 antibody immunoconjugates.* J Control Release 2001, 74: 249-53.

40. Duncan, R., Kopeček, J., Rejmanová, P., Lloyd, J.B. *Targeting of N-(2-hydroxypro-pyl)methacrylamide copolymers to liver by incorporation of galactose residues.* Biochim Biophys Acta 1983, 755: 518-21.

41. Duncan, R., Kopeček, J., Lloyd, J.B. *Drug targeting to lysosomes.* Biochem Soc Trans 1984, 12: 913-5.

42. Duncan, R., Seymour, L.C.W., Scarlett, L., Lloyd, J.B., Rejmanová, P., Kopeček, J. *Fate of N-(2-hydroxypropyl)-methacrylamide copolymers with pendent galactosamine residues after intravenous administration to rats.* Biochim Biophys Acta 1986, 880: 62-71.

43. Duncan, R., Kopečková-Rejmanová, P., Strohalm, J. et al. *Anticancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. 1. Evaluation of daunomycin and puromycin conjugates in vitro.* Br J Cancer 1987, 55: 165-74.

44. Duncan, R., Kopečková, P., Strohalm, J., Hume, I.C., Lloyd, J.B., Kopeček, J. *Anticancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. II. Evaluation of daunomycin conjugates in vivo against L1210 leukaemia.* Br J Cancer 1988, 57: 147-56.

45. Duncan, R., Hume, I.C., Yardley, H.J., Flanagan, P.A., Ulbrich, K., Šubr, V., Strohalm, J. *Macromolecular prodrugs for use in targeted cancer chemotherapy: Melphalan covalently coupled to N-(2-hydroxypropyl)methacrylamide copolymers.* J Control Release 1991, 16: 121-36.

46. Říhová, B., Krinick, N.L., Kopeček, J. *Targetable photoactivatable drugs. 3. In vitro efficacy of polymer bound chlorin e_6 toward human hepatocarcinoma cell line (PLC/PRF/5) targeted with galactosamine and to mouse splenocytes targeted with anti-Thy 1.2 antibodies.* J Control Release 1993, 25: 71-87.

47. Seymour, L.W., Flanagan, P.A., Al-Shamkhani, A., Šubr, V., Ulbrich, K., Cassidy, J., Duncan, R. *Synthetic polymers conjugated to monoclonal antibodies: Vehicles for tumour-targeted drug delivery.* Select Cancer Ther 1991, 7: 59-73.

48. Rathi, R.C., Kopečková, P., Říhová, B., Kopeček, J. *N-(2-Hydroxypropyl)methacrylamide copolymers containing pendant saccharide moieties. Synthesis and bioadhesive properties.* J Polym Sci Part A: Polym Chem 1991, 29: 1895-902.

49. Wroblewski, S., Berenson, M., Kopečková, P., Kopeček, J. *Biorecognition of HPMA copolymer-lectin conjugates as an indicator of differentiation of cell-surface glycoproteins in development, maturation and diseases of human, and rodent gastrointestinal tissues.* J Biomed Mater Res 2000, 329-42.

50. Wroblewski, S., Říhová, B., Rossmann, P., Hudcovicz, T., Řeháková, Z., Kopečková, P., Kopeček, J. *The influence of a colonic microbiota on HPMA copolymer-lectin conjugates binding in rodent intestine.* J Drug Targ 2001, 9: 85-94.

51. O'Hare, K.B., Duncan, R., Strohalm, J., Ulbrich, K., Kopečková, P. *Polymeric drug-carriers containing doxorubicin and melanocyte-stimulating hormone: In vitro and in vivo evaluation against murine melanoma.* J Drug Targ 1993, 1: 217-29.

52. Omelyanenko, V., Kopečková, P., Prakash, R., Ebert, Ch. D., Kopeček, J. *Biorecognition of HPMA copolymer-adriamycin conjugates by lymphocytes mediated by synthetic receptor binding epitopes.* Pharm Res 1999, 16: 1010-9.

