Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3023–3029

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Characterization of a Catalytic Antibody–HPMA Copolymer-Conjugate as a Tool for Tumor Selective Prodrug Activation

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Received 13 December 2001; accepted 28 February 2002

Abstract—Selective chemotherapy remains a key issue for successful treatment in cancer therapy. The use of targeting approaches like the enhanced permeability and retention (EPR) effect of macromolecules, is consequently needed. Here, we report the preparation of a novel catalytic antibody–polymer conjugate for selective prodrug activation. HPMA copolymer was conjugated to catalytic antibody 38C2 through an amide bond formation between ε-amino group of lysine residue from the antibody molecule and a *p*-nitrophenyl ester of the polymer. The conjugate was purified over a size exclusion column using FPLC. In the isolated fraction, one or two molecules of polymer were conjugated to one molecule of antibody based on gel analysis. The resulting conjugate retained most of its catalytic activity (75–81%) in comparison to the free antibody. The activity was monitored with a fluorogenic substrate and a prodrug activation assay using HPLC. Furthermore, the conjugate was evaluated in vitro for its ability to activate an etoposide prodrug using two different cancer cell lines. Cells growth inhibition using the prodrug and the conjugate was almost identical to inhibition by the free antibody and the prodrug. For the first time, a catalytic antibody was conjugated to a passive targeting moiety while retaining its catalytic ability to activate a prodrug. The conjugate described in this work can be used for selective activation of prodrug in the PDEPT (polymer directed enzyme prodrug therapy) approach by replacing the enzyme component with catalytic antibody 38C2. © 2002 Published by Elsevier Science Ltd.

Introduction

Prolonged administration of effective concentrations of chemotherapeutic agents is usually not possible because of dose-limiting systemic toxicities. Furthermore, strong side effects involving non-malignant tissues are observed. Thus, new strategies to target cytotoxic agents specifically to sites of metastatic cancer are required. One such targeting approach involves the use of polymer molecules. Water-soluble synthetic polymers such as *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers are biocompatible, non-immunogenic[1] and non-toxic. Moreover, their in vivo body distribution is well characterized[2] and they are known to accumulate selectively at tumor sites due to the enhanced permeability and retention (EPR) effect.[3] This effect occurs due to the difference between the vasculature physiology of solid tumors and normal tissues. Compared with the regular ordered vasculature of normal tissues, blood vessels in tumors are often highly abnormal. The growth of the tumor creates a constant need for the continuous supply of new blood vessels. This process, termed angiogenesis, often results in the construction of vessels with leaky walls, which allows enhanced permeability of macromolecules within the tumor. In addition, poor lymphatic drainage at the tumor site promotes accumulation of large molecules. Several polymer–drug conjugates are already in early clinical trials.[4] This includes HPMA copolymer–anticancer conjugates, PK1,[5] PK2,[6] PNU (PNU 166945),[7] and PEG–camptothecin conjugate.[8] Reduced toxicity and activity in chemotherapy refractory patients has been described. Moreover, several polymer–enzyme conjugates, such as PEG-asparaginase,[9] have been shown to reduce or even abrogate immunogenicity of the conjugated protein.

Polymer-directed enzyme prodrug therapy (PDEPT) is a two-step antitumor approach in which both the prodrug

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0968-0896/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0968-0896(02)00156-6

