Acknowledgements—We are grateful to the Wellcome Trust and Medical Research Council for financial support.

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Biochemical Pharmacology, Vol. 42, No. 10, pp. 2062–2065, 1991.
Printed in Great Britain.
0006–2952/91 $3.00 + 0.00
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Anti-neoplastic glucuronide prodrug treatment of human tumor cells targeted with a monoclonal antibody–enzyme conjugate

(*Received* 19 *April* 1991; *accepted* 17 *July* 1991)

Treatment of cancer with anti-neoplastic prodrugs that are specifically activated to cytotoxic agents at the tumor site is a conceptually attractive strategy. For example, a glucuronide prodrug [(*p*-di-2-chloroethylaminophenol-β-D-glucopyranosid) uronic acid, HAMG*] of *p*-hydroxyaniline mustard [*N,N*-di-(2-chloroethyl)-4-hydroxyaniline, HAM] cured mice bearing well-established PC5 plasma tumors containing high levels of β-glucuronidase [1–3]. The relatively nontoxic prodrug HAMG was apparently formed *in vivo* in the liver of mice treated with aniline mustard [*N,N*-di-(2-chloroethyl)aniline] and subsequently converted to highly cytotoxic *p*-hydroxyaniline mustard by β-glucuronidase at the tumor site [2]. Clinical trials using aniline mustard [4, 5], however, have been disappointing, likely due to insufficient elevations of β-glucuronidase in most human tumors [4].

Inadequate differences in enzyme levels between normal and tumor cells for specific prodrug activation can be alleviated by targeting appropriate enzymes to tumor cells with monoclonal antibodies that bind to tumor-associated antigens [6–8]. Anti-neoplastic prodrugs susceptible to enzymatic activation can then be administered and converted to cytotoxic agents at the tumor site. We report here the development of a glucuronide prodrug/enzyme-antibody system and show that cytotoxic drug can be selectively generated at tumor cells using this strategy.

* Abbreviations: HAM, *p*-hydroxyaniline mustard; HAMG, *p*-hydroxyaniline mustard glucuronide; BHAMG, tetra-*n*-butyl ammonium salt of HAMG; IC_{50}, concentration of drug resulting in 50% inhibition of cellular protein synthesis; PBS, phosphate-buffered saline; and SPDP, *N*-succinimidyl-3(2-pyridyldithio)propionate.

Materials and Methods

Materials and cells. β-Glucuronidase (EC 3.2.1.31) from *Escherichia coli* and pepsin were purchased from the Sigma Chemical Co., St. Louis, MO. *N*-Succinimidyl-3(2-pyridyldithio)propionate (SPDP) was from Pharmacia LKB Biotechnology, Uppsala, Sweden. [^{3}H]Leucine (50 Ci/mmol) was purchased from ICN Biomedicals Inc., Costa Mesa, CA. COLO 205 human colon and WISH human amnion cell lines were obtained from the American Type Culture Collection, Rockville, MD. Cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin.

Drug synthesis. p-Hydroxyaniline mustard and the tetra-*n*-butyl ammonium salt of HAMG (BHAMG) were synthesized as previously described [9]. Structures were confirmed by NMR and melting point determination.

β-Glucuronidase conjugation to monoclonal antibody. Mab 12.8 is a murine IgG_3 monoclonal antibody that binds strongly to COLO 205 human colon carcinoma cells but does not react with WISH cells. Mab 12.8 also reacts positively with about 40% of human colon carcinoma tissues but has limited reaction with normal human tissues [10]. Mab 12.8 F(ab′)$_2$ fragments were generated by pepsin digestion [11].

β-Glucuronidase was linked to Mab 12.8 F(ab′)$_2$ via a disulfide bond with the heterobifunctional cross-linking agent SPDP [12]. An average of 2.4 and 2.0 2-pyridyl groups, measured as described [12], were introduced into Mab 12.8 F(ab′)$_2$ and β-glucuronidase molecules, respectively. Derivatized enzyme was reduced with 1 mM dithiothreitol, and after removing excess reducing agent by gel filtration, derivitized enzyme and antibody were mixed and incubated overnight at room temperature.

