Tumor-targeting prodrug-activating bacteria for cancer therapy

C-M Cheng1,8, Y-L Lu2,8, K-H Chuang1, W-C Hung3,4, J Shiea4,5, Y-C Su6, C-H Kao1, B-M Chen7, S Roffler7 and T-L Cheng4,6

1Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; 2Chia Nan University of Pharmacy and Science, Tainan, Taiwan; 3Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan; 4National Sun Yat-Sen, University-Kaohsiung medical University Joint Research Center, Kaohsiung, Taiwan; 5Department of Chemistry, National Sun Yat-Sen University, Kaohsiung, Taiwan; 6Faculty of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan and 7Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan

Increasing the specificity of chemotherapy may improve the efficacy of cancer treatment. Toward this aim, we developed a strain of bacteria to express enzymes for selective prodrug activation and non-invasive imaging in tumors. β-glucuronidase and the luxCDABE gene cluster were expressed in the DH5α strain of Escherichia coli to generate DH5α-lux/βG. These bacteria emitted light for imaging and hydrolyzed the glucuronide prodrug 9ACG to the topoisomerase I inhibitor 9-aminocamptothecin (9AC). By optical imaging, colony-forming units (CFUs) and staining for βG activity, we found that DH5α-lux/βG preferentially localized and replicated within CL1-5 human lung tumors in mice. The intensity of luminescence, CFU and βG activity increased with time, indicating bacterial replication occurred in tumors. In comparison with DH5α-lux/βG, 9AC or 9ACG treatment, combined systemic administration of DH5α-lux/βG followed by 9ACG prodrug treatment significantly (P<0.005) delayed the growth of CL1-5 tumors. Our results demonstrate that prodrug-activating bacteria may be useful for selective cancer chemotherapy.


Keywords: non-invasive imaging; β-glucuronidase; glucuronide prodrug; optical imaging; luminescence; prodrug-activating bacteria

Introduction

Methods that increase the therapeutic index of chemotherapy may potentially improve treatment efficacy and reduce side effects associated with systemic drug delivery. Selective activation of prodrugs at cancer cells is an attractive method to increase treatment specificity.1 Tumor selective activation can be achieved by designing prodrugs that are activated by endogenous enzymes that are naturally enriched by cancer cells.2 Differences between enzyme expression in tumor and normal tissues, however, may be minor. Prodrug-activating enzymes can also be targeted to cancer cells as immunoenzymes3,4 or by delivery of genes that encode for specific enzymes,2 but effective delivery of these agents to tumors is inefficient. An improved approach to allow selective targeting and high expression of prodrug-activating enzymes at cancer cells is required to realize the potential of targeted prodrug therapy of cancer.

Recently, several genera of bacteria have been shown to specifically accumulate and replicate within tumors, including Clostridium,6,7 Salmonella,8,9 Bifidobacterium10 and E. coli.11 These bacteria can cause cancer cell death by competing for nutrients and/or by secreting toxic bacterial products. Systemically administered bacteria selectively replicated at tumor region may be dependent on various tumor-related factors. For example, tumor may release the regulatory cytokines (IL-10, IL-4, TGF-β),12,13 downregulation of Fas or upregulation of FasL expression to escape immune clearance14,15 reducing the expression of vascular cell adhesion molecule-1 on endothelial cell to prevent immune effector cells to enter tumor tissue16 and hypoxia-induced adenosine accumulation suppresses the function of activated immune cells17 to provide an immunosuppressive or hypoxic environment for bacteria to replicate within tumors. Based on these findings, we wished to test whether bacterial delivery of the prodrug-activating enzyme βG could be achieved to
allow selective intratumoral activation of a glucuronide prodrug 9ACG for cancer therapy (Figure 1). Targeted βG immunoenzymes and genes have been shown to selectively activate glucuronide prodrugs, allowing accumulation of high drug concentrations at the tumor site, and produce bystander killing of antigen-negative tumor cells and generate long-lasting protective immunity to subsequent tumor challenge. In addition, glucuronide derivatives of many anti-neoplastic agents have been synthesized and can be selected for a particular tumor type. Polar glucuronide prodrugs do not easily enter cells due to their charged carboxyl group. However, for E. coli bacteria, glucuronide compounds are easily transported into βG-expressing E. coli by a glucuronide transport system for conversion. Targeted βG-expressing E. coli may therefore be an attractive strategy for specific conversion of glucuronide prodrugs for cancer therapy.