and the enzyme are targeted to the tumor site with a polymer molecule.[10] In the first step, a polymer–prodrug conjugate is administered and trapped in tumor tissues through the EPR effect. The excess of the conjugate is cleared out from the blood in a relatively short time. In the second step, a polymer–enzyme conjugate is injected. The polymer molecule carries the enzyme to the tumor site, where it releases the drug from the polymer. MDEPT (macromolecular-directed enzyme prodrug therapy)[11] is a similar approach to ADEPT (antibody-directed enzyme prodrug therapy)[12,13] in which a macromolecule replaces the targeting antibody. We sought to replace the enzyme component in the MDEPT or PDEPT complexes with a catalytic antibody that is capable of catalyzing prodrug activation. The use of a catalytic antibody in this concept might offer new advantages. Since an antibody molecule, like all immunoglobins, can be humanized, minimum immunogenicity is feasible. One such antibody is the aldolase antibody 38C2, prepared by a novel reactive immunization method, which catalyzes aldol and retro-aldol reactions with a broad range of substrate.[14,15] We have chosen this antibody because it is one of the most efficient catalytic antibodies prepared to date and we have used it to develop a unique strategy for prodrug activation.[16] Antibody 38C2 catalyzes a sequence of retro-aldol retro-Michael cleavage reactions, using substrates that are not recognized by human enzymes. Therefore, non-specific prodrug activation should be minimized. Furthermore, the antibody has recently demonstrated its efficiency in activating several prodrugs in vitro and in vivo.[16,17] A dramatic 75% decrease in subcutaneous (s.c.) tumor size has been observed in mice that received intratumoral injection of antibody 38C2 and systemic treatments with an etoposide prodrug.[17]

Here, we report on a preparation of a novel conjugate between a catalytic antibody and HPMA copolymer. The coupling chemistry is performed by amide bond formation between an active ester of the HPMA copolymer and an amino-lysine residue of the catalytic antibody. We demonstrate that the antibody retains its catalytic activity after conjugation to the HPMA copolymer. Furthermore, a cell growth inhibition assay demonstrates that the conjugate retains its catalytic activity and is capable of activating prodrug in a manner analogous to the free antibody.

Material and Methods

Materials

HPMA copolymer-Gly-Gly-ONp (4.4 mol%), (ONp is *p*-nitrophenyl) was obtained from Polymer Laboratories (Church Stratton, UK). Catalytic antibody 38C2 was provided by The Scripps Research Institute (La Jolla, CA, USA). Etoposide was obtained from Sigma (St. Louis, MO, USA). Pro VP-16 was synthesised as published elsewhere.[17] Cynol was prepared as described previously.[18] Cell proliferation assay kit with XTT reagent was purchased from Biological Industries (Beit Haemek, Israel).

All other chemicals were of analytical grade.

Cell lines

MOLT-3 human acute lymphoblastic leukemia cells and NXS2 human neuroblastoma cells were grown in RPMI 10% FCS and DMEM 10% FCS, respectively (5% CO_2 37 °C).

Synthesis of HPMA copolymer–38C2 catalytic antibody conjugate

The polymeric precursor specifications for the enzyme conjugate: Feedstock ratio: 95 mol% HPMA monomer/ 5 mol% MA-GG-ONp, Total NpOH (*p*-nitrophenol): 4.4 mol%, M_w: 34,679, M_n: 21,115, M_p: 29,692.

HPMA copolymer-Gly-Gly-ONp was dissolved in double deionized water (DDW) (10 mg/mL) and the solution of 38C2 catalytic antibody in PBS pH 7.4 (10 mg/mL) was added dropwise while stirring. The reaction mixture was stirred in the dark, at 4 °C, for 30 min. Then the pH was carefully raised by adding saturated tetraborate buffer to pH 8.5. The mixture was stirred for another 8 h and the reaction was completed by adding 1-amino-2-propanol (20 μL) in order to remove unreacted ONp groups. The final yellow solution was transferred to a VivaSpin (10 KDa MW cut-off) column in order to remove any low MW compounds present in the solution. The VivaSpin was centrifuged at 4 °C at 3000*g* for 30 min. This procedure was repeated, adding phosphate buffer each time, until no ONp groups were visible (no yellow color remained). The mixture was concentrated to a final volume of 500 μL. The conjugate was purified and characterized by FPLC and SDS-PAGE (gradient gels 5–15% acrylamide), and the protein content in the conjugate was determined by the Bradford assay.

Purification by FPLC

Purification of conjugate from free polymer and free antibody was achieved by FPLC using Superdex 200HR 10/30 column from Amersham Pharmacia Biotech. Analysis was achieved using FPLC director® version 1.10 software. Samples recovered from the VivaSpin (200 μL) were passed through the FPLC column using 0.01 M phosphate buffer with 0.15 M NaCl, pH 7.4 at a flow rate of 0.5 mL/min. Purification was monitored at 280 nm. Fractions (1.0 mL) were collected and tested for activity.

