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Review article

HPMA copolymer–anticancer drug conjugates: design, activity, and mechanism of action

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Abstract

The design, synthesis and properties of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers as carriers of anticancer drugs are reviewed. Macromolecular therapeutics based on HPMA copolymers are biocompatible, preferentially accumulate in tumors, and possess a higher anticancer efficacy than low molecular weight drugs. Novel designs of HPMA copolymer carriers resulted in long-circulating conjugates and gene and oligonucleotide delivery systems. HPMA copolymer based macromolecular therapeutics were active against numerous cancer models and are in clinical trials. The data obtained indicated that macromolecular therapeutics activated different signaling pathways and possessed a different mechanism of action than free drugs. This bodes well for the success of future research aimed at identification of new intracellular molecular targets as a basis for the design of the second generation of macromolecular therapeutics.

Keywords: HPMA copolymer; Anticancer drugs; Macromolecular therapeutics; HPMA copolymer based prodrugs; Cancer treatment; Multidrug resistance

1. Introduction

The idea of using macromolecules as carriers of (anticancer) drugs developed continuously over the last 90 years. Ehrlich in 1906 coined the phrase 'magic bullet', recognizing the importance of biorecognition [1]. DeDuve discovered that many enzymes are localized in the lysosomal compartment and the lysosomotropism of macromolecules [2]. The conjugation of drugs to synthetic and natural macromolecules was initiated nearly 50 years ago. Jatzkewitz used a dipeptide spacer to attach a drug to polyvinylpyrrolidone in the early fifties [3] and Ushakov's group synthesized numerous water-soluble polymer–drug conjugates in the sixties and seventies (see, for example, Ref. [4]). Mathé et al. [5] pioneered conjugation of drugs to immunoglobulins, setting the stage for targeted delivery. Finally, Ringsdorf [6] presented the first clear concept of the use of polymers as targetable drug carriers.

The development of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers as anticancer drug carriers was the result of systematic research on hydrophilic biomedical polymers performed in one of the author's (J.K.) laboratory at the Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences (IMC) in Prague. In the late sixties and seventies, the IMC was a unique place to study hydrophilic biomedical polymers. Hydrogels were designed there by Wichterle and Lím [7] as well as soft contact lenses [8]. This was the driving force behind a detailed study of the relationship between the structure of soluble and crosslinked hydrophilic polymers and their biocompatibility [9–14]. Based on these investigations, hydrogels have been used successfully in human medicine [15] and basic data on the structure–properties relationship were obtained, which permitted the design of new water-soluble polymeric drug carriers [16,17].

A new hydrophilic, biocompatible polymer, based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) [18,19] was chosen as a candidate for a soluble polymeric drug carrier. The α-carbon substitution and the N-substituted amide bond ensured hydrolytic stability of the side-chains [13]. In addition, the crystallinity of the monomer guaranteed the absence of divinyl compounds (a problem with hydrophilic esters of the 2-hydroxyethyl methacrylate type) and the linearity of the macromolecules. The possibility to control the molecular weight distribution of macromolecular therapeutics is a prerequisite for their elimination from the organism. Oligopeptide side-chains were designed as drug

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attachment sites [20]. The important observation that oligopeptide sequences attached to HPMA copolymers were degradable in vivo and thus had potential as drug attachment/release sites was encouraging and crucial for the further development of HPMA copolymer based macromolecular therapeutics [21]. The chemistry of the synthesis of HPMA copolymer based macromolecular therapeutics was studied in detail [22–24]. Insulin [25] and ampicillin [26] were attached to HPMA copolymers by aminolysis of reactive polymeric precursors, whereas polymer conjugates containing *N*-(4-aminobenzensulfonyl)-*N′*-butylurea were prepared by copolymerization of HPMA and polymerizable derivatives of the drug [27].

The above mentioned initial studies resulted in numerous collaborations and indeed in an independent interest of other laboratories to pursue the potential of HPMA based therapeutics. This review attempts to present the state of the art developments of HPMA copolymer based macromolecular therapeutics and indicate the directions to be taken for the discovery of novel molecular targets and the design, synthesis, and evaluation of second generation conjugates. The review concentrates on HPMA copolymer conjugates only, however, the conclusions may be valid for other water-soluble macromolecular therapeutics.

2. Biological rationale

The rationale for the use of water-soluble polymers as carriers of anticancer drugs is based on the mechanism of cell entry. Whereas the majority of low molecular weight drugs enter the cell interior by diffusion via the plasma membrane, the entry of macromolecules is restricted to endocytosis [2]. Macromolecules captured by this mechanism are channeled to the lysosomal compartment of the cell. Endocytosis is a common term encompassing phagocytosis and pinocytosis. Phagocytosis describes the capture of vesicular material by specialized cells (macrophages and monocytes). Pinocytosis describes the capture of extracellular fluid, all solutes dissolved therein and any material adherent to the infolding surface [28]. It is a process common to most cell types. Depending on the structure of the macromolecule, three types of pinocytosis may occur: fluid-phase, adsorptive, and receptor-mediated pinocytosis. Fluid-phase pinocytosis occurs when no interaction of the macromolecule with cell surface takes place. Consequently, macromolecules are taken up slowly depending on their concentration in the extracellular fluid. Incorporation of hydrophobic moieties [29] or positive charges [30] into the macromolecular structure results in non-specific interactions with plasma membranes of different cells and a concomitant increase in the rate of macromolecular uptake. This process is known as adsorptive pinocytosis. Incorporation of moieties in the macromolecular structure, which are complementary to cell surface receptors or antigens of a subset of cells, renders the macromolecule biorecognizable [31,32]. The macromolecules are internalized specifically by a select subset of cells and not only the rate of uptake, but also body distribution is substantially altered.

3. Design of HPMA copolymer based macromolecular therapeutics

The design of macromolecular therapeutics must be based on a sound biological rationale. The HPMA copolymer–drug conjugate should be biorecognizable at two levels: at the plasma membrane to increase the recognition and internalization by a subset of target cells and intracellularly by lysosomal enzymes to release the drug from the carrier. The latter is a prerequisite for transport of the drug into the cytoplasm and nucleus resulting in biological activity [33–36].

3.1. Lysosomally cleavable spacers

The lysosomal membrane is not permeable to macromolecules [37]. Consequently, drug–polymer linkages have to be designed to be stable in the bloodstream and interstitial space but susceptible to hydrolysis in the lysosomal compartment. One option is to use the change of pH in subcellular vesicles during internalization and bind drugs via acid-labile bonds [38,39]. The problem is that the difference of two pH units between the blood stream and the lysosomal compartment is not high enough to ensure both the stability in the blood stream and fast hydrolysis in prelysosomal and lysosomal compartments. Consequently, the design of the bond between the HPMA copolymer carrier and the (anticancer) drug was based on the activity of lysosomal enzymes. From the several options available (oligonucleotide, oligosaccharide, oligopeptide sequences), the oligopeptide sequences were chosen as drug attachment/release points [33,34,40].

The choice of the optimal sequence was based on the detailed study of the relationship between oligopeptide sequences attached to HPMA copolymers and their degradability by proteolytic enzymes. Early studies with model enzymes, chymotrypsin [41], trypsin [42], and papain [43], resulted in recognition of the main factors responsible for the release of drugs bound at oligopeptide side-chain termini: length and detailed structure of the oligopeptide sequence, drug loading and related solution properties of the conjugate, structure of the drug, and steric hindrance [34,40]. The observation of in vivo degradability of oligopeptide sequences [21] and their stability in blood plasma and serum [44] clearly indicated lysosomal degradation. Degradation studies with isolated lysosomal enzyme mixtures [45] and individual lysosomal enzymes [46,47] followed. The study with cathepsin B [46], the most important lysosomal cysteine proteinase, resulted in the recognition of the glycylphenylalanylleucylglycine (GFLG) sequence [35], which is incorporated in all conjugates used in clinical trials (Fig. 1; see Section 7.4).

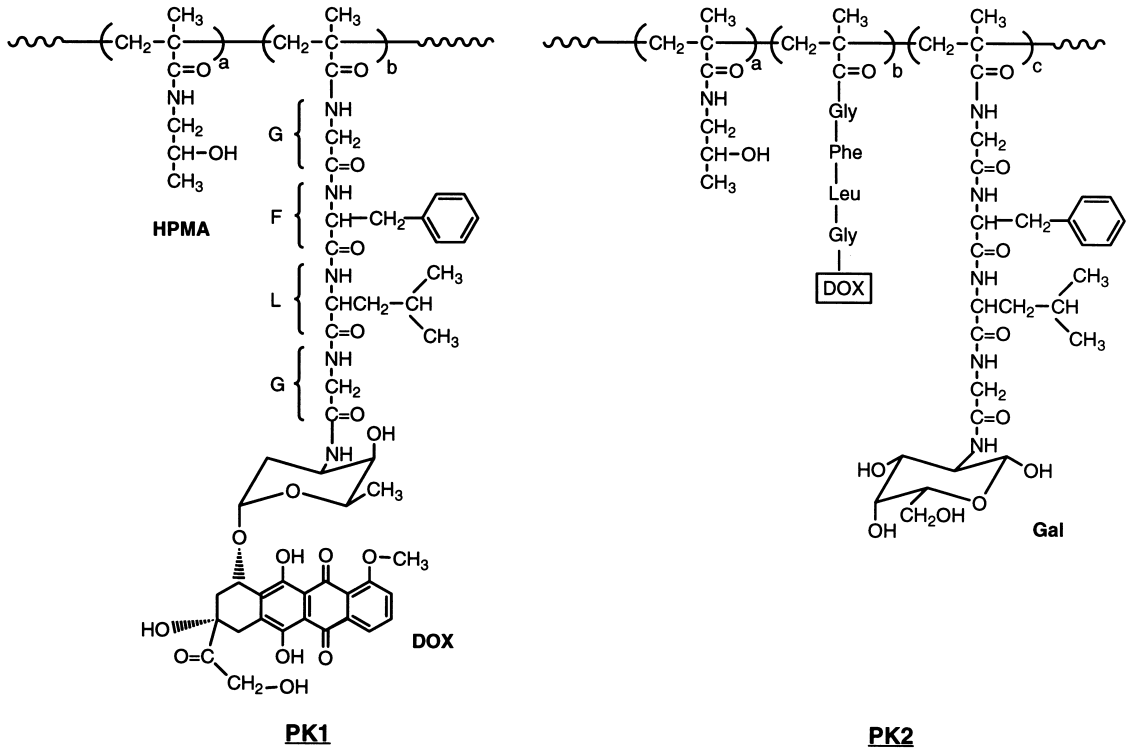

Fig. 1. Structure of HPMA copolymer–doxorubicin conjugates. Conjugate PK1 contains doxorubicin bound to HPMA copolymer via a tetrapeptide sequence stable in the blood stream but susceptible to enzymatically catalyzed hydrolysis in the lysosomes. Conjugate PK2 contains in addition side-chains terminated in *N*-acylated galactosamine complementary to the asialoglycoprotein receptor on hepatocytes. We coined the names PK1 and PK2 (P for Prague, K for Keele) to reflect the fact that they were developed as a collaboration between Kopeček's group in Prague and Duncan's group at the University of Keele. Both conjugates are in Phase I/II clinical trials.

