[CANCER RESEARCH 52, 4484–4491, August 15, 1992]

Specific Activation of Glucuronide Prodrugs by Antibody-targeted Enzyme Conjugates for Cancer Therapy[1]

Shing-Ming Wang,[2] Ji-Wang Chern, Ming-Yang Yeh, Joyce Co Ng, Edward Tung, and Steve R. Roffler[3]

Institute of Biomedical Sciences, Academia Sinica, Taipei 11529 [S-M. W., J. C. N., E. T., S. R. R.], and Institute of Pharmacy and Medical Laboratories [J-W. C.] and Department of Microbiology and Immunology [M-Y. Y.], National Defense Medical Center, Taipei 10764, Taiwan, Republic of China

ABSTRACT

Cancer chemotherapy may be improved by increasing antineoplastic drug specificity for tumor cells. We have synthesized a glucuronide prodrug that can be enzymatically converted to an antineoplastic agent at tumor cells that are able to bind β-glucuronidase-monoclonal antibody conjugates. The glucuronide prodrug BHAMG, the tetra-*n*-butyl ammonium salt of (*p*-di-2-chloroethylaminophenyl-β-D-glucopyranosid) uronic acid, was 150 times less toxic than the parent drug, *N,N*-di-(2-chloroethyl)-4-hydroxyaniline, to HepG2 human hepatoma cells and over 1000-fold less toxic than the parent drug to AS-30D rat hepatoma cells *in vitro*. In the presence of β-glucuronidase, BHAMG was activated and became as toxic as the parent drug *N,N*-di-(2-chloroethyl)4-hydroxyaniline. A conjugate (RH1-βG) was formed by linking β-glucuronidase to a monoclonal antibody which binds to an antigen expressed on the surface of AS-30D cells. The concentration of BHAMG causing 50% inhibition of AS-30D cellular protein synthesis was reduced over 1000-fold, from >770 μM to <0.74 μM after these cells were preincubated with RH1-βG. Specificity of BHAMG activation at antigen-positive cells was shown by monoclonal antibody RH1 blocking of RH1-βG conversion of BHAMG to toxic drug and by the inability of BHAMG to be converted to active drug when antigen-negative control cells were preincubated with RH1-βG. Our results show that the targeted-β-glucuronidase activation of BHAMG can increase the specificity of chemotherapy for rat hepatoma *in vitro* and suggest that the targeted activation of glucuronide prodrugs may be useful for cancer therapy.

INTRODUCTION

Chemotherapy is an important treatment modality for many cancers, although its use is often palliative rather than curative (1). The basic limitation of chemotherapy is the physiological similarity between normal and tumor cells (1). Cancer chemotherapy is thus often terminated due to normal tissue toxicity and associated side effects such as leukopenia; immunosuppression; and pulmonary, cardiac, and neurotoxicities (2). We and others have attempted to increase drug specificity by linking antineoplastic agents to monoclonal antibodies that bind to tumor-associated antigens preferentially expressed on the surface of tumor cells (3–7). While direct conjugation of drugs to antibodies can increase the specific targeting of drugs to tumor cells, this method suffers from several drawbacks. Practical limits on drug loading or slow internalization of drug conjugates into cancer cells may result in poor tumor killing (8, 9). In addition, cells expressing low levels of antigen in heterogeneous cell populations may also escape destruction (10).

A new indirect drug-targeting strategy has recently been observed to overcome problems associated with chemoimmunoconjugates while retaining the advantages of selective drug action (11–14). In this method, enzyme rather than drug is linked to an antibody that binds antigen preferentially expressed on the surface of tumor cells. The antibody-enzyme complex is then targeted to tumor cells, thus allowing the conjugated enzyme to accumulate at the cancer site. A latent, nontoxic "prodrug" is then introduced so that prodrug coming into contact with targeted enzyme at the tumor site can be enzymatically converted to the active parent compound which can then kill the tumor cells. This method may provide some advantages compared with chemoimmunoconjugates, including accumulation of higher drug concentrations at the tumor, less sensitivity to tumor cell heterogeneity, and the possibility of creating defined immunoconjugates through genetic engineering.

We have recently described a relatively nontoxic glucuronide prodrug, BHAMG,[4] that can be enzymatically converted to the parent drug HAM by βG (15). Specific activation of BHAMG at tumor cells can be obtained by targeting βG to tumor cells as an enzyme-Mab conjugate. Fig. 1 illustrates the strategy of targeted-enzyme activation of BHAMG. In this report, we examine the specific *in vitro* activation of BHAMG at rat hepatoma cells expressing a tumor-associated antigen. Hepatocellular carcinoma is the most common cancer worldwide, causing approximately 1 million deaths each year (16, 17). Hepatoma is usually lethal, with a median survival time as short as several months (17), indicating that improved therapies are needed. We demonstrate that rat and human hepatoma cells are sensitive to HAM but not to the prodrug BHAMG. Protein synthesis of hepatoma cells, however, was inhibited by BHAMG after activation by βG. We also show that BHAMG can be specifically activated and can preferentially kill antigen-positive hepatoma tumor cells that were previously exposed to a βG-Mab conjugate.

MATERIALS AND METHODS

Reagents and Cells. HAM and BHAMG were synthesized as described (18). Structures were confirmed by nuclear magnetic resonance and melting point determination. UDP-glucuronic acid, *p*-nitrophenyl β-D-glucuronide, *p*-nitrophenol, glucaro 1,4-lactone, reduced glutathione, 1-chloro-2,4-dinitrobenzene, and β-glucuronidase (EC 3.2.1.31) from *Escherichia coli* (type X-A) were purchased from Sigma Chemical Company (St. Louis, MO). Sephadex G-25 gel was from Pharmacia LKB Biotechnology (Uppsala, Sweden). SMCC was from Pierce Chemical Company (Rockford, IL). [^{3}H]Leucine (50 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). AS-30D rat hepatoma cell line (19) was generously provided by Dr. J. P. Chang (Institute of Zoology, Academia Sinica, Taipei, Taiwan, ROC). CaSki human cervical carcinoma cells were kindly provided by Dr. R. A.

Received 2/27/92; accepted 6/5/92.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by grants and allocations from the National Science Council and Academia Sinica, Taipei, Taiwan, Republic of China.

[2] Present Address: Division of Colon and Rectal Surgery, Tri-Service General Hospital, Taipei, Taiwan, Republic of China.

[3] To whom requests for reprints should be addressed.

[4] The abbreviations used are: BHAMG, tetra-*n*-butyl ammonium salt of HAMG; HAMG, glucuronide prodrug of *p*-hydroxy aniline mustard; HAM, *p*-hydroxyaniline mustard [*N,N*-di-(2-chloroethyl)-4-hydroxyaniline]; βG, β-glucuronidase; Mab, monoclonal antibody; RH1-βG, conjugate of RH1 antibody with β-glucuronidase; PBS, phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4); SMCC, succinimidyl-4-(N-maleimidomethyl) cyclohexane 1-carboxylate; UDPGT, uridine 5′-diphosphoglucuronyl transferase; GST, glutathione *S*-transferase; IC_{50}, concentration of test sample causing 50% inhibition of cellular protein synthesis.

