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Poly(ethylene glycol) modification of β-glucuronidase-antibody conjugates for solid-tumor therapy by targeted activation of glucuronide prodrugs

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Abstract Methoxypoly(ethylene glycol) (PEG) modification of *Escherichia coli* β-glucuronidase (βG) was examined as a method to improve the stability and pharmacokinetics of antibody-βG conjugates for the targeted activation of glucuronide prodrugs at tumor cells. Introduction of 3 PEG molecules did not affect βG activity whereas higher degrees of PEG modification produced progressively greater loss of enzymatic activity. The enzyme was found to be stable in serum regardless of PEG modification. PEG-modified βG was coupled via a thioether bond to mAb RH1, an IgG_{2a} antibody that binds to the surface of AS-30D hepatoma cells, to produce conjugates with 3 (RH1-βG-3PEG), 5.2 (RH1-βG-5PEG) or 9.8 (RH1-βG-10PEG) PEG molecules per βG with retention of 75%, 45% and 40% of the combined antigen-binding and enzymatic activity of the unmodified conjugate RH1-βG. In contrast to the rapid serum clearance of RH1-βG observed in mice, the PEG-modified conjugates displayed extended serum half-lives. RH1-βG-3PEG and RH1-βG-5PEG also exhibited reduced spleen uptake and greater tumor accumulation than RH1-βG. BHAMG, the glucuronide prodrug of *p*-hydroxyaniline mustard (pHAM), was relatively nontoxic in vivo. Injection of 6 mg/kg or 12 mg/kg pHAM i.v. depressed white blood cell numbers by 46% and 71% whereas 80 mg/kg BHAMG reduced these levels by 22%. Although the tumor/blood ratio of RH1-βG-5PEG was adversely affected by slow clearance from serum, combined therapy of small solid hepatoma tumors with this conjugate, followed 4 and 5 days later with i.v. injections of BHAMG, cured all of seven mice with severe combined immunodeficiency. Combined treatment with a control antibody-βG conjugate and BHAMG delayed tumor growth and cured two of six mice while treatment with pHAM or BHAMG alone was ineffective.

Key words Monoclonal antibody · β-Glucuronidase · Prodrugs · Cancer therapy · Poly(ethylene glycol)

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Introduction

Cancer chemotherapy is often terminated because of unacceptable toxicity to normal tissues and associated side-effects [1]. Drug specificity can be increased by linking antineoplastic agents to monoclonal antibodies that bind to antigens preferentially expressed on the surface of tumor cells [2, 3]. Limits on the amount of drug that can be linked to antibodies [2], slow internalization of drug conjugates into cancer cells [4], and heterogeneous antigen expression and conjugate distribution in solid tumors [5], however, combine to limit the in vivo efficacy of direct drug conjugates. An alternative two-step strategy [6, 7], in which an antibody-enzyme conjugate is targeted to tumor cells for subsequent activation of an antineoplastic prodrug, can theoretically overcome many of the limitations inherent in chemoimmunoconjugates. Antibody-enzyme conjugates are non-toxic, allowing administration of high concentrations for enhanced tumor localization and intratumor penetration. Conjugate internalization is unnecessary, as enzyme localized on the surface of tumor cells is capable of activating a subsequently administered prodrug [8]. In addition, enzymatic conversion of prodrugs in tumors can produce high concentrations of low-molecular-mass active anti-neoplastic drugs [9], which should be able to diffuse throughout the tumor to produce bystander killing [10].

Although several enzyme-prodrug combinations are currently being investigated for cancer therapy [11], our efforts have focused on the activation of the glucuronide

prodrug (BHAMG) of *p*-hydroxyaniline mustard (pHAM) by β-glucuronidase (βG) linked to monoclonal antibodies [12, 13]. βG-activated glucuronide prodrugs possess potential advantages for cancer therapy. Because the concentration of βG in human serum is very low [14], glucuronide prodrugs should be resistant to premature activation in the blood after i.v. administration. Although the liver, gastrointestinal tract, spleen and lungs contain endogenous βG [15], mammalian tissues also express UDPglucuronosyltranferases, a class of xenobiotic-detoxification enzymes that can reverse the reaction catalyzed by βG [16]. Glucuronide conjugates are major metabolites of many drugs in humans [17, 18], further indicating that glucuronide prodrugs may not be prematurely activated by endogenous βG in vivo. Activated prodrug not taken up by tumor cells may also be reconverted to the glucuronide conjugate after passing through organs containing high UDPglucuronosyltransferase activities. In addition, *Escherichia coli*-derived βG has a pH optimum at physiological pH [19] and the low specificity of the enzyme for aglycones conjugated with the glucuronide group [14] has allowed the development of a variety of glucuronide prodrugs [20–22]. Glucuronide prodrugs also possess increased water solubility [20], which may allow improved formulation of insoluble antineoplastic agents.

The aim of the present investigation was to study the effect of polyethylene glycol modification of βG on enzyme stability and the pharmacokinetics and tumor localization of antibody-βG conjugates for the targeted activation of BHAMG for solid tumor therapy. Preliminary studies employing a conjugate formed between βG and mAb RH1, an IgG_{2a} monoclonal antibody that binds to a 32-kDa antigen expressed on the surface of AS-30D hepatoma cells [23], indicated that RH1-βG rapidly disappeared from the circulation of mice. In addition, βG has been reported to be unstable in serum [20]. Proteins derivatized with monomethoxypoly(ethylene glycol) (PEG), a linear, hydrophobic, uncharged, flexible polymer [24], often exhibit increased stability, extended serum half-lives, and reduced immunogenicity [25]. We demonstrate that βG is stable in serum regardless of PEG modification. In addition, PEG modification extended the half-life of RH1-βG, decreased normal tissue uptake and increased the localization of conjugates at solid tumors in nude mice. Combined therapy with PEG-modified RH1-βG and BHAMG also produced cures in 100% of mice with severe combined immunodeficiency (scid) bearing small solid AS-30D hepatoma tumors.

Materials and methods

Reagents and cells

p-Hydroxyaniline mustard (pHAM) and its glucuronide prodrug (BHAMG) were synthesized as described [12]. Succinimidyl succinate poly(ethyleneglycol) 5 kDa, *p*-nitrophenyl β-D-glucuronide, *p*-nitrophenol, trinitrobenzenesulfonic acid, Sepharose CL-4B protein A and bovine serum albumin (BSA; fraction V) were purchased from Sigma Chemical Company, St. Louis, Mo. Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate was from Pierce Chemical Company, Rockford, Ill. Sephadex G-25 and Sephacryl S-300 HR gels were purchased from Pharmacia Biotech Far East Ltd., Taipei, Taiwan. [^{3}H]Leucine was purchased from Amersham International plc, Buckinghamshire, England. Taq DNA polymerase was from Perkin Elmer, Norwalk, and restriction enzymes were from BRL, Gaithersburg. HepG2 human hepatoma cells and FO myeloma cells were obtained from ATCC, Rockville, Md. The AS-30D rat hepatoma cell line [26] was generously provided by Dr. J. P. Chang, Institute of Zoology, Academia Sinica, Taiwan. HepG2 cells were maintained in RPMI-1640 medium (Gibco BRL, Grand Island, N.Y.) supplemented with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. AS-30D cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented as above.

Animals

BALB/c, nude and scid mice were obtained from the animal room of the Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan. Animal experiments were performed in accordance with institute guidelines.

