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ORIGINAL ARTICLE

Expression of β-glucuronidase on the surface of bacteria enhances activation of glucuronide prodrugs

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Extracellular activation of hydrophilic glucuronide prodrugs by β-glucuronidase (βG) was examined to increase the therapeutic efficacy of bacteria-directed enzyme prodrug therapy (BDEPT). βG was expressed on the surface of *Escherichia coli* by fusion to either the bacterial autotransporter protein Adhesin (membrane βG (mβG)/AIDA) or the lipoprotein (lpp) outermembrane protein A (mβG/lpp). Both mβG/AIDA and mβG/lpp were expressed on the bacterial surface, but only mβG/AIDA displayed enzymatic activity. The rate of substrate hydrolysis by mβG/AIDA-BL21cells was 2.6-fold greater than by pβG-BL21 cells, which express periplasmic βG. Human colon cancer HCT116 cells that were incubated with mβG/AIDA-BL21 bacteria were sensitive to a glucuronide prodrug (p-hydroxy aniline mustard β-D-glucuronide, HAMG) with an half maximal inhibitory concentration (IC_{50}) value of 226.53 ± 45.4 μM, similar to the IC_{50} value of the active drug (p-hydroxy aniline mustard, pHAM; 70.6 ± 6.75 μM), indicating that mβG/AIDA on BL21 bacteria could rapidly and efficiently convert HAMG to an active anticancer agent. These results suggest that surface display of functional βG on bacteria can enhance the hydrolysis of glucuronide prodrugs and may increase the effectiveness of BDEPT.

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INTRODUCTION

Tumors are composed of immunosuppressive and hypoxic microenvironments that facilitate the accumulation and replication of bacteria.[1,2] Numerous studies have thus demonstrated that bacteria can be used as selective anticancer vectors.[2–4] For example, an attenuated strain of *Salmonella typhimurium* (VNP20009) that was genetically modified to express cytosine deaminase, a prodrug-converting enzyme, successfully completed Phase I clinical trials. Some tumor colonization was observed at the highest tolerated dose (3×10^8 cfu m^{-2}), with toxicity observed in patients receiving 1×10^9 cfu m^{-2}, including thrombocytopenia, diarrhea, anemia, persistent bacteremia, vomiting, nausea, hyperbilirubinemia, hypophosphatemia and elevated alkaline phosphatase.[5,6] Recently, *Salmonella* expressing carboxypeptidase G2,[7] *Escherichia coli* expressing β-glucuronidase (βG)[8] and *Salmonella* expressing herpes simplex virus thymidine kinase[3] have been shown to generate potent and selective antitumor activity by converting systemically administered prodrugs to active anticancer agents in tumors, while minimizing exposure of normal tissues to active drug. Therefore, bacteria-directed enzyme prodrug therapy (BDEPT) is a promising therapeutic approach for treatment of solid tumors.

Inadequate colonization of bacteria may limit the conversion of prodrugs by BDEPT in some tumors.[9] Thus, methods that increase conversion efficiency of prodrug-activating bacteria within tumors are anticipated to improve the therapeutic efficacy of BDEPT. βG is an attractive prodrug-converting enzyme for selective prodrug therapy, because glucuronide prodrugs can display orders of magnitude with less toxicity than the parent drug, and most antineoplastic agents can be converted to glucuronide prodrugs by employing linkers between the drug and glucuronide moieties.[10–13] We previously found that extracellular activation of hydrophilic glucuronide prodrugs by cell membrane-anchored βG can increase the antitumor activity and bystander cytotoxic effects of glucuronide anticancer prodrugs.[14–16]

In this study, we fused βG to the amino terminus of the bacterial autotransporter protein adhesin (AIDA-I) or the carboxy terminus of the lipoprotein outermembrane protein A (lpp-ompA) to generate membrane βG (mβG)/AIDA-BL21 and mβG/lpp-BL21 bacteria, respectively. AIDA-I is Gram-negative bacterial autotransporter involved in diffuse adherence, consisting of an N-terminal signal peptide, secreted passenger domain, a linker region and a C-terminal translocator domain, which forms a β-barrel structure in the outer membrane. AIDA-I has been used for the surface expression of a variety of recombinant proteins.[17,18] the lpp-ompA consists of a signal sequence and the first nine amino acids of the *E. coli* lpp fused with amino acids 46–159 of the *E. coli* ompA,[19] previously shown to allow display of passenger proteins on *E. coli*.[20–23] Expression of βG on the surface of bacteria by lpp-ompA or AIDA-I was hypothesized to increase prodrug conversion

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efficiency and maximize the therapeutic efficacy of BDEPT (Figure 1).