Spectrophotometric assay for 38C2 catalytic antibody activity assay against the low molecular weight substrate Cynol

All reagents were prepared in PBS pH 7.4. Cynol was dissolved in acetonitrile at a concentration of 10 mM (final working concentration = 500 μM in PBS). Ab 38C2 was diluted in PBS to 2 mg/mL (final working concentration = 0.2 mg/mL).

To a 100-μL capacity well, 80 μL of PBS and 5 μL of the cynol solution were added. The 96-well microtiter plate

was placed in the spectrophotometer and baseline absorbance was monitored at 405 nm for 10 min at 25 °C. Stock solution of antibody 38C2 and conjugate (20 μL) were added to the wells. Catalytic activity (initial rate of reaction) was measured by monitoring the increase in absorbance at 405 nm due to the release of a yellow product. Specific activity (μmol/s mg) was calculated.

Drug activation assay

The generation and purification of mouse mAb 38C2 was described previously.[19] A stock solution of 12 mg/mL antibody 38C2 in PBS (pH 7.4), stored at 4 °C, was used. All antibody reactions were performed in PBS (pH 7.4) at 37 °C in microfuge tubes. Typically, reactions were carried out at concentrations of 20–200 μM of substrate and 5 μM antibody. Antibody-catalyzed reactions were monitored at 280 nm for etoposide by RP-HPLC (Hitachi L-6200A equipped with an AS-2000 autosampler and a Supelcosil LC-18 column (25 cm × 4.6 mm, 5 m) using a ratio of 35:65 of acetonitrile/water at 1 mL/min.

Cell proliferation assay with XTT reagent

A colorimetric method based on the tetrazolium salt, XTT, was first described by P. A. Scudiero in 1988. While the use of MTT produced a non-soluble formazan compound that necessitated dissolving the dye in order to measure it, the use of XTT produces a soluble dye.

MOLT-3 cells (10^4) and NXS-2 (5×10^3) cells were cultivated in a flat 96-well plate. To each well 100 μL of growth media was added. The cells were incubated in a CO_2 incubator at 37 °C. Cells were used to assay proliferation after 24 h. Cells were treated with serial descending concentrations of Pro-VP-16 + HPMA copolymer–38C2 Ab and incubated for 72 h. Control groups consisted of Pro-VP-16 alone, free and conjugated 38C2 Ab, and VP-16 alone. Activation solution (100 μL) was added to 5 mL XTT reagent. The reaction solutions (50 μL) were added to each well. The plate was incubated for 2 h, shaken gently to evenly distribute the dye in the wells. Absorbance was measured at a wavelength of 450–500 nm. In order to measure reference absorbance (to measure nonspecific readings), a wavelength of 630–690 nm was used.

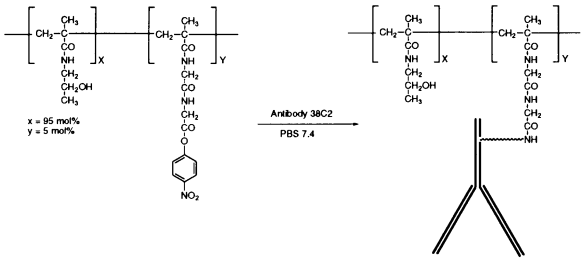

Figure 1. Schematic representation of the preparation of antibody–HPMA copolymer conjugate via an amide bond formation.

Statistical methods

All the in vitro data are expressed as the mean ± standard deviation of the mean (SD) and were performed using the Student's *t*-test. *p* Values of 0.05 or less were considered statistically significant.