Purification and characterization of β-glucuronidase-antibody conjugate. Enzyme–antibody conjugate was purified by HPLC gel filtration on two 7.5 mm × 30 cm SW 300 columns (Waters) in series. Protein concentrations were measured by the bicinchoninic acid (BCA) assay [13]. Molecular weight of the enzyme–antibody conjugate was approximately 350 kDa as estimated from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [14]. The conjugate retained an enzymatic activity of 0.25 units/μg (1 unit liberates 1.0 μmol *p*-nitrophenol from *p*-nitrophenol glucuronide/hr at 37°), about 20% of original β-glucuronidase activity, measured as described [15]. The conjugate retained full antibody activity compared with unconjugated antibody as measured by enzyme immunoassay (EIA) using fixed COLO 205 cells as antigen source [16].

In vitro *activity of prodrug and conjugate.* COLO 205 or WISH cells were plated overnight in 96-well microtiter plates at 20,000 cells per well. Serial dilutions of HAM or BHAMG in RPMI medium containing 10% fetal bovine serum were added to cells for 24 hr at 37°. Cells were subsequently washed once with sterile phosphate-buffered saline (PBS), incubated for an additional 24 hr in fresh medium, and then pulsed for 2 hr with [^{3}H]leucine (1 μCi/well) in leucine-free medium. Radioactivity of trichloroacetic acid precipitated protein was measured in a Beckman LS 6000 series liquid scintillation counter.

BHAMG conversion to cytotoxic HAM was tested by incubating BHAMG and β-glucuronidase (10 units/well) for 24 hr at 37°. Cells were washed once with PBS, and after an additional 24-hr incubation, the rate of protein synthesis was measured. The *in vitro* activation of BHAMG by Mab 12.8 F(ab')$_2$-β-glucuronidase conjugate was tested by preincubating plated cells with the indicated concentrations of conjugate for 1 hr at room temperature. After washing cells once with PBS, 200 μg/mL BHAMG was added and the assay carried out as described above. All experiments were performed in triplicate.

Results and Discussion

HAM (Fig. 1a) and BHAMG (Fig. 1b) were synthesized, and their cytotoxicity was determined by measuring the incorporation of [^{3}H]leucine into cellular protein of cells exposed to these drugs for 24 hr. Figure 2 shows that COLO 205 and WISH cells were about equally sensitive to HAM with IC_{50} values of 34 and 49 μM, respectively. The water-soluble prodrug BHAMG was less toxic to both COLO 205 and WISH cell lines with IC_{50} values of 1890 and 1320 μM, respectively. Simultaneous addition of 10 units β-glucuronidase and BHAMG to cells resulted in a cytotoxic effect as great as the addition of HAM alone (Fig. 2), indicating that enzymatic cleavage of the water-soluble glucuronide group converts BHAMG to the cytotoxic alkylating agent HAM. Addition of β-glucuronidase alone had no effect on cells.

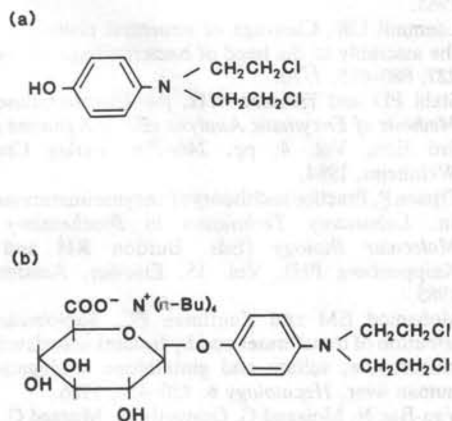

Fig. 1. Structures of HAM (a) and BHAMG (b).

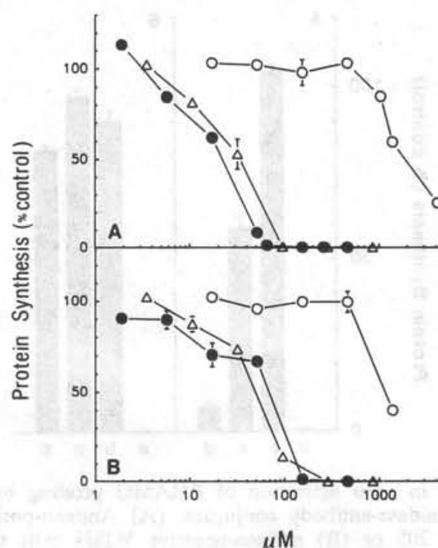

Fig. 2. Cytotoxicity of HAM (△), BHAMG (○) or BHAMG + β-glucuronidase (●) to COLO 205 (A) and WISH (B) cells. Cells were exposed to drugs for 24 hr in 96-well microtiter plates, and the rate of protein synthesis was measured after an additional 24-hr incubation in drug-free medium. Results are presented as percent protein synthesis relative to control cells, measured as [^{3}H]leucine incorporation (200,000 and 290,000 cpm for COLO 205 and WISH control cells, respectively) and represent the average of three independent wells. Standard errors of the mean are indicated.