53. Kopeček, J., Rejmanová, P., Strohalm, J. et al. *Synthetic polymeric drugs.* US 5037883.

54. Rejmanová, P., Kopeček, J., Duncan, R., Lloyd, J.B. *Stability in rat plasma and serum of lysosomally degradable oligopeptide sequences in N-(2-hydroxypropyl)methacrylamide copolymers.* Biomaterials 1985, 6: 45-8.

55. Duncan, R., Cable, H.C., Lloyd, J.B., Rejmanová, P., Kopeček, J. *Degradation of side-chains of N-(2-hydroxypropyl)methacrylamide copolymers by lysosomal thiol-proteinases.* Biosci Rep 1982, 2: 1041-6.

56. Encyclopedia of Immunology, 2nd Ed. Delves, P.J. and Roitt, I.M. (Eds.). Academic Press 1998.

57. Říhová, B., Ulbrich, K., Kopeček, J., Mančal, P. *Immunogenicity of N-(2-hydroxypropyl)methacrylamide copolymers - Potential hapten or drug carriers.* Folia Microbiol 1983, 28: 217-27.

58. Říhová, B., Kopeček, J., Ulbrich, K., Pospíšil, J., Mančal, P. *Effect of the chemical structure of N-(2-hydroxypropyl)methacrylamide copolymers on their ability to induce antibody formation in inbred strains of mice.* Biomaterials 1984, 5: 143-8.

59. Říhová, B., Kopeček, J., Ulbrich, K., Chytrý, V. *Immunogenicity of N-(2-hydroxypropyl) methacrylamide copolymers.* Makromol Chem Suppl 1985, 9: 13-24.

60. Šimečková, J., Říhová, B., Plocová, D., Kopeček, J. *The activity of complement in the presence of N-(2-hydroxypropyl)methacrylamide copolymers.* J Bioact Comp Polym 1986, 1: 20-31.

61. Volfová, I., Říhová, B., Větvička, V., Rossmann, P., Ulbrich, K. *Biocompatibility of biopolymers.* J Bioact Comp Polym 1992, 7: 175-90.

62. Šubr, V., Etrych, T., Ulbrich, K. et al. *Synthesis and properties of poly[N-(2-hydropxypropyl)methacrylamide] conjugates of superoxide dismutase.* J Bioact Comp Polym 2002, 17: 105-22.

63. Oupický, D., Ulbrich, K., Říhová, B. *Conjugates of semitelechelic poly[N-(2-hydroxypropyl)methacrylamide] with enzymes for protein delivery.* J Bioact Comp Polym 1999, 14: 213-31.

64. Abuchowski, A., McCoy, J.R., Palczuk, N.C., Van Es, T., Davis, F.F. *Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase.* J Biol Chem 1977, 252: 3582-6.

65. Matsumura, Y., Maeda, H. *A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanisms of tumoritropic accumulation of proteins and the antitumor agent.* Cancer Res 1986, 6: 6387-92.

66. Maeda, H., Matsumura, Y. *Tumoritropic and lymphotropic principles of macromolecular drugs.* CRC Crit Rev Ther Drug Carrier Syst 1992, 9: 135-87.

67. Maeda, H., Seymour, L.W., Miyamoto, Y. *Conjugates of anticancer agents and polymers: Advantages of macromolecular therapeutics in vivo.* Bioconj Chem 1992, 3: 351-5.

68. Noguchi, Y., Wu, J., Duncan, R., Strohalm, J., Ulbrich, K., Akaike, T., Maeda, H. *Early phase tumor accumulation of macromolecules: A great difference in clearance rate between tumor and normal tissues.* Jpn J Cancer Res 1998, 89: 307-14.

69. Ulbrich, K., Zacharieva, E.I., Kopeček, J., Hume, I.C., Duncan, R. *Polymer-bound derivatives of sarcolysin and their antitumour activity against mouse and human leukemia in vitro.* Makromol Chem 1987, 188: 2497-509.