Results

Synthesis of the catalytic antibody–HPMA polymer conjugate

Antibody 38C2 was conjugated to HPMA copolymer, using standard lysine chemistry. Thus, *p*-nitrophenyl ester of the polymer was reacted with amino-lysine group of the antibody in PBS 7.4 (Fig. 1). The conjugate was purified by FPLC using a superdex 200 column. After purification no free antibody was detectable, as shown by the HPLC chromatogram and gel analysis (Fig. 2). Fractions 1–6 were separated from the fractions 7 and 8, which contained multi numbers of HPMA copolymer molecules conjugated with the antibody. In fractions 1–6, one to two molecules of HPMA copolymer were conjugated per one molecule of antibody. The reaction yield calculated for the antibody molecule was 90%.

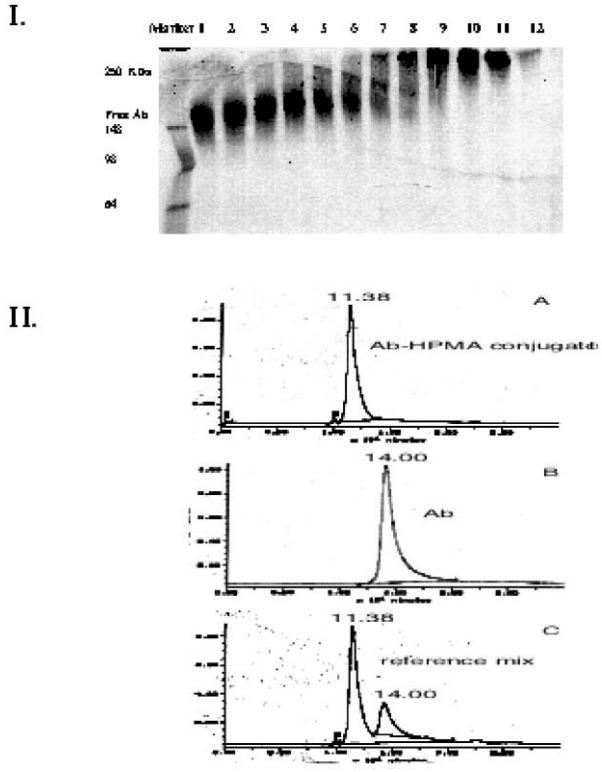

Figure 2. (**I**) Gel analysis of the antibody–HPMA copolymer conjugate collected after purification by FPLC. Different fractions are shown by SDS-PAGE (the molecular weights of the antibody and the HPMA copolymer are 150 and 30 KD, respectively). (**II**), HPLC chromatograms using size exclusion column TSKgel G2000, of the conjugate (A), the free antibody (B), and a reference mixture (C).

Catalytic activity of the antibody–HPMA polymer conjugate

The catalytic activity of the conjugate was checked against cynol **I** (fluorogenic substrate). The substrate reacts with antibody 38C2 to generate a compound with yellow color (aldehyde **II**) (Fig. 3a). The conjugate was found to be sufficiently active retaining 81% of its original catalytic activity (V_{max} Ab = 0.0021 OD/s vs V_{max} conjugate = 0.0017 OD/s) (Fig. 3b).

Prodrug activation assay

The etoposide prodrug was synthesized as described elsewhere.[17] At this point, we wanted to evaluate the ability of the conjugate to activate the etoposide prodrug. Thus, the prodrug was incubated with the free antibody and the antibody–HPMA copolymer conjugate in PBS 7.4 solutions at 37 °C. Again, the conjugate was found to be highly active, retaining 75% of its original catalytic activity as shown by detection of the free etoposide drug by HPLC assay (Fig. 4).

Cells growth inhibition

In order to evaluate the efficacy in vitro of HPMA–38C2 antibody-mediated etoposide release, the cytotoxicity of Pro-VP-16 was evaluated in the presence or absence of HPMA copolymer–38C2 antibody in cultured MOLT-3 and NXS-2 cells in vitro. XTT cytotoxicity assays in the two cell lines showed a 333-fold (0.01 μM versus 0.0003 μM VP-16 equiv in MOLT-3 cells), and 20-fold (0.01 μM versus 0.2 μM in NXS-2) decrease in IC_{50} of the combination compared to Pro-VP-16 alone (Fig. 5). Wells containing free or conjugated antibody behaved the same as control groups. There was no significant difference in the IC_{50} between combinations of prodrug with free antibody compared to conjugated antibody.