3.2. Attachment of targeting moieties

Various targeting moieties, such as carbohydrates [31,48,49] and antibodies [32,50,51], have been used to achieve the biorecognizability of HPMA copolymer conjugates at the plasma membrane. Synthetic methods were developed (Fig. 2) for binding antibodies via amino groups [32,51], oxidized saccharide moieites close to the hinge region [51] and for binding of antibody fragments to HPMA copolymers [51,52].

The internalization and subcellular trafficking of targetable HPMA copolymer–anticancer drug conjugates can be visualized by confocal fluorescence microscopy. Indeed, conjugates containing monoclonal OV-TL 16 antibodies were recognized by the OA-3 antigen on human ovarian carcinoma cells and ultimately localized in the lysosomes. The drug (doxorubicin) was released from the carrier by lysosomal enzymes and transported, via the cytoplasm, into the cell nuclei [53].

4. Biocompatibility of macromolecular therapeutics

Binding of anticancer drugs and targeting moieties such as antibodies to polymeric carriers improves their biocompatibility [36]. Using a non-toxic and non-immunogenic polymeric carrier, the decrease of non-specific toxicity of an attached drug (when compared to an unbound drug) may be mainly attributed to the change in body distribution. For example, anthracycline antibiotics have a non-specific cardiotoxicity and bone marrow toxicity, limiting the dose which can be administered. Binding of anthracyclines to polymeric carriers decreases their rate of uptake in heart tissue. Consequently, a smaller fraction of drug administered as a macromolecular conjugate will localize in the heart. On the other hand, a low molecular weight drug bound to a polymeric carrier may act as a hapten, facilitating an immune response. On the contrary, conjugation of high molecular weight compounds, such as (targeting) antibodies to non-immunogenic polymeric carriers, usually decreases the anti-antibody immune response due to steric hindrance and impaired biorecognition of the conjugate by the immune system [54].

A prerequisite for developing a biocompatible drug carrier is to start with a biocompatible polymer. This does not imply that the conjugates will also be biocompatible, but it will considerably improve the chances. The biocompatibility of poly[*N*–(2–hydroxypropyl)methacrylamide] was evaluated using procedures developed for blood plasma expanders [55–59]. The next step was to evaluate the biocompatibility of HPMA copolymers containing attached haptens (arsanilic acid (ARS), fluorescein isothiocyanate

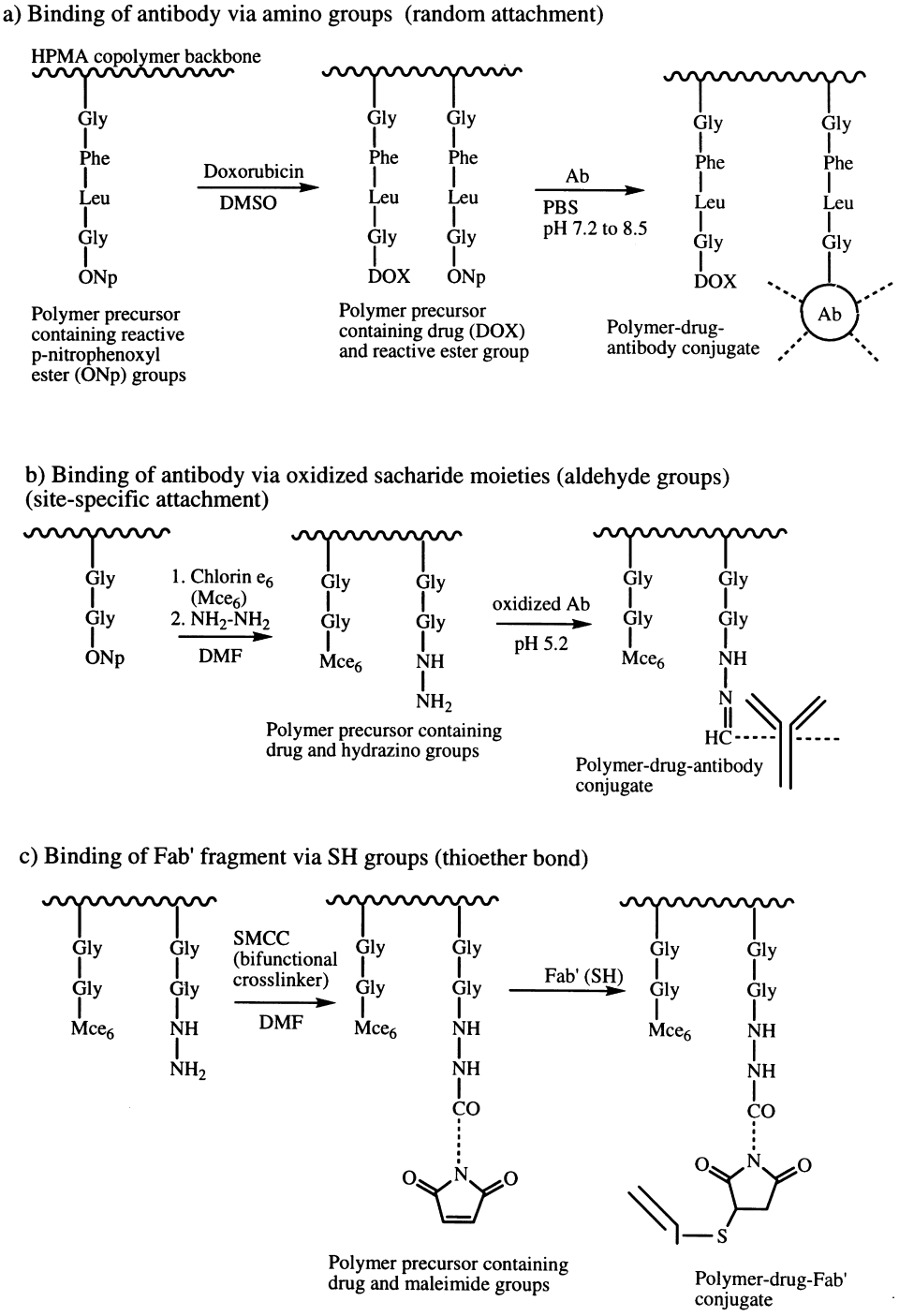

Fig. 2. Examples of synthesis of polymer–drug–antibody conjugates.

(FITC)) as drug models [60] and oligopeptide side-chains [61]. It was shown [60–62] that the homopolymer was not recognized as a foreign macromolecule in any of the five inbred strains of mice used, and no detectable antibodies were found against it. Attachment of oligopeptide side-chains gave rise to compounds possessing very weak immunogenic activity. The intensity of response depended on the structure of the side-chain, dose, and genetic background of the mice. Neither the homopolymer nor the HPMA copolymers possessed mitogenic activity. The intensity of response increased with increasing molecular weight of the conjugate. While the ARS–HPMA copolymer conjugate behaved as a high dose tolerogen, tolerogenicity was not observed with the FITC conjugate. In summary, HPMA copolymers behaved as thymus-independent antigens with low immunogenicity [50]. The immune response towards HPMA

copolymers was about four orders of magnitudes lower when compared with human gamma globulin [34]. However, the results obtained strongly indicated that the biocompatibility of each drug–HPMA copolymer conjugate would have to be evaluated independently.

HPMA homopolymer and copolymer conjugates did not have a prominent effect on the complement system in vitro [63]. An important observation was the fact that the attachment of HPMA copolymers to antibodies decreases their immunogenicity [32,64]. As mentioned above, attachment of anticancer drugs to HPMA copolymer carriers decreases the drug's toxicity. The effects of free daunomycin (DNM) and DNM bound to HPMA copolymer–anti-Thy 1.2 antibody conjugates on the depletion of hematopoietic stem cells from bone marrow were compared [50]. Conjugation of DNM to the targetable carrier substantially decreased its non-specific toxicity. Histopathological evaluation has shown that the free drug was irritating to Kupffer cells while HPMA copolymer conjugates had no such effect [50].

HPMA based polymeric prodrugs containing doxorubicin bound to the polymeric backbone via GFLG side-chains, and optionally galactosamine bound via the same sequence, were evaluated for immunogenicity after i.v., s.c., and oral administration in two inbred strains of mice. It was found that antibodies were produced in very small amounts [65]. Attachment of doxorubicin to HPMA copolymers considerably decreased its toxicity against hematopoietic precursors in bone marrow as determined by the in vivo colony-forming unit-spleen assay and its ability to inhibit [^{3}H]thymidine incorporation by mouse splenocytes and human peripheral blood lymphocytes in vitro [50]. The decreased cardiotoxicity of HPMA copolymer–doxorubicin conjugates was evaluated in rats using a dose of 4 mg/kg. Whereas in those animals to which free drug was administered, a time-dependent decrease in cardiac output was observed and these animals subsequently died, animals receiving the polymer conjugate showed no change in cardiac output and no overt signs of toxicity [66]. These results were corroborated during the preclinical evaluation of the HPMA copolymer conjugates [67,68].