Production of recombinant βG

Plasmid pRAJ260 [19], generously provided by Dr. R. A. Jefferson, Department of Molecular, Cellular and Developmental Biology, University of Colorado, was used as the template for polymerase chain reaction (PCR) amplification of βG with the primers 5′-CGAGATCTGCAGGTCAGTCCCTTATGTTA-3′ and 3′-TCGTCCCTCCGTTTGTTACTTTTCGAATGAGAGG-5′ to introduce *Pst*I and *Hin*dIII restriction sites before the initiation and after the stop codons of βG respectively. Amplification was performed in a Perkin-Elmer thermocycler by touchdown PCR [27]. The amplified fragment was digested with *Pst*I and *Hin*dIII and subcloned in-frame into pRSETB (Invitrogen, The Netherlands) to introduce a histidine tag at the N-terminus of βG. Recombinant βG was produced by isopropyl β-D-thiogalactopyranoside induction of BL21(DE3) bacteria (Novagen, Madison, Wis.) that had been transformed with the pRSETB-βG vector. βG was purified from crude bacterial lysates by metal chelate chromatography on His-Bind resin (Novagen). After washing of the column with 30 mM imidazole, 0.5 M NaCl, 20 mM TRIS/HCl, pH 7.9, βG was eluted with 500 mM imidazole, 250 mM NaCl, 10 mM TRIS/HCl, pH 7.9. The purified enzyme was desalted on a Sephadex G-25 column equilibrated with phosphate-buffered saline (PBS) and stored at –80 °C. Recombinant βG, obtained in yields of 70–80 mg/l bacterial culture, ran as a single band on sodium dodecylsulfate/polyacrylamide gels and possessed activity equivalent to a highly purified commercial preparation of βG (Sigma Type X-A).

Antibodies

mAb RH1 is a murine IgG_{2a} monoclonal antibody, developed in our laboratory, which binds a 32-kDa antigen expressed on the surface of AS-30D cells [23]. Control hybridoma H16-L10-4R5, which secretes an IgG_{2a} antibody (mAb HB65) against a nucleoprotein of influenza A virus, was obtained from the American Type Culture Collection (Rockville, Md.). A hybridoma secreting mAb 1E8 (IgG_1) was generated as described [28] by fusing FO myeloma cells with spleenocytes isolated from a BALB/c mouse that had been immunized with recombinant βG. Polyclonal rabbit serum against βG was produced by standard techniques. mAb were purified from ascites produced in BALB/c mice by protein-A affinity chromatography. Secondary antibodies were from Organon (Durham, N.C.).

Pharmacokinetics of RH1-βG in mice

Groups of two BALB/c mice were i.v. injected with 300 µg βG, RH1-βG or mAb RH1 in sterile PBS. βG and RH1-βG concentrations in

serum, recovered from whole blood by centrifugation, were estimated by measuring serum βG activity. A 20-µl sample of serum diluted in PBS containing 0.05% BSA was added to 200 µl βG assay buffer (100 mM acetic acid, 50 mM BISTRIS, 50 mM triethanolamine, pH adjusted to 7 with NaOH) in 96-well microtiter plates. A 25-µl sample of 32 mM *p*-nitrophenyl β-D-glucuronide substrate was added for 30 min at 37 °C and the absorbance of wells was then measured in a microtiter plate reader (Molecular Devices, Menlo Park, Calif.) at 405 nm. mAb RH1 concentrations were determined by ELISA using AS-30D cells coated in 96-well plates as antigen source [2]. Half-lives of conjugates were estimated by linear regression analysis of data plotted as log concentration versus time.

PEG modification of βG

Various weight ratios of succinimidyl succinate poly(ethylene glycol) were added to βG (1.7 mg/ml in deoxygenated PBS containing 1 mM EDTA) for 2 h at room temperature. After extensive dialysis against PBS (five changes), samples were analyzed for protein concentration by the bicinchoninic acid assay (Pierce, Rockford, Ill.) with BSA employed as the reference protein. βG activity was measured as described above with recombinant βG employed as a standard. The number of PEG molecules attached to βG was estimated by measuring the reduction of trinitrobenzesulfonic-acid-reactive amine groups in βG after PEG modification [29]. A molar absorption coefficient for free amino groups (ε_{335}) of 1.46×10^4 M^{-1} cm^{-1} was estimated by reacting known concentrations of recombinant βG with trinitrobenzenesulfonic acid.

βG stability

Recombinant βG and PEG-modified βG (500 µg/ml) were incubated in PBS at 4 °C or 37 °C or in PBS containing 50% bovine, mouse or human serum at 37 °C for 24 h. Samples were serially diluted in PBS or in PBS containing 0.5% BSA and 20-µl samples were assayed for βG activity as described above, except that the assays were carried out for 20 min (PBS-diluted samples) or 10 min (samples diluted in PBS+0.5% BSA) before the absorbance was read at 405 nm. The in vivo stability of βG was determined by i.v. injecting 500 µg βG into a BALB/c mouse. Serum samples recovered before and 5 min, 30 min, 1 h, 2 h, and 4 h after injection were electrophoresed on a reduced sodium dodecyl sulfate/polyacrylamide gel, transferred to nitrocellulose paper and immunoblotted with mAb 1E8 or polyclonal rabbit serum against βG. After incubation with the appropriate horseradish-peroxidase-coupled second antibodies, immunoblots were developed with the SuperSignal CL-HRP substrate system (Pierce).

Antibody-βG conjugates

Conjugates with different degrees of PEG modification were generated by reacting βG with 3.5, 7, or 14 mg succinimidyl succinate poly(ethylene glycol)/mg βG before conjugation. These conjugates are referred to as mAb-βG-3PEG, mAb-βG-5PEG, and mAb-βG-10PEG respectively. One-twentieth volume of saturated glycine in PBS was added to PEG-modified βG for 15 min to deactivate free PEG before separation on Sephadex G-75 equilibrated with PBS containing 1 mM EDTA. βG-PEG was coupled to mAbs RH1 and HB65 via thioether bonds as described [13]. Conjugates were purified by size-exclusion chromatography on Sephacryl S-300 HR equilibrated with PBS. The molecular mass of conjugates was greater than 220 kDa as estimated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Protein concentrations were measured by the bicinchoninic acid assay and enzyme activities of conjugates were measured as described above. The combined antigen-binding and βG activities of conjugates were determined by adding 50 µl serially diluted conjugate to microtiter plates coated with AS-30D cells [2]. After incubation for 1 h at 37 °C, plates were washed with PBS and then assayed for βG activity as described above.

In vitro activation of prodrug by RH1-βG-3PEG

AS-30D or HepG2 cells were plated overnight in 96-well microtiter plates at 20000 cells/well. A 1-µg/ml sample of RH1-βG-3PEG was added to the cells in triplicate for 30 min at room temperature; controls received no RH1-βG-3PEG. After washing cells once with PBS, serial dilutions of pHAM or BHAMG in medium containing 5% fetal calf serum were added to cells for 2 h at 37 °C. Cells were subsequently washed once with sterile PBS, incubated for 48 h in fresh medium and then pulsed for 2 h with [^{3}H]leucine (1 µCi/well) in leucine-free medium. Radioactivity of trichloroacetic-acid-precipitated protein was measured in a Beckman LS 6000 series liquid scintillation counter. Results are expressed as percentage inhibition of [^{3}H]leucine incorporation (cpm) compared with untreated cells by the following formula:

$$\text{Inhibition } (\%) = 100 \times \frac{^3\text{H in sample} - {}^3\text{H background}}{^3\text{H in control} - {}^3\text{H background}}$$

Pharmacokinetics of PEG-modified conjugates in mice

Samples containing 300 µg RH1-βG or RH1-βG-PEG (different degrees of modification) were injected into the lateral tail vein of groups of five mice. Serum, isolated from blood samples, was serially diluted in PBS containing 0.05% BSA. Known concentrations of RH1-βG or RH1-βG-PEG, employed as standards, were likewise diluted and 50-µl samples were added to microtiter plates coated with AS-30D cells for 1 h at 37 °C. Plates were washed three times with PBS and assayed for βG activity. This assay measures intact conjugates that display both antigen-binding and enzymatic activities.