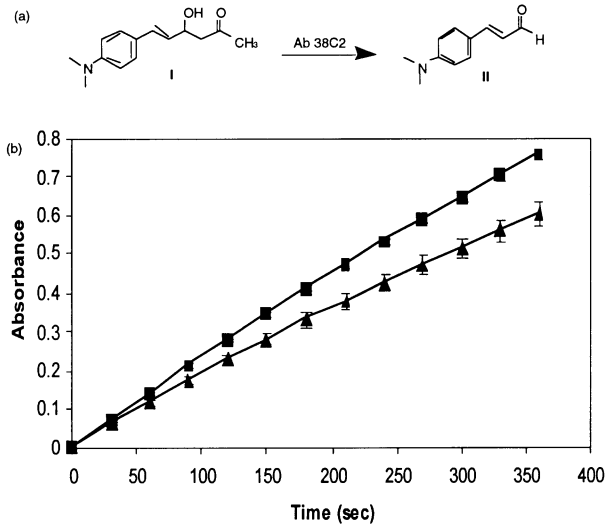

Figure 3. Comparison of the catalytic activity of free 38C2 antibody versus antibody–HPMA copolymer conjugate using cynol as a fluorogenic substrate (the absorbance at 405 nm was monitored versus time). Panel (A): schematic representation of the retro-aldol reaction catalyzed by antibody 38C2. Panel (B): the catalytic activity of antibody–HPMA copolymer conjugate [▲; ($y = 0.017x + 0.0195$)] and free antibody [■; ($y = 0.021x + 0.075$)] measured by the release of compound **II** at 405 nm.

Discussion

Antibody 38C2 has a lysine residue with a free ε-amino group in the catalytic site with perturbed pK_a of 5.8. This lysine participates directly in the antibody's catalytic reaction and is essential for its activity. Therefore, this amine group is much more reactive as a nucleophile than any other amine residues in the antibody molecule. The conjugation reaction that we used is based on an amide bond formation between an amino-lysine from the antibody and a *p*-nitrophenyl active ester of the HPMA copolymer. It was found that the ε-amino group of the lysine in the active site of the antibody retained its catalytic ability despite its preferred reactivity. In order to explain this phenomenon, we looked at the interaction of the original antigen with the binding site of the catalytic antibody (Fig. 6). The ε-amino group of the lysine reacts with the diketone antigen to form a stable *N*-aminone moiety. The resulting nitrogen-carbon positions a methyl group of the antigen at the inner side of the antibody pocket, and the rest on the external side. Therefore, it is expected that the antibody structure will limit the space in the binding site at the methyl location. The *p*-nitrophenyl group of the HPMA copolymer active ester, is much larger in size than a methyl group. Thus, it is likely that the *p*-nitrophenyl ester will not be able to react with the amino-lysine in the catalytic site because of space limitations.

Antibody 38C2 was generated using a novel strategy termed 'reactive immunization'. In this method of immunization, the antigen is reacting with functional group of the antibody molecule and this reaction becomes part of the catalytic mechanism of the

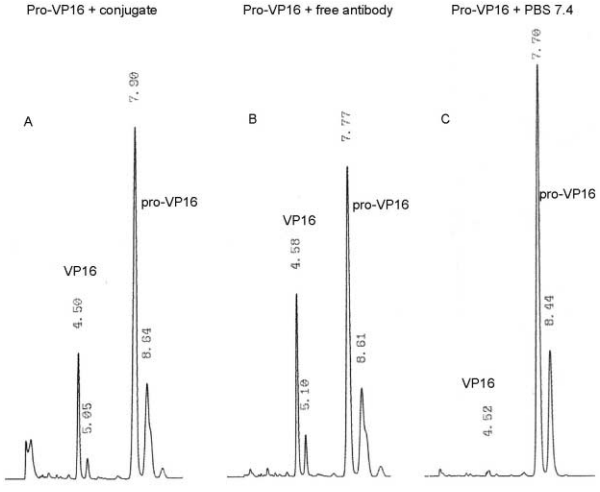

Figure 4. (A) HPLC chromatograms of the etoposide prodrug (500 μM) with antibody–HPMA copolymer conjugate (20 μM) versus (B) the etoposide prodrug (500 μM) with the free antibody (20 μM) and (C) etoposide prodrug (500 μM) in PBS 7.4. The peaks appearing at 8.6 and 5.1 min are satellites peaks of the prodrug and the drug, respectively, which are generated upon side reaction between the buffer and etoposide moiety. Reaction were incubated at 37 °C for 12 h prior to injection into the HPLC.