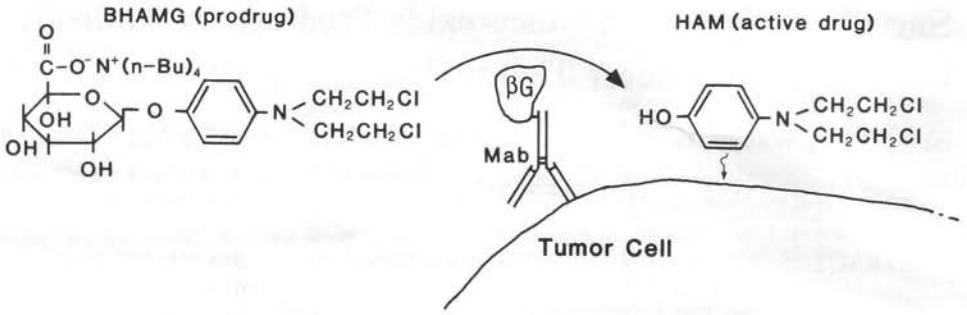

Fig. 1. A glucuronide prodrug system. Mab-βG conjugate is first allowed to bind antigen that is preferentially expressed on the tumor cell membranes, resulting in accumulation of βG at the cancer site. The glucuronide prodrug BHAMG is subsequently activated by βG to the potent alkylating agent HAM which can kill the tumor cell.

Pattillo (Medical College of Wisconsin, Milwaukee, WI). HepG2 human hepatoma and COLO 205 human colon carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). Human cells were maintained in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. AS-30D cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented as above.

β-Glucuronidase Conjugation to Monoclonal Antibody. Mab RH1 is a murine IgG_{2a} monoclonal antibody developed in our laboratory that binds strongly to AS-30D cells but does not bind HepG2 cells. RH1-βG was formed by linking β-glucuronidase to Mab RH1 via a thioether bond. A maleimido group was first introduced into the immunoglobulin molecule with the heterobifunctional cross-linking agent SMCC. A 7-fold molar excess of SMCC dissolved in dioxane (3 mg/ml) was added to Mab RH1 (5–10 mg/ml) in PBS for 45 min at 37°C. Excess SMCC was removed by gel filtration on Sephadex G-25, and the number of maleimido groups was measured (20). Modified RH1 antibody was then reacted with thiol groups present in βG. Lypholized βG was dissolved in PBS (3 mg/ml) and passed through Sephadex G-25. Free thiol groups were measured (21), and βG was mixed with derivatized IgG, concentrated by ultrafiltration, and reacted overnight at 4°C. All coupling reactions were performed in PBS containing 1 mM EDTA, deoxygenated by boiling and sparging with nitrogen.

Purification and Characterization of β-Glucuronidase-Antibody Conjugate. RH1-βG was purified in a two-step process. Uncoupled βG was removed from the conjugate by protein A-Sepharose affinity chromatography. Free Mab RH1 was then removed by ion exchange chromatography on a DEAE 5 PW high-performance liquid chromatography column (Waters) by eluting with a linear gradient of NaCl in 20 mM bis-tris, pH 6.0. Eluted conjugates were concentrated by ultrafiltration, and after adding 1 mg/ml human serum albumin they were filter sterilized and stored at −70°C. Protein concentrations were measured by the bicinchoninic acid assay (22). Apparent molecular weight of the enzyme-antibody conjugate was calculated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis (23, 24). The antigen-binding activities of Mab RH1 and RH1-βG were determined by enzyme-linked immunosorbent assay using whole AS-30D cells coated on 96-well plates as antigen (7). βG enzyme activity was measured in a microtiter assay. Serial dilutions of βG standard or sample (20 μl/well) were added to wells of 96-well microtiter plates containing 200 μl reaction buffer (100 mM acetic acid, 50 mM bis-tris, 50 mM triethanolamine, pH 7.0, with NaOH) and 20 μl of 40 mM *p*-nitrophenyl β-D-glucuronide. Plates were incubated at 37°C for 30 min before addition of 12 μl of 1 N NaOH to each well. The absorbance of the wells was immediately read at 405 nm in a Molecular Devices (Menlo Park, CA) microplate reader. Antigen-binding and enzyme activities of RH1-βG were also measured simultaneously by first incubating RH1-βG in plates coated with AS-30D cells for 1 h at 37°C and then carrying out the βG activity assay after washing plates three times with PBS.

***In Vitro* Activity of Prodrug and Conjugate.** Protein synthesis of cell cultures was measured as described (24). Briefly, AS-30D or HepG2 cells were plated overnight in 96-well microtiter plates at 20,000 cells/well. Serial dilutions of HAM or BHAMG in medium containing 5% fetal calf serum were added to cells for 1 or 24 h at 37°C. Cells were subsequently washed once with sterile PBS, incubated until hour 48 in fresh medium, and then pulsed for 2 h with [^{3}H]leucine (1 μCi/well) in leucine-free medium. The radioactivity of trichloroacetic acid-precipitated protein was measured in a Beckman LS 6000 series liquid scintillation counter.

BHAMG conversion to cytotoxic HAM was tested by incubating AS-30D and HepG2 cells with BHAMG plus βG (10 units/well) for 24 h at 37°C. One unit of βG can hydrolize 1 μmol *p*-nitrophenyl β-D-glucuronide in 1 h at 37°C. Cells were washed once with PBS and incubated in fresh medium for an additional 24 h before measuring the rate of protein synthesis. The *in vitro* activation of BHAMG by RH1-βG was examined by preincubating plated AS-30D and HepG2 cells with the indicated concentrations of conjugate for 30 min at room temperature. After washing cells once with PBS, BHAMG was added, and the assay was carried out as described above.

Competitive blocking of RH1-βG with Mab RH1 $F(ab')_2$ fragments was also examined. RH1 $F(ab')_2$ (50 μg/ml), prepared as described (25) except for increasing the pepsin:antibody ratio to 40:1 (wt/wt) and digesting for 1 h, was added with 1 μg/ml RH1-βG to AS-30D or HepG2 cells for 30 min at room temperature. Cells were then washed once with PBS and exposed to 90 μM BHAMG for 24 h at 37°C. Fresh medium was added to the cells for 24 h before measuring cellular protein synthesis rate. All experiments were performed in triplicate.

Enzyme Activities. Enzyme activities of cell homogenates were measured. Cultured cells were trypsinized, washed twice with PBS, and transferred to 20 mM bis-tris, pH 6.0, containing 0.1% (v/v) triton X-100 for 45 min at 4°C. Cells were broken in a glass Dounce homogenizer, and homogenates were frozen at −76°C or immediately assayed for enzymatic activity.

βG activity was measured using *p*-nitrophenyl glucuronide as substrate (26). One hundred μl of cell homogenate and 50 μl of 40 mM *p*-nitrophenyl glucuronide were added to 300 μl reaction buffer (100 mM acetic acid, 50 mM bis-tris, 50 mM triethanol amine, pH adjusted to 7 with NaOH) for 1 h at 37°C. The reaction was terminated by adding 500 μl 0.5 M trichloroacetic acid and heating to 100°C for 5 min. Samples were clarified by centrifugation, and 0.7 ml was then transferred to a 3-ml cuvette. Sample pH was adjusted to >11 by addition of 250 μl of 1 N NaOH and 1 ml distilled water. After mixing, absorbance was measured in a Beckman DU-70 spectrophotometer at 405 nm. Specific activities were calculated from a standard curve of absorbance *versus* *p*-nitrophenol concentration.