70. Minko, T., Kopečková, P., Kopeček, J. *Efficacy of chemotherapeutic action of HPMA copolymer-bound doxorubicin in a solid tumor model of ovarian carcinoma.* Intl J Cancer 2000, 86: 108-17.

71. Cassidy, J., Duncan, R., Morrison, G.J., Strohalm, J., Plocová, D., Kopeček, J., Kaye, S.B. *Activity of N-(2-hydroxypropyl)methacrylamide copolymers containing daunomycin against a rat tumour model.* Biochem Pharmacol 1989, 38: 875-9.

72. Shiah, J.-G., Sun, Y., Peterson, C.M., Kopeček, J. *Biodistribution of free and N-(2-hydroxypropyl)methacrylamide copolymer-bound mesochlorin e_6 and adriamycin in nude mice bearing human ovarian carcinoma OVCAR-3 xenografts.* J Control Release 1999, 61: 145-57.

73. Vasey, P.A., Kaye, S.B., Morrison, R. et al. *Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)-methacrylamide copolymer-doxorubicin]: First member of a new class of chemotherapeutic agents-drug-polymer conjugates.* Clin Cancer Res 1999, 5: 83-94.

74. Říhová, B. *Receptor-mediated targeted drug or toxin delivery.* Adv Drug Del Rev 1998, 29: 273-89.

75. Říhová, B., Strohalm, J., Plocová, D., Ulbrich, K. *Selectivity of antibody-targeted anthracycline antibiotics on T lymphocytes.* J Bioact Comp Polym 1990, 5: 249-66.

76. Kovář, M., Mrkvan, T., Strohalm, J., Etrych, T., Ulbrich, K., Šťastný, M., Říhová, B. *HPMA copolymer-bound doxorubicin targeted to tumor specific antigen of BCL1 mouse B cell leukemia.* J Control Release 2003, 29: 315-30.

77. Jelínková, M., Strohalm, J., Etrych, T., Ulbrich, K., Říhová, B. *Starlike versus classical macromolecular prodrugs: Two different antibody-targeted HPMA copolymers of doxorubicin studied in vitro and in vivo as potential anticancer drugs.* Pharm Res 2003, 20: 1558-64.

78. Press, O.W., Vitetta, E., Farr, A., Hansen, J., Martin, P. *Evaluation of ricin A chain immunotoxins directed against human T cells.* Cell Immunol 1986, 102: 10-20.

79. Thorpe, P.E., Blakey, D.C., Brown, A.N. et al. *Comparison of two anti-Thy 1.1-abrin A-chain immunotoxins prepared with different cross-linking agents: Antitumor effects, in vivo fate, and tumor cell mutants.* J Natl Cancer Inst 1987, 79: 1101-12.

80. Press, O.W., Martin, J.P., Thorpe, P.E., Vitetta, E.S. *Ricin A-chain containing immunotoxins directed against different epitopes on the CD2 molecule differ in their ability to kill normal and malignant T cells.* J Immunol 1988, 141: 4410-7.

81. Manetti, C. Le Doussal, J.M., Rouvier, E., Gruaz-Guyon, A., Barbet, J. *Intracellular uptake and catabolism of anti-IgM antibodies and bispecific antibody-targeted hapten by B-lymphoma cells.* Intl J Cancer 1995, 63: 250-6.

82. Matthay, K.K., Abai, A.M., Cobb, S., Hong, K., Papahadjopoulos, D., Straubinger, R.M. *Role of ligand in antibody-directed endocytosis of liposomes by human T-leukemia cells.* Cancer Res 1989, 49, 4879-86.

83. Ali, S.A., Waren, S.D., Richter, K.Y. et al. *Improving the tumor retention of radioiodinated antibody: Aryl carbohydrate aducts.* Cancer Res 1990, 50: 783s-8s.