antibody. This approach has several advantages. In other research works regarding prodrug activation by catalytic antibodies, researchers have used the 'transition state analogue' approach to generate antibodies capable of activating a specific prodrug.[20,21] Antibody 38C2 is able to accept broad substrate scope due to the immunization method it was generated. Therefore, the chemistry developed to mask drugs into prodrugs allows the potential to use almost any drug for the described application. Three different prodrugs have been reported to date as substrates for antibody 38C2; doxorubicin prodrug,[16] camptothecin prodrug[16] and etoposide prodrug.[17] For our application, we chose the etoposide prodrug. The mechanism by which this prodrug is activated by catalytic antibody 38C2 is shown in Figure 7. The first two steps are retro-aldol and retro-Michael transformations, which are both catalyzed by the antibody. The other two steps occur spontaneously and include a decarboxylation and a cyclization step to form the free etoposide drug.

The conjugate of the catalytic antibody and the HPMA copolymer was synthesized in an excellent yield (about 90% after purification). According to gel analysis (Fig. 2), in fractions 1–6 collected from the FPLC, 1–2 molecules of HPMA copolymer were conjugated per one molecule of antibody. Furthermore, the retention of the catalytic activity in the conjugate was very high. Conjugation between a protein and a polymer has a clear advantage in terms of yield consideration. In comparison to other conjugates between two proteins such as an enzyme–antibody conjugate in the application of ADEPT, the overall yields of purified products are typically 10–15%.[22]

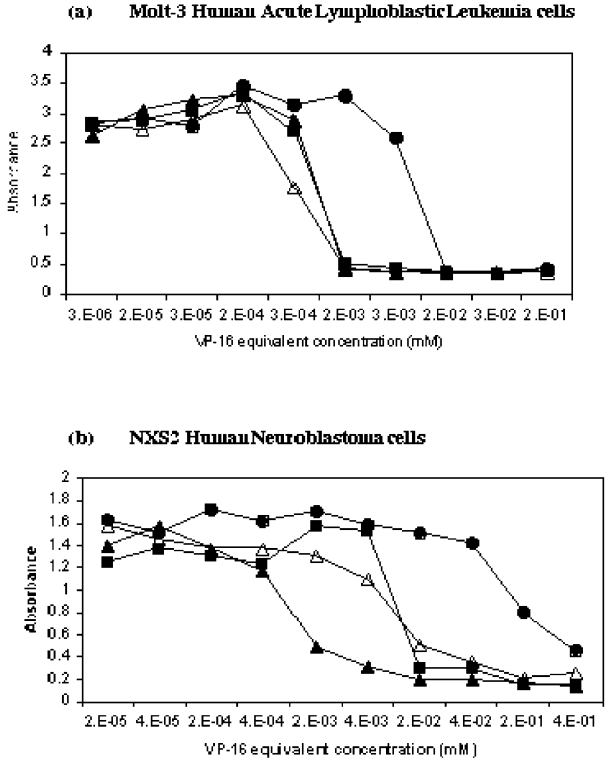

Figure 5. XTT cytotoxicity assay of Pro-VP-16 in the presence and absence of HPMA-38C2 Ab compared to VP-16 alone and untreated groups. Panel (A) assay on MOLT-3 leukemia and panel (B) on NXS-2 neuroblastoma cells. VP-16 (-▲-); Pro-VP-16 (-●-); Pro-VP-16 + 38C2 Ab (-△-); Pro-VP-16 + HPMA-38C2 Ab (-■-).