Molecular weight and molecular weight distribution are important factors in a polymer carrier's biocompatibility. There is a clear relationship between the molecular weight and rate of elimination [17,69]. The molecular weight distribution of synthetic carriers has to be below the renal threshold to ensure its elimination. Biodegradable polymeric drug carriers are traditionally derived from natural products (polysaccharides, poly(amino acids)) in the hopes that the body's natural catabolic mechanisms will act to break down the macromolecular structure into small, easily eliminated fragments. However, the substitution of natural macromolecules with covalently linked drug molecules generally results in the hampering of the host's ability to effectively degrade the polymeric carrier enzymatically [70]. It is apparent that enzymes that cleave peptide or saccharide bonds have a considerably large active site to accommodate several 'monomer' units. Substitution along the macromolecular backbone renders the formation of the enzyme–substrate complex energetically less favorable. Therefore, drug substitution of a polymeric carrier may result in the inability of a naturally occurring, normally biodegradable macromolecule to be degraded into easily eliminated fragments. Degradation of drug modified macromolecules, such as poly(amino acids) [71] or polysaccharides [72], may yield fragments which cannot penetrate the lysosomal membrane. Moreover, they may possess biological activity. At the present, it is safe to treat any macromolecular drug carrier as non-degradable [73].

The biocompatibility of HPMA copolymer based therapeutics was validated in clinical trials (see Section 7.4).

5. Preferential accumulation of macromolecular therapeutics in solid tumor

5.1. Enhanced permeability and retention effect

It is now well accepted that the enhanced permeability and retention (EPR) effect is the predominant mechanism by which soluble macromolecular anticancer drugs exhibit their therapeutic effect on solid tumors. The phenomenon is attributed to high vascular density of the tumor, increased permeability of tumor vessels, defective tumor vasculature, and defective or suppressed lymphatic drainage in the tumor interstitium [74,75]. Other factors, however, may have an opposite effect. For example, a high interstitial pressure may result in a convective fluid flow from the center of the tumor to the periphery, which might carry macromolecules [76]. Nevertheless, a number of studies showed increased accumulation of macromolecules in tumors as compared to that in normal tissue [74,75,77–83]. The degree of accumulation was dependent on molecular weight [75,81], charge [82,83], and their overall hydrophobic–hydrophilic character. It appears that the permeability and accessibility of a particular tumor will depend on many factors [84], one of the very important ones being the size of the macromolecule. The tumor type and microenvironment may influence its transport characteristics (pore cut-off size). The effective permeability of small macromolecules, such as albumin, was independent of the tumor pore cut-off size [85]. It is important to note that HPMA copolymers used as drug carriers possessed a small size ($<$10 nm).

5.1.1. Long-circulating HPMA copolymer–doxorubicin conjugates

Molecular weight and molecular weight distribution (MWD) of a drug carrier conjugate are crucial for effective functioning. Polymeric carriers with a molecular weight below the renal threshold may be rapidly lost from the circulation. High-molecular weight (long-circulating) polymer conjugates accumulate efficiently in tumor tissue [86] due to the EPR effect [74]. However, if they possess a non-

degradable backbone, they may deposit and accumulate in various organs [55]. We have previously proposed to connect HPMA chains via lysosomally degradable oligopeptide sequences [46] to form water-soluble branched conjugates [21,42,43,87–89]. Following intravenous (i.v.) administration to rats, the oligopeptide cross-links were cleaved and the resulting lower molecular weight polymer chains were excreted into the urine [21]. However, these copolymers [21,87–89] were synthesized by cross-linking of HPMA copolymer precursors (containing oligopeptide side-chains terminated in a reactive ester group) with diamines. A reproducible synthesis of high-molecular weight (branched) HPMA copolymers by these reactions was difficult. It was practically impossible to stop the reaction at a particular conversion below the gel point.

We designed a new, reproducible synthetic pathway for long-circulating HPMA copolymers [90,91]. New cross-linking agents were synthesized and high-molecular weight copolymers prepared by cross-linking copolymerization (Fig. 3). This method is also suitable for the synthesis of HPMA copolymers which contain, in addition to oligopeptide cross-links, oligopeptide side-chains terminated in doxorubicin (DOX) (or other anticancer drugs) [90,91]. The composition of the monomer mixture, however, has to be such that at the end of the polymerization the system is short of the gel point (water-soluble).

Recently, we evaluated the influence of the molecular weight of such conjugates on their biological activity [92,93]. Copolymerization of HPMA, a polymerizable derivative of DOX (*N*-methacryloylglycylphenylalanylleucylglycyl doxorubicin) and a newly designed cross-linking agent, N^2,N^5-bis(*N*-methacryloylglycylphenylalanylleucylglycyl)ornithine, resulted in high-molecular weight, branched, water-soluble HPMA copolymers containing lysosomally degradable oligopeptide sequences in the cross-links as well as in side-chains terminated in DOX. Four conjugates with average molecular weight (Mw) of 22, 160, 895 and 1230 kDa were prepared. Biodistribution of the conjugates and their treatment efficacy in nu/nu mice bearing s.c. human ovarian OVCAR-3 carcinoma xenografts were evaluated (Fig. 3). The half-life of conjugates in the blood was up to five times longer and the elimination rate from the tumor was up to 25 times slower as the Mw of conjugates increased from 22 to 1230 kDa. The treatment with HPMA copolymer-bound DOX possessing a Mw higher than 160 kDa inhibited the tumor growth more efficiently than that of 22 kDa or free DOX ($P < 0.02$). The data clearly indicated that the higher the molecular weight of the conjugate the higher the treatment efficacy of human ovarian xenografts in nu/nu mice [92,93].

5.1.2. Influence of the cytotoxicity of macromolecules on their accumulation in solid tumors

In vitro [94–97] and in vivo [77] data demonstrated that HPMA copolymer-bound doxorubicin (P(GFLG)-DOX) possessed a higher anticancer activity when compared to free doxorubicin (DOX), especially in DOX-resistant cells or tumors (see Section 7.3). We hypothesized that the EPR effect for macromolecules containing cytotoxic drugs might significantly differ from macromolecules without drug. To verify the hypothesis, the influence of the cytotoxicity of HPMA copolymers, vascular endothelial growth factor (VEGF) gene expression and vascular permeability on the

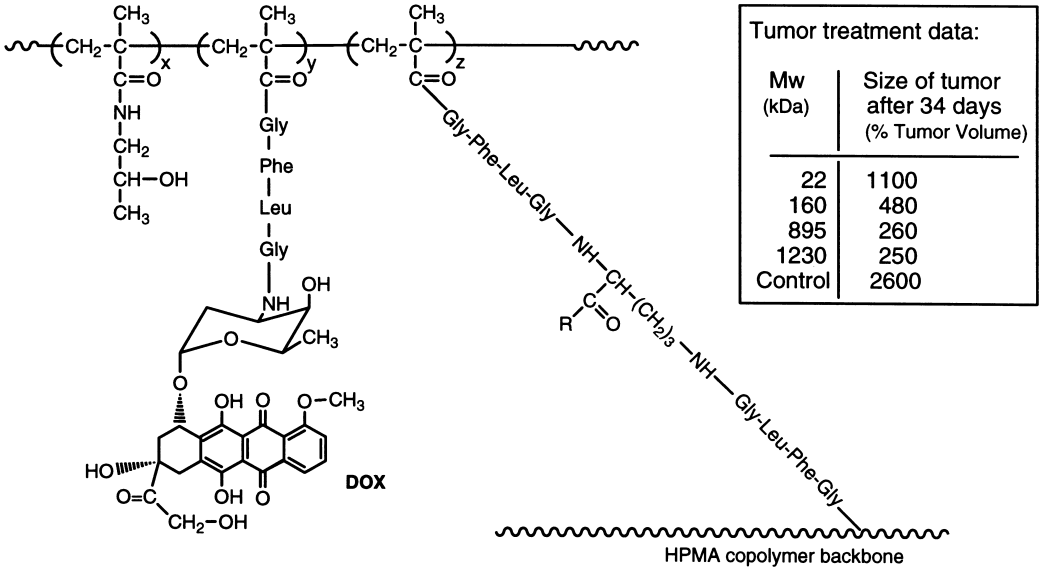

Fig. 3. Synthesis of long-circulating HPMA copolymer–doxorubicin conjugates and the influence of their molecular weight on growth inhibition of OVCAR-3 human ovarian carcinoma heterotransplanted in nude mice. Modified from Refs. [90–93].

enhanced permeability and retention (EPR) effect was studied [120].

To this end, mice bearing xenografts of A2780/AD multidrug resistant (MDR) human ovarian carcinoma were treated by free DOX and P(GFLG)-DOX, HPMA copolymer–Texas Red conjugate (P-TR), and HPMA copolymer–FITC conjugate (P-FITC). Antitumor activity, drug distribution in tumor, vascular permeability, VEGF gene expression, and DNA fragmentation were studied [120].

The accumulation of free DOX led to VEGF gene overexpression and increased the vascular permeability, which in turn enhanced the drug accumulation in the same location. This positive feedback led to a highly inhomogeneous distribution of the drug within the tumor. In contrast, P(GFLG)-DOX down-regulated the VEGF gene and decreased vascular permeability. This negative feedback seemed to prevent additional drug accumulation in dead necrotic tissue, resulting in a more uniform drug distribution, and enhanced the antitumor activity P(GFLG)-DOX.

It appeared that the EPR effect significantly differed for macromolecules containing DOX when compared to macromolecules without drug. The cytotoxicity of P(GFLG)-DOX amplified the EPR effect, led to a more homogenous distribution of the drug, increased the average drug concentration in tumor and augmented its efficacy [120].

6. New targeting strategies

There is a possibility of applying concepts of cell biology in targeted drug delivery. The incorporation of certain chemical features (targeting moieties) into a macromolecule can enormously enhance its rate of uptake by cells, by causing it to adhere to the plasma membrane being internalized [36,98]. New developments in the design of targetable macromolecular therapeutics are discussed below.