UDPGT activity was measured in a similar fashion using *p*-nitrophenol and UDP-glucuronic acid as substrates (27). Briefly, 125 μl cell homogenate, 65 μl 50 mM UDP-glucuronic acid, and 65 μl 2 mM *p*-nitrophenol were added to 400 μl reaction buffer (80 mM sodium phosphate, pH 7.0, 8.0 mM glucaro-1,4-lactone, 0.8 g/liter Triton X-100). After reaction at 37°C for 1 h, samples were processed as above for βG activity.

Glutathione *S*-transferase activity was measured by using reduced glutathione and 1-chloro-2,4-dinitrobenzene as substrates. One hundred μl cell homogenate, 40 μl 50 mM glutathione, and 40 μl 50 mM 1-chloro-2,4-dinitrobenzene (in ethanol) were added to 1.8 ml buffer (80 mM sodium phosphate, pH 7). The absorbance at 340 nm was detected in a spectrophotometer every 10 s for 2 min using a kinetic data program of the Beckman DU-70 spectrophotometer. The slope of the linear curve was determined by least squares regression. Specific GST activities were calculated assuming an ϵ^{340} of 9.6 mM^{-1} cm^{-1} (28).

Cell Growth Rate. The growth rate of tumor cells *in vitro* was determined by trypsinizing cells from triplicate wells of 6-well plates and counting viable cells once a day for 5 days. Doubling times were calculated from the slope of log (cell number) *versus* time as determined by least-squares regression.

RESULTS

β-Glucuronidase Activation of Glucuronide Prodrug. BHAMG was designed as a glucuronide prodrug of the potent alkylating agent HAM. The effect of BHAMG and HAM on several tumor cell lines was determined by measuring [^{3}H]-leucine incorporation into the protein of cells after drug exposure. Comparison of IC_{50} values revealed that BHAMG was over 1000 times less toxic than HAM to AS-30D rat hepatoma cells (Fig. 2*A*) and about 150-fold less toxic to HepG2 human hepatoma cells (Fig. 2*B*) after 24 h of drug exposure. The simultaneous addition of βG (10 units/well) and BHAMG to tumor cells resulted in a cytotoxic effect equal to that of HAM alone, indicating that cleavage of the glucuronide functional group converted BHAMG to HAM (Fig. 2). Addition of βG alone did not affect [^{3}H]leucine incorporation into cellular protein (data not shown).

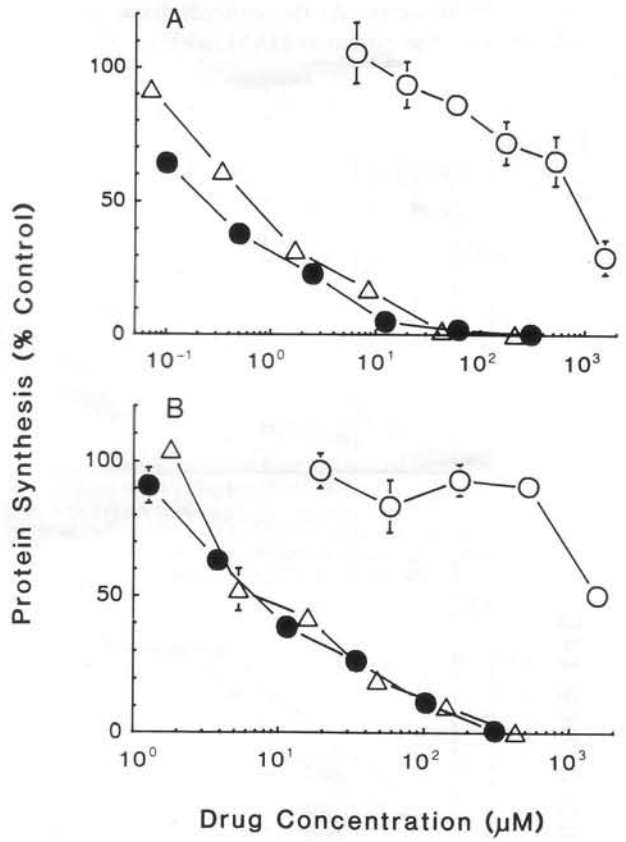

Fig. 2. *In vitro* growth inhibition of hepatoma cells by HAM and BHAMG. AS-30D rat hepatoma (*A*) and HepG2 human hepatoma (*B*) cells were exposed to HAM (△), BHAMG (○), or BHAMG plus 10 units βG (●) for 24 h, washed with PBS, and then incubated in fresh medium for an additional 24 h. The cellular protein synthesis rate of drug-treated cells is compared to that of untreated control cells at 48 h. Note that the scales of the abscissas are different. *Bars*, SE of triplicate determinations.

Table 1 summarizes the effects of HAM and BHAMG on cellular protein synthesis in several cell lines. AS-30D cells were most sensitive to HAM with a mean IC_{50} value of 0.85 μM. Other cell lines were more resistant to HAM, with CaSki human cervical carcinoma cells being the most resistant (IC_{50}, 53.5 μM). Prodrug latency, a measure of the difference in toxicities between prodrug and the parent compound, was also greatest for AS-30D cells; BHAMG was an average of 1280 times less toxic than HAM to AS-30D cells. The effect of drug exposure time on cell cytotoxicity was also examined in AS-30D cells. HAM and BHAMG were both about 2 times more toxic in a 24-h exposure assay compared to a 1-h exposure (Table 1). Drug latency, however, was relatively insensitive to drug exposure time (1300 *versus* 1160 for 1-h and 24-h exposure times, respectively).

Endogenous Enzyme Activities of Cells. Cell line sensitivities to HAM or BHAMG plus βG varied by nearly 100-fold. Variation of cellular sensitivity to HAM or BHAMG was hypothesized to be due to the relative activities of the endogenous detoxification enzymes GST and UDPGT and the prodrug-activating enzyme βG. Table 2 summarizes specific enzyme activities in whole cell homogenates prepared from the cell lines shown in Table 1. The sensitivity of cells to HAM appeared to be inversely related to cellular GST activity. Cells with lower GST activities (AS-30D and HepG2) were most sensitive to HAM, while cells expressing high GST activity (COLO 205 and CaSki) were relatively resistant to HAM. Linear regression analysis of HAM IC_{50} values *versus* the GST activity of these cells gave a positive correlation coefficient of 0.976. No correlation was found between cell sensitivity to HAM and UDPGT activity or cell growth rate. The sensitivity of AS-30D cells to intermediate concentrations of BHAMG (20% inhibition of protein synthesis at 100 μM BHAMG) is likely due to the high βG activity of these cells. The βG activity of AS-30D cells was significantly greater than that of other cells shown in Table 2 ($P < 0.005$). Similarly, AS-30D cells were significantly more

Table 1 *In vitro effect of HAM and BHAMG*

The effect of HAM and its glucuronide prodrug BHAMG on protein synthesis in AS-30D rat hepatoma and human HepG2 hepatoma, Colo 205 colon carcinoma, or CaSki cervical carcinoma cells was calculated by interpolation of dose-response curves similar to those in Fig. 2.

