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SHORT COMMUNICATION

Characterization of an Antineoplastic Glucuronide Prodrug

Tian-Lu Cheng,† Wan-Chih Chou,*† Bing Mae Chen,† Ji-Wang Chern‡ and Steve R. Roffler†§*

*Graduate Institute of Life Sciences, National Defense Medical School, †Institute of Biomedical Sciences, Academia Sinica, and ‡School of Pharmacy, National Taiwan University, Taipei, Taiwan

ABSTRACT. The specificity of tumor therapy may be improved by preferentially activating antineoplastic prodrugs at tumor cells pretargeted with antibody–enzyme conjugates. In this study, the conditions required for the efficient activation of *p*-hydroxyaniline mustard glucuronide (BHAMG) to *p*-hydroxyaniline mustard (pHAM) were investigated. pHAM induced cross-links in linearized double-stranded DNA at about 180-fold lower concentrations than BHAMG, indicating that the nucleophilicity of pHAM was decreased by the presence of a glucuronide group. The partition coefficient of BHAMG was about 1890 times lower than pHAM in an octanol–water two-phase system, suggesting that the reduced toxicity of BHAMG was due to both hindered diffusion across the lipid bilayer of cells and decreased reaction with nuclear DNA. BHAMG was significantly less toxic to BHK cells that expressed cytosolic *Escherichia coli*-derived β-glucuronidase (βG) compared with cells that were engineered to secrete βG, demonstrating that extracellular localization of βG was required for optimal activation of BHAMG. The extended retention of mAb RH1 on the surface of AS-30D cells was also consistent with extracellular activation of BHAMG. Taken together, our results indicate that the low toxicity of BHAMG was due to hindered cellular uptake and low alkylating activity. BHAMG must be enzymatically activated outside of tumor cells for maximum cytotoxicity, and non-internalizing antibodies are preferred for human tumor therapy by targeted antibody–enzyme activation of BHAMG. BIOCHEM PHARMACOL **58**;2:325–328, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. monoclonal antibody; ADEPT; prodrugs; β-glucuronidase; cancer; partition coefficient; internalization

Increasing the specificity of chemotherapy may improve the efficacy of cancer treatment. One approach to increase drug selectivity is to target an antibody–enzyme conjugate to tumor cells to activate a prodrug to a cytotoxic agent [1, 2]. We have developed a glucuronide prodrug (BHAMG‖) that can be activated to pHAM by *Escherichia coli*-derived βG (EC 3.2.1.31) [3]. BHAMG is up to 1000 times less toxic than pHAM to cultured tumor cells [4] and possesses low toxicity *in vivo* [5]. Malignant ascites [5] and solid tumors [6] have been cured by combined treatment with antibody–βG conjugates and BHAMG in rodent models. Targeted activation of BHAMG also produces a bystander effect *in vitro* and *in vivo*, which may contribute to the efficacy of cancer therapy [7]. Rational translation of BHAMG to human cancer treatment would benefit from a better understanding of the parameters important for the observed efficacy of tumor therapy with BHAMG. Towards this aim, we investigated the conditions required for efficient activation of BHAMG at tumor cells.

MATERIALS AND METHODS AND RESULTS

Alkylating Activities of Drugs

The alkylating activities of pHAM and BHAMG were examined by reacting the drugs for 1 hr at 37° with 2 μg of linear double-stranded DNA (*Hin*dIII-linearized pRSETB, Invitrogen) in 20 μL of 1 mM phosphate buffer, pH 7.4, containing 3 mM NaCl before electrophoresis on an alkaline gel to separate single-stranded and cross-linked DNA. Figure 1 shows that incubation of DNA with pHAM or BHAMG resulted in DNA cross-links at low drug concentrations and DNA degradation at higher drug concentrations. Analysis of the intensity of the single-stranded DNA band at different drug concentrations with the public-domain NIH Image program revealed that pHAM induced DNA cross-links at concentrations about 180 times lower than BHAMG.

§ Corresponding author: Dr. Steve Roffler, Box 78, Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan. Tel. 886-22-652-3079; FAX 886-22-782-9142; E-mail: sroff@ibms.sinica.edu.tw

‖*Abbreviations*: BHAMG, tetra-*n*-butyl ammonium salt of the glucuronide prodrug of *p*-hydroxyaniline mustard; βG, β-glucuronidase; and pHAM, *p*-hydroxyaniline mustard.