6.1. Synthetic epitopes

6.1.1. Polyvalent interactions

It appears that the use of small synthetic peptides as receptor-binding epitopes would be an advantageous strategy in targetable conjugate design. One of the main advantages is the possibility of attaching numerous biorecognizable moieties to one macromolecule. Resulting polyvalent interactions can collectively be much stronger than corresponding monovalent interactions [99]. The biorecognition of HPMA copolymers containing side-chains terminated in *N*-acylated galactosamine by the asialoglycoprotein receptor was dependent on the amount of bound ligand [100]. A similar advantage of polyvalent interactions (cooperative binding) was observed in the inhibition of virus mediated agglutination of erythrocytes by polyacrylamides with pendant sialoside groups [101], lectin recognition of HPMA copolymers with pendant fucosylamine residues [102], and BCL_1 mouse lymphoma binding of short peptides fused via a semi-rigid hinge region with the coiled-coil assembly domains into a multivalent binding molecule [103]. Another advantage of macromolecular carriers containing multiple small epitopes may be in easier transcompartmental transport when compared to large antibody conjugates.

The Epstein–Barr virus (EBV) gp350/220 envelope glycoprotein mediates virus attachment to the EBV/C3dg receptor on human B lymphocytes [104] and specific binding to some receptors on human T cell lymphomas [105]. Two regions of amino acid similarity were found in the gp350 and C3d coding sequences and it was suggested that they may represent CD21 (CR2) binding sites of gp350/220. It was shown that multimeric forms of the N-terminal gp350/220 peptide, composed from nine amino acid residues (EDPGFFNVE), conjugated to albumin efficiently blocked recombinant gp350/220 and C3dg binding to B cells [104].

The biorecognizability of a (covalently attached) EDPGFFNVE nonapeptide (NP) as a targeting moiety for HPMA copolymers by B- and T-lymphocytes has been evaluated [106]. The NP was attached to the HPMA copolymer backbone (P) via dipeptide (GG) or tetrapeptide (GFLG) side-chains [40], and covalently attached Cascade Blue was used as the fluorescence marker of the conjugate. Flow cytometry studies revealed that binding was homogeneous to both cell types. The apparent binding constants to T-cells were about two times higher when compared to B-cells. The binding and cytotoxicity increased with increased amount of epitopes per polymer chain – polyvalent interactions resulted in a one order of magnitude change in binding constant. Attachment of the NP via a GFLG spacer resulted in increased biorecognition when compared with conjugates containing NP bound via a GG spacer. HPMA copolymer–NP–ADR conjugates possessed specific cytotoxicity to T- and B-malignant cells. Concentrations which were lethal to the latter were not toxic for peripheral blood lymphocytes. The data obtained [107] seem to indicate the potential of synthetic receptor-binding epitopes as targeting moieties.

6.1.2. Self-assembled monolayers as scaffolds for epitope display

Detailed studies are needed to find the optimal epitope-receptor pair for the best biorecognition and targeting. To this end, we established a model for biorecognition studies based on an epitope presentation scaffold – a coiled-coil stem loop (CCSL) peptide self-assembled on a solid substrate [107, 108]. A bifunctional *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer containing nitrilotriacetic acid (NTA) and benzophenone (BP) groups was synthesized by free radical copolymerization of HPMA, 2-methacrylamidobutyl nitrilotriacetic acid (MABNTA), and 4-methacrylamido benzophenone (MABP). A His-tagged coiled-coil stem loop peptide containing a tridecapeptide (TDP) epitope (GFLGEDPGFFNVE) in the loop domain (CCSL-TDP) was designed and synthesized genetically by expressing an artificial gene in *E. coli* BL21 (DE3). The peptide was char-

acterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), size exclusion chromatography (SEC), and circular dichroism spectrometry. Surfaces containing self-assembled CCSL-TDP peptide were prepared by first covalently grafting poly(-HPMA-*co*-MABNTA-*co*-MABP) onto a polystyrene (PS) surface by UV irradiation, then charging the surface with nickel through NTA groups, and finally attaching the CCSL-TDP peptide through Ni-histidine chelation. The modified PS surfaces with and without self-assembled CCSL-TDP peptide were characterized by X-ray photoelectron spectroscopy and time-of-flight secondary ion mass spectrometry (TOF-SIMS). Cell attachment studies with human Burkitt's lymphoma Raji B cells showed that the cells selectively bound to the self-assembled CCSL-TDP peptide surfaces, but not to the surfaces of PS, PS with grafted copolymer, and PS with the grafted copolymer and a self-assembled coiled-coil peptide with a similar structure but without the epitope [107,108]. This indicates that the cell attachment was mediated by the CCSL-TDP peptide, most probably by the TDP epitope domain. The CCSL peptide self-assembly presented here may represent a feasible model of exposing epitopes for biorecognition studies.

6.2. Star-like conjugates

Ulbrich and coworkers [109–111] designed star-like HPMA copolymer–drug–antibody conjugates. In this new construct, semitelechelic polyHPMA chains are linked to the antibody via terminal carboxyl groups. The advantage of these constructs is the narrow molecular weight distribution of the conjugate. The release of DOX by lysosomal enzymes is unchanged. The biorecognition of star-like conjugates by target cells was comparable to free (anti-Thy 1.2) antibody [111].

6.3. Polymer-directed enzyme prodrug therapy

Satchi and Duncan [112,113] modified the well-established concepts of antibody directed enzyme prodrug therapy (ADEPT) [114] and gene directed enzyme prodrug therapy (VDEPT) [115] and proposed a new scheme, polymer directed enzyme prodrug therapy (PDEPT). The administration of an HPMA copolymer–anticancer drug conjugate containing an enzymatically cleavable spacer is followed by the administration of HPMA copolymer-bound enzyme (e.g. cathepsin B). This approach is feasible, since it is known that an interaction of HPMA copolymer-bound enzyme with HPMA copolymers containing oligopeptide side-chains terminated in leaving groups results in biorecognition and cleavage [40,41].

6.4. Polymerizable antibody fragments

We designed a new pathway for the synthesis of targeted polymeric drug delivery systems using polymerizable antibody fragments [52]. A new macromonomer, a polymerizable antibody Fab′ fragment (MA-Fab′) of the OV-TL 16 antibody (IgG1), has been synthesized and copolymerized with HPMA to produce poly(HPMA-*co*-MA-Fab′). Both MA-Fab′ and poly(HPMA-*co*-MA-Fab′) can effectively bind to OVCAR-3 human ovarian carcinoma cells. A targetable mesochlorin e_6 (Mce_6) containing conjugate, poly(HPMA-*co*-MA-Fab′-*co*-MA-GFLG-Mce_6), was synthesized by copolymerization of HPMA, MA-Fab′ and MA-GFLG-Mce_6 (MA-Mce_6) (Fig. 4). The targeted conjugate exhibited a higher cytotoxicity toward OVCAR-3 cells than the non-targeted poly(HPMA-*co*-MA-Mce_6) or free Mce_6. The cytotoxicity data correlated well with the efficacy of internalization by OVCAR-3 cells observed by confocal fluorescence microscopy – the targeted copolymer was internalized more efficiently than the non-targeted copolymer (Fig. 5). The concept of using polymerizable Fab′ fragments as macromonomers provides a new paradigm for the synthesis of targeted polymeric drug delivery systems, and may have unique applications in other areas, such as immunoassays, biosensor technology and affinity chromatography.

7. Treatment of cancer

7.1. Experimental cancer

HPMA copolymer conjugates have shown activity toward various tumor models. HPMA copolymer–daunomycin conjugate was active in the treatment of experimental Walker sarcoma [78] and L1210 leukemia [116,117]. HPMA copolymer–doxorubicin conjugates were active against L1210 leukemia [118], B16F10 melanoma [119], M5076 [67], LS174T human colorectal carcinoma xenografts [67] and sensitive and resistant human ovarian carcinoma models [77,94,95,97,120]. HPMA copolymer platinates have shown biological activity in vivo against B16F10 melanoma [121]. Binding of taxol to HPMA copolymers through an ester bond via its 2′ OH group resulted in a soluble and active conjugate [122,123].

Targeted HPMA copolymer conjugates were also studied in detail. Anthracycline antibiotic conjugates targeted with monoclonal antibodies toward different T-cell surface antigens were effective [50,111,124–126]. The same antibodies were used for the delivery of mesochlorin e_6 [127,128] and cyclosporin A [129–131]. In addition to improved efficacy, a substantial decrease of non-specific toxicity of the conjugates was observed when compared to free drugs. Other targeting moieties successfully used were N-acylated galactosamine [31,88,132], transferrin [133], and melanocyte stimulating hormone [134].

7.2. Combination chemotherapy and photodynamic therapy

Photodynamic therapy (PDT) uses the combination of light and certain absorbing molecules, called photosensitizers, which in the presence of oxygen can lead to rapid cell