84. Jelínková, M., Strohalm, J., Plocová, D., Šubr, V., Šťastný, M., Ulbrich, K., Říhová, B. *Targeting of human and mouse T-lymphocytes by monoclonal antibody-HPMA copolymer-doxorubicin conjugates directed against different T-cell surface antigens.* J Control Release 1998, 52: 253-70.

85. Říhová, B., Vereš, V., Fornůsek, L. et al. *Action of polymeric prodrugs based on N-(2-hydroxypropyl)methacrylamide copolymers. II. Body distribution and T-cell accumulation of free and polymer bound [^{125}I]daunomycin.* J Control Release 1989, 10: 37-49.

86. Duncan, R. *Drug-polymer conjugates: Potential for improved chemotherapy.* Anticancer Drugs 1992, 3: 175-210.

87. Říhová, B.,Větvička, V., Strohalm, J., Ulbrich, K., Kopeček, J. *Action of polymeric prodrugs based on N-(2-hydroxypropyl)-methacrylamide copolymers. I. Suppression of the antibody response and proliferation of mouse splenocytes in vivo.* J Control Release 1989, 9: 21-32.

88. Duke-Cohan, J.S., Morimoto, Ch., Schlossman, S.F. *Targeting of an activated T-cell subset using a bispecific antibody-toxin conjugate directed against CD4 and CD26.* Blood 1993, 82: 2224-34.

89. Kummer, U., Thierfelder, S., Mysliwietz, J. *Antigen density on target cells determines the immunosuppressive potential of rat IgG_{2b} monoclonal antibodies.* Eur J Immunol 1990, 20: 107-12.

90. Rejmanová, P., Kopeček, J., Pohl, J., Baudyš, M., Kostka, V. *Polymers containing enzymatically degradable bonds, 8. Degradation of oligopeptide sequences in N-(2-hydroxypropyl) methacrylamide copolymers by bovine spleen cathepsin B.* Makromol Chem 1983, 184: 2009-20.

91. Flanagan, P.A., Duncan, R., Šubr, V., Ulbrich, K., Kopečková, P., Kopeček, J. *Evaluation of protein-N-(2-hydroxypropyl)-methacrylamide copolymer conjugates as targetable drug-carriers. 2. Body distribution of conjugates containing transferrin, antitransferrin receptor antibody or anti-Thy 1.2 antibody and effectiveness of transferrin-containing daunomycin conjugates against mouse L1210 leukaemia in vivo.* J Control Release 1992, 18: 25-38.

92. Ulbrich, K., Strohalm, J., Šubr, V., Plocová, D., Duncan, R., Říhová, B. *Polymeric conjugates of drugs and antibodies for site-specific drug delivery.* Makromol Symp 1996, 103: 177-92.

93. Hovorka, O., Šťastný, M., Etrych, T., Šubr, V., Strohalm, J., Ulbrich, K., Říhová, B. *Differences in the intracellular fate of free and polymer-bound doxorubicin.* J Control Release 2002, 80: 101-17.

94. Etrych, T., Jelínková, M., Říhová, B., Ulbrich, K. *New HPMA copolymers containing doxorubicin bound via pH-sensitive linkage: Synthesis and preliminary in vitro and in vivo biological properties.* J Control Release 2001, 73: 89-102.

95. Říhová, B., Etrych, T., Pechar, M. et al. *Doxorubicin bound to a HPMA copolymer carrier through hydrazone bond is effective also in a cancer cell line with a limited content of lysosomes.* J Control Release 2001, 74: 225-32.

96. Ulbrich, K., Etrych, T., Chytil, P., Jelínková, M., Říhová, B. *HPMA copolymers with pH-controlled release of doxorubicin in vitro cytotoxicity and in vivo antitumor activity.* J Control Release 2003, 87: 33-47.