In the present study, we generated E. coli DH5α-lux/βG that express βG and the luxCDABE gene cluster for prodrug activation and luminescence emission, respectively. We showed that the DH5α-lux/βG can hydrolyze glucuronide substrates and emit luminescence. They also allowed specific conversion of the glucuronide prodrug 9ACG to the topoisomerase I poison 9-aminocamptothecin (9AC). Through optical imaging and colony-forming unit we showed that the bacteria localize and replicate in human tumor xenografts and produce substantial antitumor activity in combination with systemic 9ACG prodrug therapy.

Materials and methods

Bacteria, cells and animals

E. coli DH5α (F’ φ80 lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK− mK−) phoA supE44 thi-1 gyrA96 relA1 tonA, Gibco Laboratories, Grand Island, NY, USA) were used in this study. CL1-5 cells, a highly invasive subline selected from CL1-0 human lung adenocarcinoma cells, were kindly provided by Dr PC Yang, Department of Internal Medicine, National Taiwan University Hospital (Taipei, Taiwan), and were grown in Dulbecco’s Minimal Essential Medium (Sigma, St Louis, MO, USA) supplemented with 5% heat-inactivated bovine serum, 100 units ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Gibco Laboratories, Grand Island, NY, USA) at 37°C in a 5% CO₂ humidified atmosphere. Six to eight-week-old nude mice were purchased from the National Laboratory Animal Center, Taipei, Taiwan. Animal experiments were performed in accordance with institute guidelines.

Plasmid construction

pGHΛ23 was used as template for polymerase chain reaction amplification of the fen promoter with primers 5’-ATCCGCCACCGGTGATGAAATTAATATG-3’ and 5’-GCTCTAGAGTCGACCCCTCAAATCTTAA TT-3’ to introduce EaeI and XbaI restriction sites. The fen promoter is derived from the fengycin synthetase operon and drives the expression of the antifungal antibiotics, fengycin, from Bacillus subtilis F29-3. The PCR fragment was digested with EaeI and XbaI, and cloned into pRSETB (Invitrogen, The Netherlands) in place of the T7 promoter to form pRSETB-fen. The gene coding E. coli βG (pRSETB-βG) was digested with PstI and HindIII and subcloned into pRSETB-fen to create pRSETB-fen-βG. The XLEPT7.3 plasmid containing the luxCDABE gene cluster was a generous gift from Dr EA Meighen (Department of Biochemistry, McGill University, Montreal, Quebec, Canada). The luxCDABE gene cluster was excised with EcoRI and subcloned into pRSETB-fen to create pRSETB-fen-lux. Finally, the pRSETB-fen-βG plasmid was used as template for PCR amplification of fen-fG with 5’-ATCCACGTAGTGGCC GCGGTGTTGCCC-3’ and 5’-CTGCGACACTACGTGC TAGTTATGGCTCAG-3’ primers. The PCR fragment was digested with DraIII and subcloned into pRSETB-fen-lux to form pRSETB-lux/βG. The pRSETB-lux/βG plasmid was used to transform E. coli DH5α to produce light-emitting DH5α-lux/βG strains.

Analysis of luciferase and βG activity

E. coli DH5α was transformed with pRSETB-fen, pRSETB-βG, pRSETB-lux or pRSETB-lux/βG. The 50 μl of transformed DH5α (OD₆₀₀ = 0.5) were transferred into a 96-well white Plate (Nalge Nunc International, Naperville, IL) and the continuous bioluminescence emission was measured on a Top Count Luminescence Counter (Perkin-Elmer Life Sciences, Boston, USA), and the units were counts per second (CPS) per well. The βG activity of transformed DH5α strains was measured by monitoring the release of p-nitrophenol (PNP) from p-nitrophenyl-β-D-glucopyranoside (PNPG) conversion. Transformed DH5α strains were washed twice with PBS and 50 μl of transformed DH5α (OD₆₀₀ = 0.5) was immediately mixed with 50 μl 3.2 mM PNPG in PBS containing 0.05% BSA, pH 7.4 at

Figure 1 Bacteria-targeted activation of glucuronide prodrugs. Selective replication in tumors of DH5α-Lux/βG, a light-emitting and βG-expressing E. coli, may allow accumulation of high levels of βG to activate a glucuronide prodrug (9ACG) to an anti-neoplastic drug (9AC). Diffusion of 9AC within the tumor may maximize therapeutic efficacy.
37°C for 30 min. The absorbance of wells was then measured in microtiter plate reader at 405 nm.