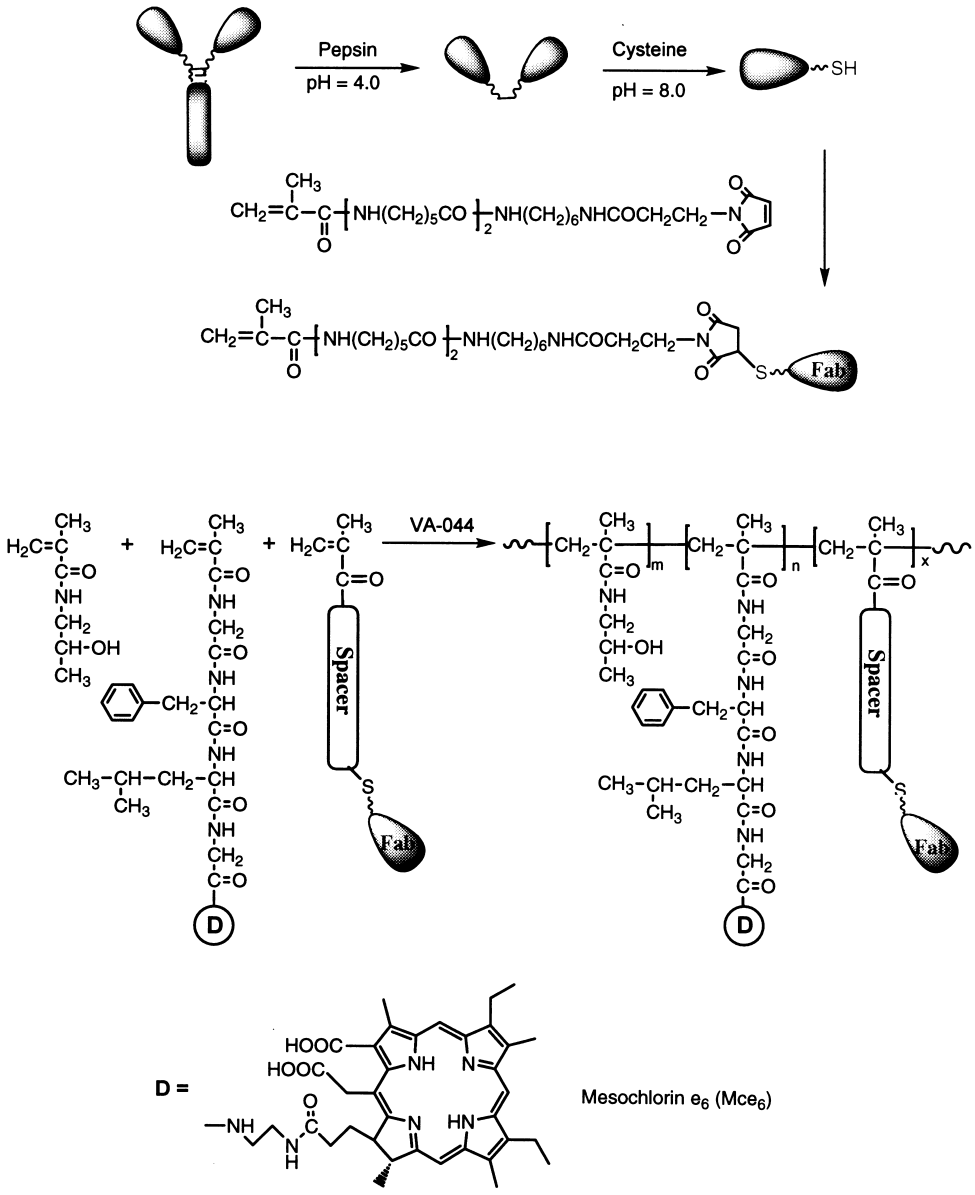

Fig. 4. Synthesis of polymerizable antibody Fab′ fragment and its copolymerization to produce a targeted HPMA copolymer–Mce_6 conjugate [52].

destruction [127,135]. The generation of singlet oxygen is ultimately responsible for the majority of such phototoxic effects, although other reactions, for example, the formation of radicals, do indeed occur. A reactive excited state of molecular oxygen, singlet oxygen, lies only 90 kJ mol^{-1} above the triplet ground state, enabling photosensitizers to efficiently catalyze its formation [136]. Because the lifetime of singlet oxygen is short ($\sim 10^{-6}$ s) its site of action is largely determined by its location. Polymer-bound photosensitizers will end up in the lysosomal compartment of target cells where they will be inactive without light. A double targeting effect can be achieved by biorecognition and tumor accumulation followed by the subsequent localized application of light [137].

A novel concept of combination chemotherapy and photodynamic therapy (PDT) using HPMA copolymer-bound drugs was developed [138]. On two cancer models, Neuro 2A neuroblastoma [139] and human ovarian carcinoma heterotransplanted in nude mice [140], we have shown that combination therapy with HPMA copolymer–anticancer drug (DOX and mesochlorin e_6 mono(*N*-2-aminoethylamide) (Mce_6)) conjugates showed tumor cures which could not be obtained with either chemotherapy or PDT alone. Cooperativity of the action of both drugs contributed to the observed effect [141].

Recently, we performed a series of studies to optimize the efficacy of chemotherapy and photodynamic therapy in the treatment of experimental ovarian carcinoma. An association of hydrophobic side-chains terminated with the drug may result in a decreased quantum yield of singlet oxygen

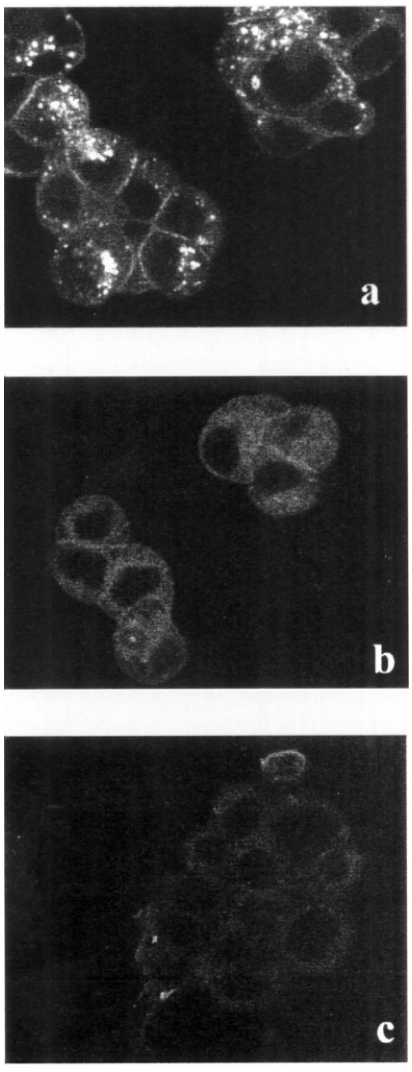

Fig. 5. The confocal fluorescence microscopic images of OVCAR-3 cells incubated for 6 h at 37°C with 15 μM Mce_6 equivalent of: (a) poly(HPMA-*co*-MA-Fab′-*co*-MA-Mce_6) (IC_{50} 2.6 μM, expressed in Mce_6 equivalents) was localized in the lysosomal compartment of OVCAR-3 cells; (b) free Mce_6 (IC_{50} 7.9 μM) did not show lysosomal accumulation, but the fluorescence intensity was brighter than that of the cells incubated with poly (HPMA-*co*-MA-Mce_6); (c) poly(HPMA-*co*-MA-Mce_6) (IC_{50} 230 μM) did not show detectable lysosomal accumulation at experimental conditions used. Modified from Ref. [52].

formation (P(GFLG)-Mce_6) or a modified rate of DOX release (P(GFLG)-DOX). Consequently, we optimized the structure of the conjugates based on their solution properties [142,143]. A biodistribution study of P(GFLG)-Mce_6 and P(GFLG)-DOX revealed the optimum time lag between the administration of both macromolecular therapeutics and irradiation of the tumor [79]. Based on these data, we hypothesized that combination therapies of s.c. human ovarian carcinoma OVCAR-3 xenografts in nude mice using multiple doses/irradiation of P(GFLG)-Mce_6 and P(GFLG)-DOX may acquire low effective doses without sacrificing the therapeutic efficacy. Indeed, ten out of 12 tumors exhibited complete responses in the group of mice receiving multiple PDT plus multiple chemotherapy [144,145]. The results (Fig. 6) demonstrated that (a) HPMA copolymer-bound drugs exhibited selective tumor accumulation contrary to free drugs, (b) PDT using an HPMA copolymer–Mce_6 conjugate (P(GFLG)-Mce_6) with multiple light irradiations was a better therapy than that with single light irradiation, and finally (c) combination chemotherapy and photodynamic therapy with HPMA copolymer–DOX and HPMA copolymer–Mce_6 conjugates was the most effective regimen [140,145].

7.3. Multidrug resistant tumors

7.3.1. Multidrug resistance

Resistance of malignant tumors to chemotherapeutic agents remains the major cause of failure in cancer therapy. A membrane glycoprotein, termed P-glycoprotein, has been shown to be responsible for cross-resistance to a broad range of structurally and functionally distinct cytotoxic agents. This glycoprotein, encoded in humans by the *MDR1* gene, functions as an energy-dependent efflux pump to remove cytotoxic agents from the resistant cells. The elucidation of the function of P-glycoprotein [146], other ATP-driven efflux pumps [147] as well as other mechanisms of multidrug resistance [148] have had a major impact on the understanding of multidrug resistance in human tumors.

It is well known that macromolecular drug delivery systems have a potential to overcome multidrug resistance. In various in vitro model systems the advantage of water-soluble polymeric carriers [149–152], micelle forming block copolymers [153], liposomes [154], and nanospheres [155] in delivering anticancer drugs to MDR cells was demonstrated.

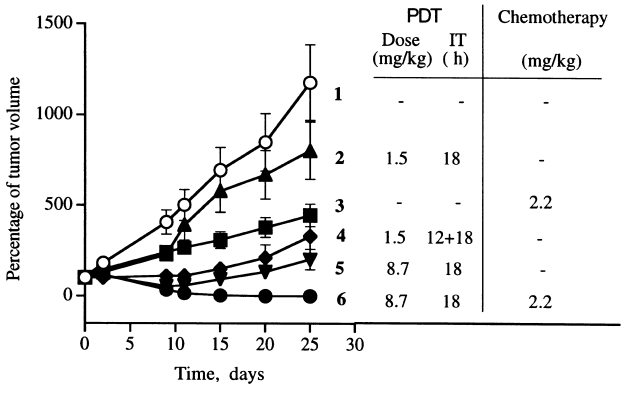

Fig. 6. Growth inhibition of OVCAR-3 human ovarian carcinoma heterotransplanted in nude mice by combination chemotherapy and photodynamic therapy with HPMA copolymer-bound anticancer drugs (DOX and Mce_6). Drug doses are expressed in drug equivalents. Irradiation time (IT) is the time lag between drug administration and irradiation. (1) Control; (2) HPMA copolymer–Mce_6 conjugate (1.5 mg/kg; IT 18 h); (3) HPMA copolymer–DOX conjugate (2.2 mg/kg); (4) HPMA copolymer–Mce_6 conjugate (1.5 mg/kg; IT 12 and 18 h); (5) HPMA copolymer–Mce_6 conjugate (8.7 mg/kg; IT 18 h); (6) combination therapy with HPMA copolymer–DOX conjugate (2.2 mg/kg) and HPMA copolymer–Mce_6 conjugate (8.7 mg/kg; IT 18 h). Modified from Refs. [144,145].

