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 β-glucuronidase-monoconal antibody conjugates. The glucuronide prodrug BHAMG, the tetra-t-buty1 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. 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 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 chemoinmunconjugates 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 enzyme-antibody 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 chemoinmunconjugates, 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, 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. Hepato-cellular 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 both 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 glucuronic acid, 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). [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.

4The abbreviations used are: BHAMG, tetra-t-buty1 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 KH2PO4, 8.1 mM NaH2PO4); SMCC, succinimidyl-4-(N-maleimidomethyl)cyclohexane 1-carboxylate; UDPGT, uridine 5'-diphosphoglucuronyl transferase; GST, glutathione S-transferase; IC50, concentration of test sample causing 50% inhibition of cellular protein synthesis.

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fetal calf serum were added to cells for 1 or 24 h at 37°C. Cells were
well. Serial dilutions of HAM or BHAM in medium containing 50% 

Human cells were maintained in RPMI 1640 (Gibco BRL., Grand Is-
land, NY) supplemented with 5% heat-inactivated fetal bovine serum.

Polyacrylamide gel electrophoresis and Western blot analysis (23,24).

Purification and Characterization of β-Glucuronidase-Antibody Con-
jugate. RH1-βG was purified in a two-step process. Uncoupled βG
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bis-tris, pH 6.0. Eluted conjugates were concentrated by ultrafiltration,
and after adding 1 mg/ml human serum albumin they were filter ster-
diluted and stored at -70°C. Protein concentrations were measured by
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conjugate was calculated by sodium dodecyl sulfate-

protein synthesis of cells was measured as described (24). Briefly,

Animals. Enzyme activities of cell homogenates were mea-
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transferred to 20 mM bis-tris, pH 6.0, containing 0.1% (v/v) triton
X-100. All experiments were performed in triplicate.

HAM (active drug)

BHAGM (prodrug)

TARGETED-ENZYME ACTIVATION OF A GLUCURONIDE PRODRUG

Fig. 1. A glucuronide prodrug system. Mab-βG conjugate is first allowed to bind an-
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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.

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.

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 PBS, and then incubated in fresh medium for an additional 24 h. The cellular protein synthesis rate of drug-exposed 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

Table 1 In vitro effect of HAM and BHAMG

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HAM</th>
<th>BHAMG</th>
<th>BHAMG + βG</th>
<th>Latency[^b] (BHAMG/HAM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-30D</td>
<td>0.85 ± 0.15 (5)</td>
<td>1090 ± 180 (4)</td>
<td>0.69 ± 0.25 (4)</td>
<td>1280</td>
</tr>
<tr>
<td>1 h exposure</td>
<td>1.18 ± 0.08 (2)</td>
<td>1370 ± 200 (2)</td>
<td>0.55 ± 0.09 (2)</td>
<td>1160</td>
</tr>
<tr>
<td>24 h exposure</td>
<td>0.62 ± 0.12 (3)</td>
<td>899 ± 9 (2)</td>
<td>0.82 ± 0.38 (2)</td>
<td>1300</td>
</tr>
<tr>
<td>HepG2</td>
<td>7.9 ± 1.6 (8)</td>
<td>1185 ± 128 (7)</td>
<td>10.8 ± 2.9 (9)</td>
<td>150</td>
</tr>
<tr>
<td>Colo 205</td>
<td>37 ± 6.4 (3)</td>
<td>1880 ± 18 (3)</td>
<td>15.9 ± 2.5 (7)</td>
<td>51</td>
</tr>
<tr>
<td>CaSki</td>
<td>53.5 ± 2.2 (3)</td>
<td>2790 ± 190 (2)</td>
<td>126 ± 42 (3)</td>
<td>52</td>
</tr>
</tbody>
</table>

[^a] Unless otherwise indicated, cells were exposed to drugs for 24 h.
[^b] Latency is the ratio of mean IC₅₀ 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.