Toxicity of 9AC and pHAM to DH5α-lux/βG strains
DH5α-lux/βG strains were cultured up to OD600 = 0.35, then the DH5α-lux/βG were subdivided into triplicate and incubated with graded amounts of 9AC, a topoisomerase I inhibitor, or p-hydroxyaniline mustard (pHAM),25 an alkylating agent, at 37°C for 3 and 6 h. The growth inhibition of DH5α-lux/βG was measured by the spectrophotometer at OD600 nm.

Activation of 9-ACG by DH5α-lux/βG in vitro
CL1-5 cells (5 × 10^5 cells per well) grown overnight in 96-well microtiter plates were treated with graded concentrations of 9AC, 9ACG, βG (2 µg well⁻¹) + 9ACG or DH5α-lux/βG (2 × 10^5 bacteria per well) + 9ACG in medium that contained 25 µgml⁻¹ gentamycin and then incubated at 37°C for 24 h. The cells were washed three times with PBS and then incubated an additional 24 h in fresh medium that contained 25 µgml⁻¹ gentamycin to kill residual bacteria. Cell viability was determined by the ATPLite luminescence ATP detection assay system (Perkin-Elmer Life and Analytical Science, Boston, MA). Results are expressed as percent inhibition of luminescence as compared to untreated cells by the following formula:

\[
\text{% inhibition} = 100 \times \frac{\text{sample luminescence} - \text{background luminescence}}{\text{background luminescence}}
\]

In vivo bioluminescence imaging
Group of three BALB/c nude mice bearing established CL1-5 tumor (200–300 mm³) in their right flank was intravenously (i.v.) (tail vein) injected with 4 × 10⁷ DH5α-lux/βG bacteria. Mice were anesthetized with isoflurane using the XGI-8 gas anesthesia system (Xenogen, Alameda, USA) and whole-body images were acquired at the indicated times (24, 48, and 72 h) by a cooled Charge Coupled Device camera (Xenogen IVIS 50, Xenogen, Alameda, USA). After 72 h, different tissues, CL1-5 tumor, liver, kidney, stomach, colon, intestine, were removed and imaged by Charge Coupled Device camera.

Histological analysis of DH5α-lux/βG bacteria in tumors
CL1-5 tumors (200–300 mm³) were excised at 0, 24, 48, 72 h after i.v. tail vein injection of 4 × 10⁷ DH5α-lux/βG bacteria, embedded in Tissue-Tek OCT compound (Sakura, USA) in liquid nitrogen, and cut into 20 µm sections. Tumor sections were stained for βG activity with the GUS β-Glucuronidase Reporter Gene Staining kit (Sigma). Briefly, the sections were placed in fixation solution (10 mM MES, pH 5.6, with 300 mM mannitol and 0.3% formaldehyde) at room temperature for 45 min, washed three times with wash solution (10 mM sodium phosphate, pH 7.0, with 0.2 mM EDTA), and stained with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-GlC₆A) at 37°C for 3 h. The sections were then stained with nuclear fast red (Sigma) as a counter stain. All sections were examined on an upright microscope (Olympus BX41, Japan).

Analysis of DH5α-lux/βG in tumor and normal tissues
DH5α-lux/βG was administered by i.v. tail vein injection into BALB/c nu/nu mice (n = 3) bearing established CL1-5 tumor (200–300 mm³). At 24, 48, 72 h after injection, the tumors and organs were removed, weighed and homogenized in sterile LB medium. The homogenate was then plated onto LB plate that contains 100 µg ml⁻¹ ampicillin and incubated for 24 h at 37°C. The colony-forming units per mg of tissues were determined by counting colonies and dividing them by the weight of the tumors and specific organs.