β-Glucuronidase was linked to Mab 12.8 F(ab')$_2$ via a disulfide bond. This conjugate binds to antigen-positive COLO 205 cells but is unable to bind antigen-negative WISH cells. Figure 3A shows that pretreatment of COLO 205 cells with enzyme–antibody conjugate greatly increased the cytotoxic effect of BHAMG. Antigen-negative WISH cells, in contrast, were still resistant to BHAMG after pretreatment with conjugate (Fig. 3B). The addition of antibody, enzyme or conjugate alone had no effect on cells. These results indicate that sufficient β-glucuronidase can be targeted to tumor cells to convert glucuronide prodrugs to active anti-neoplastic agents. In addition, the lack of toxicity to antigen-negative cells indicates that prodrug activation is specific.

Specific activation of glucuronide prodrugs at tumor cells with β-glucuronidase–antibody conjugates may possess advantages over other prodrug–enzyme combinations. The absence of high serum β-glucuronidase activities in humans [15] should minimize premature activation of prodrug. β-Glucuronidase is highly specific for the glucuronyl residue but has little specificity for the conjugated aglycone [15], suggesting that a wide variety of glucuronide prodrugs can be synthesized. Glucuronide prodrugs also appear to be less toxic than sulfate or phosphate prodrugs [9]. In addition, any activated drug not taken up by tumor cells may be reconverted to glucuronide prodrug by UDP-glucuronosyltransferase present in the liver [17]. Several investigators have shown that aglycone anti-neoplastic drugs are converted to glucuronides in rodents [18, 19] and humans [20].

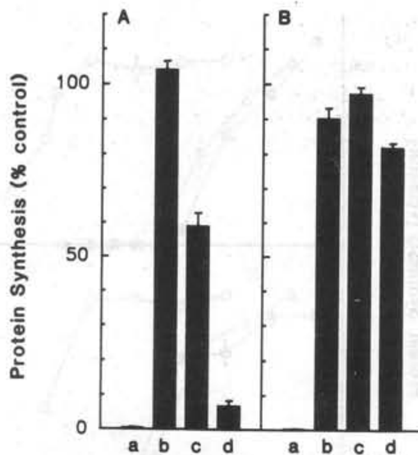

Fig. 3. *In vitro* activation of BHAMG prodrug by β-glucuronidase-antibody conjugate. (A) Antigen-positive COLO 205 or (B) antigen-negative WISH cells were preincubated with (a, b) medium alone, (c) 20 μg/mL β-glucuronidase-antibody conjugate or (d) 50 μg/mL conjugate for 1 hr at room temperature. Cells were then washed and incubated with (a) 200 μg/mL HAM or (b, c, d) 200 μg/mL BHAMG for 24 hr. The cellular protein synthesis rate was measured 24 hr later. Results are presented as percent protein synthesis relative to control cells, measured as [^{3}H]leucine incorporation (140,000 and 290,000 cpm for COLO 205 and WISH control cells, respectively) and represent the mean of results from three separate wells. Error bars show the standard error of the mean.

In summary, BHAMG is a latent form of HAM that can be converted to HAM by β-glucuronidase at neutral pH. Tumor cells expressing tumor-associated antigen on their surface can be selectively killed by first targeting β-glucuronidase to the cells as an antibody–enzyme conjugate before introduction of BHAMG prodrug. Antibody-directed enzymatic activation of glucuronide prodrugs may lead to more specific chemotherapy treatments.

Acknowledgements—This research was supported by grants from the National Science Council and Academia Sinica, Taipei, Taiwan, R.O.C. The technical assistance of Bing-Mae Chen, Joyce C. Ng, Huang-Chun Tseng, Tian-Chun Chang and Shu-Fen Chiu is gratefully acknowledged.