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Table 2 Enzyme activities of tumor cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Doubling time (h)</th>
<th>βG (μmol/h-cell)</th>
<th>UDpG (μmol/h-cell)</th>
<th>GST activity (μmol/min-cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-30D</td>
<td>21</td>
<td>10.1 ± 0.6</td>
<td>7.6 ± 0.1</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>HepG2</td>
<td>25</td>
<td>2.7 ± 0.1</td>
<td>2.9 ± 0.3</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>Colo 205</td>
<td>20</td>
<td>2.6 ± 0.2</td>
<td>2.6 ± 0.3</td>
<td>36.5 ± 0.7</td>
</tr>
<tr>
<td>CaSki</td>
<td>24</td>
<td>1.5 ± 0.1</td>
<td>1.3 ± 0.3</td>
<td>3.6 ± 0.3</td>
</tr>
</tbody>
</table>

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

Table 1 illustrates the enzyme activities of tumor cells.

Sensitivity of BHAMG to RHI-βG.

The ability of BHAMG to specifically activate BHAMG at antigen-positive AS-30D cells was examined by preincubating cells with 1 or 10 μg/ml RHI-βG and subsequently exposing the cells to varying concentrations of BHAMG for 24 h. Preincubation of AS-30D cells with 1 μg/ml RHI-βG decreased the IC50 of BHAMG by about 200-fold (Fig. 6A). In contrast, RHI-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 RHI-βG concentration to 10 μg/ml. At this concentration of RHI-βG, BHAMG was about as potent as HAM, with an IC50 of <0.75 μM.

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

Competing antibody fragment (Fig. 5, Lane a). Blocking of RHI-βG with excess Mab RHI 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 RHI-βG to specifically activate BHAMG at antigen-positive AS-30D cells was also examined by preincubating cells with 1 or 10 μg/ml RHI-βG and subsequently exposing the cells to varying concentrations of BHAMG for 24 h. Preincubation of AS-30D cells with 1 μg/ml RHI-βG decreased the IC50 of BHAMG by about 200-fold (Fig. 6A). In contrast, RHI-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 RHI-βG concentration to 10 μg/ml. At this concentration of RHI-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 RHI by a stable thioether linkage. We previously linked βG to the Fab' fragment of an IgG Mab via a disulfide bond formed by introducing 2-pyridyl disulfide groups into both E. coli βG and immunoglobulin molecules with the heterobifunctional cross-linking agent N-succinimidyl 3-(2-pyridylthio)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 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 RHI 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 RHI-βG consisted of a major band at M, 221,000, corresponding to a conjugate containing one molecule each of Mab RHI and βG (data not shown). Several minor bands with higher molecular weights were also visible. RHI-βG retained almost complete enzymatic activity (Fig. 3A) as well as antigen-binding activity (Fig. 3B). The enzyme and antibody activities of RHI-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). RHI-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 RHI-βG for 30 min, washing the cells, and then exposing the cells to 90 μM BHAMG for 24 h. Fig. 4A shows that protein synthesis was reduced by up to 95% in AS-30D cells preincubated with RHI-βG and then exposed to BHAMG. Even at a RHI-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 RHI-βG. RHI-βG activation of BHAMG was specific for antigen-positive cells; preincubation of antigen-negative HepG2 cells with RHI-βG did not increase the toxicity of BHAMG to these cells (Fig. 4B).

RHI-βG specificity for AS-30D cells was further verified by a competition assay. The addition of 50 μg/ml Mab RHI F(ab')2 during the preincubation of AS-30D cells with 1 μg/ml RHI-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 RHI-βG with excess Mab RHI 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 RHI-βG to specifically activate BHAMG at antigen-positive AS-30D cells was also examined by preincubating cells with 1 or 10 μg/ml RHI-βG and subsequently exposing the cells to varying concentrations of BHAMG for 24 h. Preincubation of AS-30D cells with 1 μg/ml RHI-βG decreased the IC50 of BHAMG by about 200-fold (Fig. 6A). In contrast, RHI-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 RHI-βG concentration to 10 μg/ml. At this concentration of RHI-βG, BHAMG was about as potent as HAM, with an IC50 of <0.75 μM.
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-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...
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 alleviated 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 that regenerating 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-β-amidase conjugate (44) and the conversion of 5-fluorocytosine into the antineoplastic agent 5-fluorouracil by a cytosine deaminase-Mab conjugate (45).

Four 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-β-lactamase 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 aline mustard (32), 9-hydroxycyclophrine (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 recovered 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 glycone (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.

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