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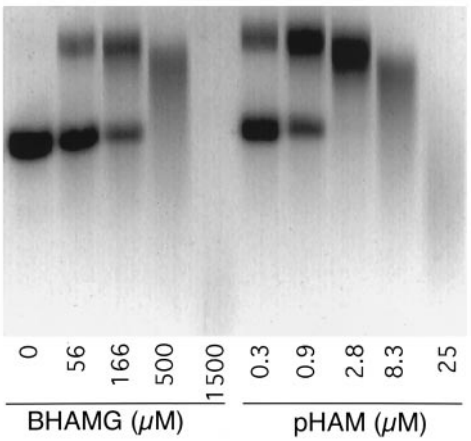

FIG. 1. Alkylating activity of pHAM and BHAMG. Linearized double-stranded DNA was reacted with various concentrations of pHAM or BHAMG for 1 hr before electrophoresis on an alkaline gel to separate single (lower band) and cross-linked DNA (upper band).

Partition Coefficients of Drugs

Two hundred fifty micrograms of pHAM or BHAMG was added to a two-phase system consisting of 0.5 mL of distilled water and 0.5 mL of water-saturated octanol. After shaking for 2 hr at 4°, the phases were separated, and the concentration of drug in each phase was quantified on a Waters μBondapak C_{18} (3.9 x 300 mm) column at a flow rate of 1 mL/min with a 20-min linear gradient from methanol:water:acetic acid (50:50:1) to 100% methanol with detection at 254 nm. The retention times and recoveries of pHAM and BHAMG were 15.4 min/99.3% and 11.3 min/91.6%, respectively. The concentrations of drugs were calculated by comparison of peak area ratios with standard drug solutions. Partition coefficients were defined as the ratio of drug concentrations in the octanol and aqueous phases. BHAMG favored distribution into the aqueous phase with a partition coefficient of 0.15 ± 0.02. In contrast, pHAM strongly favored partition into the octanol phase with a partition coefficient of 280 ± 11.5.

Antibody Internalization

Internalization of mAb RH1 [8] into AS-30D rat hepatoma cells was examined as this antibody has been employed to target βG to AS-30D cells for activation of BHAMG [4–7]. Ten million AS-30D cells were incubated in 2 mL PBS containing 50 μg/mL of mAb RH1 for 1 hr at 4°. Cells were washed three times with cold PBS and incubated in Dulbecco's modified Eagle's medium containing 5% bovine serum at 37° in a CO_2 incubator. Samples of 10^5 AS-30D cells were transferred to an ice bath after 0, 4, and 18 hr. Then all samples were washed twice with PBS and incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (1:200) for 1 hr at 4°. After washing cells twice with cold PBS, the surface fluorescence of 10,000 cells was measured with a FACSCalibur flow cytometer. Figure 2 shows that mAb RH1 remained on the surface of AS-30D cells for at least 18 hr, indicating minimal internalization of mAb RH1.

Prodrug Activation by βG-Transfectants

The low partition coefficient of BHAMG and the minimal internalization of mAb RH1 suggested that BHAMG and βG must interact extracellularly for efficient prodrug activation. This hypothesis was directly tested by constructing transgenes to express βG either intracellularly (pNeo/Gus) or in a secreted form (pLS/Gus). Both transgenes employed a mutant GUS gene that was modified to destroy the cryptic N-linked glycosylation site in the GUS coding region (pGUSN358-S, Clonetech). pNeo/Gus was constructed by ligating the *Hin*dIII-*Eco*RI fragment of pGUSN358-S in the corresponding sites of the expression vector pcDNA3 (Invitrogen). pLS/Gus was constructed by first introducing a *Cla*I site at the 5′ end and an *Eco*RI site immediately after the stop codon of GUSN358-S by polymerase chain reaction (PCR) amplification of pGUSN358-S with the primers 5′-CAGTCATCGATGTTACGTCCTGTAGAAACC-3′ and 5′-GGAGAGGAATTCATTCATTGTTTGCCTCC-3′, respectively. The PCR product was digested with *Cla*I and *Eco*RI and ligated in pLTM-1 [9] to produce a transgene with the ICAM-1 leader sequence at the N-terminal of GUSN358-S. The transgene was excised by digestion with *Xba*I and ligated into pcDNA3 to produce pLS/Gus.