Cell line	Mean IC_{50} (μM)[a]			Latency[b] (BHAMG/HAM)
	HAM	BHAMG	BHAMG + βG	
AS-30D	0.85 ± 0.15 (5)	1090 ± 180 (4)	0.69 ± 0.25 (4)	1280
1 h exposure	1.18 ± 0.08 (2)	1370 ± 200 (2)	0.55 ± 0.09 (2)	1160
24 h exposure	0.62 ± 0.12 (3)	809 ± 9 (2)	0.82 ± 0.58 (2)	1300
HepG2	7.9 ± 1.6 (8)	1185 ± 138 (7)	10.8 ± 2.9 (9)	150
Colo 205	37 ± 6.4 (3)	1880 ± 18 (3)	15.9 ± 2.5 (7)	51
CaSki	53.5 ± 2.2 (3)	2790 ± 190 (2)	126 ± 42 (3)	52

[a] Unless otherwise indicated, cells were exposed to drugs for 24 h.
[b] Latency is the ratio of mean IC_{50} values for BHAMG to HAM.
[c] Numbers in parentheses, number of independent assays, each carried out in triplicate, used to determine mean values. SEMs are also indicated.

Table 2 *Enzyme activities of tumor cells*

The activities of β-glucuronidase, glutathione *S*-transferase, and UDP-glucuronosyltransferase in whole cell lysates were measured as described in "Materials and Methods."

Cell	Doubling time (h)	10^9 × enzyme activity[a] (μmol/h-cell) βG	UDPGT	10^9 × GST acivity (μmol/min-cell)
AS-30D	21	10.1 ± 0.6	7.6 ± 0.1	6.4 ± 0.5
HepG2	25	1.5 ± 0.1	1.3 ± 0.3	3.6 ± 0.3
Colo 205	20	2.7 ± 0.1	11.4 ± 0.3	29.8 ± 0.8
CaSki	24	2.6 ± 0.2	0.26 ± 0.37	36.5 ± 0.7

[a] Results are mean values of duplicate samples. SD of mean values are also shown.

sensitive to BHAMG, compared by IC_{50} values, than HepG2, Colo 205, or CaSki cells ($P < 0.10$, 0.005, and 0.005, respectively).

Mab-βG Conjugate. To test the feasibility of specifically activating BHAMG at antigen-positive hepatoma tumor cells, βG was conjugated to Mab RH1 by a stable thioether linkage. We previously linked βG to the $F(ab')_2$ fragment of an IgG_3 Mab via a disulfide bond formed by introducing 2-pyridyldisulfide groups into both *E. coli* βG and immunoglobulin molecules with the heterobifunctional cross-linking agent *N*-succinimidyl-3-(2-pyridyldithio)proprionate (15). Eighty % of original βG activity, however, was lost in this conjugate (15). We subsequently found that it is unnecessary to introduce thiol groups into *E. coli* βG, since this enzyme possesses nine cysteine residues (29) and has three or four apparent free thiol groups when probed with 4,4′-dithiodipyridine (data not shown). In this work, an average of 1.5 maleimido groups was introduced into Mab RH1 with the bifunctional cross-linking agent SMCC, and the derivatized IgG was directly reacted with free thiol groups present in βG. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis revealed that RH1-βG consisted of a major band at M_r 221,000, corresponding to a conjugate containing one molecule each of Mab RH1 and βG (data not shown). Several minor bands with higher molecular weights were also visible. RH1-βG retained almost complete enzymatic activity (Fig. 3*A*) as well as antigen-binding activity (Fig. 3*B*). The enzyme and antibody activities of RH1-βG were also simultaneously assayed by first allowing the conjugate to bind to AS-30D cells and then assaying for bound βG activity (Fig. 3*C*). RH1-βG was active at concentrations of less than 200 ng/ml.

Specific Activation of Prodrug. The specific activation of BHAMG at antigen-positive AS-30D cells was examined by first incubating cells with different concentrations of RH1-βG for 30 min, washing the cells, and then exposing the cells to 90 μM BHAMG for 24 h. Fig. 4*A* shows that protein synthesis was reduced by up to 95% in AS-30D cells preincubated with RH1-βG and then exposed to BHAMG. Even at a RH1-βG concentration of only 60 ng/ml, protein synthesis of BHAMG-treated AS-30D cells was inhibited by 44% compared to cells not exposed to RH1-βG. RH1-βG activation of BHAMG was specific for antigen-positive cells; preincubation of antigen-negative HepG2 cells with RH1-βG did not increase the toxicity of BHAMG to these cells (Fig. 4*B*).

RH1-βG specificity for AS-30D cells was further verified by a competition assay. The addition of 50 μg/ml Mab RH1 $F(ab')_2$ during the preincubation of AS-30D cells with 1 μg/ml RH1-βG protected the cells from BHAMG; cellular protein synthesis was inhibited by only 30% (Fig. 5, *Lane b*) compared to 90% inhibition of protein synthesis in the absence of competing antibody fragment (Fig. 5, *Lane a*). Blocking of RH1-βG with excess Mab RH1 $F(ab')_2$ did not affect the protein synthesis of antigen-negative HepG2 cells exposed to BHAMG (Fig. 5, *Lanes c* and *d*).

The ability of RH1-βG to specifically activate BHAMG at antigen-positive AS-30D cells was also examined by preincubating cells with 1 or 10 μg/ml RH1-βG and subsequently exposing the cells to varying concentrations of BHAMG for 24 h. Preincubation of AS-30D cells with 1 μg/ml RH1-βG decreased the IC_{50} of BHAMG by about 200-fold (Fig. 6*A*). In contrast, RH1-βG was ineffective at potentiating the activity of BHAMG at HepG2 cells (Fig. 6*B*). Table 3 shows that BHAMG toxicity to AS-30D cells was further increased by raising the RH1-βG concentration to 10 μg/ml. At this concentration of RH1-βG, BHAMG was about as potent as HAM, with an IC_{50} of <0.75 μM.

