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

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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 $\beta$-glucuronidase-monoclonal antibody conjugates. The glucuronide prodrug BHAMG, the tetra-$n$-butyl ammonium salt of $(\rho$-di-2-chloroethylaminophenyl-$\beta$-$n$-glucopyranos-$\omega$) 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 $\beta$-glucuronidase, BHAMG was activated and became as toxic as the parent drug $N,N$-di-(2-chloroethyl)-4-hydroxyaniline. A conjugate (RH1-$\beta$G) was formed by linking $\beta$-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 $>770 \mu M$ to $<0.74 \mu M$ after these cells were preincubated with RH1-$\beta$G. Specificity of BHAMG activation at antigen-positive cells was shown by monoclonal antibody RH1 blocking of RH1-$\beta$G 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-$\beta$G. Our results show that the targeted-$\beta$-glucuronidase activation of BHAMG can increase the specificity of chemistry 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 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 cell populations, however, was inhibited by BHAMG after activation by $\beta$G. We also show that BHAMG can be specifically activated and can preferentially kill antigen-positive hepatoma tumor cells that were previously exposed to a $\beta$G-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 $\beta$-$o$-glucuronide, $p$-nitrophenol, glucaro 1,4 lactic acid, reduced glutathione, 1-chloro-2,4-dinitrobenzene, and $\beta$-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). $[3H]$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.

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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]; $\beta$G, $\beta$-glucuronidase; Mah, monoclonal antibody; RH1-$\beta$G, conjugate of RH1 antibody with $\beta$-glucuronidase; PBS, phosphate-buffered saline (0.14 $M$ NaCl, 2.7 $M$ KCl, 1.5 $M$ K$_2$HPO$_4$, 8.1 $M$ Na$\cdot$HPO$_4$); SMCC, succinimidyl-4-(N-maleimidomethyl) cyclohexane 1-carboxylic acid; UDPGST, uridine 5'-diphosphoglucuronyl transferase; GST, glutathione S-transferase; $IC_{50}$, concentration of test sample causing 50% inhibition of cellular protein synthesis.

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

Pattullo (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 \(\mu\)g/ml streptomycin. AS-30D cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco BRL) supplemented as above.

\(\beta\)-Glucuronidase Conjugation to Monoclonal Antibody. Mab RH1 is a murine IgG2a monoclonal antibody developed in our laboratory that binds strongly to AS-30D cells but does not bind HepG2 cells. RH1-\(\beta G\) was formed by linking \(\beta\)-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 \(\beta G\). Lyophilized \(\beta G\) was dissolved in PBS (3 mg/ml) and passed through Sephadex G-25. Free thiol groups were measured (21), and \(\beta G\) 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 \(\beta\)-Glucuronidase-Antibody Conjugate.** RH1-\(\beta G\) was purified in a two-step process. Uncoupled \(\beta G\) 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-\(\beta G\) were determined by enzyme-linked immunosorbent assay using whole AS-30D cells coated on 96-well plates as antigen (7).** \(\beta G\) enzyme activity was measured in a Beckman DU-70 spectrophotometer at 405 nm. One hundred \(\mu\)l of cell homogenate, 40 mM glutathione, and 40 mM 50 mM UDP-glucuronic acid as substrates (27). Briefly, 125 \(\mu\)l cell homogenate, 65 \(\mu\)l 50 mM UDP-glucuronic acid, and 65 \(\mu\)l 2 mM \(p\)-nitrophenol were added to 400 \(\mu\)l reaction buffer (80 mM sodium phosphate, pH 7.0, 8.0 mM glucose-1,4-lactone, 0.8 g/liter Triton X-100). After reaction at 37°C for 1 h, samples were processed as above for \(\beta G\) activity.

**UDPGT activity was measured in a similar fashion using \(p\)-nitrophenol and UDP-glucuronic acid as substrates (27).** Briefly, 125 \(\mu\)l cell homogenate, 65 \(\mu\)l 50 mM UDP-glucuronic acid, and 65 \(\mu\)l 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 \(e^{340}\) of 9.6 mm\(^{-1}\) cm\(^{-1}\) (28).

BHAMG conversion to cytotoxic HAM was tested by incubating AS-30D and HepG2 cells with BHAMG plus \(\beta G\) (10 units/well) for 24 h at 37°C. One unit of \(\beta G\) can hydrolyze 1 \(\mu\)mol \(p\)-nitrophenyl \(\beta\)-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-\(\beta G\)** 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-\(\beta G\) with Mab RH1 F(ab')\(_2\); fragments was also examined. RH1 F(ab')\(_2\); (50 \(\mu\)g/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 \(\mu\)g/ml RH1-\(\beta G\) to AS-30D or HepG2 cells for 30 min at room temperature.** Cells were then washed once with PBS and exposed to 90 \(\mu\)M 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.

**\(\beta G\) activity was measured using \(p\)-nitrophenyl glucuronide as substrate (26).** One hundred \(\mu\)l of cell homogenate and 50 \(\mu\)l of 40 mM \(p\)-nitrophenyl glucuronide were added to 300 \(\mu\)l 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 \(\mu\)l 0.5% 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 \(\mu\)l 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 \(\mu\)l cell homogenate, 65 \(\mu\)l 50 mM UDP-glucuronic acid, and 65 \(\mu\)l 2 mM \(p\)-nitrophenol were added to 400 \(\mu\)l reaction buffer (80 mM sodium phosphate, pH 7.0, 8.0 mM glucose-1,4-lactone, 0.8 g/liter Triton X-100). After reaction at 37°C for 1 h, samples were processed as above for \(\beta G\) activity.