Surface expression of βG on the BL21 cells was confirmed by enzyme-linked immunosorbent assay. We then examined whether surface-expressed βG can hydrolyze p-nitrophenyl-β-D-glucuronide (PNPG) to p-nitrophenol. In addition, the hydrolytic efficiency of p-nitrophenyl-β-D-glucuronide (PNPG) in mβG/AIDA-BL21, pβG-BL21 (periplasmic βG) and pβG-BL21 lysates were investigated. Finally, the cytotoxic effects of adding mβG/AIDA-BL21 or pβG-BL21 bacteria and HAMG prodrug to HCT116 human colon cancer cells were examined. Our results show that surface expression of βG may provide a strategy to enhance the potency of BDEPT for glucuronide prodrug therapy.

MATERIALS AND METHODS

Bacteria and cell line

E. coli BL21 (F- ompT hsdSB (rB^-,mB^-) gal dcm (DE3); Novagen, San Diego, CA, USA) was used in this study. HCT116 human colorectal adenocarcinoma cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's minimal essential medium (Sigma, St Louis, MO, USA) supplemented with 5% heat-inactivated bovine serum, 100 units per ml penicillin and 100 μg ml^{-1} streptomycin (Gibco Laboratories, Grand Island, NY, USA) at 37 °C in 5% CO_2 in humidified air.

Plasmid construction

The coding sequence of βG was amplified by PCR, using the plasmid pRSETB-βG[8] as template and the primers 5′-CGGGATCCGGCGGCCGC GTATCCATATGATGTTCCA-3′ and 5′-GGGGTACCTTACTCGAGATCGATCCCG GGTGTCGACTACTTTCGGCG CCTGAGCATC-3′, to introduce a hemagglutinin epitope tag and unique restrictions sites as follows: *Bam*HI–*Not*I–hemagglutinin–*Sfi*I–βG–*Sal*I–*Sma*I–*Cla*I–*Kpn*I. The PCR fragment was digested with *Bam*HI and *Kpn*I, and cloned into pRSETB (Invitrogen, Grand Island, NY, USA) to form pRSETB-sfiI-βG. The gene coding *E. coli* AIDA-I (*pMK14*[24]), a kind gift from Dr Benz Inga (University of Münster, Münster, Germany), was digested with *Sma*I and *Cla*I, and then subcloned into pRSETB-sfiI-βG to create pRSETB-mβG-AIDA. The plasmid pTX101,[25] a kind gift from Dr George Georgiou (University of Texas, Austin, TX, America), was used as a template to amplify the coding sequence of lpp–membrane protein A with the following primers: 5′-CATATGATGAAAGCTACTAAT-GAAAGCTACTAAACTG-3′ and 5′-GGCCCAGCCGGCCGTTGTCCGGACGAGT GCC-3′, and was subcloned into pRSETB to form pRSETB-lpp-βG.

Bacterial βG expression

BL21 *E. coli* were transformed with pRSETB-βG-AIDA, pRSETB-lpp-βG or pRSETB-βG to form mβG/AIDA-BL21, lpp/mβG-BL21 and pβG-BL21 cells, respectively. βG expression was detected by western blotting, using a polyclonal rabbit anti-βG antibody. Transformed BL21 cells ($OD_{600\,nm} = 0.1$, 100 μl) were immediately mixed with 20 μl of reducing sample buffer and loaded onto an SDS-polyacrylamide gel electrophoresis (3% stacking gel; 8% running gel). Proteins were transferred onto nitrocellulose membranes (Hybond C-extra; Amersham, Piscataway, NJ, USA). Membranes were blocked in phosphate buffered saline–0.05% Tween, containing 5% nonfat milk for 1 h. Blocked membranes were then incubated with rabbit anti-*E. coli* βG antibody (G5420; Sigma-Aldrich, Schnelldorf, Germany) at a dilution of 1:2000 in phosphate buffered saline–0.05% Tween and 2.5% nonfat milk for 1 h. Membranes were washed and incubated with horseradish-conjugated goat antirabbit IgG (1:2000) (Jackson ImmunoResearch, Soham, UK) in the same buffer for 1 h. After extensive washing in phosphate buffered saline–0.05% Tween, membranes were developed by an ECL luminescence kit (Millipore, Bedford, MA, USA) and exposed to X-ray film.