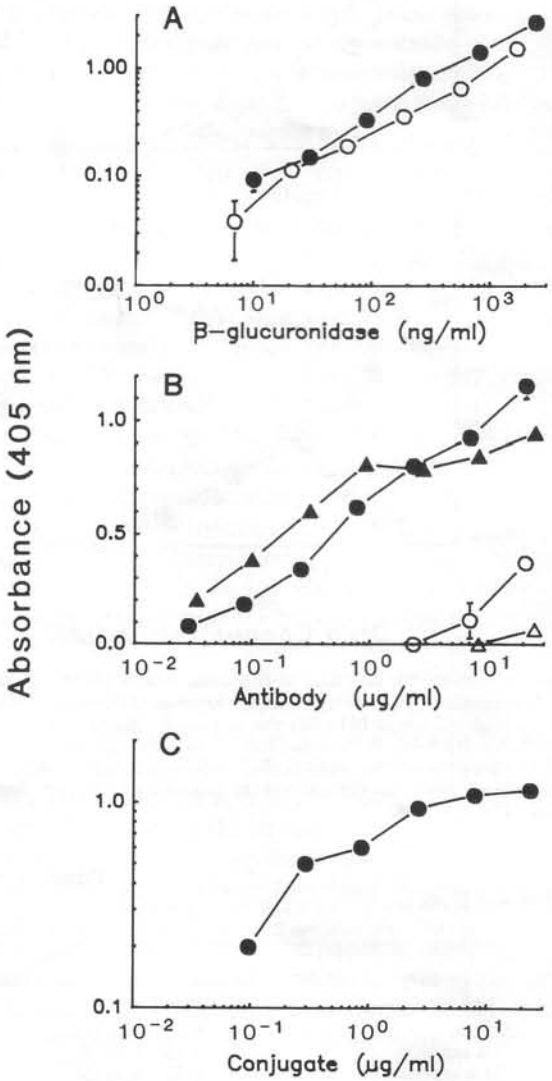

Fig. 3. Enzyme and antibody activities of RH1-βG. *A*, enzyme activity of βG before (○) or after (●) coupling to Mab RH1, measured by monitoring the release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucuronide at 405 nm. *B*, enzyme-linked immunosorbent assay measurement of antigen-binding activities of Mab RH1 (△, ▲) or RH1-βG (○, ●) to antigen-positive AS-30D cells (●, ▲) or antigen-negative HepG2 cells (○, △). *Bars*, SE of triplicate determinations. *C*, combined immunosorbent and βG activity measurement of RH1-βG. RH1-βG was incubated in wells coated with AS-30D cells and then assayed for βG activity using *p*-nitrophenyl β-D-glucuronide as the substrate.

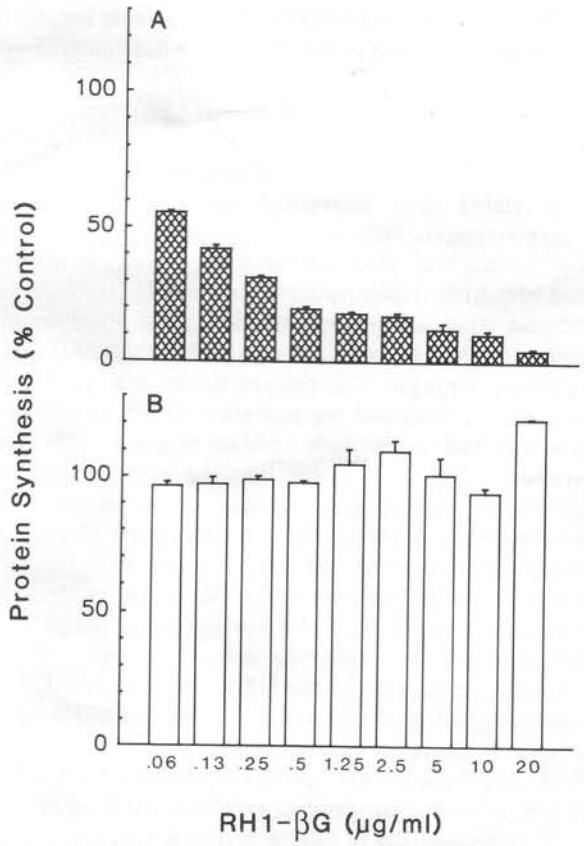

Fig. 4. *In vitro* activation of BHAMG by RH1-βG. AS-30D (*A*) or HepG2 (*B*) cells were preincubated with different concentrations of RH1-βG for 30 min at room temperature. Cells were then washed once with PBS and exposed to 90 μM BHAMG (60 μg/ml) for 24 h. The cellular protein synthesis rate was measured 24 h later and is compared to the protein synthesis rate of control cells exposed to 90 μM BHAMG only. *Bars*, SE of triplicate determinations.

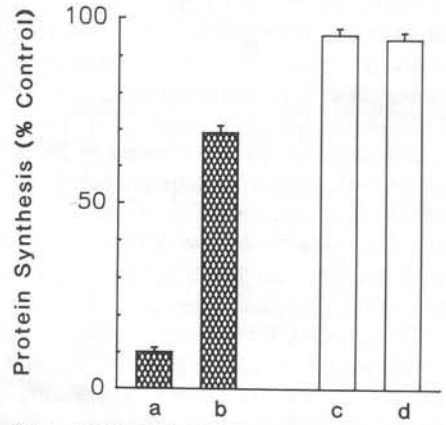

Fig. 5. Specificity of RH1-βG for AS-30D cells. Antigen-positive AS-30D cells (*a*, *b*) and antigen-negative HepG2 cells (*c*, *d*) were preincubated with 1 μg/ml RH1-βG alone (*a*, *c*) or 1 μg/ml RH1-βG plus 50 μg/ml RH1 $F(ab')_2$ (*b*, *d*) for 1 h at room temperature. Cells were washed once with PBS, exposed to 90 μM BHAMG for 24 h, and incubated for an additional 24 h in fresh medium before measuring the cellular protein synthesis rate. Results are expressed as percentage protein synthesis in treated cells compared to control cells exposed to 90 μM BHAMG without the addition of RH1-βG. *Bars*, SE of triplicate determinations.

DISCUSSION

The impetus for examining targeted-enzyme activation of glucuronide prodrugs for cancer therapy came from earlier reports that mice bearing well-established PC5 plasma tumors containing high levels of β-glucuronidase were cured by treatment with aniline mustard (30–32). Aniline mustard was apparently converted to HAM and then to HAMG *in vivo* in the liver of treated mice. HAMG was subsequently converted to highly cytotoxic HAM by endogenous βG present at the tumor site (31). Clinical trials using aniline mustard for cancer chemotherapy (33, 34), however, were disappointing, likely due to insufficient activity of βG in most human tumors (33). We hypothesized that targeting βG to the cancer site could allow specific activation of glucuronide prodrugs at tumor cells. Our results show that it is possible to specifically kill cancer cells expressing tumor-associated antigen by first targeting a β-glucuronidase-monoclonal antibody conjugate to tumor cells to elevate the activity of βG and then treating the cells with a glucuronide prodrug.

The purpose of converting a prodrug to an antineoplastic agent at tumor cells but not normal tissues is to increase the specificity and lower the toxicity of cancer chemotherapy. The generation of local high concentrations of drug at tumor sites

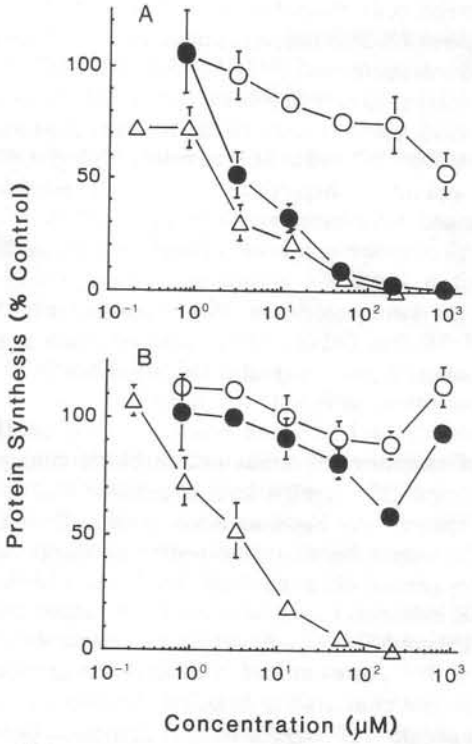

Fig. 6. Specificity of BHAMG activation at antigen-positive tumor cells. AS-30D (*A*) and HepG2 (*B*) cells were preincubated with 1 μg/ml RH1-βG (●) or medium (△, ○) for 1 h at room temperature, washed once with PBS, and incubated for 24 h in the presence of HAM (△) or BHAMG (○, ●). The cellular protein synthesis of treated cells is compared to that of untreated control cells, measured at 48 h. *Bars*, SE of triplicate determinations.