Conjugate localization

Samples containing 10^7 AS-30D cells were injected s.c. into 8-week old nude mice. After tumors had grown to approximately 1 cm^3, 300 µg RH1-βG, HB65-βG-3PEG, RH1-βG-3PEG or RH1-βG-5PEG was i.v. injected into the lateral tail vein of mice. Groups of three to ten mice for RH1-βG and RH1-βG-5PEG and one mouse for HB65-βG-3PEG and RH1-βG-3PEG were sacrificed 48, 72 and 96 h later. Blood and tumor, liver, kidney, spleen, stomach and lung tissues were removed and washed in PBS. Individual tissue samples were weighed on an analytical balance, cut into small pieces and suspended in 2 ml βG assay buffer. Samples were sonicated four times for 20 s on ice and then assayed for βG activity as described above. Uptake of βG in tumors and organs was determined by subtracting the activity of endogenous βG in tissues obtained from tumor bearing mice that did not receive conjugate.

Drug toxicity

Acute toxicity of drugs in BALB/c mice was determined by i.v. injecting 0, 3, 6, or 12 mg/kg pHAM in dimethylsulfoxide or 0, 10, 20 or 40 mg/kg BHAMG in PBS on 2 consecutive days and observing animal survival for 100 days. To determine hematological toxicity, groups of BALB/c mice (n = 5–10) were i.v. injected with 0, 3, 6, or 12 mg/kg pHAM in dimethylsulfoxide or 0, 10, 20 or 40 mg/kg BHAMG in PBS on days 0 and 1. A 50-µl sample of blood from the tail vein of mice was collected into Labcraft disposable 100-µl calibrated pipettes that had been prefilled with 50 µl PBS containing 1.5 mg/ml EDTA. Blood was immediately expelled into microfuge tubes containing 50 µl of the same buffer and the number of platelets, lymphocytes and red blood cells were measured with a Coulter T-540 cell counter.

Solid-tumor therapy

Groups of six or seven scid mice were s.c. injected on the right flank with 5×10^6 AS-30D cells on day 1. On day 5, mice were left untreated

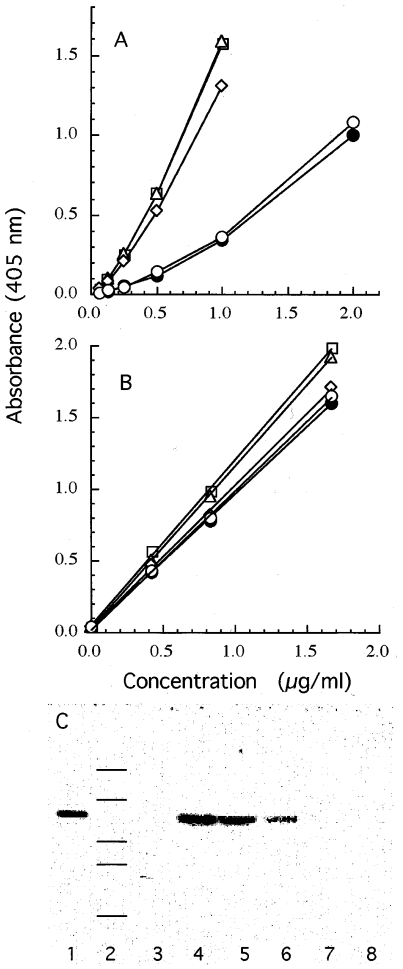

Fig. 1A–C Stability of β-glucuvonidase (βG) in serum. βG (500 μg/ml) was incubated for 24 h in phosphate-buffered saline (PBS) at 4 °C (●) or in 50% bovine (□), mouse (△) or human (◇) serum or PBS (○) at 37 °C. Samples were then serially diluted in PBS (**A**) or in PBS containing 0.5% bovine serum albumin BSA (**B**) before assay of βG activity. Results represent mean values of duplicate determinations. **C** Serum samples recovered from a BALB/c mouse after i.v. injection of βG were electrophoresed on a reduced polyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with mAb 1E8. Lanes: *1* recombinant βG; *2* position of prestained molecular mass standard proteins (112, 86, 70, 57, and 39.5 kDa); *3* serum sample before injection of βG; and serum samples 5 min (*4*), 30 min (*5*), 1 h (*6*), 2 h (*7*) or 4 h (*8*) after injection of βG

(control, pHAM, and BHAMG groups) or i.v. injected via the lateral tail vein with 200 μg RH1-βG-5PEG or HB65-βG-5PEG. On days 9 and 10, mice were i.p. injected with 3 mg/kg pHAM in dimethylsulfoxide (pHAM group) or i.v. injected with PBS (control group) or 15 mg/kg BHAMG in PBS (BHAMG, RH1-βG-5PEG and HB65-βG-5PEG groups). Tumor volumes were calculated as length × width × height × 0.5. Mice were killed when they displayed signs of morbidity or when the tumor size exceeded 2.5 cm^3.

Statistical analysis

Statistical significance of differences between mean values was estimated with the shareware program Schoolstat (White Ant Occasional Publishing, West Melbourne, Australia) using the independent *t*-test for unequal variances.

Results

In vivo pharmacokinetics of RH1-βG

The serum concentration of RH1-βG after i.v. injection into BALB/c mice rapidly decreased with an initial half-life of 1.4 h (results not shown). βG was also rapidly eliminated with an initial serum half-life of less than 1.3 h. mAb RH1, in contrast, was eliminated more slowly with an initial half-life of 6.3 h, suggesting that rapid elimination of RH1-βG from the circulation of mice was due to loss of βG activity or removal of βG from the blood pool.

βG stability in serum

To determine whether deactivation of βG was responsible for the rapid decline of βG and RH1-βG observed in mice, the stability of βG in serum was investigated. Figure 1A shows that the activity of βG incubated in PBS at 37 °C for 24 h was essentially identical to enzyme incubated at 4 °C. Incubation of βG in human serum increased activity by 250% whereas activity increased 290% in bovine or mouse serum. In this experiment, all samples were diluted in PBS before assaying βG activity. βG incubated in PBS for 24 h was also serially diluted in PBS containing 0.5% BSA before assaying βG activity. Under these conditions, enzymatic activity increased to the levels found in human serum (Fig. 1B), demonstrating that serum stimulated rather than stabilized βG activity. The activity of βG was also stimulated in solutions containing immunoglobulin, mucin, or the detergent CHAPS (results not shown). Immunoblot analysis of serum obtained after i.v. injection of βG into a BALB/c mouse did not detect any degradation of the enzyme (Fig. 1C). Identical results were obtained on immunoblots probed with rabbit polyclonal antibodies against βG (data not shown). The half-life of βG, estimated by densiometric analysis of immunoblots probed with monoclonal or polyclonal antibodies, was 0.8 h and 2.3 h respectively, bracketing the estimated half-life of βG determined by enzyme assay of blood samples (less than 1.3 h). These results indicate that the rapid decline of RH1-βG in serum after i.v. injection in mice was not due to deactivation or degradation of βG.

PEG modification of βG

Reaction of βG with increasing weight ratios of succinimidyl succinate poly(ethyleneglycol) resulted in a dose-related increase in the number of PEG molecules conjugated to βG with a corresponding decrease in enzymatic activity (Fig. 2A). Introduction of 3 PEG molecules did not decrease enzyme activity whereas linkage of 5 or 10 PEG molecules to βG resulted in 25% and 35% reduced activity respectively. PEG-modified βG was stable in serum (Fig. 2B).