In vivo antitumor activity
Groups of 4–5 Balb/c nude mice bearing 100–300 mm³ CL1-5 tumors were i.v. (tail vein) injected with 4 × 10⁷ DH5α-lux/βG on day 12 followed by i.v. tail vein injections of 50 mg kg⁻¹ 9ACG on days 14 and 15. Control groups of tumor-bearing mice were i.v. (tail vein) injected with PBS, 50 mg kg⁻¹ 9ACG, 4 × 10⁷ DH5α-lux/βG or subcutaneously injected with 5 mg kg⁻¹ 9AC as a suspension in lipiodol. Tumor volumes (length × width × height × 0.5) and weight were estimated two times a week.

Statistical analysis
Statistical significance of differences between mean values was estimated with Excel (Microsoft, Redmond, WA, USA) using the independent t-test for unequal variances. P-values of <0.05 were considered statistically significant.

Results
Characterization of luminescence and βG-expressing E. coli (DH5α).
The plasmid pRSETB-lux/βG, which contains the luxCDABE-based bioluminescent reporter and E. coli βG genes are under the control of two fen promoters (Figure 2a), was transformed into DH5α bacteria to generate the DH5α-lux/βG strain. The activities of the luxCDABE and βG genes in DH5α-lux/βG were analyzed by bioluminescent and βG microassays. DH5α-lux/βG bacteria displayed stable luminescence emission without addition of exogenous substrate; the bioluminescence intensity in both DH5α-lux/βG and DH5α-lux bacteria was about 100-fold higher than in control DH5α-vector or DH5α-βG bacteria (Figure 2b). DH5α-Lux/βG and DH5α-βG bacteria hydrolyzed the β-glucuronidase substrate PNPG, and converted it to PNP, showing that these strains expressed functional βG (Figure 2c).

The toxicity of 9AC and pHAM to bacteria
Active drug released by hydrolysis of glucuronide anticancer prodrug may also influence the viability of DH5α-lux/βG bacteria. Therefore, we investigated the toxicity of two glucuronide prodrug products, the mammalian
topoisomerase I inhibitor 9AC and the alkylating agent pHAM, on DH5α-lux/βG viability. We incubated DH5α-lux/βG with graded concentrations of 9AC or pHAM for 3 and 6 h and measured bacterial density at an absorbance of 600 nm. pHAM, but not 9AC, inhibited the growth of DH5α-lux/βG in a time-dependent manner (Figure 3), indicating that 9AC does not inhibit prokaryotic topoisomerase. This suggests that 9ACG is a good prodrug candidate for DH5α-lux/βG targeted activation.

Activation of 9ACG by DH5α-lux/βG in vitro
To examine if DH5α-lux/βG hydrolysis of 9ACG could inhibit the growth of human cancer cells in vitro, CL1-5 human lung cancer cells were incubated with 9AC, 9ACG, 9ACG mixed with E. coli βG, or 9ACG mixed with DH5α-lux/βG bacteria. The cellular ATP synthesis rate after drug treatment was measured as an index of cell viability. The IC50 value of cells treated with 9ACG plus DH5α-lux/βG bacteria (138 nM) was near to the IC50 value observed when cells were treated with 9AC (52 nM).
or with 9ACG plus βG (96 nM) but not with 9ACG (6285 nM), showing that DH5z-lux/βG bacteria efficiently converted 9ACG to 9AC to inhibit the growth of CL1-5 tumor cells in vivo (Figure 4).