97. Omelyanenko, V., Kopečková, P., Gentry, C., Shiah, J.-G., Kopeček, J. *HPMA copolymer - anticancer drug - OV-TL16 antibody conjugates. 1. Influence of the method of synthesis on the binding affinity to OVCAR-3 ovarian carcinoma cells in vitro.* J Drug Target 1996, 3: 357-73.

98. Ulbrich, K., Pechar, M., Strohalm, J., Šubr, V., Říhová, B. *Polymeric carriers of drugs for site-specific therapy*. Makromol Symp 1997, 118: 577-85.

99. Ulbrich, K., Šubr, V., Strohalm, J., Plocová, D., Jelínková, M., Říhová, B. *Polymeric drugs based on conjugates of synthetic and natural macromolecules. I. Synthesis and physico-chemical characterization.* J Control Release 2000, 64: 63-79.

100. Lu, Z.-R., Kopečková, P., Kopeček, J. *Polymerizable Fab' antibody fragments for targeting of anticancer drugs.* Nature Biotechnol 1999, 17: 1101-4.

101. Waldor, M.K., Sriram, S., McDevitt, H.O., Steinman, L. *In vivo therapy with monoclonal anti-I-A antibody suppresses immune responses to acetylcholine receptor.* Proc Natl Acad Sci USA 1983, 80: 2713-7.

102. Tarnowski, G.S., Ralph, P., Stock, C.Ch. *Sensitivity to chemotherapeutic and immunomodulating agents of two mouse lymphomas and of a macrophage tumor.* Cancer Res 1979, 39: 3964-7.

103. Otsuji, E., Yamaguchi, T., Tsuruta, H. et al. *Applicability of monoclonal antibody Fab fragments as a carrier of neocarzinostatin in targeting chemotherapy.* J Surg Oncol 1996, 61: 149-54.

104. Lang, L., Jagoda, E., Wu, C. et al. *Factors influencing the in vivo pharmacokinetics of peptides and antibody fragments: The pharmacokinetics of two PET-labeled low molecular weight proteins.* Q J Nucl Med 1997, 41: 53-61.

105. Waldmann, T.A. *Monoclonal antibodies in diagnosis and therapy.* Science 1991, 252: 1657-62.

106. Juweid, M., Sharkey, R.M., Behr, T.M. et al. *Clinical evaluation of tumor targeting with the carcinoembryonic antigen murine monoclonal antibody fragment, MN-14F(ab)$_2$.* Cancer 1996, 78: 157-68.

107. Martell, L.A., Agrawal, A., Ross, D.A., Muraszko, K.M. *Efficacy of transferrin receptor-targeted immunotoxins in brain tumor cell lines and pediatric brain tumors.* Cancer Res 1993, 53: 1348-53.

108. Jiang, A., Chu, T.H., Nocken, F., Cichutek, K., Dornburg, R. *Cell-type-specific gene transfer into human cells with retroviral vectors that display single-chain antibodies.* J Virol 1998, 72: 10148-56.

109. Denis, O., Macedo Soares, F., Latinne, D., Nisol, F., Bazin, H. *In vivo study of mIgM and mIgD cross-linking on murine B cells.* Scand J Immunol 1994, 39: 625-32.

110. Van Niekerk, C.C., Rameakers, F.C.S., Hanselaar, A.G.J.M., Aldeweireldt, J., Poels, L.G. *Changes in expression of differentiation markers between normal ovarian cells and derived tumors.* Am J Pathol 1993, 142: 157-77.

111. Boerman, O., Massuger, L., Makkink, K., Thomas, C., Kenemans, P., Poels, L. *Comparative in vitro binding characteristics and biodistribution in tumor-bearing athymic mice of anti-ovarian carcinoma monoclonal antibodies.* Anticancer Res 1990, 10: 1289-96.