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Biochemical Pharmacology, Vol. 42, No. 10, pp. 2065–2067, 1991.
Printed in Great Britain.
0006-2952/91 $3.00 + 0.00
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Modulation of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine induced inhibition of cell-free protein synthesis by sulfur compounds*

(*Received 22 April* 1991; *accepted* 3 *August* 1991)

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG†), which is a potent mutagen and carcinogen [1–4] has been demonstrated to inhibit protein synthesis *in vitro* and *in vivo* [5, 6]. Since proteins are important biomacromolecules involved in many regulatory processes of the cell, an inhibition of protein synthesis may severely perturbate normal cellular activities and may also be connected to carcinogenesis. An attempt was made to find out whether thiocompounds which were proven to reduce toxic and carcinogenic effects of *N*-nitrosamines [7–9] may affect the inhibitory action of MNNG on protein synthesis. We report here the influence of PDTC, DDTC, GSH and GS-DDTC on [^{14}C]amino acid incorporation into protein using rat liver postmitochondrial supernatant (S30 fraction) in an *in vitro* protein synthesizing system.

Materials and Methods

ATP, GTP, creatine phosphate, creatine phosphokinase (2.7.3.2), and dithiothreitol were purchased from Serva Feinbiochemikalien (Heidelberg, Germany). MNNG was purchased from Fluka AG (Buchs SG, Switzerland). L-[U-^{14}C]Leucine (sp. act. 308 mCi/mmol), was obtained from the Radiochemical Centre (Amersham, U.K.). The dithiocarbamates used were synthesized in this laboratory [9]. All other chemicals used were commercially available with the highest degree of purity. Male Sprague–Dawley rats (Wiga) weighing 100–120 g were used. They were killed by cervical dislocation and the liver homogenate (25% w/v) was prepared in STKM buffer. Postmitochondrial supernatant (S30 fraction), containing all the components required for *in vitro* incorporation of amino acids into protein, was prepared from the homogenate by centrifugation at 30,000 *g* for 15 min. Amino acid incorporation studies were carried out following the procedure of Richardson *et al.* [10] with slight modifications as described [5]. Each assay mixture (250 μL) contained 10 mM HCl (pH 7.2), 80 mM KCl, 5 mM magnesium acetate, 2 mM ATP, 1 mM GTP, 20 mM creatine phosphate, 2.5 μg creatine phosphokinase, 1 μCi [^{14}C]leucine (sp. act. 308 mCi/mmol) and S30 fraction (2 mg protein). After incubation at 37° for 15 min, a 50 μL aliquot was spotted on Whatman No. 3 MM filter paper circles (2.5 cm diameter) and processed before counting [11]. Protein concentration in S30 fraction was measured by the method of Lowry *et al.* [12].

Results

Each incorporation assay was conducted in duplicate or triplicate, and the result given as the average value. Each experiment was then repeated with S30 fraction from another rat. Variation was found to be within ±10%. Reproducible results were obtained with different preparations.

The inhibitory activity of various amounts of MNNG for [^{14}C]leucine incorporation was increased by the addition of PDTC into the assay system. At lower concentrations of MNNG (0.08 and 0.2 mM) no inhibition was observed (Fig. 1). However, the addition of PDTC (2.0 mM) along with MNNG showed significant inhibition. This increase in inhibition depended on the concentration of MNNG as well as of PDTC (PDTC data not shown). In contrast to PDTC, the addition of DDTC to the assay system stimulated the cell-free protein synthesis in a dose-dependent way up to 143% of the control value (Fig. 2, empty columns). The stimulation of amino acid incorporation by DDTC is blocked by MNNG, in contrast to PDTC where the inhibition is increased. Two other compounds were tested for their effect on the MNNG-induced inhibition of leucine incorporation into protein, namely GSH and the mixed disulfide GS-DDTC. They increase the inhibitory activity of MNNG upon leucine incorporation in a dose-dependent way (Table 1). Both these compounds show effects which are comparable to those produced by PDTC. However, these compounds also inhibit cell-free protein synthesis without MNNG.

Discussion

The thiocompounds showed a different behavior with respect to cell-free protein synthesis. DDTC stimulated the incorporation of amino acids whereas GSH or GS-DDTC showed an inhibition of it. These alterations depend on the concentration of the dithiocarbamates. However, PDTC did not show any significant alteration. The effects observed with PDTC and DDTC on the MNNG-induced inhibition of amino acid incorporation are opposite in action. Since both compounds, PDTC and DDTC, are dithiocarbamates, a qualitatively different effect was not to be expected. There have been few experiments up to

* Dedicated to the late Professor Dr. Dietrich Schmähl.

† Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PDTC, prolinedithiocarbamate; DDTC, diethyldithiocarbamate; GSH, glutathione; GS-DDTC, glutathionyldiethyldithiocarbamate.