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Use of a genetically engineered *E. coli* overexpressing β-glucuronidase accompanied by glycyrrhizic acid, a natural and anti-inflammatory agent, for directed treatment of colon carcinoma in a mouse model

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ABSTRACT

Bacteria-directed enzyme prodrug therapy (BDEPT), is an emerging alternative directed and tumor-specific approach. The basis of this method is the conversion of a non-toxic prodrug by a bacterial enzyme to a toxic drug within the tumor-microenvironment (TME). In the present study, the therapeutic efficacy of BDEPT was investigated based on the ability of *Escherichia coli* DH5α-lux/βG in activation of glycyrrhizic acid (GL), a natural and non-toxic compound purified from licorice, to glycyrrhetinic acid (GA) only in TME. To do so, the antibacterial effects of GL on bacteria and the cytotoxic effects of the produced GA on survival rate of CT26 mouse colon carcinoma cells were evaluated. The IC_{50} values of the produced GA and cisplatin were determined as 210 μM and 100 μM, respectively. Comparing these values to GL treatment (1305 μM) indicates that bacteria could have efficiently activated GL to GA to inhibit the growth of tumor cells. Afterward, the anti-cancer effects of bacteria used in combination with GL was investigated in a mouse model of colon carcinoma. Results were indicative of targeted homing and even proliferation of luminescent bacteria in TME. Moreover, combined treatment greatly inhibited tumor growth. Histopathological analysis of dissected tissues also demonstrated increased apoptosis rate in tumor cells after combined treatment and interestingly, showed no obvious damage to the spleen and liver of treated mice. Accordingly, this BDEPT approach could be considered as an effective alternative tumor-specific therapy utilizing prodrug-activating enzymes expressing from tumor-targeting bacteria to allow the development of new tumor-specific pharmacotherapy protocols.

1. Introduction

Cancer is the rapid and invasive growth of abnormal cells which have lost the control of cell proliferation and can migrate to other tissues and establish secondary tumors. In most cases, cancer initiates and develops through a multi-stage process in which a precancerous lesion becomes a malignant tumor mass. The initiation and also progression rate of this process are related to some genetic aberrations and environmental factors such as physical carcinogens including ultraviolet and ionizing radiations, chemical agents like asbestos, tobacco, aflatoxins (food contaminants) and arsenic (drinking water pollutant) as well as biological agents such as certain types of viral, bacterial and parasitic infections (WHO, 2019). Despite many advancements in diagnosis and treatment, cancer has remained as one of the deadliest diseases in the world and its incidence is steadily increasing. According to the report of the International Agency for Research on Cancer, the most common cancers in the world in 2018 were lung, breast, and colorectal cancers. Overall, colorectal cancer (CRC) which ranks third

Abbreviations: BDEPT, Bacteria-directed enzyme prodrug therapy; TME, tumor-microenvironment; GL, glycyrrhizic acid; GA, glycyrrhetinic acid; CRC, colorectal cancer; βG, β-glucuronidase; RPMI, Roswell Park Memorial Institute; FBS, Fetal Bovine Serum; EDTA, ethylenediamine tetraacetic acid; RLU/sec, relative luminescent emission per second; MTT, 3- (4, 5- dimethylthiazol- 2- yl) - 2, 5- diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; LB, Lysogeny broth; H&E, hematoxylin and eosin; MIC, minimum inhibitory concentration; IC, inhibitory concentration; 11β-HSD2, 11 β-hydroxysteroid dehydrogenase type 2; PGE2, prostaglandin E2; COX-2, cyclooxygenase 2

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in terms of incidence but second in terms of mortality (Bray et al., 2018), originates mainly with lesions called polyps which are developed in the internal wall of colon or rectum (National Cancer Institute, 2019). Finding polyps by methods such as colonoscopy and their complete removal by laparoscopic surgery have significantly reduced the risk of death from CRC, in recent years (NCCN, 2019). However, standard approaches including surgery, radiation therapy, and chemotherapy are ineffective in advanced tumor stages, and therefore, developing new, targeted and alternative treatments which have desired therapeutic effects with the least adverse side effects is important in CRC therapy.