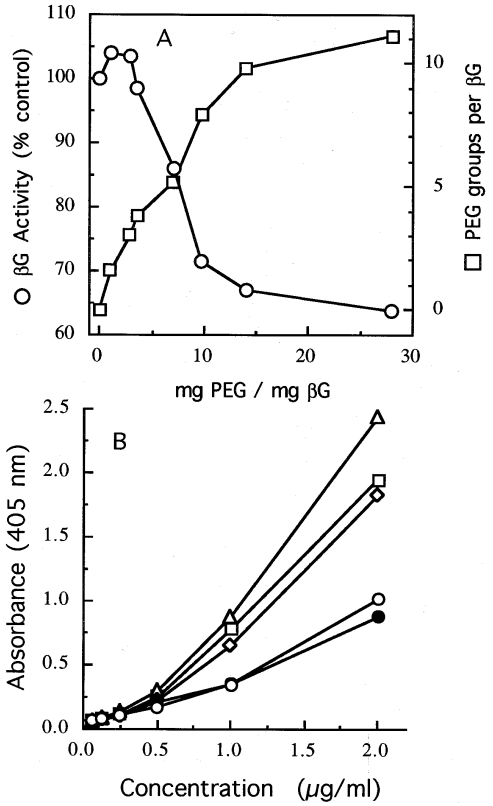

Fig. 2A,B Poly(ethylene glycol) (*PEG*) modification of βG. **A** βG was reacted with different weight ratios of succinimidyl succinate monomethoxypoly(ethylene glycol) for 2 h at room temperature. The number of PEG molecules introduced into βG (□) and mean values of βG-PEG activity (○) from duplicate determinations are compared to the activity of native βG. **B** βG-3PEG (500 μg/ml) was incubated for 24 h in PBS at 4 °C (●) or in 50% bovine (□), mouse (△) or human (◇) serum or PBS (○) at 37 °C before samples were serially diluted in PBS and assayed for βG activity

PEG-modified antibody-βG conjugates

PEG-modified βG was covalently linked to mAb RH1 and HB65 to form the conjugates RH1-βG-3PEG, HB65-βG-3PEG, RH1-βG-5PEG and RH1-βG-10PEG, containing βG modified with an average of 3, 5.2, or 9.8 molecules of PEG. The enzymatic activity of RH1-βG-3PEG and the control antibody conjugate HB65-βG-3PEG was similar to that of RH1-βG whereas the βG activity of RH1-βG-5PEG and RH1-βG-10PEG was about 25% lower than that of the unmodified conjugate (data not shown). The combined antigen-binding and enzymatic activities of RH1-βG-3PEG, RH1-βG-5PEG, and RH1-βG-10PEG were approximately 25%, 55% and 60% lower than that of RH1-βG (Fig. 3A). Conjugate binding to AS-30D cells was antibody-mediated, as shown by the low binding of the control conjugate HB65-βG-3PEG to these cells. None of the conjugates bound antigen-negative HepG2 cells (data not shown), confirming that the conjugates retained antigen-binding specificity.

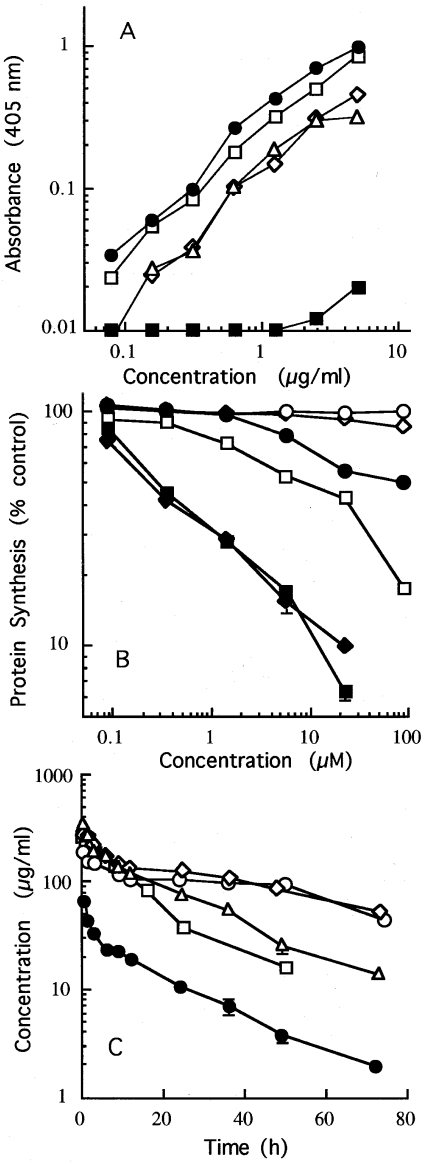

Fig. 3A–C Activity and in vivo elimination of PEG-modified conjugates. **A** Serial dilutions of RH1-βG (●), RH1-βG-3PEG (□), RH1-βG-5PEG (△), RH1-βG-10PEG (◇), or HB65-βG-3PEG (■) were incubated in microtiter plates coated with antigen-positive AS-30D cells before assay of βG activity. Results represent mean values of duplicate determinations. **B** Antigen-positive AS-30D (*solid symbols*) or antigen-negative HepG2 cells (*open symbols*) were incubated with *p*-hydroxy aniline mustard (pHAM; ■, □) or the glucuronide prodrug of *p*-hydroxy aniline mustard (BHAMG; ●, ○) or with medium containing 1 μg/ml RH1-βG-3PEG for 30 min before cells were washed and BHAMG (◆, ◇) was added for 2 h. Cells were incubated in fresh medium for an additional 46 h and incorporation of [^{3}H]leucine into cellular protein was measured. Results represent mean values of triplicate determinations. **C** 300 μg of mAb RH1 (○), RH1-βG (●), RH1-βG-3PEG (□), RH1-βG-5PEG (△), or RH1-βG-10PEG (◇) was injected i.v. into groups of five BALB/c mice at time zero. Antibody and conjugate concentrations were determined in serum samples obtained at the indicated times. *Bars* SE of the mean

Table 1 Comparison of conjugate half-life and localization in tumor and spleen. *NM* not meaningful, *ND* not determined, *βG* β-glucuronidase, *RH1* RH1 antibody, *PEG* poly(ethylene glycol), $\tau_{1/2\alpha}$ initial half-life, $\tau_{1/2\beta}$ terminal half-life. Specific βG activity was determined 96 h after conjugate administration

Conjugate	Half-life		Specific βG activity (10^{-3} μmol mg^{-1} h^{-1})			Activity ratio	
	$\tau_{1/2\alpha}$ (h)	$\tau_{1/2\beta}$ (h)	Tumor	Spleen	Serum	Tumor/blood	Tumor/spleen
RH1-βG	<1.3	17.7	0.7±1.6	6.5±1.6	0.0	NM	0.26
RH1-βG-3PEG[a]	7.3	15.4	6.1	1.0	0.55	11	6
HB65-βG-3PEG[a]	ND	ND	0.04	0.8	0.45	0.09	0.05
RH1-βG-5PEG	7.2	19.1	8.2±5.3*	0.2±0.6**	3.8±0.87	2.2	41
RH1-βG-10PEG	10.4	43.6	ND	ND	ND	NM	NM
RH1	7.3	51.9	NM	NM	NM	NM	NM

* $P = 0.10$ compared with RH1-βG
** $P < 0.05$ compared with RH1-βG
[a] Values represent the results of one mouse

The IC_{50} value of BHAMG to AS-30D cells was about 90 μM (Fig. 3B). AS-30D cells preincubated with 1 μg/ml RH1-βG-3PEG, however, were killed by BHAMG with an IC_{50} value of 0.28 μM, similar to the IC_{50} value produced by pHAM (0.22 μM), showing that RH1-βG-3PEG efficiently converted BHAMG to pHAM at antigen-positive tumor cells. In contrast, RH1-βG-3PEG did not appreciably convert BHAMG to pHAM at HepG2 antigen-negative cells (Fig. 3B), confirming the specificity of this conjugate. Protein synthesis was not affected in AS-30D cells that were preincubated with HB65-βG-3PEG before addition of BHAMG (data not shown), indicating that the control conjugate did not substantially bind to AS-30D cells.