BHK cells were transfected with pNeo/Gus and pLS/Gus using Lipofectamine reagent (Bethesda Research Laboratories). Cells resistant to 0.5 mg/mL of G418 were expanded and cloned by limiting dilution. Clones expressing pNeo/Gus (BHK-iβG) or pLS/Gus (BHK-sβG) were assayed for secreted and intracellular βG by culturing the cells in

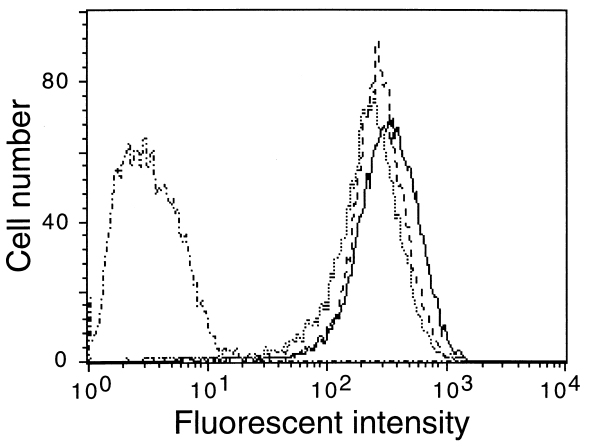

FIG. 2. Antibody internalization. Viable AS-30D cells were incubated with PBS (— · — · —) or mAb RH1, washed, and then incubated at 37° for 0 hr (—), 4 hr (— — —), or 18 hr (···) before transfer to ice. After 18 hr, all samples were stained with FITC-conjugated goat anti-mouse IgG, and the surface immunofluorescence of 10,000 cells was determined in a flow cytometer.