It has been demonstrated that some microorganisms including *Clostridium, Salmonella, Bifidobacterium, Listeria* and *E. coli* (Cheng et al., 2008; Hsieh et al., 2015; Huang et al., 2007; King et al., 2009; Quispe-Tintaya et al., 2013), have the ability to accumulate and even proliferate within the tumor-microenvironment (TME). This ability offers a tremendous advantage to targeted cancer treatment (Patyar et al., 2010). Genetically engineered bacteria can specifically target tumors and induce controllable cell death of cancer cells (Chen et al., 2013). This specific accumulation of systemically administered bacteria in TME depends on some tumor-related factors. Hypoxia condition in the TME is well suited to facultative anaerobic bacteria such as *E. coli* while reducing the anti-bacterial activity of macrophages and neutrophils (Cheng et al., 2008; Nemunaitis et al., 2003). On the other hand, the immunosuppressive microenvironment within the tumor is advantageous for growth and proliferation of tumor-tropic bacteria. This microenvironment is produced by secretion of some immunomodulatory cytokines such as IL-4, IL-10 and TGF-β, downregulation of Fas receptor or upregulation of FasL, reducing the expression of VCAM-1 on endothelial cells, as well as the accumulation of hypoxia-induced adenosine induced by tumor cells (Cheng et al., 2013, 2008; Hsieh et al., 2015; Zu and Wang, 2014). Among various approaches used for bacterial cancer therapy, the most reliable method is the Bacteria-directed enzyme prodrug therapy (BDEPT), which even succeeded to undergo some clinical trials (ClinicalTrials.gov identifiers NCT01118819 and NCT00006254). The purpose of this therapeutic approach is to overcome the adverse side effects of bacterial therapies (Hsieh et al., 2015; Lehouritis et al., 2013). It is based on harnessing a bacterial vector to deliver a specific enzyme-encoding gene able to convert a non-toxic prodrug into a toxic drug in the margin or inside of cancer cells (Stritzker et al., 2008; Friedlos et al., 2008). BDEPT is a two-step treatment: in the first step, the genetically engineered bacteria, which act here as the delivery system (vector), are administrated to the patient and specifically target the TME, where they are colonized and the gene of the prodrug converting enzyme would be expressed. In the second step, when the enzyme reaches an optimum level, the prodrug is administrated which would then be converted to the active drug specifically within the TME. This approach, results in selective toxicity of the therapeutic system, only for cancer cells.

The bacterium selected for this study was *E. coli* carrying pRSETB-lux/βG plasmid which contains the β-glucuronidase (βG) enzyme encoding gene as the prodrug converting enzyme and also a cluster of bacterial bioluminescent genes (*luxCDABE* gene cluster) for constitutive bioluminescent emission (Cheng et al., 2008). *E. coli* is a component of the natural flora in the gastrointestinal tract and can be controlled by antibiotics if it became a pathogen. The level of βG enzyme in human's serum is very low, and therefore glucuronide prodrugs administrated systemically cannot be activated by host enzymes (Renoux et al., 2017; Tranoy-Opalinski et al., 2014; Yang et al., 2011). Glycyrrhizic acid (GL, PubChem CID: 14982), is a glucuronide compound purified from *Glycyrrhiza glabra* roots. This natural compound contains two glucuronic acid groups covalently linked to the main structure of pentacyclic triterpenoid (Fig. 1). In the presence of βG, the two glucuronide units are removed from GL and it would be converted to its metabolically active form, glycyrrhetinic acid (GA). The therapeutic effects of GA are about 200 times more powerful than GL (Lee et al., 2008; Stewart and Prescott, 2009; Zhang et al., 2009). GL has a wide range of pharmacological effects, including anti-inflammatory, anti-cancer, anti-hypercholesterolemia, anesthetic, anti-ulcer, anti-diabetic, anti-allergic, anti-viral and anti-*Helicobacter pylori* effects. GL has also been known as expectorant, hepatoprotective against ulcers, useful in treatment of viral hepatitis and myocarditis, Alzheimer's, rheumatoid arthritis, wound healing, increasing blood pressure and modulating the immune system (Li et al., 2011; Utsunomiya et al., 1997; Zhang et al., 2009). Both the licorice extract and GL are approved as food additives by the Food and Drug Administration (FDA) of the United States, the European Union and also by the World Health Organization (WHO) (Lee et al., 2008).

In the present study, the suitability of GL, as a natural and non-toxic prodrug in combination with *E. coli* DH5α-lux/βG using BDEPT approach was evaluated in a mouse model of colon carcinoma.

2. Material and methods

2.1. Bacteria and plasmid

E. coli DH5α and pRSETB-lux/βG plasmid were kindly provided by Dr. Tian-Lu Cheng (Kaohsiung Medical University, Taiwan). This construct, contains the gene encoding beta-glucuronidase (βG), luxCDABE-based bioluminescent reporter system, and the ampicillin resistant gene as a selectable marker (Cheng et al., 2008). *E. coli* DH5α competent cells were transformed with pRSETB-lux/βG and plasmid extraction was carried out by miniprep method (Hanahan and Harbor, 1983).