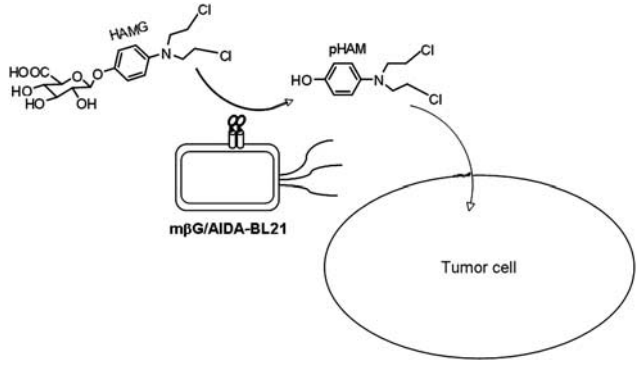

Figure 1. β-glucuronidase (βG) expressed on the bacteria surface improves conversion efficacy of glucuronide prodrugs. The hydrophilic glucuronide prodrugs are cell impermeable. βG expressed on bacteria surfaces (membrame βG (mβG)/AIDA-BL21) may increase conversion of prodrugs into active drugs in the extracellular space to maximize bacteria-directed enzyme prodrug therapy efficacy.

Surface-expressed βG on bacteria by enzyme-linked immunosorbent assay

Transformed bacteria mβG/AIDA-BL21, lpp/mβG-BL21 and pβG-BL21 were grown until $OD_{600\,nm} = 0.35$. Bacteria were collected and coated on a 96-well microtiter plate (1×10^7 cfu per 50 μl per well) in 0.1 M $NaHCO_3$ (pH 7.8) at 4 °C overnight. After removing uncoated bacteria by extensive washing, the plates were blocked overnight with 2% nonfat milk at 4 °C and then incubated with 1 μg ml^{-1} anti-hemagglutinin tag antibody (MMS-101P; Covance, Berkeley, CA, USA) in dilution buffer (PBS contains 2% nonfat milk) at room temperature for 1 h. The plates were washed with PBS and 50 μl ml^{-1} horseradish-peroxidase-conjugated goat antimouse IgG Fc (0.5 μg ml^{-1}; Jackson ImmunoResearch) was added at room temperature for 1 h. The plates were washed as described above, and bound peroxidase was measured by adding 150 μl per well of 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) at 0.4 mg ml^{-1} in the presence of 0.003% H_2O_2 at room temperature for 30 min. Color development was measured at 405 nm by a microplate reader.

Analysis of βG activity

βG activity was measured by monitoring the release of p-nitrophenol from PNPG (Sigma) Transformed BL21 cells ($OD_{600\,nm} = 0.1$, 50 μl) were washed five times with PBS, broken by ultrasonication and were immediately incubated with 100 μl of 0.625 mM PNPG in PBS containing 0.05% bovine serum albumin in a microtiter plate for 10 min. The absorbance at 405 nm was measured on a plate reader.

Cytotoxicity by pβG-BL21- or βG/AIDA-BL21-mediated conversion of prodrug

HCT116 cells (5×10^3 cells per well) grown overnight in 96-well microtiter plates were treated with graded concentrations of pHAM and HAMG provided by Dr Lu (Chia Nan University of Pharmacy and Science, Tainan, Taiwan), βG/AIDA-BL21 (2×10^7 bacteria per well) + HAMG or pβG-BL21 (2×10^7 bacteria per well) + HAMG at 37 °C for 15 min. The cells were washed three times with PBS and then cultured for an additional 72 h in fresh medium containing 25 μg ml^{-1} gentamycin (Sigma), to kill residual bacteria. Cell viability was determined by the ATPlite luminescence ATP detection assay system (Perkin-Elmer Life and Analytical Science, Boston, MA, USA). Results are expressed as percent inhibition of luminescence as compared with untreated cells by the following formula:

% inhibition = 100 × (sample luminescence − background luminescence/control luminescence − background luminescence).