The catalytic activity of the conjugate was evaluated in three different assays. The first involved a low molecular weight substrate in which a retro-aldol cleavage reaction generated an aldehyde that has a yellow color. This assay showed that the conjugate retained 81% of the catalytic activity in comparison to the free antibody. The second assay was a prodrug activation reaction. Etoposide prodrug was incubated with either the conjugate or the free antibody. The release of the free drug was monitored by HPLC and again similar results were obtained. The third assay was cell growth inhibition. The conjugate's ability to activate the etoposide prodrug in vitro was tested using two different cell lines. The results in both cell lines were similar. The conjugate was able to activate the etoposide prodrug and consequently to inhibit proliferation in MOLT3 T-cell and NXS2 neuroblastoma cells. The assay results were almost identical in the case of the conjugate and the free antibody.

The recently described concept of polymer directed enzyme prodrug therapy (PDEPT) might be an intriguing application for the catalytic antibody–HPMA copolymer conjugate. PDEPT already offers the possibility to conjugate a non-mammalian enzyme[23] in order to avoid non-specific prodrug activation in the body. The ability of the catalytic antibody to activate several prodrugs in vitro and in vivo has been already proven. The advantage of an antibody molecule in comparison to an enzyme in the ADEPT/PDEPT complex has been discussed.[16] Application of this strategy to human therapy requires access to humanized antibodies, because of immunogenicity problems. Catalytic antibody 38C2 has been humanized recently by Tanaka et al.[24] Furthermore, the antibody catalyzes a unique retro-aldol retro-Michael tandem reaction that is not catalyzed by any known enzyme. Therefore, non-specific prodrug activation by other enzymes is minimized. Several protein–polymer conjugates have already been prepared and showed improved EPR effect for the conjugate compared with the free protein.[25–27] Specifically, Rihova et al. have prepared an IgG conjugate with HPMA copolymer–doxorubicin, which showed superior anti-tumor effect.[28] Functional in vivo study of the PDEPT approach with the catalytic antibody and prodrug–HPMA copolymer conjugates will be reported soon.

In summary, we have prepared the first catalytic antibody–HPMA copolymer conjugate based on amide bond formation between an external lysine of the antibody and an active ester of the HPMA copolymer. The conjugation yield was very high and the antibody retained most of its catalytic activity in the conjugate. Furthermore, we have shown that the antibody–polymer conjugate can activate an etoposide prodrug in vitro and consequently inhibit proliferation in two different cancer cell lines (Molt3-T-cell leukemia and