There is enough information available to permit a rational design of novel polymeric anticancer drugs effective in the treatment of multidrug resistant cells. The exclusion of the polymer–drug conjugate from the cytoplasm of the cell, due to the fact that intracellular trafficking occurs in membrane limited organelles, should render the efflux pumps ineffective. We have hypothesized that the administration of anticancer drugs as water-soluble polymeric conjugates will result in a change in the direction of the gradient of drug distribution inside cancer cells and in an increased intracellular drug concentration in P-glycoprotein (Pgp) expressing MDR cells when compared to free drug [156]. Whereas in the case of free drugs the concentration gradient is directed from the plasma membrane to the perinuclear region, in the case of polymer-bound drugs a gradient in the opposite direction is achieved. The drug released from the carrier in the lysosomal compartment enters the cytoplasm in the perinuclear region. Consequently, the probability of its interaction with nuclear DNA and/or topoisomerase II is higher than the probability of its recognition by the Pgp pump [156].

7.3.2. In vitro activity of HPMA copolymer–doxorubicin conjugates

The effect of free and HPMA copolymer-bound DOX on the viability of A2780 sensitive and A2780/AD multidrug resistant human ovarian carcinoma cells was studied in vitro [94]. As expected, the IC_{50} dose for the HPMA copolymer–DOX conjugate (P(GFLG)-DOX) was higher than for free DOX reflecting the difference in the mechanism of cell entry. The resistant A2780/AD cells demonstrated about 40 times higher resistance to free DOX than the sensitive A2780 cells. On the contrary, there was only a small difference in cytotoxicity of the HPMA copolymer–DOX conjugate toward sensitive A2780 or multidrug resistant A2780/AD cells. The IC_{50} value for A2780/AD was only about 20% higher than the value for sensitive A2780 cells [94]. These data seem to indicate that the HPMA copolymer–DOX conjugate may, at least partially, avoid the ATP-driven P-glycoprotein (Pgp) efflux pump. The analysis of the expression of the *MDR1* gene which encodes the Pgp has shown that free DOX in high doses stimulated *MDR1* gene expression in sensitive A2780 cells. However, the HPMA copolymer–DOX conjugate did not induce the expression of the *MDR1* gene. At the same time both free DOX and HPMA copolymer–DOX conjugate partially inhibited the expression of the *MDR1* and $\beta_2 m$ genes in multidrug resistant A2780/AD cells.

Based on the acute experiments described above, we hypothesized that HPMA copolymer-bound DOX would behave differently than free DOX during long-term incubation with cancer cells. To verify the hypothesis, we have studied the effect of free DOX and HPMA copolymer-bound DOX (P(GFLG)-DOX) on the induction of multidrug resistance and changes in metabolism in human ovarian carcinoma A2780 cells during repeated cyclic (chronic) exposure [95]. Such experiments are of therapeutic relevance. The development of multidrug resistance and changes in cellular energy metabolism during adaptation of sensitive human ovarian carcinoma A2780 cells to free DOX and P(GFLG)-DOX was analyzed. Adaptation of sensitive A2780 cells to repeated action of free DOX augmented cellular resistance to DOX and finally led to the over-expression of the *MDR1* gene. It is interesting to note that the first registered level of *MDR1* expression in adapted cells was almost the same as the level in the resistant A2780/AD cells. In fact, within 5 days (days 37–42 of incubation) the level of *MDR1* mRNA expression increased from a non-detectable limit ($<10\%$ of $\beta_2 m$ mRNA) to the level present in resistant cells ($>150\%$ of $\beta_2 m$ mRNA). These data clearly indicate that multidrug resistance in A2780/AD cells and its development in the sensitive A2780 cells, during adaptation to free DOX, is closely connected with the over-expression of the *MDR1* gene and the Pgp pump. Furthermore, it suggests the existence of a trigger mechanism which may initiate the *MDR1* over-expression. On the other hand, HPMA copolymer-bound DOX (P(GFLG)-DOX) induced neither the multidrug resistance with or without *MDR1* gene expression, nor the adaptation of the sensitive A2780 cells to free DOX.

The *MRP* gene encoding the multidrug resistance-associated protein was over-expressed in A2780/AD resistant cells. At the same time, no significant increase in its expression was found after incubation of A2780 sensitive cells with free and HPMA copolymer-bound DOX. Moreover, P(GFLG)-DOX partially down-regulated its expression, which may be the result of limited cell energy metabolism in the experimental conditions used. These data show that the *MRP* gene, which may play a certain role in the resistance of A2780/AD cells, does not appear to significantly participate in the cell resistance during adaptation to DOX [95].

7.3.3. HPMA copolymer–doxorubicin conjugates in the treatment of resistant ovarian carcinoma

Anticancer activities of free DOX and HPMA copolymer-bound DOX (P(GFLG)-DOX) were studied in solid tumor mice models of DOX sensitive (A2780) and resistant (A2780/AD) human ovarian carcinoma [77]. Free DOX was effective only in sensitive tumors decreasing the tumor size about three times, while P(GFLG)-DOX decreased the tumor size 28 and 18 times in the sensitive and resistant tumors, respectively (Fig. 7). An enhanced accumulation of P(GFLG)-DOX in the tumor was observed, whereas only low concentrations of DOX were detected in other organs (brain, liver, kidney, lung, spleen, and heart) following P(GFLG)-DOX administration. This effect was dependent on the high permeability of blood vessels in untreated tumors. After treatment with P(GFLG)-DOX the permeability decreased concomitantly with the down-regulation of *VEGF* gene expression.

The permeability of tumor vessels was studied by extra-

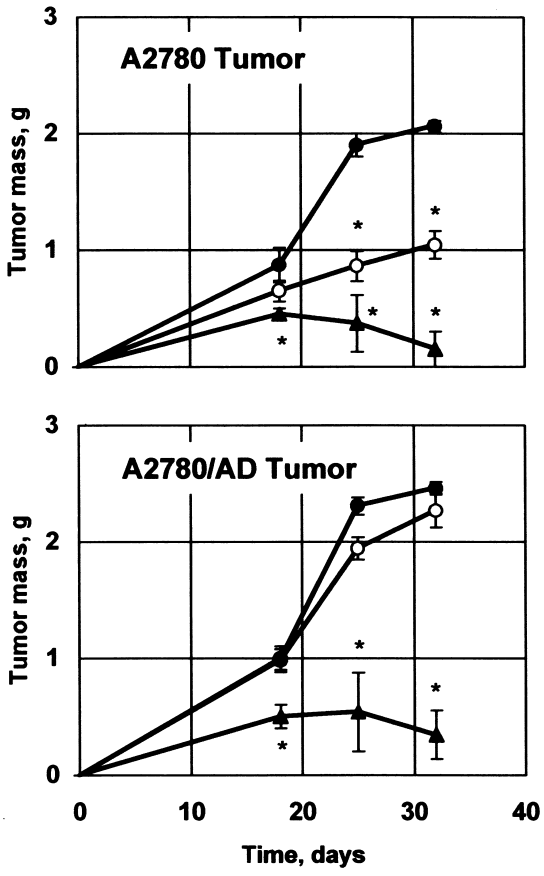

Fig. 7. Effect of free DOX (open circles) and HPMA copolymer-bound DOX (triangles) on the growth of A2780 and A2780/AD tumors in mice. Closed circles, control tumor. Means ± SD are shown. $*P < 0.05$ compared with control. Modified from Ref. [77].

vasation of an Evans Blue–albumin complex in tumor and surrounding normal tissue [77,120]. The vascular permeability in untreated tumors was more than four times higher than in the neighboring tissue. No significant differences in vascular permeability between A2780 sensitive and A2780/AD DOX resistant tumors were found. Administration of free DOX and P(GFLG)-DOX resulted in opposite changes in vascular permeability. While treatment with free DOX increased the permeability by 30–40%, HPMA copolymer-bound DOX reduced vascular permeability both in sensitive and resistant tumors approximately six times [77]. The data permitted preliminary conclusions to be drawn. We as well as others [157] have found high irregularities and disturbances in tumor vascularization. Blood vessels in untreated tumors were characterized by frequent abnormalities including blind ends, shunting, occlusions, and defects of the vascular walls. All these peculiarities combined with high vascular permeability could lead to accumulation of HPMA copolymer-bound DOX in tumor tissues. While free DOX did not produce significant changes in tumor vascularization, HPMA copolymer-bound DOX dramatically changed the morphology of tumors. After the action of P(GFLG)-DOX, the major part of the tumor tissue appeared to be dead, with obstructed vessels containing hyaline thrombi. Consequently, tumor blood flow seemed to be significantly reduced [120].

As a result, the accumulation of P(GFLG)-DOX in sensitive and resistant tumors was more significant and more homogenous when compared to free drug (Fig. 8). P(GFLG)-DOX effectively killed both types of tumors by inducing apoptosis and necrosis. All data relevant to the mechanism of anticancer action of P(GFLG)-DOX indicated a higher antitumor activity and lower systemic toxicity of HPMA copolymer-bound DOX when compared to free DOX [77].

7.4. Clinical trials

Three HPMA copolymer–anticancer drug conjugates reached the stage of clinical trials. The structure of DOX conjugates (PK1 and PK2) is shown in Fig. 1. The published results are summarized below.

7.4.1. Conjugate PK1

HPMA copolymer–doxorubicin (adriamycin) conjugate (PK1) was evaluated in Phase I clinical trials in the United Kingdom. The aim was to determine the maximum tolerated dose, toxicity profile, and pharmacokinetics of PK1 administered as an intravenous infusion every 3 weeks to patients with refractory or resistant cancers. Altogether, 100 cycles were administered (range 20–320 mg/m^2 drug equivalent) to 36 patients. The maximum tolerated dose was 320 mg/m^2, and the dose-limiting toxicities were febrile neutropenia and mucositis. No congestive cardiac failure was seen despite individual cumulative doses up to 1680 mg/m^2. The maximum tolerated dose is substantially higher than the clinically used dose of free DOX (60–80 mg/m^2). Other anthracycline-like toxicities were attenuated. PK1 demonstrated antitumor activity in refractory cancers, no polymer-related toxicity, and proof of the principle that polymer–drug conjugation decreases doxorubicin dose-limiting toxicities [158]. The data obtained strongly indicate the biocompatibility of the HPMA copolymer carrier. PK1 contained 8 wt. % of DOX. Consequently, patients in the highest dose group (320 mg/m^2) were administered about 30 g of HPMA copolymer (in six infusions every 3 weeks) without serious side-effects. The pharmacokinetic data of PK1 in 33 patients were recently analyzed [159]. A Phase II clinical study with a dose of 280 mg/m^2 every 3 weeks mainly on colorectal, NSCLC (non-small cell lung cancer), and breast cancer patients is underway.