**Glutathione S-transferase activity** was measured by using reduced glutathione and 1-chloro-2,4-dinitrobenzene as substrates. One hundred \(\mu\)l cell homogenate, 40 \(\mu\)l 50 mM glutathione, and 40 \(\mu\)l 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 \(e^{340}\) 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 [3H]-leucine incorporation into the protein of cells after drug exposure. Comparison of IC50 values revealed that BHAMG was over 1000 times less toxic than HAM to AS-30D rat hepatoma cells (Fig. 2A) and about 150-fold less toxic to HepG2 human hepatoma cells (Fig. 2B) 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 [3H]leucine incorporation into cellular protein (data not shown).

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 IC50 value of 0.85 μM. Other cell lines were more resistant to HAM, with CaSki human cervical carcinoma cells being the most resistant (IC50 = 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 more sensitive to HAM, while cells expressing high GST activity (COLO 205 and CaSki) were relatively resistant to HAM. Linear regression analysis of HAM IC50 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...

![Fig. 2. In vitro growth inhibition of hepatoma cells by HAM and BHAMG. AS-30D rat hepatoma (○) and HepG2 human hepatoma (□) 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.](image_url)
ronosyltransferase in whole cell lysates were measured as described in “Materials and Methods.”

We previously linked βG to the F(ab’)2 fragment of an IgG, Mab RH1, to activate BHAMG at antigen-positive hepatoma tumor cells. βG was conjugated to Mah RH1 by a stable thioether linkage. Conjugation consisted of a major band at Mr 221,000, corresponding to a disulfide bond formed by introducing 2-pyridyldisulfide 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 the derivatized IgG was directly reacted with free thiol groups of p-nitrophenyl /β-D-glucuronide as the substrate.

Specific Activation of Prodrug. The specific activation of BHAMG at antigen-positive AS-30D cells was 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 IC50 of BHAMG by about 200-fold (Fig. 6A). In contrast, RH1-βG was ineffective at potentiating the activity of BHAMG at HepG2 cells (Fig. 6B). 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 IC50 of <0.75 µM.

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, 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)propionate (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 both E. coli βG and immunoglobulin molecules, 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 Mr 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. 3A) as well as antigen-binding activity (Fig. 3B). 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. 3C). RH1-βG was active at concentrations of less than 200 ng/ml.

Table 2. Enzyme activities of tumor cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Doubling time (h)</th>
<th>10^5 × enzyme activity (µmol/h-cell)</th>
<th>10^8 × GST activity (µmol/min-cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-30D</td>
<td>21</td>
<td>10.1 ± 0.6</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>HepG2</td>
<td>25</td>
<td>1.5 ± 0.1</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Colo 205</td>
<td>20</td>
<td>2.7 ± 0.1</td>
<td>29.8 ± 0.8</td>
</tr>
<tr>
<td>CaSki</td>
<td>24</td>
<td>2.6 ± 0.2</td>
<td>36.5 ± 0.7</td>
</tr>
</tbody>
</table>

*Results are mean values of duplicate samples. SD of mean values are also shown.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody activity (ng/ml)</th>
<th>Antibody activity (µg/ml)</th>
<th>Antibody activity (µg/ml)</th>
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<tbody>
<tr>
<td>RH1-βG</td>
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<td>0.01</td>
<td>10^3</td>
</tr>
<tr>
<td>RH1-βG</td>
<td>10^2</td>
<td>10^1</td>
<td>10^0</td>
</tr>
<tr>
<td>HAM</td>
<td>10^3</td>
<td>10^2</td>
<td>10^1</td>
</tr>
</tbody>
</table>

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 (△), α 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. 4A 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. 4B).
TARGETED-ENZYME ACTIVATION OF A GLUCURONIDE PRODRUG

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-monomoclonal 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...
TARGETED-ENZYME ACTIVATION OF A GLUCURONIDE PRODRUG
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 ap-
pears 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 localiza-
tion of Mab-βG conjugate also allowed differentiation of target
and nontarget cells, demonstrated by the specific killing of an-
tigen-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 resis-
tance 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
detoxication (37). A high level of cellular βG, on the other
hand, appeared to be associated with cell sensitivity to
HAMG, suggesting that glucuronide prodrugs can be con-
verted 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 associ-
ated 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 gener-
age a great number of drug molecules at the tumor site, increas-
ing 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 acti-
vated at the surface of antigen-positive tumor cells can in prin-
ciple 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 pro-
tein 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 modu-
ation (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 phospha-
tase conjugates. They were able to demonstrate the regres-
sion 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 pro-
drug with a Mab-penicillin-V-amidase conjugate (44) and the
conversion of 5-fluorouracil into the antineoplastic agent
5-fluorouracil by a cytosome deaminase-Mab conjugate (45).
Bagshaw 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 cephalospor-
in-VInca alkaloid prodrug activated by a β-lactamase-antibody
fragment conjugate has also been described (47).

βG-activated glucuronide prodrugs possess potential advan-
tages over other enzyme-prodrug combinations for cancer ther-
apy. βG concentration in human serum is very low (26), sug-
gest 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 endog-
ogenous 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 me-
tabolites of several drugs including aniline mustard (32), 9-hy-
droxylleptilicine (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 ac-
tivation 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
tumor cells, reducing the chance of inducing an immune
response against the Mab-enzyme conjugate in humans, a po-
tential problem with conjugates containing exogenous enzyme.
In addition, although βG is highly specific for the glucuronsyl
residue of glucuronide conjugates, it has little specificity for the
conjugated aglycone (26), suggesting that a wide variety of glu-
curonide prodrugs could be used for cancer therapy. Gluco-
rone conjugates also appear to be less toxic than similar pro-
drugs (19, 46).

In summary, we have demonstrated that the glucuronide pro-
drug 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 con-
jugate 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 in vitro 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.

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