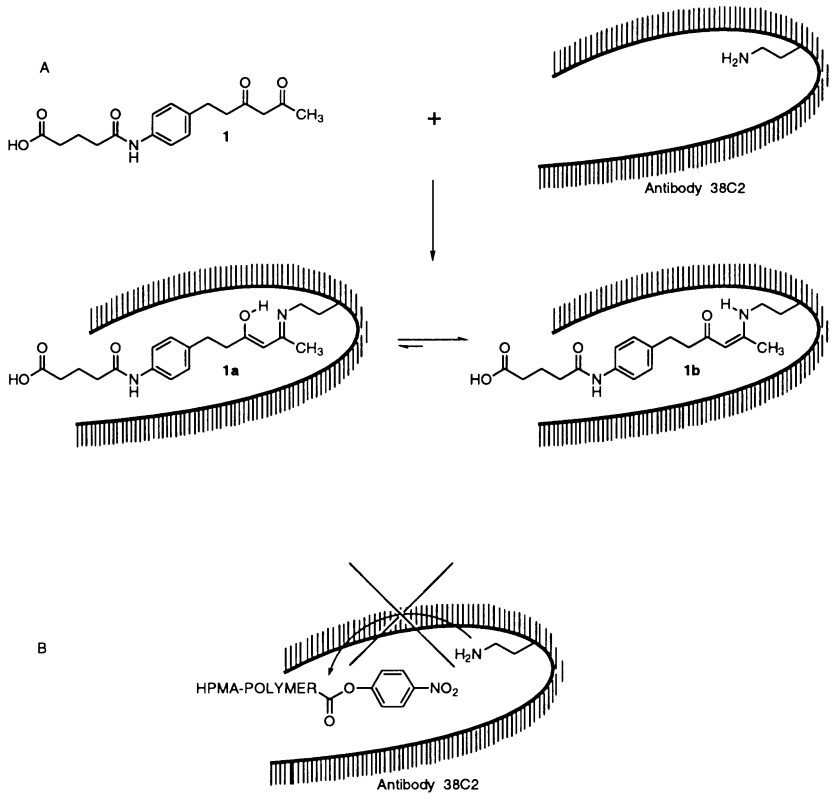

Figure 6. (A) Schematic delineating the interaction between the original antigen and catalytic antibody 38C2. (B) The interaction of the *p*-nitrophenyl ester of the HPMA copolymer with the amino lysine in the catalytic pocket of antibody 38C2.

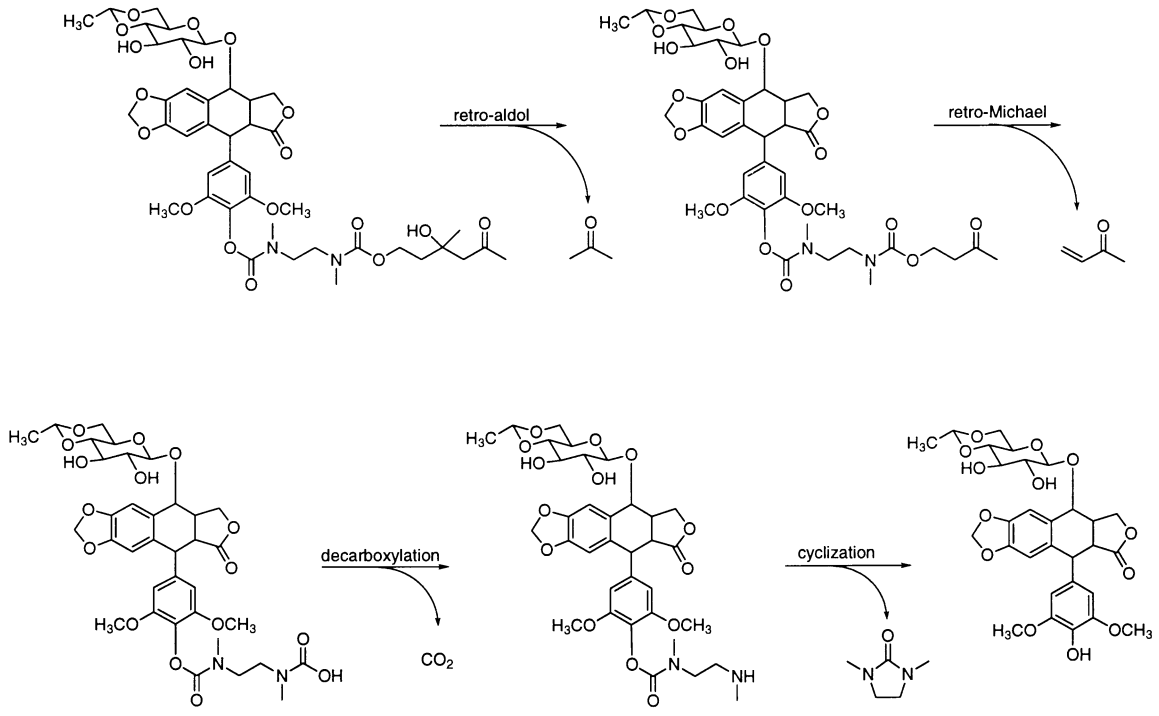

Figure 7. Mechanism of the etoposide prodrug activation by catalytic antibody 38C2.

NXS2-neurtoblastoma). The design of in vivo experiments models for PDEPT or MDEPT combinations is currently in progress.

Acknowledgements

We wish to thank Dr. Richard A. Lerner and Dr. Carlos F. Barbas III of The Scripps Research Institute for providing antibody 38C2 and TEVA Pharmaceutical Industries Ltd., Israel for a gift of etoposide.

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