In vivo tumor localization and distribution of DH5z-lux/βG
To determine whether DH5z-lux/βG bacteria could specifically target to tumors in vivo, Balb/c nu/nu mice bearing established CL1-5 tumors (∼200 mm³) in the right flank were i.v. injected with DH5z-lux/βG. The mice were then imaged after 24, 48, and 72 h. Figure 5a shows that DH5z-lux/βG was selectively retained in CL1-5 tumors and the intensity of luminescence increasing with time, indicating that DH5z-lux/βG could replicate within tumors. In addition, to verify the luminescence results, tumor sections were stained with X-GlcA to examine for the presence of functional βG activity. Figure 5b shows that tumor sections displayed a time-dependent increase in blue X-GlcA staining, indicating βG activity also increased in tumors with time. These results demonstrate that functional DH5z-lux/βG could be selectively retained in tumors to allow prodrug activation. Although DH5z-lux/βG preferentially accumulates in CL1-5 tumors, it may also localize on normal tissues. Therefore, the luminescence of isolated tissues of tumor-bearing mice at 72 h after DH5z-lux/βG injection was imaged with the IVIS® Imaging System 50. Figure 5c shows that CL1-5 tumor display obvious luminescence as compared with other tissues (liver, kidney, stomach, colon, intestine, CL1-5 tumor) and only little luminescence remained in colon. In addition, the tissues distribution pattern of DH5z-lux/βG bearing mice was also measured at different times by counting the colony-forming unit per mg tissues. Figure 5d also shows that DH5z-lux/βG accumulates preferentially in tumors and the tumor-to-normal tissues ratios were about 12–62:1, 47–450:1 and 33–1054:1 at 24, 48 and 72 h, respectively. Lastly, CL1-5 tumors but not colons or other organs, remained luminescent up to 120 h after DH5z-lux/βG inoculation with or without prodrug therapy (data not shown). In conclusion, these results suggested that DH5z-lux/βG could selectively replicate in CL1-5 tumor in vivo.

Targeted activation of 9ACG prodrug in vivo
The antitumor activity of DH5z-lux/βG in combination with 9ACG prodrug was examined in Balb/c nu/nu mice bearing established 100–300 mm³ CL1-5 tumors. The combined prodrug therapy consisted of an i.v. tail vein injection of 4 × 10⁷ c.f.u. DH5z-lux/βG on day 12 and fractionated i.v. tail vein injection administration of 50 mg kg⁻¹ 9ACG on days 14 and 15. Control groups of tumor-bearing mice were also treated with PBS, DH5z-lux/βG alone, 9ACG alone or subcutaneously injected with 9AC. Combined treatment with 9ACG and DH5z-lux/βG significantly (P < 0.005) delayed tumor growth as compared to each individual treatment with DH5z-lux/βG alone, 9ACG alone, 9AC or PBS (Figure 6a). The toxicity of combined treatment was minimal with a maximum weight loss of 7% and no significant (P > 0.05) weight loss was observed compared to each individual treatment with DH5z-lux/βG alone, 9ACG alone (Figure 6b). In contrast, 9AC treatment caused a maximum weight loss of 15%. These results demonstrate that bacteria-targeted activation of glucuronide prodrugs may be useful for selective cancer chemotherapy.

Discussion
We developed a tumor-targeted luminescence-emitting and βG-expressing bacteria (DH5z-lux/βG) as a prodrug-activating factory to convert glucuronide prodrugs to antineoplastic agents for selective chemotherapy. In the presence of DH5z-lux/βG, the glucuronide prodrug 9ACG was effectively hydrolyzed to release 9AC with similar toxicity as the parent drug 9AC. The intensity of luminescence and βG activity in CL1-5 tumors increased with time, indicating the light-emitting βG-expressing DH5z-lux/βG preferentially replicated in CL1-5 tumor in vivo. In comparison with control bacteria and prodrug treatment, combination treatment of DH5z-lux/βG and 9ACG significantly delayed the growth of tumors, showing that tumor targeted prodrug-activating bacteria may be useful for selective cancer chemotherapy.