RESULTS

Construction and expression of surface-expressed βG

To express βG on the bacterial surface, the gene coding *E. coli* βG was fused to the N terminus of the bacterial autotransporter *AIDA-I* gene or the C terminus of the bacterial *lppompA* gene to form the fusion proteins βG/AIDA and lpp/βG, respectively (Figure 2a). In addition, a plasmid for expression of βG in the periplasmic space (pβG) was used as a control.[8] To confirm the expression of the different forms of βG, these plasmids were transformed into BL-21(DE3) bacteria to form mβG/AIDA-BL21, lpp/mβG-BL21 and pβG-BL21 cells, respectively. Western blotting with an anti-*E. coli* βG antibody showed that mβG/AIDA-BL21, lpp/mβG-BL21 and pβG-BL21 cells expressed βG/AIDA, lpp/βG and pβG, with the expected sizes of 125, 100 and 75 kDa, respectively (Figure 2b).

Surface display of a functional βG

To investigate whether βG could be expressed on the surface of *E. coli*, mβG/AIDA-BL21 and lpp/mβG-BL21, cells were coated in 96-well microtiter plates, and the presence of βG on the cell surface was detected by enzyme-linked immunosorbent assay using an anti-hemagglutinin epitope tag antibody. The absorbance (representing bound antibody) in the wells coated with mβG/AIDA-BL21 (1.51 ± 0.08) and mβG/lpp-BL21 (0.43 ± 0.04) cells were significantly higher than in the wells coated with pβG-BL21 (0.06 ± 0.01) cells (Figure 3a), indicating that AIDA-I and lpp-ompA could direct βG to the bacterial surface. To verify whether the recombinant βG fusion proteins on the bacterial surface retained enzymatic activity, BL21, mβG/AIDA-BL21, mβG/lpp-BL21 and pβG-BL21 cells were incubated with the βG substrate p-nitrophenyl β-D-glucuronide. Figure 3b shows that mβG/AIDA-BL21 cells (2.81 ± 0.04) hydrolyzed more substrate than mβG/lpp-BL21 (0.04 ± 0.02) and pβG-BL21 (0.85 ± 0.01) cells, indicating that mβG/AIDA more effectively hydrolyzed substrate as compared with mβG/lpp or pβG. The enzymatic activities of mβG/AIDA-BL21, pβG-BL21 and pβG-BL21 were further compared by incubating bacteria or bacterial lysates with p-nitrophenyl-β-D-glucuronide and by measuring the absorbance at 405 nm at defined times. Figure 4 shows that generation of p-nitrophenol in mβG/AIDA-BL21 cells (slope $= 0.0237 \pm 0.0002$) was 2.6-fold faster than in pβG-BL21 cells (slope $= 0.009 \pm 0.0002$). By contrast, PNPG hydrolysis in pβG-BL21 lysates (slope $= 0.052 \pm 0.001$) was 5.7-fold faster than by intact pβG-BL21 cells, indicating that substrate hydrolysis in pβG-BL21 cells is limited by substrate entry into the cells, and expression of βG on the surface of bacteria can enhance the hydrolytic efficiency of glucronide substrate.