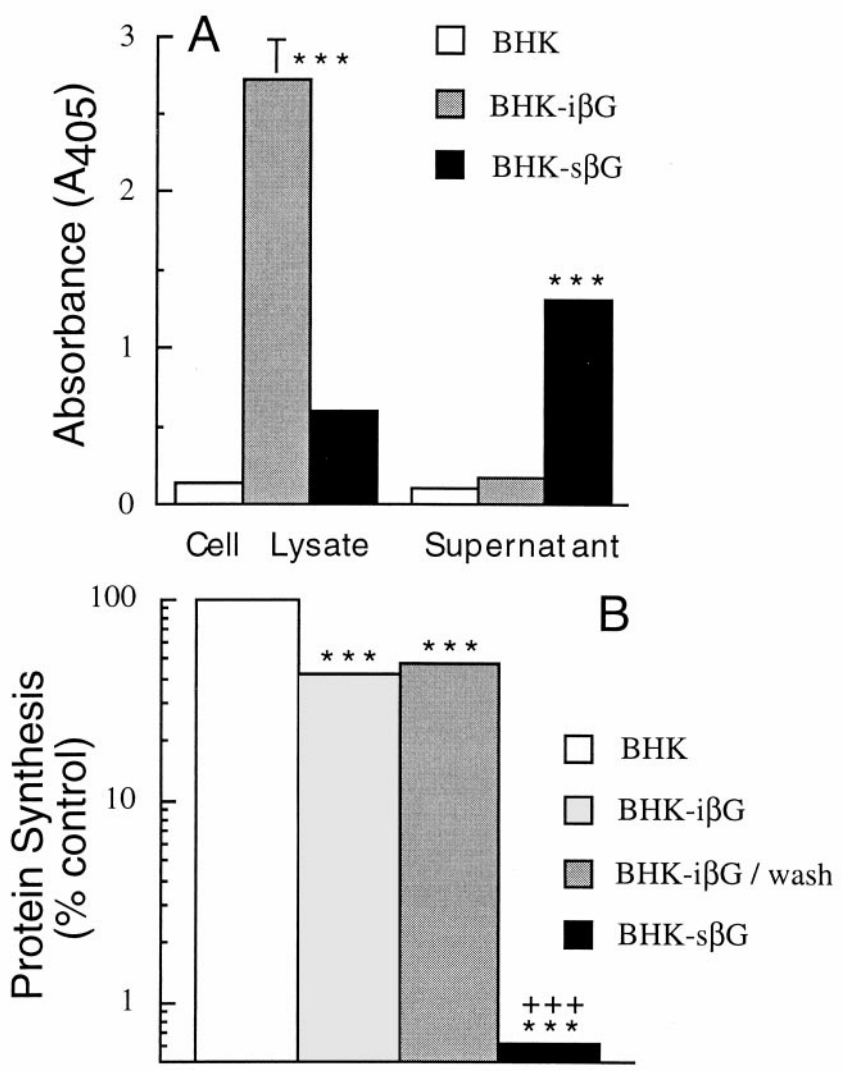

FIG. 3. Extracellular prodrug activation required for efficient cell killing. (A) BHK, BHK-sβG, or BHK-iβG cells were cultured for 96 hr before the βG activity of the culture medium and cell lysates was measured with *p*-nitrophenol β-D-glucuronide. Results represent the mean absorbance values (405 nm) of six samples. Significant differences between mean absorbance values of BHK-iβG cell lysates and other cell lysates or BHK-sβG cell supernatant and other supernatants are indicated: (***) $P \leq 0.0005$. (B) Cells were cultured for 96 hr before exposure to BHAMG for 4 hr. The medium from one group of BHK-iβG cells was replaced with fresh medium before exposure to BHAMG (BHK-iβG/wash). The rate of protein synthesis was measured after incubation in drug-free medium for 48 hr. Results are presented as percent protein synthesis relative to untreated cells, measured as [^{3}H]leucine incorporation (5.8×10^5, 5.4×10^5, and 5.3×10^5 cpm for BHK, BHK-iβG, and BHK-sβG cells, respectively) and represent the mean values of triplicate determinations. Significant differences between mean values of engineered and BHK cells are indicated: (***) $P \leq 0.0005$. Significant differences between BHK-sβG and BHK-iβG cells are also indicated: (+++) $P \leq 0.0005$. Bars = SEM.

96-well plates for 96 hr before measuring the βG activity in the culture supernatant and cell lysate as described [4]. Figure 3A shows that BHK-sβG cells secreted significantly ($P \leq 0.0005$) more βG than either BHK or BHK-iβG cells in the supernatant, whereas BHK-iβG cells produced high levels of intracellular βG. The protein synthesis of BHK cells, measured as incorporation of [^{3}H]leucine into cellular protein 48 hr after addition of drug, was unaffected by 100 μM BHAMG. BHK-iβG cells were moderately sensitive to BHAMG, with 60% reduction of cellular protein synthesis compared with untreated cells. Replacement of the culture supernatant with fresh medium immediately before addition of BHAMG did not alter the sensitivity of BHK-iβG cells to prodrug (Fig. 3B), demonstrating that cell killing was not due to release of intracellular βG by dead cells. The protein synthesis of BHK-sβG cells was inhibited significantly ($P \leq 0.0005$) by BHAMG compared with that of BHK and BHK-iβG cells.

DISCUSSION

BHAMG was over 1000 times less toxic than pHAM to AS-30D rat hepatoma cells *in vitro* [4] and significantly less toxic to blood cells than pHAM in both rats [5] and mice [6]. The low toxicity of BHAMG can be partially explained by hindered diffusion across the cell membrane, as sug-

gested by the preference of BHAMG for the aqueous phase of an octanol–water mixture as well as by the significantly greater toxicity of BHAMG to BHK-sβG compared with BHK-iβG cells. The higher toxicity of the prodrug to BHK-iβG compared with BHK cells, however, demonstrated that some BHAMG does enter cells. The absence of toxicity to BHK cells shows that intracellular BHAMG is not activated, either because of hindered diffusion into lysosomes and microsomes that contain endogenous βG [10] or by rapid detoxification by UDP-glucuronosyltransferases [11]. We favor the first mechanism because the activity of βG is higher than UDP-glucuronosyltransferase in AS-30D cells, and these cells are highly sensitive to pHAM and extracellularly activated BHAMG [4]. Intracellular BHAMG possesses low toxicity, likely due to the poor DNA alkylating activity of the prodrug.

The prolonged retention of mAb RH1 on the surface of AS-30D cells, the hydrophilicity of BHAMG, and the significantly greater cytotoxicity of BHAMG to BHK cells that secrete βG relative to cells that express cytosolic βG all support the notion that efficient enzymatic conversion of BHAMG to pHAM requires extracellular localization of βG. Human tumor therapy by targeted activation of BHAMG therefore should employ antibody conjugates that do not rapidly internalize after binding to antigen. Tumor-associated antigens, such as the L6 antigen expressed on lung, breast, colon, and ovarian tumors [12] and the TAG-72 antigen expressed on a wide range of carcinomas [13], remain on the cell surface after antibody binding [14, 15] and may represent good targets for human cancer therapy with BHAMG.

In summary, our results indicate that the low toxicity of BHAMG is due to hindered cellular uptake and reduced alkylating activity. BHAMG must be enzymatically activated outside of tumor cells for maximum cytotoxicity, and non-internalizing antibodies are preferred for human tumor therapy by targeted antibody–enzyme activation of BHAMG.

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