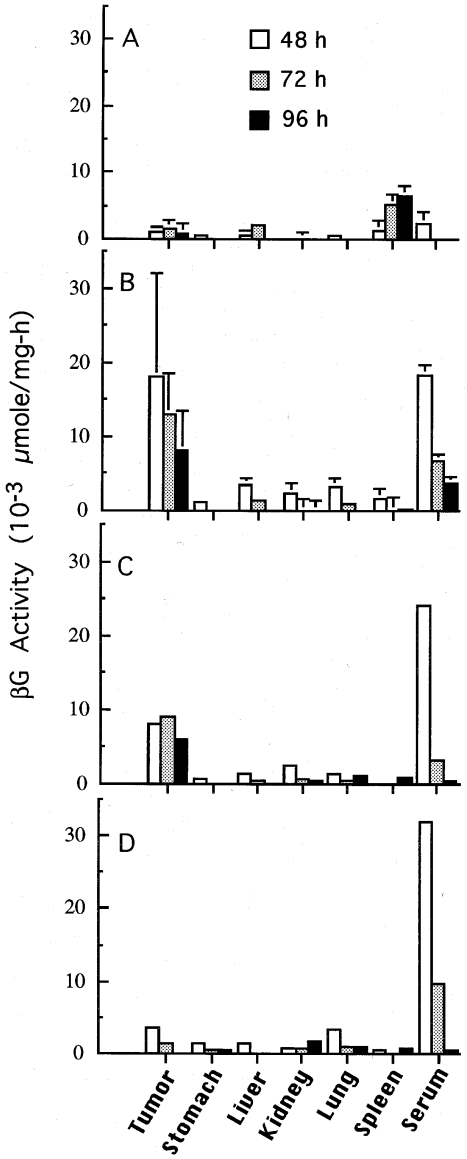

Fig. 4A–D Distribution of antibody-βG conjugates in tumor-bearing mice. Specific βG activity was measured in tumors, serum, and normal tissues removed 48, 72 and 96 h after i.v. injection of 300 μg of RH1-βG (**A**) or RH1-βG-5PEG (**B**) into groups of three to ten nude mice or injection of 300 μg RH1-βG-3PEG (**C**) or HB65-βG-3PEG (**D**) into individual nude mice bearing established s.c. solid AS-30D tumors. *Bars* SE of the mean

Serum half-lives of RH1-βG-PEG

PEG-modified conjugates displayed extended circulation times in BALB/c mice compared with RH1-βG (Fig. 3C). The elimination of RH1-βG-10PEG was similar to mAb RH1 elimination whereas conjugates modified with less PEG (RH1-βG-5PEG and RH1-βG-3PEG) were eliminated more rapidly than mAb RH1 but more slowly than RH1-βG. Attachment of 3 or 5 PEG molecules to βG increased the initial half-lives of conjugates compared to RH1-βG, whereas incorporation of 10 PEG molecules also extended the terminal half-life of RH1-βG-10PEG (Table 1). The rapid elimination of RH1-βG could not be attributed to any instability of the conjugate since blood levels were similar regardless of whether enzyme activity (data not shown) or intact conjugate (Fig. 3C) was measured.

Tumor localization of RH1-βG-PEG

Low levels of tumor localization were observed after injection of 300 μg RH1-βG (Fig. 4A). RH1-βG-5PEG,

Table 2 Hematological toxicity of the glucuronide prodrug of *p*-hydroxyaniline mustard (*BHAMG*) and *p*-hydroxyaniline mustard (*pHAM*) in mice. Values represent the percentage of white blood cells (*WBC*), red blood cells (RBC) and platelets (*PLT*) in mice i.v. injected on day 0 with BHAMG or pHAM relative to untreated control mice (100%)

Day	BHAMG 80 mg/kg			pHAM					
				6 mg/kg			12 mg/kg		
	WBC	RBC	PLT	WBC	RBC	PLT	WBC	RBC	PLT
2	100	103	106	56**	95*	95	64*	90*	66**
6	96	96	116	54**	79***	161	29**	68**	63*
9	78	99	90	90	78**	86	72	46***	42*

*–*** Significant differences between the BHAMG group (80 mg/kg) and the corresponding pHAM groups are indicated: * $P \leq 0.05$; ** $P \leq 0.005$; *** $P \leq 0.0005$

on the other hand, exhibited enhanced tumor localization (Fig. 4B) compared with RH1-βG, although the results were not statistically significant ($P = 0.10$) because of the large variation in measured enzyme activities. Results employing limited numbers of mice indicated that RH1-βG-3PEG also displayed enhanced tumor localization (Fig. 4C). Although the absolute tumor uptake of RH1-βG was low after 96 h, a high tumor/blood ratio was achieved through the rapid clearance of conjugate from the blood pool (Table 1). The tumor/blood ratio for RH1-βG-3PEG was also substantial 96 h after administration. Although the highest absolute tumor uptake was achieved with RH1-βG-5PEG, the tumor/blood ratio was only 2.2 after 96 h because of the slower clearance of this conjugate from serum. In contrast to the tumor/blood ratio, the tumor/spleen ratio was inversely related to the degree of PEG modification; spleen uptake of RH1-βG-5PEG (tumor/spleen = 41) was significantly ($P < 0.05$) lower than that of RH1-βG (tumor/spleen = 0.26). The control conjugate HB65-βG-3PEG did not specifically accumulate in solid AS-30D tumors (Fig. 4D).

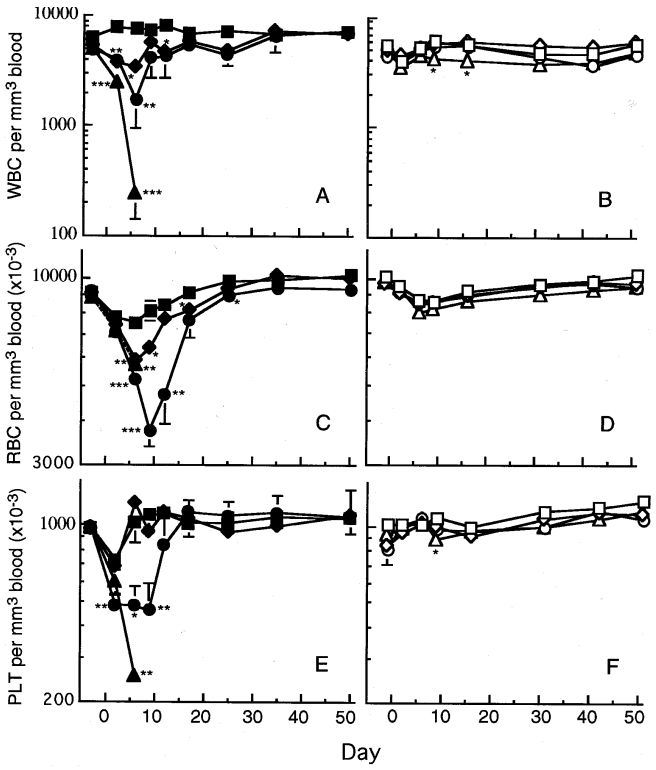

Fig. 5A–F Toxicity of pHAM and BHAMG in BALB/c mice. The mean numbers of white blood cells (*WBC*; **A**, **B**), red blood cells (*RBC*; **C**, **D**) and platelets (*PLT*; **E**, **F**) of groups of five to ten BALB/c mice after i.v. injection on days 0 and 1 of 0 (■), 3 (◆), 6 (●) or 12 (▲) mg/kg pHAM or 0 (□), 10 (◇), 20 (○) or 40 (△) mg/kg BHAMG are shown. Significant differences between drug-treated and control groups are indicated: *$P \leq 0.05$; **$P \leq 0.005$; ***$P \leq 0.0005$. *Bars* SE of the mean