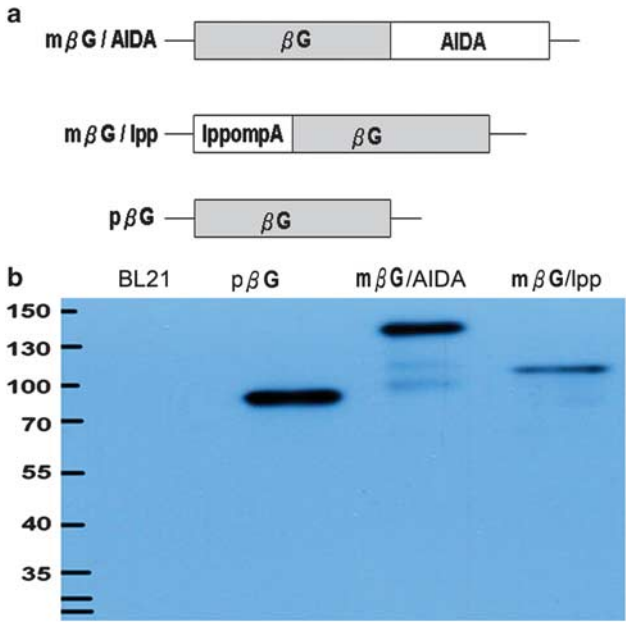

Figure 2. Construction and expression of surface β-glucuronidase (βG). (**a**) βG was fused to the N-terminal of the bacterial *AIDA-I* gene, the C terminus of the bacterial *lppompA* gene or a periplasmic signal to form membrame βG (mβG)/AIDA, mβG/lipoprotein (lpp) and periplasmic βG (pβG). pβG was used as a control. (**b**) mβG/AIDA, mβG/lpp and pβG plasmids were transformed into BL-21 to form mβG/AIDA-BL21, mβG/lpp-BL21 and pβG-BL21 cells, respectively. The expression of βG was confirmed by western blotting using an anti-*E. coli* βG antibody. Lane 1, BL21 as negative control; lane 2, pβG-BL21 cells; lane 3, βG/AIDA-BL21cells; lane 4, mβG/lpp-BL21cells.

mβG/AIDA-BL21-mediated cytotoxicity

To examine whether mβG/AIDA-BL21 cells could efficiently convert a glucuronide anticancer prodrug to active drug, human colorectal cancer HCT116 cells were incubated with graded concentrations of pHAM, HAMG, mβG/AIDA-BL21 + HAMG or pβG-BL21 + HAMG for 15 min. The cells were then washed three times with PBS and were cultured for an additional 72 h in fresh medium. Cellular ATP synthesis was measured as an index of cell viability. Figure 5 shows that in the presence of mβG/AIDA-BL21 cells, the glucuronide prodrug HAMG (half maximal inhibitory concentration (IC_{50}): 226.53 ± 45.4 μM) was nearly as toxic as active drug pHAM (IC_{50}: 70.6 ± 6.75 μM). By contrast, the IC_{50} of HAMG (> 500 μM) in the presence of pβG-BL2 cells was similar to HAMG alone (> 500 μM). These results show that surface expression of βG on mβG/AIDA-BL21 cells facilitated more effective conversion of HAMG to pHAM, to kill HCT116 cancer cells.

DISCUSSION

We successfully expressed functional βG on the surface of bacteria by fusion to the autotransporter AIDA-I, to increase the conversion efficiency of glucuronide prodrugs. mβG/AIDA-BL21 cells rapidly and efficiently converted a hydrophilic glucuronide prodrug to an active anticancer drug to inhibit tumor cell growth. Thus, surface expression of βG increased the conversion efficiency of glucuronides, which may help to compensate low colonization efficiency of bacteria in some tumors.

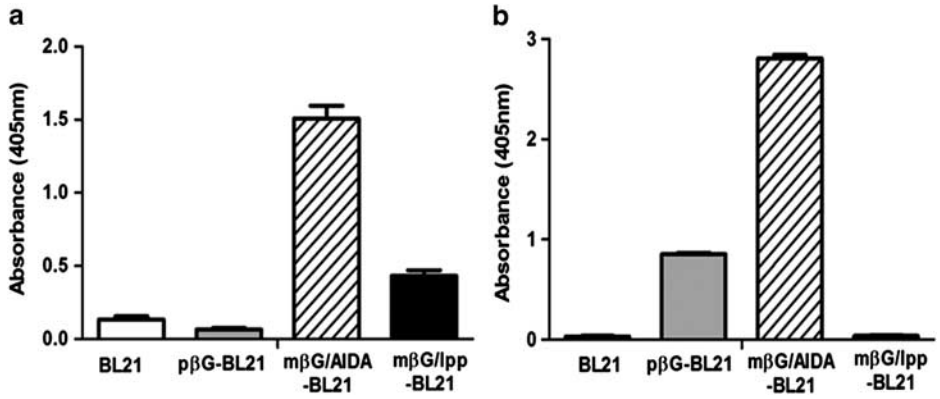

Figure 3. Surface display of a functional β-glucuronidase (βG). (**a**) Membrane βG (mβG)/AIDA-BL21, mβG/lpp-BL21 and periplasmic βG (pβG)-BL21 cells were coated on enzyme-linked immunosorbent assay plates, and the surface expression of βG was analyzed by an anti-hemagglutinin tag antibody. (**b**) mβG/AIDA-BL21, mβG/lipoprotein (lpp)-BL21 and pβG-BL21 cells (1×10^5 cfu per well) were incubated with 0.625 mM p-nitrophenyl-β-D-glucuronide (PNPG) at 37 °C for 10 min, and the absorbance at 405 nm was measured by a plate reader.