7.4.2. Conjugate PK2

An HPMA copolymer bearing both doxorubicin and galactose (PK2) for targeted delivery to the asialoglycoprotein receptor positive cells was designed (Fig. 1) and its biorecognizability has been widely documented in animals. A Phase I clinical study with the aim to verify the concept in

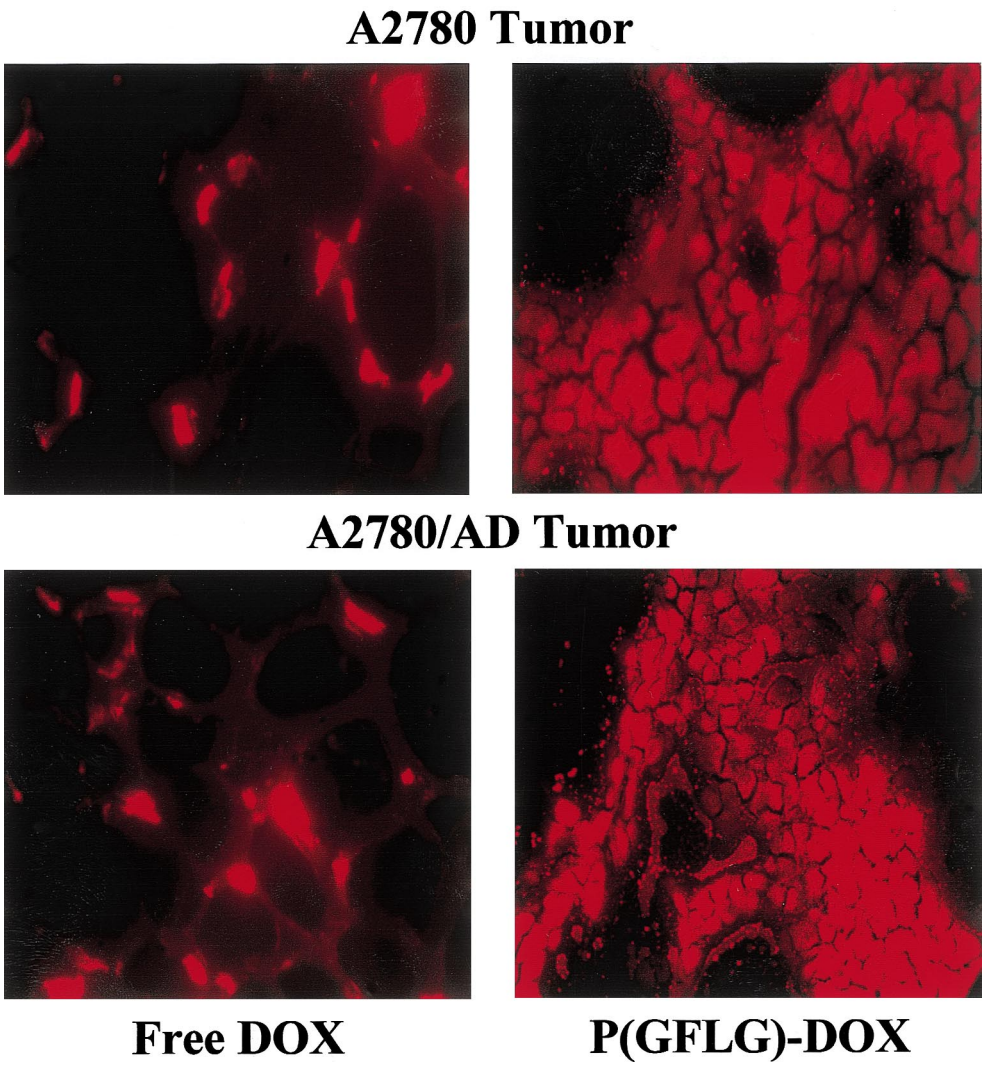

Fig. 8. Distribution of free DOX and HPMA copolymer-bound DOX in A2780 and A2780/AD tumors: fluorescence microscopy of frozen sections. Sections were taken at the end of the experiment after six injections over 3 weeks of the free DOX and HPMA copolymer-bound DOX. Objective, 60×. Modified from Ref. [77].

humans is underway. Preliminary data have been recently published [160].

7.4.3. HPMA copolymer–camptothecin conjugate

Pharmacia-Upjohn is evaluating this conjugate in Phase I clinical trials [161,162].

All three conjugates mentioned above contain a tetrapeptide spacer, Gly-Phe-Leu-Gly, which is stable in the blood stream but susceptible to enzymatically catalyzed hydrolysis by cysteine proteinases present in the lysosomal compartment of the cells [35,46].

8. Gene and oligonucleotide delivery

Many laboratories are studying strategies to deliver genes or antisense oligonucleotides into somatic cells of patients [163]. With increasing interest in non-viral delivery systems, ways to overcome membrane barriers for gene delivery into the cytoplasm and/or nucleus are being evaluated [164]. Antisense oligonucleotides have been covalently bound to HPMA copolymers via disulfide [165] and amide [166] bonds.

Novel vectors, block and graft copolymers of HPMA, and comonomers containing quaternary ammonium bonds have been synthesized [167–169] and the factors controlling formation of complexes with DNA were evaluated [169, 170]. The development of non-viral, biocompatible vectors with high transfection efficacy and the manipulation of the subcellular fate of genes and oligonucleotides are the focuses of current investigations.

9. Mechanism of action of macromolecular therapeutics

Data on the mechanism of action of HPMA copolymer–DOX conjugates on human ovarian carcinomas in vitro and in vivo seem to support our hypothesis that macromolecular

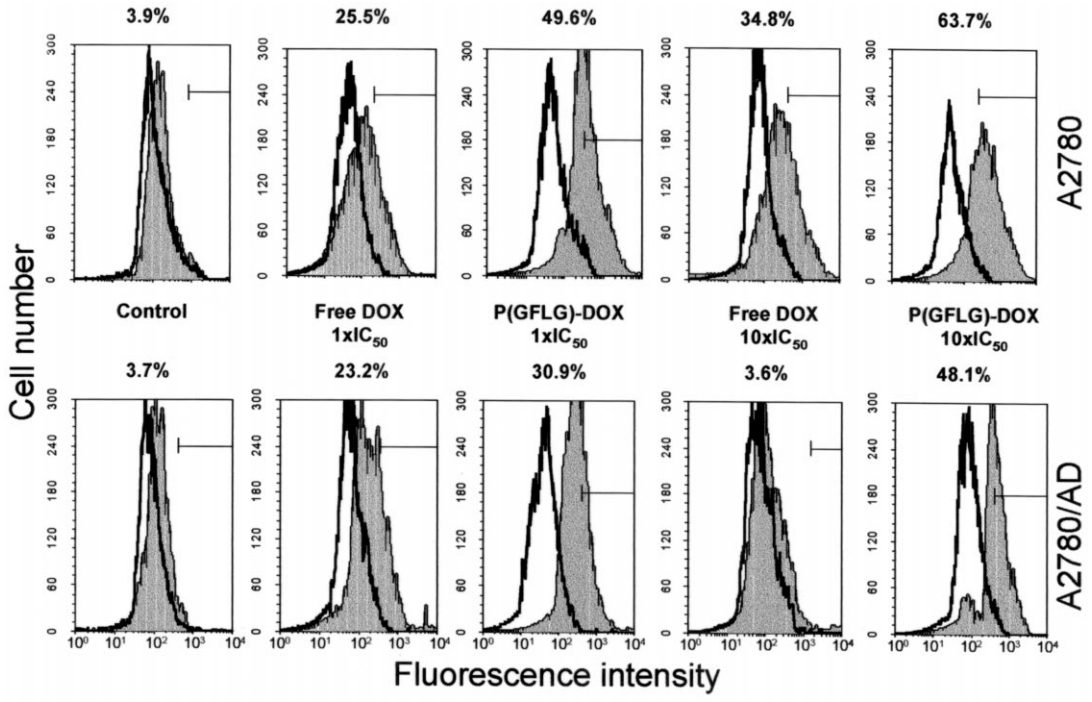

Fig. 9. Typical flow cytometry curves of TUNEL labeled A2780 and A2780/AD human ovarian carcinoma cells (1×10^7) after exposure to free DOX and HPMA copolymer-bound DOX. Numbers above the panes show percentage of apoptotic cells.

therapeutics activate different signaling pathways and possess different anticancer effects than free drugs. As a result of different pathways of internalization and subcellular trafficking, the HPMA copolymer–DOX conjugate might be more protected from cell detoxification mechanisms, resulting in an enhanced activation of apoptosis, lipid peroxidation and DNA damage when compared to free DOX. Our recent study on sensitive (A2780) and resistant (A2780/AD) human ovarian carcinoma cells [97] was devoted to verifying this hypothesis. HPMA copolymer–DOX conjugate possessed a comparable or higher toxicity than free DOX when the comparison was based on intracellular concentrations. While free DOX up-regulated genes encoding ATP-driven efflux pumps (*MDR1*, *MRP*), HPMA copolymer–DOX conjugate overcame existing pumps and down-regulated the *MRP* gene. Free DOX also activated cell metabolism and expression of the genes responsible for detoxification and DNA repair. HPMA copolymer–DOX conjugate down-regulated *HSP-70*, *GST-π*, *BUDP*, *Topo-IIα,β* and *TK1* genes. Apoptosis, lipid peroxidation and DNA damage were significantly higher after exposure to the HPMA copolymer–DOX conjugate, as reflected by simultaneous activation of *p53*, *c-fos* (in A2780 cells) or *c-jun* (in A2780/AD cells) signaling pathways and inhibition of the *bcl-2* gene (Figs. 9 and 10). In summary, the P(GFLG)-DOX conjugate overcame drug efflux pumps, more significantly induced apoptosis and lipid peroxidation, and inhibited DNA repair, replication, and biosynthesis when compared to free DOX [97].