Toxicity of pHAM and BHAMG

A total dose of 80 mg/kg BHAMG was not lethal to BALB/c mice. In contrast, a dose of 24 mg/kg pHAM resulted in the death of 100% of the mice by day 9 and 12 mg/kg pHAM was lethal to 40% of mice. All mice survived after receiving 6 mg/kg pHAM. Intravenous injection of pHAM significantly depressed numbers of white blood cells within 2 days (Fig. 5A). White blood cell nadirs were reached 6 days after pHAM administration, at which time the mean number of these cells in mice treated with 6, 12, or 24 mg/kg pHAM corresponded to 54%, 29% and 4% of the mean numbers in control mice injected with vehicle (dimethylsulfoxide). White blood cell levels in surviving mice recovered to control levels by day 16. Red blood cell toxicity at all pHAM doses was also significant by day 6 (Fig. 5C) but pHAM was less toxic to platelets (Fig. 5E).

BHAMG doses of 20 mg/kg and 40 mg/kg did not induce significant decreases in white or red blood cells or platelet numbers compared to those in control mice (Fig. 5). A total dose of 80 mg/kg BHAMG resulted in significant depression of white cells to 78% and 81% of control levels on days 9 and 16 respectively (Fig. 5B). This dose of BHAMG did not significantly affect red cell levels (Fig. 5D), but did decrease platelet numbers to 90% of control levels on day 9 (Fig. 5F).

Table 2 compares the hematological toxicity observed in mice after i.v. injection of BHAMG and pHAM. Mice that were i.v. injected with 6 mg/kg pHAM had significantly lower numbers of white blood cells on days 2 and 6 and significantly fewer red cells on days 2, 6 and 9 compared with mice that received 80 mg/kg BHAMG. Injection of

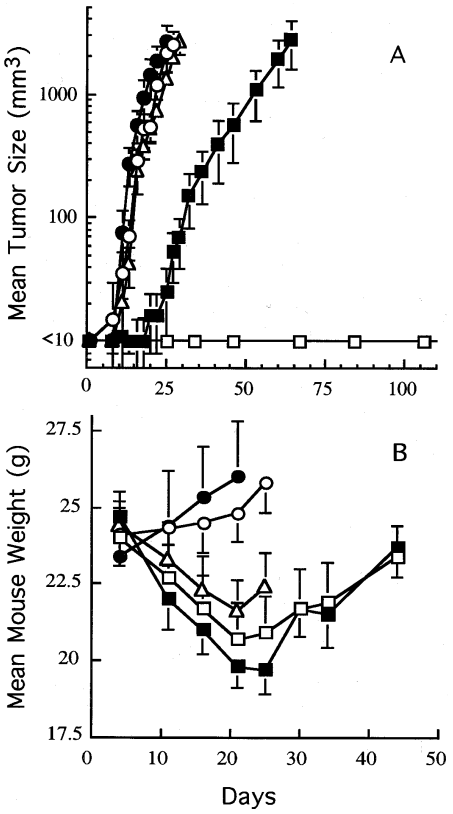

Fig. 6A,B Cure of mice bearing solid hepatoma tumors by antibody directed enzyme prodrug therapy (ADEPT). Scid mice were injected with 5×10^6 AS-30D hepatoma cells on day 1. Groups of six or seven mice were untreated (●), injected on days 9 and 10 with 3 mg/kg pHAM (△), injected on days 9 and 10 with 15 mg/kg BHAMG (○), injected on day 5 with 200 μg RH1-βG-5PEG and 15 mg/kg BHAMG on days 9 and 10 (□) or injected with 200 μg HB65-βG-5PEG on day 5 and with 15 mg/kg BHAMG on days 9 and 10 (■). Mean tumor sizes (**A**) and animal weights (**B**) of each group are shown. *Bars* SE of the mean

12 mg/kg pHAM significantly reduced the levels of all blood cells compared to mice injected with 80 mg/kg BHAMG on days 2 and 6.

Therapy of AS-30D xenografts

The efficacy of combined treatment with RH1-βG-5PEG and BHAMG was examined in scid mice bearing AS-30D rat hepatoma xenografts. Mice injected s.c. with 5×10^6 AS-30D cells developed solid tumors that rapidly progressed to a mean size of 2700 mm^3 by day 25 (Fig. 6A). Treatment of tumor-bearing mice with 3 mg/kg pHAM or 15 mg/kg BHAMG on days 9 and 10 did not significantly delay tumor growth. Combined treatment with 200 μg HB65-βG-5PEG on day 5 followed by 15 mg/kg BHAMG on days 9 and 10 produced significant delay of tumor growth ($P<0.05$ on day 25) but tumors in four of six mice progressed and had a mean size of more than 2500 mm^3 by day 60, whereas two mice achieved tumor cures. Combined treatment with RH1-βG-5PEG on day 5 and BHAMG on days 9 and 10 resulted in complete tumor regression in all of seven mice, all mice being tumor-free on day 150.

Combined treatment with RH1-βG-5PEG or HB65-βG-5PEG and BHAMG produced toxicity, as measured by decreased mouse weight (Fig. 6B). Maximum weight loss was observed around day 25 with weights returning to normal values around day 50. pHAM also caused similar toxicity (weight loss). Control and BHAMG-treated mice, in contrast, experienced rapid weight gains, reflecting the progressive growth of large AS-30D tumors.

Discussion

PEG-modified proteins have been found to exhibit increased stability and resistance to proteolytic degradation, extended serum half-lives, and reduced immunogenicity [25]. We therefore examined the effect of linking PEG groups to RH1-βG because βG has been reported to be unstable in serum [20] and initial localization studies of radiolabled RH1-βG failed to show clear tumor images in scid mice (data not shown), even though radiolabeled mAb RH1 has been shown to localize in solid AS-30D tumors [30]. Our results show that (1) βG is stable in serum regardless of PEG modification, (2) PEG modification increased the serum half-life of RH1-βG conjugates, (3) tumor uptake of RH1-βG was increased by PEG modification with a commensurate decrease in normal tissue (spleen) uptake, and (4) combined therapy with a PEG-modified antibody-enzyme conjugate and BHAMG produced complete regressions of small solid tumors.

βG has been reported to be rapidly deactivated in human serum or in medium containing fetal calf serum [20]. In contrast, we found that βG activity was not only stable in human, mouse and bovine sera, but that the addition of serum to βG increased enzyme activity. Serum did not prevent the loss of βG activity, since enzyme maintained in PBS for 24 h could be stimulated by addition of BSA to levels similar to those of enzyme maintained in serum. BSA has also been shown to stimulate the activity of enzymes such as human liver α-glucosidase [31]. Stimulation of βG is unlikely to be mediated by specific protein-protein interactions since several proteins as well as detergent (CHAPS) also stimulated βG activity. The divergent results between our study and an earlier report [20] may have resulted from technical differences, since it seems unlikely that recombinant βG is more stable than the wild-type enzyme.