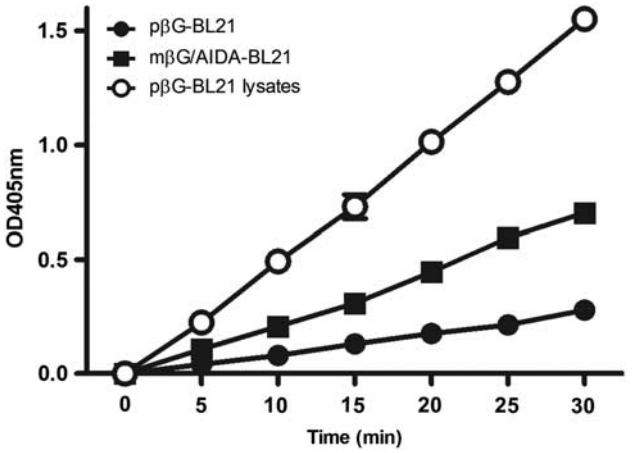

Figure 4. Hydrolytic efficiency of membrane βG (mβG)/AIDA-BL21 and periplasmic βG (pβG)-BL21 cells. mβG/AIDA-BL21 and pβG-BL21 cells (1×10^5 cfu) or pβG-BL21 lysates were incubated with 1.25 mM p-nitrophenyl-β-D-glucuronide (PNPG) at 37 °C for different time (0–35 min) and the absorbance of 405 nm was measured.

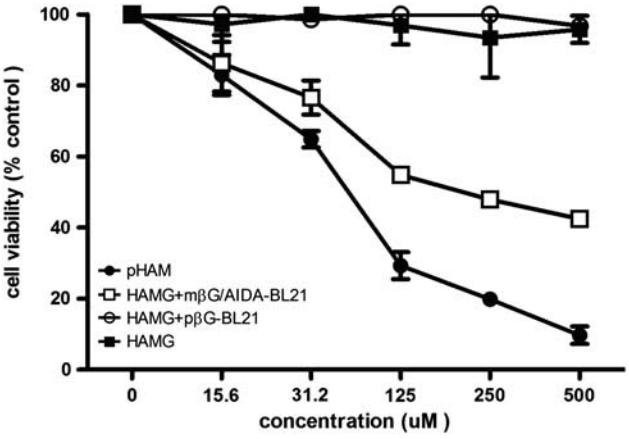

Figure 5. Activation of glucuronide prodrug by membrane βG (mβG)/AIDA-BL21 and periplasmic βG (pβG)-BL21cells. HCT116 tumor cells were treated with graded concentration of p-hydroxy aniline mustard β-D-glucuronide (HAMG; ■), p-hydroxy aniline mustard (pHAM; □), mβG/AIDA-BL21 cells + HAMG (●) or pβG-BL21cells + HAMG (○) for 15 min. The cellular ATP synthesis of treated cells was compared with that of untreated control cells after an additional 48 h incubation in drug-free medium. Bar, s.d. of triplicate determinations.

Many tumors evolve to evade control by the immune system through mechanisms, such as increased expression of FasL,[26,27] decreased expression of vascular cell adhesion molecule-1[28] and hypoxia-induced adenosine accumulation.[29] Such mechanisms lead to impaired immune surveillance and provide a microenvironment supportive of bacterial survival and replication. Pawelek *et al.*[3] showed that *Salmonella* preferentially accumulate within tumors in mice, reaching ratios of bacteria in tumor and normal tissues as high as 1000:1. Bacteria can compete for nutrients, secret toxic products or activate prodrugs to cause cancer cell death. These observations have incited interest in developing bacteria-based tumor therapies. For example, Hoffman[30] developed a tumor-seeking *S. typhimurium* that demonstrated antitumor efficacy. Other experiments demonstrated that bacteria can transfer therapeutic genes[31–33] or produce therapeutic proteins[34,35] to effectively inhibit tumor growth. Considering the ease of genetic manipulation in bacteria and that many bacteria can be cleared (or at least suppressed) by antibiotics when needed, BDEPT seems a promising treatment for cancer patients.