Table 3 *Selective activation of BHAMG by RH1-βG at AS-30D cells*

Both antigen-positive AS-30D and antigen-negative HepG2 cells were preincubated with RH1-βG, washed, and incubated with BHAMG for 24 h. Protein synthesis of cells was assayed after an additional 24 h incubation in fresh medium.

Cell line	RH1-βG (μg/ml)	IC_{50} (μM) HAM	IC_{50} (μM) BHAMG	Selectivity[a] BHAMG (BHAMG + RH1-βG)
AS-30D	0	0.41	>770	1
	1		3.6	>210
	10		<0.75	>1000
HepG2	0	3.6	>770	1
	1		>770	1
	10		>770	1

[a] Selectivity is defined as the ratio of IC_{50} values for BHAMG before and after incubation of cells with RH1-βG conjugate.

could increase the killing of cancer cells with a commensurate decrease in normal tissue toxicity. Several conditions, however, must be met for this strategy to be feasible. The most basic requirements are that (*a*) the prodrug should be less toxic than the corresponding parent drug; (*b*) prodrug can be converted under defined conditions into the active parent compound; and (*c*) tumor and normal cells should display sufficient differences in the cellular property used to activate prodrug to parent drug. The specific activation of BHAMG by Mab-targeted βG appears to meet these criteria. BHAMG was over 1000 times less toxic than HAM to tumor cells but could be enzymatically converted to HAM by βG. More importantly, by linking βG to a Mab against rat hepatoma cells, sufficient βG was targeted to antigen-positive cells to activate BHAMG to HAM *in vitro*. AS-30D cells preincubated with RH1-βG were at least 200 times more sensitive to BHAMG than untreated AS-30D cells. Elevation of βG enzyme activity at tumor cells by the localization of Mab-βG conjugate also allowed differentiation of target and nontarget cells, demonstrated by the specific killing of antigen-positive AS-30D but not antigen-negative HepG2 cells by combined treatment with RH1-βG and BHAMG.

Preliminary results indicate that tumor cells expressing lower levels of GST, an important family of detoxification enzymes, are more susceptible to HAM than cells with high GST activity. Both rat and human hepatoma cell lines expressed lower GST activities and were more sensitive to HAM or βG-activated prodrug than either colon or cervical carcinoma cells tested. This result is in agreement with reports that tumor cell resistance to alkylating agents is often associated with high GST activities (35, 36). Other factors, however, such as the rate of DNA damage repair, may also be important in determining cellular sensitivity to HAM. No correlation was found between cell sensitivity to HAM and cellular activity of UDPGT, a family of enzymes important in xenobiotic conjugation and detoxification (37). A high level of cellular βG, on the other hand, appeared to be associated with cell sensitivity to BHAMG, suggesting that glucuronide prodrugs can be converted to parent drug by high levels of endogenous βG. BHAMG, however, was several orders of magnitude less toxic than HAM to AS-30D cells which expressed the highest βG activity of the cells examined, indicating that endogenous βG was ineffective at activating BHAMG *in vitro*.

The application of targeted-enzyme activation of prodrugs to cancer chemotherapy may solve some of the problems associated with the direct linkage of antineoplastic agents to Mabs. Chemoimmunoconjugate drug loading, even when using linkers such as dextran or albumin, appears to be limited to less than 100 drug molecules/antibody (7, 38, 39). Insufficient drug may be internalized into cancer cells to totally eradicate the tumor (8, 9). A single Mab-enzyme conjugate, in contrast, can generate a great number of drug molecules at the tumor site, increasing the chance of attaining therapeutic concentrations of drug. Activated prodrugs have a low molecular weight and should diffuse more readily into the tumor mass (40). They should also be less sensitive to antigen heterogeneity, since prodrug activated at the surface of antigen-positive tumor cells can in principle diffuse to neighboring antigen-negative tumor cells (10). Chemoimmunoconjugates are also difficult to standardize and require extensive characterization. Mab-enzyme conjugates, in contrast, lend themselves to genetic engineering. A fusion protein between immunoglobulin variable chains and enzyme could be produced on a large scale as a standard product (41). This type of chimeric molecule should also clear more rapidly from the blood pool (42) and be less likely to induce antigen modulation (43).

Besides the specific activation of BHAMG described here, other targeted-enzyme-activated prodrugs have been described. Senter and colleagues (13, 14) investigated the activation of phosphorylated drug derivatives with Mab-alkaline phosphatase conjugates. They were able to demonstrate the regression of human lung adenocarcinoma xenografts in nude mice treated with Mab-alkaline phosphatase conjugates followed by mitomycin phosphate administration (14). Phosphorylated prodrug alone and in combination with a control Mab-alkaline phosphatase conjugate also delayed tumor growth. The same group has also described the activation of a doxorubicin prodrug with a Mab-penicillin-V-amidase conjugate (44) and the conversion of 5-fluorocytosine into the antineoplastic agent 5-fluorouracil by a cytosine deaminase-Mab conjugate (45). Bagshawe and coworkers (12) have developed glutamic acid prodrugs which could be converted to toxic bis-chlorobenzoic acid mustards by carboxypeptidase G2. These prodrugs were able to inhibit or eliminate human choriocarcinoma (12) or colon carcinoma (46) xenografts in nude mice after treatment with antibody-carboxypeptidase G2 conjugates. A cephalosporin-*Vinca* alkaloid prodrug activated by a β-lactamase-antibody fragment conjugate has also been described (47).

βG-activated glucuronide prodrugs possess potential advantages over other enzyme-prodrug combinations for cancer therapy. βG concentration in human serum is very low (26), suggesting that glucuronide prodrugs should be stable in the blood after i.v. administration. Although several organs, including the liver, gastrointestinal tract, spleen, and lung, do contain endogenous βG (48, 49), mammalian tissues also express UDPGT, a class of xenobiotic detoxification enzymes that can reverse the reaction catalyzed by βG (27, 37). In studies carried out in rodents and humans, glucuronide conjugates were major metabolites of several drugs including aniline mustard (32), 9-hydroxyellipticine (50), 4′-epidoxorubicin (51), 1-naphthol (52), and AZT (3′-azido-3′-deoxythymidine) (53). These studies and our own results showing the low toxicity of BHAMG to cells expressing high endogenous βG activity support the hypothesis that glucuronide prodrugs should be resistant to premature activation by endogenous βG *in vivo*. These studies also suggest that activated prodrug not taken up by tumor cells may be reconverted to the glucuronide conjugate after passing through organs containing high UDPGT activities. Also, because βG is an endogenous enzyme, it may be possible to target human βG to tumor cells, reducing the chance of inducing an immune response against the Mab-enzyme conjugate in humans, a potential problem with conjugates containing exogenous enzyme. In addition, although βG is highly specific for the glucuronyl residue of glucuronide conjugates, it has little specificity for the conjugated aglycone (26), suggesting that a wide variety of glucuronide prodrugs could be used for cancer therapy. Glucuronide prodrugs also appear to be less toxic than similar prodrugs (19, 46).