Bacteria that selectively replicate in tumors have shown promising preliminary results. Several genetically-engineered attenuated microorganisms, including Listeria, Clostridium, Salmonella, Bifidobacterium, and E. coli, have demonstrated potential as tumor targeting vectors both for their tumoricidal activity and for their ability to deliver therapeutic genes to inaccessible regions of tumors.6–10 For example, Yu et al.11 demonstrated the real-time visualization of the location, survival and replication of light-emitting bacteria in implanted tumors and their metastases in live animals. Our results also show that DH5z-lux/βG preferentially localized and replicated in CL1-5 tumor in vivo. The mechanisms that result bacteria to accumulate at tumor regions are likely to be various tumor-related factors. Solid tumor contains hypoxic region,16,17 which is well adapted by facultative anaerobes such as E. coli but decreases the bactericidal activity of macrophages and neutrophils. In addition, the tumor may provide an immunosuppressive environment that contributes to the ability of E. coli to multiply within tumors. For example, Griffioen et al. indicated that tumors downregulate vascular cell adhesion molecule-1 expression on endothelial cells, which modulate immune cells-vessel wall interaction and decrease the effector's cells to enter tumor tissue.18 Sitkovsky et al.19 showed that hypoxia-induced adenosine and adenosine receptor-triggered signaling can block innate and adaptive immune responses to prevent tissues damage from inflammatory damage and also protect cancerous tissues by inhibiting incoming antitumor T lymphocytes.28 Le et al. showed that a novel hypoxia-induced galectin-1 protein modulates tumor immune privilege in vivo.29 These results suggest that tumor microenvironment may provide a sanctuary for a small number of bacteria that will escape
In vivo tumor localization and biodistribution of the DH5α-lux/IIG. (a) DH5α-lux/IIG was i.v. injected into nude mice bearing CL1-5 tumor and images were acquired at the indicated times (0, 24, 48, and 72 h) on a cooled Charge Coupled Device camera (IVIS 50, Xenogen). The pseudocolour scale shows relative photon flux on each image. (b) After DH5α-lux/IIG injection, sections of CL1-5 tumors at the indicated times were stained with X-GlCNA and nuclear fast red (NFR) and then viewed under phase contrast microscopy. (c) The luminescence biodistribution of different organs was measured at 72 h after i.v. injection of $4 \times 10^7$ DH5α-lux/IIG. The luminescence of different organs were imaged with the IVIS® Imaging System 50 (Xenogen, Alameda, CA). (d) Results of administration of $4 \times 10^7$ DH5α-lux/IIG by i.v. injection to CL1-5-bearing mice ($n = 3$). At 24, 48, 72 h after injection, tumors and different organs were homogenized, and the number of colony-forming units per gram of tissues was determined.
the immune clearance. Therefore, bacteria may be a good candidate for tumor-targeting vector to carry therapeutic genes for the cancer treatment.

βG is an attractive enzyme for specific conversion of glucuronide prodrugs for cancer therapy. A wide variety of glucuronide prodrugs are available, including prodrugs of effective antineoplastic agents such as doxorubicin,30 etoposide,31 paclitaxel,32 docetaxel33 and alkylating agents.34 Glucuronide derivatives of most antineoplastic agents can be synthesized by employing linkers or prodrug chemotherapy alone. These data provided a new rationale for expressing of prodrug-activating enzymes from conditionally replicating bacteria to allow development of tumor-specific gene therapy protocols.

In summary, we demonstrated that the light emitting and βG-expressing DH5α-lux/βG could specifically convert 9ACG to 9AC in vitro and replicate and accumulate in tumors in vivo. Combination treatment with DH5α-lux/βG and 9ACG significantly delayed tumor growth with minimal toxicity in comparison to therapy with bacteria or prodrug chemotherapy alone. These data provided a new rationale for expressing of prodrug-activating enzymes from conditionally replicating bacteria to allow development of tumor-specific gene therapy protocols.

Acknowledgements

This work was supported by the National Research Program for Genomic Medicine (NRPGM), National Science Council, Taipei, Taiwan (NSC95-3112-B-037-001) and the National Health Research Institutes (NHRI-EX96-9420BI, NHRI-EX96-9624SI). The National Sun Yat-Sen University-Kaohsiung Medical University joint research center is also gratefully acknowledged.

References

3 Wallace PM, MacMaster JF, Smith VF, Kerr DE, Senter PD, Cosand WL. Intratumoral generation of 5-fluorouracil mediated by an antibody-cytosine deaminase


6. Agrawal(706,323),(992,328)


40 Wei MQ, Ellem KA, Dunn P, West MJ, Bai CX, Vogelstein B. Facultative or obligate anaerobic bacteria have the potential for multimodality therapy of solid tumours. *Eur J Cancer* 2007; **43**: 490–496.