A wide variety of glucuronide prodrugs are available for cancer treatment, because they possess several potential advantages: (1) glucuronide prodrugs do not become activated systemically, because βG levels are very low in human serum;[36] (2) glucuronidation is an important detoxification metabolic process in mammals,[37,38] reducing premature activation of glucuronide prodrugs by endogeneous βG *in vivo*; (3) glucuronide prodrugs are relatively nontoxic;[14] (4) glucuronide derivatives of most antineoplastic agents, such as doxorubicin,[39] etoposide,[40] paclitaxel[41] and alkylating agents,[16] can be synthesized by employing a linker between the drug and glucuronide moieties:[16,42] therefore, a variety of potent glucuronide prodrugs are available for cancer treatment and (5) human βG displays optimal activity at pH 4.5, as it is mainly located in the lysosomes and microsomes of cells. By contrast, bacterial βG displays optimal activity at pH 7, consistent with its localization to the cytoplasm.[43] The pH in the interstitial tumor space is near 7,[44,45] which is suitable for bacteria but not human βG-catalyzed hydrolysis of glucuronide prodrugs. Combining the advantages of glucuronide prodrugs with the tumor-targeting ability of some bacteria may be beneficial for clinical therapy of tumors.

Extracellular activation of glucuronide prodrugs by βG has been shown to increase therapeutic efficacy for cancer. Glucuronide derivatives do not permeate cells easily due to the presence of a charged carboxyl group. Accordingly, interaction of glucuronide prodrugs with lysosomal βG is minimal.[46] We previously showed that glucuronide prodrugs must be enyzmatically activated outside of tumor cells to achieve maximum cytotoxicity.[47] We also demonstrated that anchoring βG on the surface of tumor cells promoted effective activation of glucuronide prodrugs for enhanced cytotoxicity, which could overcome low transgenic efficiency *in vivo*.[16] In the present study, we expressed βG on the bacterial surface (mβG/AIDA-BL21) to enhance the conversion of a hydrophilic glucuronide prodrug. We observed that mβG/AIDA-BL21 cells could more rapidly and efficiently convert prodrug into active drugs as compared with pβG. Therefore, mβG/AIDA-BL21 cells may help overcome inadequate bacterial colonization in tumors and enhance the therapeutic efficacy of BDEPT.

Lpp-ompA-mediated surface expression has been demonstrated to allow expression of enzymes,[25,48] single-chain antibodies[49] or antigenic pepitdes[50] on the bacterial surface. AIDA-I is a component of the bacterial β-autotransporter system, which has been used to express a variety of proteins on bacterial surfaces, including antigenic proteins,[51,52] enzymes[17] and dimeric adrenodoxin.[18] In our study, we constructed two kinds of mβG (mβG/AIDA and mβG/lpp) and examined their surface expression and function. Our results showed that both mβG/AIDA and mβG/lpp were expressed on the bacterial surface, but only mβG/AIDA-BL21 cells displayed βG activity. We speculate that the eight transmembrane domains of lpp-ompA may have prevented proper formation of βG tetramers, which is required for enzyme activity.[53] On the other hand, Jose *et al.*[18] demonstrated that the dimeric bovine adrenodoxin protein was readily expressed on the bacterial surface using AIDA-I, similar to our results with βG. Therefore, AIDA-I mediates more efficient expression of functional βG on bacterial surfaces than lpp-ompA.

In summary, we demonstrated that AIDA-I mediated efficient expression of functional βG on bacterial surfaces (mβG/AIDA-BL21). mβG/AIDA-BL21 cells more rapidly and efficiently converted the glucuronide substrate PNPG to p-nitrophenol than pβG (pβG/lpp-BL21), and it enhanced the cytoxicity of the glucuronide prodrug HAMG to active drug pHAM. These data suggest that surface expression of βG on bacteria can enhance prodrug conversion efficiency and help compensate inadequate colonization of bacteria to maximize the therapeutic efficacy of BDEPT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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