112. Putnam, D., Kopeček, J. *Polymer conjugates with anticancer activity.* Adv Polym Sci 1995, 122: 55-123.

113. Timour, Q., Nony, P., Lang, J., Lakhal, M., Trillet, V., Faucon, G. *Doxorubicin concentration time course in the myocardium after single administration to the dog.* Cancer Chemother 1987, 20: 267-9.

114. Rossi C., Gasparini, G., Canobbio, L. et al. *Doxorubicin distribution in human breast cancer.* Cancer Treat Rep 1987, 71: 1221-6.

115. Brenner, D.E., Wiernik, P.H., Wesley, M., Bachur, N.R. *Acute doxorubicin toxicity. Relationship to pretreatment, liver function, response and pharmacokinetics in patients with acute nonlymphocytic leukemia.* Cancer 1984, 53: 1042-8.

116. Terasaki, T., Iga, T., Sugiyama, Y., Hanano, M. *Pharmacokinetic study of the mechanism of tissue distribution of doxorubicin. Inter-organ and inter-species variation in tissue to plasma partition coefficients in rats, rabbits and guinea pigs.* J Pharm Sci 1984, 73: 1359-62.

117. Seymour, L.W., Duncan, R., Strohalm, J., Kopeček, J. *Effect of molecular weight (MW) of N-(2-hydroxypropyl)methacrylamide copolymers on body distribution and rate of excretion after subcutaneous, intraperitoneal, and intravenous administration to rats.* J Biomed Mater Res 1987, 21: 1341-58.

118. Duncan, R., Cable, H.C., Rejmanová, P., Kopeček, J., Lloyd, J.B. *Tyrosinamide residues enhance pinocytic capture of N-(2-hydroxypropyl)methacrylamide copolymers.* Biochim Biophys Acta 1984, 799: 1-8.

119. De Duve, C., De Barsy, T., Poole, B. et al. *Lysosomotropic agents.* Biochem Pharmacol 1974, 23: 2495-531.

120. Hertler, A.A., Frankel, A.E. *Immunotoxins: A clinical review of their use in the treatment of malignancies.* J Clin Oncol 1989, 7: 1932-42.

121. Říhová, B., Bilej, M., Větvička, V., Ulbrich, K., Strohalm, J., Kopeček, J., Duncan, R. *Biocompatibility of N-(2-hydroxypropyl)methacrylamide copolymers containing adriamycin.* Biomaterials 1989, 10: 335-42.

122. Yeung, T.K., Hopewell, J.W., Simmonds, R.H. et al. *Reduced cardiotoxicyty of doxorubicin given in the form of N-(2-hydroxypropyl)methacrylamide cojugates: An experimental study in the rat.* Cancer Chemother Pharmacol 1991, 29: 105-11.

123. Hopewell, J.W., Duncan, R., Wilding, D., Chakrabarti, K. *Preclinical evaluation of the cardiotoxicity of PK2: A novel HPMA copolymer-doxorubicin-galactosamine conjugate antitumour agent.* Hum Exp Toxicol 2001, 20: 461-70.

124. Říhová, B., Strohalm, J., Hoste, K. et al. *Immunoprotective therapy with targeted anticancer drugs.* Makromol Symp 2001, 172: 21-8.

125. Paluska, E., Hrubá, A., Šterba, O., Kopeček, J. *Effect of a synthetic poly-N-(2-hydroxypropyl)methacrylamide (DUXON) on haemopoiesis and graft-versus-host reaction*. Folia Biol 1986, 32: 91-102.

126. Endicott, J.A., Ling, V. *The biochemistry of P-glycoprotein-mediated multidrug resistance.* Annu Rev Biochem 1989, 58: 137-71.

127. Gottesman, M.M. *How cancer cells evade chemotherapy: Sixteenth Richard and Hinda Rosenthal Foundation Award Lecture.* Cancer Res 1993, 53: 747-54.