In summary, we have demonstrated that the glucuronide prodrug BHAMG is much less toxic than the corresponding parent compound HAM to several tumor cell lines, including both human and rat hepatoma cells. A monoclonal antibody-βG conjugate was constructed and shown to preferentially accumulate at cancer cells that express tumor-associated antigen. Antigen-positive tumor cells were also specifically killed by BHAMG

after cells were exposed to antibody-βG conjugate. These results show that a glucuronide prodrug of low toxicity can be converted to a highly toxic drug *in vitro* at tumor cells in which βG activity has been elevated. Taken together, these results suggest that targeted βG activation of glucuronide prodrugs is potentially useful for cancer therapy. More work is required, however, to examine the behavior of βG-activated glucuronide prodrugs *in vivo* and to address questions such as the mechanism of reduced glucuronide prodrug toxicity, the stability of glucuronide prodrugs *in vivo*, and the effect of antibody internalization on the ability of conjugates to activate prodrug at tumor cells. Only when these and other questions are answered can the potential of this strategy be realized.

ACKNOWLEDGMENTS

We thank Bing-Mae Chen, Hui-Ru Chen, and Y-J. Luo for technical assistance and J-C. Chou for preparation of the illustrations.

REFERENCES

1. Kauffman, J. M., and Foye, W. O. Cancer chemotherapy. *In:* W. O. Foye (ed.), Principles of Medicinal Chemistry, pp. 837–861. Philadelphia: Lea and Febiger, 1981.
2. Haskell, C. M. Principles and practice of cancer chemotherapy. *In:* C. M. Haskell (ed.), Cancer Treatment, pp. 21–43. Philadelpia: W. B. Saunders Co., 1990.
3. Deguchi, T., Chu, T. M., Leong, S. S., Horozsewicz, J. S., and Lee, C. L. Potential therapeutic effect of Adriamycin-monoclonal anti-prostatic acid phosphatase antibody conjugate on human prostate tumor. J. Urol., *137:* 353–358, 1987.
4. Yu, D-S., Chu, T-M., Yeh, M-Y., Chang, S-Y., Ma, C-P., and Han, S. H. Antitumor activity of doxorubicin-monoclonal antibody conjugate on human bladder cancer. J. Urol., *140:* 415–421, 1988.
5. Dillman, R. O., Johnson, D. E., Shawler, D. L., and Koziol, J. A. Superiority of an acid-labile daunorubicin-monoclonal antibody immunoconjugate compared to free drug. Cancer Res., *48:* 6097–6102, 1988.
6. Tjandra, J. J., Pietersz, G. A., Teh, J. G., Cuthbertson, A. M., Sullivan, J. R., Penfold, C., McKenzie, I. F., and Smyth, M. Phase I clinical trial of drug-monoclonal antibody conjugates in patients with advanced colorectal carcinoma: a preliminary report. Surgery (St. Louis), *106:* 533–545, 1989.
7. Yeh, M-Y., Roffler, S. R., and Yu, M-H. Doxorubicin-monoclonal antibody conjugate for therapy of human cervical carcinoma. Int. J. Cancer, *51:* 274–282, 1992.
8. Bumol, T. F. Workshop summary: monoclonal antibody conjugates with drugs or toxins. *In:* R. A. Reisfeld and S. Sell (eds.), Monoclonal Antibodies and Cancer Therapy, pp. 257–259. New York: Alan R. Liss, Inc., 1985.
9. Schneider, Y-J., Abarca, J., Aboud-Pirak, E., Baurain, R., Ceulemans, F., Deprez-De Campeneere, D., Lesur, B., Masquelier, M., Otte-Slachmuylder, C., Rolin-van Swieten, D., and Trouet, A. Drug targeting in human cancer chemotherapy. *In:* G. Gregoriadis, G. Poste, J. Senior, and A. Trouet (eds.), Receptor-Mediated Targeting of Drugs, pp. 1–25. New York: Plenum Press, 1984.
10. Pirker, R. Immunotoxins against solid tumors. J. Cancer Res. Clin. Oncol., *114:* 385–393, 1988.
11. Bagshawe, K. D. Antibody directed enzymes revive anti-cancer prodrugs concept. Br J. Cancer, *56:* 531–532, 1987.
12. Bagshawe, K. D., Springer, C. J., Searle, F., Antoniw, P., Sharma, S. K., Melton, R. G., and Sherwood, R. F. A cytotoxic agent can be generated selectively at cancer sites. Br. J. Cancer, *58:* 700–703, 1988.
13. Senter, P. D., Saulnier, M. G., Schreiber, G. J., Hirschberg, D. L., Brown, J. P., Hellstrom, I., and Hellstrom, K. E. Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate. Proc. Natl. Acad. Sci. USA, *85:* 4842–4846, 1988.
14. Senter, P. D., Schreiber, G. J., Hirschberg, D. L., Ashe, S. A., Hellström, K. E., and Hellström, I. Enhancement of the *in vitro* and *in vivo* antitumor activities of phosphorylated mitomycin C and etoposide derivatives by monoclonal antibody-alkaline phosphatase conjugates. Cancer Res., *49:* 5789–5792, 1989.
15. Roffler, S. R., Wang, S-M., Chern, J-W., Yeh, M-Y., and Tung, E. Antineoplastic glucuronide prodrug treatment of human tumor cells targeted with a monoclonal antibody-enzyme conjugate. Biochem. Pharmacol., *42:* 2062–2065, 1991.
16. Yeh, F-S., and Shen, K-N. Epidemiology and early diagnosis of primary liver cancer in China. Adv. Cancer Res., *47:* 297–329, 1986.
17. Haskell, C. M., Selch, M. T., and Ramming, K. P. Primary neoplasms of the liver. *In:* C. M. Haskell (ed.), Cancer Treatment, pp. 276–286. Philadelpia: W. B. Saunders Co., 1990.
18. Bukhari, M. A., Everett, J. L., and Ross, W. C. J. Aryl-2-halogenoalkylamines—XXVI. Glucuronic, sulphuric and phosphoric esters of *p*-di-2-chloroethylaminophenol. Biochem. Pharmacol., *21:* 963–967, 1972.
19. Smith, D. F., Walborg, E. F., and Chang, J. P. Establishment of a transplantable ascites variant of a rat hepatoma induced by 3'-methyl-4-dimethylaminoazobenzene. Cancer Res., *30:* 2306–2309, 1970.
20. Ishikawa, E., Hashida, S., Kohno, T., Kotani, T., and Ohtaki, S. Modification of monoclonal antibodies with enzymes, biotin, and fluorochromes and their applications. *In:* L. B. Schook (ed.), Monoclonal Antibody Production Techniques and Applications, pp. 113–137. New York: Marcel Dekker, Inc., 1987.
21. FitzGerald, D. J. P. Construction of immunotoxins using *Pseudomonas* exotoxin A. Methods Enzymol., *151:* 139–145, 1987.
22. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. Measurement of protein using bicinchoninic acid. Anal. Biochem., *150:* 76–85, 1985.
23. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.
24. Roffler, S. R., Yu, M-H., Chen, B. M., Tung, E., and Yeh, M-Y. Therapy of human cervical carcinoma with monoclonal antibody-*Pseudomonas* exotoxin conjugates. Cancer Res., *51:* 4001–4008, 1991.
25. Yu, M-H., Roffler, S. R., Hwang, W-S., Tung, E., Chen, W-L., and Yeh, M-Y. Localization and therapy of human cervical tumor xenografts with radiolabeled monoclonal antibody 1H10. Gynecol. Oncol., in press, 1992.
26. Stahl, P. D., and Fishman, W. H. β-D-Glucuronidase. *In:* J. Bergmeyer and M. Graßl (eds.), Methods in Enzymatic Analysis, Vol. 5, pp. 246–256. Weinheim: Verlag Chemie, 1984.
27. Hallinan, T. UDP-glucuronosyltranferases. *In:* J. Bergmeyer and M. Graßl (eds.), Methods of Enzymatic Analysis, Vol. 3, pp. 365–374. Weinheim: Verlag Chemie, 1983.
28. Jensson, H., Alin, P., and Mannervik, B. Glutathione transferase isoenzymes from rat liver cytosol. Methods Enzymol., *113:* 504–507, 1985.
29. Jefferson, R. A., Burgess, S. M., and Hirsh, D. β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. USA, *83:* 8447–8451, 1986.
30. Whisson, M. E., and Connors, T. A. Cure of mice bearing advanced plasma cell tumours with aniline mustard. Nature (Lond.), *206:* 689–691, 1965.
31. Connors, T. A., and Whisson, M. E. Cure of mice bearing advanced plasma cell tumours with aniline mustard: the relationship between glucuronidase activity and tumour sensitivity. Nature (Lond.), *210:* 866–867, 1966.
32. Connors, T. A., Farmer, P. B., Foster, A. B., Gilsenan, A. M., Jarman, M., and Tisdale, M. J. Metabolism of aniline mustard [*N,N*-di-(2-chloroethyl)-aniline]. Biochem. Pharmacol., *22:* 1971–1980, 1973.
33. Kyle, R. A., Costa, G., Cooper, M. R., Ogawa, M., Silver, R. T., Glidewell, O., and Holland, J. F. Evaluation of aniline mustard in patients with multiple myeloma. Cancer Res., *33:* 956–960, 1973.
34. Young, C. W., Yagoda, A., Bittar, E. S., Smith, S. W., Grabstald, H., and Whitmore, W. Therapeutic trial of aniline mustard in patients with advanced cancer. Comparison of therapeutic response with cytochemical assessment of tumor cell β-glucuronidase activity. Cancer (Phila.), *38:* 1887–1895, 1976.
35. Hill, B. T. Modulation of antitumour drug resistance: experimental laboratory data and results of clinical evaluation. Cancer Treat. Rev., *17:* 197–202, 1990.
36. Black, S. M., Beggs, J. D., Hayes, J. D., Bartoszek, A., Muramatsu, M., Sakai, M., and Wolf, C. R. Expression of human glutathione *S*-transferases in *Saccharomyces cerevisiae* confers resistance to the anticancer drugs Adriamycin and chlorambucil. Biochem. J., *268:* 309–315, 1990.
37. Bock, K. W. Roles of UDP-glucuronosyltransferases in chemical carcinogenisis. Crit. Rev. Biochem. Mol. Biol., *26:* 129–150, 1991.
38. Trouet, A., Masquelier M., Baurain, R., and Deprez-De Campeneere, D. A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier conjugate: *in vitro* and *in vivo* studies. Proc. Natl. Acad. Sci. USA, *79:* 626–629, 1982.
39. Shen, W-C., Ballou, B., Ryser, H. J-P., and Hakala, T. R. Targeting, internalization, and cytotoxicity of methotrexate-monoclonal anti-stage-specific embryonoic antigen-1 antibody conjugates in cultured F-9 teratocarcinoma cells. Cancer Res., *46:* 3912–3916, 1986.
40. Jain, R. K. Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. Cancer Res., *50:* 814s-819s, 1990.
41. Plückthun, A. Antibody engineering: advances from the use of *Escherichia coli* expression systems. Bio/Technology, *9:* 545–551, 1991.
42. Colcher, D., Bird, R., Roselli, M., Hardman, K. D., Johnson, S., Pope, S., Dodd, S. W., Pantoliano, M. W., Milenic, D. E., and Schlom, J. *In vivo* tumor targeting of a recombinant single-chain antigen-binding protein. J. Natl. Cancer Inst., *82:* 1191–1197, 1990.
43. Glennie, M. J., and Stevenson, G. T. Univalent antibodies kill tumour cells *in vitro* and *in vivo*. Nature (Lond.), *295:* 712–714, 1982.
44. Kerr, D. E., Senter, P. D., Burnett, W. V., Hirschberg, D. L., Hellström, I., and Hellström, K. E. Antibody-penicillin-V-amidase conjugates kill antigen-positive tumor cells when combined with doxorubicin phenoxyacetamide. Cancer Immunol. Immunother., *31:* 202–206, 1990.
45. Senter, P. D., Su, P. C. D., Katsuragi, T., Sakai, T., Cosand, W. L., Hellström, I., and Hellström, K. E. Generation of 5-fluorouracil from 5-fluorocytosine by monoclonal antibody-cytosine deaminase conjugates. Bioconjugate Chem., *2:* 447–451, 1991.
46. Bagshawe, K. D. Towards generating cytotoxic agents at cancer sites. Br. J.