PEG-modified conjugates were produced by attaching PEG to βG before coupling to monoclonal antibodies. We limited PEG modification to βG for several reasons. First, many enzymes [25], including bovine βG, have been successfully modified with PEG, implying that βG should be amenable to PEG modification. Second, the active sites of many enzymes, including human β-glucuronidase [32], are located in clefts, suggesting that the catalytic site of βG might be resistant to deactivation by PEG. Although attach-

ment of PEG groups to enzymes may hinder the diffusion of substrate to the active site [33], complete loss of conjugate activity could result if PEG reacted with lysine residues present in the relatively accessible antigen-binding sites of the antibody [34]. In fact, PEG was found to deactivate mAb RH1 (results not shown). Although some antibodies have been successfully derivatized with PEG, albeit with varying losses of antigen-binding activity [35–37], modification of βG allows the use of any antibody for conjugate formation. Finally, modification of βG was expected to provide better control of conjugate pharmacokinetics since preliminary experiments revealed that βG was responsible for the rapid disappearance of RH1-βG from the circulation. Limiting attachment to βG also minimizes the final conjugate size, which could otherwise adversely affect tumor penetration and localization.

Succinimidyl succinate poly(ethylene glycol), which preferentially reacts with lysine groups, was employed to attach PEG molecules covalently to βG. Increased modification of βG led to a progressive loss of enzyme activity but the majority of βG activity was maintained after the attachment of up to 11 PEG molecules. The number of PEG molecules that could be introduced into βG reached a plateau at around 11, suggesting that 11 or the 28 lysine residues of recombinant βG are accessible to modification. The decrease in the combined antigen-binding and enzymatic activity of conjugates formed by linking βG-PEG to mAb RH1 (Fig. 3A) was greater than the loss of βG activity due to PEG modification (results not shown), implying that some (approx. 25%) antibody activity was lost upon conjugation with βG-PEG. Antibody activity may have been adversely affected by failure to remove free PEG completely before coupling βG-PEG to mAb RH1 or by steric hindrance of antigen binding by PEG groups extending from the surface of βG. RH1-βG-3PEG, however, efficiently activated BHAMG in an antigen-dependent manner in vitro, indicating that retention of 50%–75% of the combined enzyme and antigen-binding activities of the conjugates was sufficient for targeted prodrug activation.

PEG modification of βG slowed the serum clearance of conjugates compared to RH1-βG. RH1-βG was rapidly cleared from serum immediately after i.v. injection, indicating that the conjugate rapidly distributed into tissues. RH1-βG accumulated in the spleen, suggesting that spleenic retention of βG contributed to the short initial half life ($\tau_{1/2\alpha}$) of unmodified conjugate in mice. PEG modification of βG prevented the initial rapid clearance of conjugates and significantly reduced accumulation in the spleen. Reduced spleen uptake could be important for combined therapy with antibody-βG conjugates and BHAMG, since hematological toxicity is often dose-limiting for alkylating agents such as pHAM [38]. Kitamura and colleagues also found that introduction of 5 PEG groups into mAb A7 or its $F(ab)'_2$ fragment increased the circulation time of the antibodies, primarily by increasing $\tau_{1/2\alpha}$ [35]. Reduced uptake in the spleen and liver was also observed. PEG modification was also shown to extend the $\tau_{1/2\alpha}$ of a $F(ab')_2$-enzyme conjugate, presumably by reducing the uptake of the conjugate by parenchymal cells of the liver [39]. The terminal elimination of RH1-βG-10PEG, in contrast to RH1-βG-3PEG and RH1-βG-5PEG, was also reduced compared to RH1-βG, indicating that high levels of PEG modification can also slow the excretion or catabolism of βG conjugates.

Besides displaying reduced accumulation in the spleen, PEG-modified conjugates also exhibited a trend of increasing tumor uptake with higher degrees of PEG modification, even though the activity of PEG-modified conjugates was reduced compared to RH1-βG. mAb RH1 does not internalize after binding to AS-30D cells (unpublished data), allowing conjugates to remain at the tumor for extended periods. The benefits of enhanced tumor uptake, however, were offset by the slower clearance of PEG-modified conjugates from the serum, resulting in decreased tumor-to-blood ratios for RH1-βG-5PEG. Initial studies of the pharmacokinetics of RH1-βG-3PEG indicate that this degree of PEG modification represents a good compromise between tumor uptake and serum clearance with a good tumor/blood ratio of 11 after 96 h.

Although the tumor/blood ratio 96 h after injection of RH1-βG-5PEG was only about 2, combined therapy with this conjugate and BHAMG provided therapeutic efficacy, with complete tumor regressions achieved in all of seven mice. Combined treatment with RH1-βG-5PEG and BHAMG was superior to treatment with the parent drug even though pHAM was administered near its maximum tolerated dose on the basis of hematological toxicity (Fig. 5). The poor efficacy of pHAM may be attributable to rapid deactivation by hydrolysis [40], rapid sequestration due to protein binding [41], or failure to reach the tumor in adequate concentrations. The therapeutic efficacy of combined treatment with RH1-βG-5PEG and BHAMG required localization of the conjugate in the tumors, as shown by the significantly better efficacy compared to combined treatment with the control antibody conjugate HB65-βG-5PEG and BHAMG. The significant delay of tumor growth and cure of two mice after combined treatment with HB65-βG-5PEG and BHAMG indicate that some pHAM was generated by non-specific activation of BHAMG by conjugate still present in the serum of the mice, since specific tumor localization of control conjugate was not observed. This is supported by the increased toxicity observed after treatment with HB65-βG-5PEG and BHAMG compared to the toxicity of BHAMG in naive mice (Fig. 5). Reducing the concentration of conjugate in serum before administration of prodrug by employing a clearing agent [39] or optimizing the conjugate formulation should reduce non-specific prodrug activation, thereby further improving the therapeutic index and efficacy of treatment.

Generation of human antibodies against βG could preclude repeated administration of conjugate [42]. Attachment of PEG to proteins, however, often reduces their immunogenicity [25]. In addition, recent studies, showing that intravenous administration of cyclosporin A reduced the generation of antibodies against carboxypeptidase G2 in humans [43] and that deoxyspergualin suppressed and delayed the human anti-(mouse Ig) antibody response against a murine monoclonal antibody [44], suggest that

multiple rounds of conjugate and prodrug treatment may be feasible.

In summary, PEG modification of βG provides the means to adjust the serum half-life, increase tumor uptake, and decrease normal tissue binding of antibody-βG conjugates. The in vivo efficacy observed after combined treatment with RH1-βG-5PEG and BHAMG suggests that employing PEG-modified antibody-βG conjugates for the targeted activation of glucuronide prodrugs warrants further investigation for solid-tumor therapy.