128. Pinedo, H.M., Giaccone, G. *P-glycoprotein-a marker of cancer-cell behavior.* New Engl J Med 1995, 333: 1417-9.

129. Minko, T., Kopečková, P., Kopeček, J. *Chronic exposure to HPMA copolymer-bound adriamycin does not induce multidrug resistance in a human ovarian carcinoma cell line.* J Control Release 1999, 59: 133-48.

130. Suzuki, H., Zelphati, O., Hildebrand, G., Leserman, L. *CD4 and CD7 molecules are targets for drug delivery from antibody bearing liposomes.* Exp Cell Res 1991, 193: 112-9.

131. Van Oosterhout, Y.V., van den Herik-Oudijk, I.E., Wessels, H.M., de Witte, T., van de Winkel, J.G., Preijers, F.W. *Effect of isotype on internalization and cytotoxicity of CD19-ricin A immunotoxins.* Cancer Res 1994, 54: 3527-32.

132. Venne, A., Li, S., Mandeville, R., Kabanov, A., Alakhov, V. *Hypersensitizing effect of pluronic L61 on cytotoxic activity, transport and subcellular distribution of doxorubicin in multi-drug resistant cells.* Cancer Res 1996, 56: 3626-9.

133. Alakhov, V.Y., Moskaleva, E.U., Batrakova, E.V. Kabanov, A. *Hypersensitization of multidrug resistant human ovarian carcinoma cells by pluronic P85 block copolymer*. Bioconj Chem 1996, 7: 209-16.

134. Dallas, G. et al. *Drug transporters in the central nervous system: Brain barriers and brain parenchyma consideration.* Pharmacol Rev 2001, 53: 569-96.

135. Hunter, A.Ch., Moghimi, S.M. *Therapeutic synthetic polymers: A game of Russian roulette?* DDT 2002, 7: 998-1001.

136. Luo, W., Tapolsky, M., Earley, K., Wood, C.G., Wilson, D.R., Logothetis, C.J., Lin, S.H. *Tumor-suppressive activity of CD66a in prostate cancer.* Cancer Gene Ther 1999, 6: 313-21.

137. Asea, A., Kraeft., S.K., Kurt-Jones, E.A. et al. *HSP70 stimulates cytokine production through a CD 14-dependent pathway, demonstrating its dual role as a chaperone and cytokine.* Nature Med 2000, 6: 435-42.

138. Srivastava, P.K., Menoret, A., Basu, S., Binder, R.J., McQuade, K.L. *Heat shock proteins come of age: Primitive functions acquire new roles in an adaptive world.* Immunity 1998, 8: 657-65.

139. Srivastava, P.K., Udono, H. *Heat shock protein-peptide complexes in cancer immunotherapy.* Curr Opin Immunol 1994, 6: 728-32.

140. Demoy, M., Minko, T., Kopečková, P., Kopeček, J. *Time- and concentration-dependent apoptosis and necrosis induced by free and HPMA copolymer-bound doxorubicin in human ovarian carcinoma cells.* J Control Release 2000, 69: 185-96.

141. O´Connell, J., Bennett, M.W., O´Sullivan, G.C., Collins, J.K., Shanahan, F. *The Fas counterattack: Cancer as a site of immune privilege.* Immunol Today 1999, 20: 46-52.

142. Friesen, C., Herr, I., Krammer, P.H., Debatin, K.M. *Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells.* Nature Med 1996, 2: 574-7.

143. Friesen, C., Fulda, S., Debatin, K.M. *Cytotoxic drugs and the CD95 pathway.* Leukemia 1999, 13: 1854-8.

144. Shoenfeld, Y., Fishman, P. *Gamma-globulin inhibits tumor spread in mice.* Intl Immunol 1999, 11: 1247-52.

145. Thomson, A.H., Vasey, P.A., Murray, L.S., Cassidy, J., Fraier, D., Frigerio, E., Twelves, C. *Population pharmacokinetics in phase I drug development: A phase I study of PK1 in patients with solid tumours.* Br J Cancer 1999, 81: 99-107.