These important features of P(GFLG)-DOX were preserved in vivo [77,120]. P(GFLG)-DOX inhibited *MDR1* and *MRP* genes encoding drug efflux pumps, and down-regulated genes responsible for drug detoxification and non-specific resistance. As a result, HPMA copolymer-bound DOX preserved its high activity and produced more DNA damage and more significantly up-regulated the

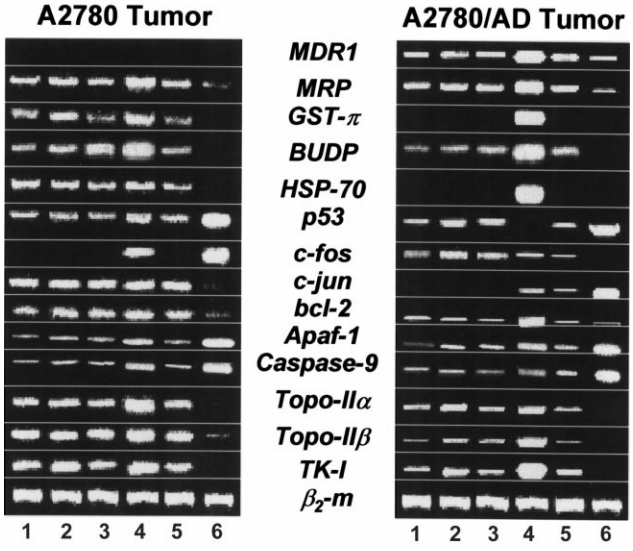

Fig. 10. Typical images of gel electrophoresis of RT-PCR products of mRNA isolated from A2780 and A2780/AD tumors. (1,2) Untreated tumor; (3,4) tumor treated with free DOX; (5,6) tumor treated with HPMA copolymer-bound DOX; (1,3,5) 18 days; (2,4,6) 32 days after subcutaneous injection of cancer cells; β_2-microglobulin (β_2-m) was used as internal standard. Modified from Ref. [77].

main trigger of apoptosis, the *p53* gene, when compared to free DOX. In addition, P(GFLG)-DOX activated another important apoptosis pathway associated with caspase-9. It is known [171] that one of the important mechanisms of apoptosis induction is associated with the release of cytochrome c from the mitochondria, which binds in the cytoplasm to the Apaf-1 protein and activates caspase-9. Once active, caspase-9 then triggers caspase activation events leading to apoptosis. We found that this mechanism of apoptosis induction was more significantly activated after the action of HPMA copolymer-bound DOX than after the action of free DOX.

While both P(GFLG)-DOX and free DOX activated signaling pathways of apoptosis, only the HPMA copolymer-bound DOX suppressed cell death protective mechanisms. It down-regulated the expression of the main cell apoptosis defensive system associated with the bcl-2 protein family, which prevents the release of cytochrome c from mitochondria. In addition, unlike free DOX, which activated mechanisms of DNA repair, replication and synthesis, P(GFLG)-DOX inhibited them (Fig. 10).

The main mechanisms of the antitumor action of HPMA copolymer-bound DOX in vivo are summarized in Fig. 11. After the initial accumulation in a tumor by the EPR effect, P(GFLG)-DOX enters the cell. It overcame existing P-glycoprotein (Pgp) and multidrug resistant protein (MRP) drug efflux pumps. During subcellular trafficking towards the nucleus after release from the lysosomes, activity of the HPMA copolymer-bound DOX was decreased by glutathione (GST) and BUDP transferases. DNA damage produced by the drug was diminished by heat-shock

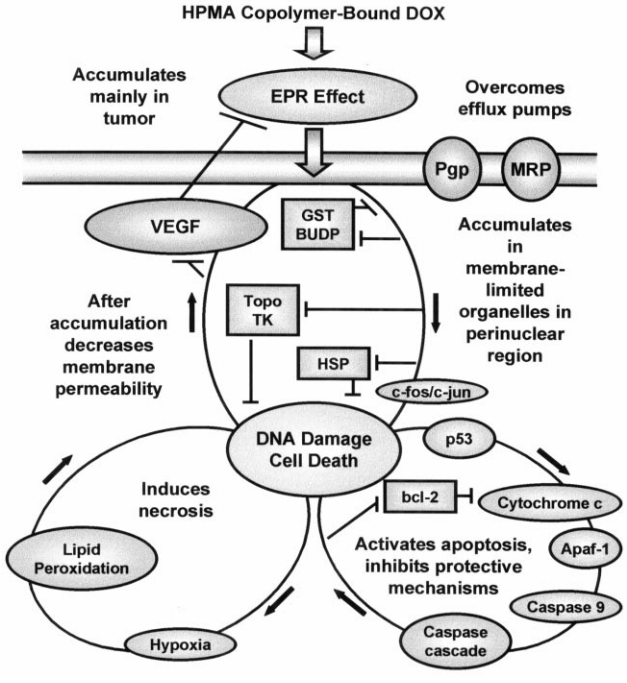

Fig. 11. Mechanism of action of HPMA copolymer-bound DOX in vivo. A detailed description is in the text. Modified from Ref. [77].

proteins (HSP), topoisomerases (Topo) and thymidine kinases (TK). However, after initiating the DNA damage, P(GFLG)-DOX down-regulated genes which encode proteins responsible for detoxification and repair of DNA damage. This in turn weakened the cellular defensive systems. As a result, the first vicious cycle which greatly augments the DNA damage was formed [77]. The question of whether P(GFLG)-DOX interferes directly with signal transduction and/or gene regulation is complex. From the fact that the cleavage of P(GFLG)-DOX in the lysosomes is a prerequisite for its anticancer activity [50], one may conclude that interaction of the HPMA copolymer-bound DOX with the plasma membrane may be minor. The question of whether such interactions may nevertheless result in the activation of signal transduction pathways cannot be answered at this time.

We found that HPMA copolymer-bound DOX down-regulated the *VEGF* gene responsible for the high vascular permeability of tumor blood vessels [77,120]. This decreased the permeability and prevented the additional worthless accumulation of the polymer drug in dead tissues. This may result in the amplification of the EPR effect and normalization of the drug distribution in the whole tumor. DNA damage produced by HPMA copolymer-bound DOX activated *c-fos*/*c-jun* and *p53* genes encoding the central cell death signal, and inhibited the bcl-2 family proteins, which are channel forming proteins responsible for maintaining normal mitochondrial physiology [172]. Inhibition of bcl-2 expression leads to altered mitochondrial membrane permeability resulting in the release of cytochrome c into the cytosol (reviewed in Ref. [173]). In the cytosol, cytochrome c is bound to the apoptotic protease-activating factor Apaf-1 [174], which also binds caspase-9 [175] and dATP [174,175]. The binding of cytochrome c triggers activation of caspase-9, which then induces apoptosis by activating other caspases. Apoptotic DNA damage in turn amplifies the apoptotic cell death signal, which forms the second vicious cycle (Fig. 11). In addition to apoptosis, HPMA copolymer-bound DOX induces cellular hypoxia and lipid peroxidation, which produces DNA damage, forming the third cycle. The formation of these vicious cycles of DNA damage finally leads to both apoptosis and necrosis in cancer cells. In contrast, free DOX activates drug efflux pumps and cellular defensive systems and increases vascular permeability. These events lead to the highly inhomogeneous distribution of free drug in tumor tissue and less induction of cell death when compared to P(GFLG)-DOX.

The data obtained confirmed our previous in vitro findings and demonstrated the potential of HPMA copolymer-bound DOX as an efficient anticancer drug. The following mechanisms were responsible for the higher anticancer activity of P(GFLG)-DOX. First, free DOX accumulated both in the tumor and normal tissues, while HPMA copolymer-bound DOX was highly toxic for the tumor and significantly less toxic for other tissues. This phenomenon was caused by an amplified EPR effect specific to P(GFLG)-

DOX. Second, free DOX was able to induce cell death mainly in DOX sensitive tumors. In contrast, HPMA copolymer-bound DOX overcame multidrug resistance and induced apoptosis and necrosis in both DOX sensitive and DOX resistant tumors. The degree of induction was significantly higher for HPMA copolymer-bound DOX than for free DOX. Third, in contrast to free DOX, which activates cellular detoxification and DNA repair mechanisms, HPMA copolymer-bound DOX inhibits mechanisms of cellular drug defense. These features of HPMA copolymer-bound DOX make it a promising new antitumor preparation targeted mainly to solid tumors.

10. Conclusions and future directions

HPMA copolymers [18] and their conjugates with drugs (reviewed in Ref. [36]) have been one of the most extensively studied systems [176]. HPMA copolymer–anticancer drug conjugates have been found to be active against numerous cancer models and are in clinical trials. The scientific evidence as well as the results of clinical trials seem to indicate the great potential of macromolecular therapeutics in cancer treatment. The fact that the maximum tolerated dose of DOX in patients was several times higher when compared to free DOX [158,159] bodes well for their potential to treat some forms of resistant cancers. Moreover, due to decreased immunotoxicity and myelotoxicity of macromolecular therapeutics, cancer patients will not suffer from recurrent viral and fungal infections, which are common after intensive conventional chemotherapy.

The field of macromolecular therapeutics reached a stage of maturity and is at a cross-roads. Further development is possible in two directions. First, there is the potential to synthesize more conjugates using numerous low molecular weight (e.g. poorly soluble) drugs and to evaluate them on various cancer models. This is a feasible option and needs to be pursued. The other option, looking further in the future, is to initiate detailed studies on the differences in the mechanism of anticancer action of free and polymer-bound drugs on the cellular and subcellular levels. The possibility to evaluate alterations of gene expression profiles in cancer cells using microarray technology [177,178] will permit the identification of signaling pathways specific for macromolecular therapeutics. The identification of new intracellular molecular targets will permit the second generation of macromolecular therapeutics to be designed. The attachment of anticancer drugs to macromolecular carriers offers a unique opportunity to direct and control the subcellular localization of the drug based on its mechanism of action and signaling pathways involved.

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