Cancer, *60:* 275–281, 1989.
47. Shepherd, T. A., Jungheim, L. N., Meyer, D. L., and Starling, J. J. A novel targeted delivery system utilizing a cephalosporin-oncolytic prodrug activated by an antibody β-lactamase conjugate for the treatment of cancer. J. Biomed. Chem. Lett., *1:* 21–26, 1991.
48. Dixon, M., and Webb, E. C. Enzymes, pp. 634–635. New York: Academic Press, 1979.
49. Anghileri, L. J., and Miller, E. S. β-Glucuronidase activity in tumors: accumulation of radioiodinated phenolphthalein. Oncology (Basel), *25:* 19–32, 1971.
50. Maftouh, M., Monsarrat, B., Rao, R. C., Meunier, B., and Paoletti, C. Identification of the glucuronide and glutathione conjugates of the antitumor drug N^2-methyl-9-hydroxyellipticinium acetate (celiptium): comparative disposition of this drug with its olivacinium isomer in rat bile. Drug Metab. Dispos., *12:* 111–119, 1984.
51. Weenen, H., Van Maanen, J. M. S., de Planque, M. M., McVie, J. G., and Pinedo, H. M. Metabolism of 4′-modified analogs of doxorubicin. Unique glucuronidation pathway for 4′-epidoxorubicin. Eur. J. Cancer Clin. Oncol., *20:* 919–926, 1984.
52. Redegeld, F. A., Hofman, G. A., and Noordhoek, J. Conjugative clearance of 1-naphthol and disposition of its glucuronide and sulfate conjugates in the isolated perfused rat kidney. J. Pharmacol. Exp. Ther., *244:* 263–267, 1988.
53. Blum, M. R., Liao, S. H., Good, S. S., and de Miranda, P. Pharmacokinetics and bioavailability of zidivudine in humans. Am. J. Med., *85:* 189–194, 1988.