References

1. Haskell CM (1990) Principles and practice of cancer chemotherapy. In: Haskell CM (ed) Cancer treatment. Saunders, Philadelpia, p 21
2. Yeh MY, Roffler SR, Yu MH (1992) Doxorubicin-monoclonal antibody conjugate for therapy of human cervical carcinoma. Int J Cancer 51:274–282
3. Trail PA, Willner D, Lasch SJ, Henderson AJ, Hofstead S, Casazza AM, Firestone RA, Hellström I, Hellström KE (1993) Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. Science 261:212–215
4. Schneider YJ, Abarca J, Aboud-Pirak E, Baurain R, Ceulemans F, Deprez-De Campeneere D, Lesur B, Masquelier M, Otte-Slachmuylder C, Rolin-van Swieten D, Trouet A (1984) Drug targeting in human cancer chemotherapy. In: Gregoriadis G, Poste G, Senior J, Trouet A (eds) Receptor-mediated targeting of drugs. Plenum, New York, p 1
5. Pirker R (1988) Immunotoxins against solid tumors. J Cancer Res Clin Oncol 114:385–393
6. Bagshawe KD (1987) Antibody directed enzymes revive anticancer prodrugs concept. Br J Cancer 56:531–532
7. Senter PD, Saulnier MG, Schreiber GJ, Hirschberg DL, Brown JP, Hellström I, Hellström KE (1988) Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate. Proc Natl Acad Sci USA 85:4842–4846
8. Blakey DC, Pinder PE, Wright AF (1994) Comparison of the cellular internalization of antibodies used either as immunotoxins or in ADEPT. Cell Biophys 24–25:175–183
9. Wallace PM, MacMaster JF, Smith VF, Kerr DE, Senter PD, Cosand WL (1994) Intratumoral generation of 5-fluorouracil mediated by an antibody-cytosine deaminase conjugate in combination with 5-fluorocytosine. Cancer Res 54:2719–2723
10. Baxter LT, Jain RK (1996) Pharmacokinetic analysis of the microscopic distribution of enzyme-conjugated antibodies and prodrugs: comparison with experimental data. Br J Cancer 73:447–456
11. Sherwood RF (1996) Advanced drug delivery reviews: enzyme prodrug therapy. Adv Drug Delivery Rev 22:269–288
12. Roffler SR, Wang SM, Chern JW, Yeh MY, Tung E (1991) Antineoplastic glucuronide prodrug treatment of human tumor cells targeted with a monoclonal antibody-enzyme conjugate. Biochem Pharmacol 42:2062–2065
13. Wang SM, Chern JW, Yeh MY, Ng JC, Tung E, Roffler SR (1992) Specific activation of glucuronide prodrugs by antibody-targeted enzyme conjugates for cancer therapy. Cancer Res 52:4484–4491
14. Stahl PD, Fishman WH (1984) β-D-glucuronidase. In: Bergmeyer J, Gral M (eds) Methods in enzymatic analysis, vol 5. Verlag Chemie, Weinheim, p 246
15. Dixon M, Webb EC (1979) Enzymes. Academic Press, New York, p 634
16. Hallinan T (1983) UDPglucuronosyltranferases. In: Bergmeyer J, Gral M (eds) Methods in enzymatic analysis, vol 3. Verlag Chemie, Weinheim, p 365
17. Weenen H, Van Maanen JMS, De Planque MM, McVie JG, Pinedo HM (1984) Metabolism of 4′-modified analogs of doxorubicin. Unique glucuronidation pathway for 4′epidoxorubicin. Eur J Cancer Clin Oncol 20:919–926
18. Rivory LP, Robert J (1995) Identification and kinetics of a β-glucuronide metabolite of SN-38 in human plasma after administration of the camptothecin derivative irinotecan. Cancer Chemother Pharmacol 36:176–179
19. Jefferson RA, Burgess SM, Hirsh D (1986) β-glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc Natl Acad Sci USA 83:8447–8451
20. Haisma HJ, Boven E, Muijen M van, Jong J de, Vijgh WJ van der, Pinedo HM (1992) A monoclonal antibody-β-glucuronidase conjugate as activator of the prodrug epirubicin-glucuronide for specific treatment of cancer. Br J Cancer 66:474–478
21. Nolen H, Fedorak RN, Friend DR (1995) Budesonide-β-D-glucuronide: a potential prodrug for treatment of ulcerative colitis. J Pharm Sci 84:677–681
22. Houba PH, Leenders RG, Boven E, Scheeren JW, Pinedo HM, Haisma HJ (1996) Characterization of novel anthracycline prodrugs activated by human β-glucuronidase for use in antibody-directed enzyme prodrug therapy. Biochem Pharmacol 52: 455–463
23. Roffler SR, Chan J, Yeh MY (1994) Potentiation of radioimmunotherapy by inhibition of topoisomerase I. Cancer Res 54: 1276–1285
24. Nucci ML, Shorr R, Abuchowski A (1991) The therapeutic value of poly(ethylene glycol)-modified proteins. Adv Drug Delivery Rev 6:133–151
25. Delgado C, Francis GE, Fisher D (1992) The uses and properties of PEG-linked proteins. Crit Rev Ther Drug Carrier Syst 9:249–304
26. Smith DF, Walborg EF, Chang JP (1970) Establishment of a transplantable ascites variant of a rat hepatoma induced by 3′-methyl-4-dimethylaminoazobenzene. Cancer Res 30: 2306–2309
27. Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) Touchdown PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008
28. Yeh MY, Hellstrom I, Brown JP, Warner GA, Hansen JA, Hellstrom KE (1979) Cell surface antigens of human melanoma identified by monoclonal antibody. Proc Natl Acad Sci USA 76:2927–1931
29. Habeeb AFSA (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal Biochem 14:328–336
30. Wang SM (1992) Treatment of cancer with prodrugs activated by antibody targeted enzyme conjugates. PhD thesis, National Defence Medical School, Taipei
31. Koster JF, Slee RG (1977) Some properties of human liver acid α-glucosidase. Biochim Biophys Acta 482:89–97
32. Jain S, Drendel WB, Chen ZW, Mathews FS, Sly WS, Grubb JH (1996) Structure of human β-glucuronidase reveals candidate lysosomal targeting and active-site motifs. Nat Struct Biol 3:375–381
33. Banci L, Bertini I, Caliceti P, Monsu-Scolaro L, Schiavon O, Veronese FM (1990) Spectroscopic characterization of polyethyleneglycol modified superoxide dismutase: ^{1}H NMR studies on its Cu_2Co_2 derivative. J Inorg Biochem 39:149–159
34. Mariuzza RA, Phillips SE, Poljak RJ (1987) The structural basis of antigen-antibody recognition. Annu Rev Biophys Biophys Chem 16:139–159
35. Kitamura K, Takahashi T, Yamaguchi T, Noguchi A, Noguchi A, Takashina K, Tsurumi H, Inagake M, Toyokuni T, Kakomori S (1991) Chemical engineering of the monoclonal antibody A7 by polyethylene glycol for targeting cancer chemotherapy. Cancer Res 51:4310–4315
36. Pedley RB, Boden JA, Boden R, Begent RHJ, Turner A, Haines AMR, King DJ (1994) The potential for enhanced tumour localisation by poly(ethylene glycol) modification of anti-CEA antibody. Br J Cancer 70:1126–1130

37. Delgado C, Pedley RB, Herraez A, Boden R, Boden JA, Keep PA, Chester KA, Fisher D, Begent RHJ, Francis GE (1996) Enhanced tumour specificity of an anti-carcinoembryonic antigen Fab′ fragment by poly(ethylene glycol) (PEG) modification. Br J Cancer 73:175–182
38. Earhart RH, Koeller JM, Davis HL (1981) Phase I trial of PCNU administered by 5-day courses. Cancer Treat Rep 65:835–840
39. Eno-Amooquaye EA, Searle F, Boden JA, Sharma SK, Burke PJ (1996) Altered biodistribution of an antibody-enzyme conjugate modified with polyethylene glycol. Br J Cancer 73:1323–1327
40. Wilman DE, Palmer BD, Denny WA (1995) Application of 15N nuclear magnetic resonance spectroscopy to the determination of the stability of aryl nitrogen mustards. J Med Chem 38:2256–2258
41. Gera S, Musch E, Osterheld HK, Loos U (1989) Relevance of the hydrolysis and protein binding of melphalan to the treatment of multiple myeloma. Cancer Chemother Pharmacol 23:76–80
42. Sharma SK, Bagshawe KD, Melton RG, Sherwood RF (1992) Human immune response to monoclonal antibody-enzyme conjugates in ADEPT pilot clinical trial. Cell Biophys 21:109–120
43. Sharma SK (1996) Immune response in ADEPT. Adv Drug Delivery Rev 22:369–376
44. Dhingra K, Fritsche H, Murray JL, Lobuglio AF, Khazaeli MB, Kelley S, Tepper MA, Grasela D, Buzdar A, Valero V, Booser D, Whealin H, Collins TJ, Pursley JM, Hortobagyi G (1995) Phase I clinical and pharmacological study of suppression of human antimouse antibody response to monoclonal antibody L6 by deoxyspergualin. Cancer Res 55:3060–3067