2.2. Cell line and culture conditions

CT26 mouse colon carcinoma cells were purchased from Pasteur Institute (Tehran, Iran) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, Germany) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Thermo Fisher Scientific, Germany) at 37 °C and 5% CO_2 in 97% atmosphere humidity (Memmert, Germany). When required cells were passaged with 0.25% trypsin and 1 mM EDTA (Thermo Fisher Scientific, Germany).

2.3. Prodrug preparation

Glycyrrhizic acid (glycyrrhizin, GL) white powder purified from licorice roots (Golexir Pars, Mashhad, Iran) was dissolved in sterile deionized water and different concentrations of GL were prepared as required.

2.4. Animals

6–8-week-old inbred Balb/c mice (body weights ranging from 20 to 25 g) were purchased from Royan Institute (Tehran, Iran). Mice were kept and bred with free and unrestricted access to water and food in an animal house at Ferdowsi University of Mashhad. Animal experiments were performed in accordance with university guidelines. Protocols were approved by the animal care and ethics committee.

2.5. Bioluminescent emission analysis

The intensity of bioluminescent emissions for *E. coli* DH5α and *E. coli* DH5α-lux/βG (OD_{600} = 0.5) were measured in an FB12 Tube Luminometer (Titertek-Berthold, Germany), and the units were expressed as relative luminescent emission per second (RLU/sec).

2.6. Anti-bacterial activity

To investigate if βG overexpression and conversion of GL to GA has any anti-bacterial effects, both *E. coli* DH5α and *E. coli* DH5α-lux/βG were treated by graded concentrations (29, 58, 117, 234, 468, 937,

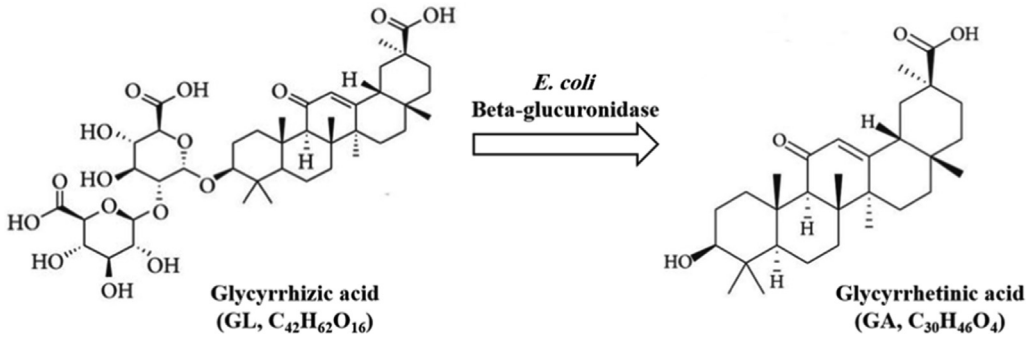

Fig. 1. The chemical structures of glycyrrhizic acid (GL) and glycyrrhetinic acid (GA) and conversion of GL to GA by the gut bacteria after consumption of GL containing foods or additives.

1875, 3750 and 7500 μM) of GL. Broth microdilution assay (Wiegand et al., 2008) was used to determine the MIC_{50} values of GL. At first, both bacteria were cultured to reach OD_{600} = 0.5 and the luminescent emission was measured by the luminometer. In the next step, bacteria were subdivided into triplicate and 5×10^5 cfu ml^{-1} were transferred to each well of 96 well plates containing graded concentrations of GL and incubated overnight at 37 °C and 120 rpm. Finally, the absorbance of each well was determined using an ELISA reader (Awareness Technology, USA) at 630 nm wavelength.

2.7. In vitro prodrug activation

One of the most common methods for measuring cell survival is the MTT assay. The basis of this method is on the ability of mitochondrial enzymes of living cells to convert yellow tetrazolium salt (3- (4, 5- dimethylthiazol- 2- yl) - 2, 5- diphenyl tetrazolium bromide) or MTT to purple formazan crystals (Riss et al., 2016; van Meerloo et al., 2011). CT26 cells (1×10^4 cells $well^{-1}$) grown overnight in 96-well plates were treated by graded concentrations of GL (23.43, 46.87, 93.75, 187.5, 375, 750, 1500 and 3000 μM), *E. coli* DH5α-lux/βG (2×10^7 cfu $well^{-1}$), GL accompanied by *E. coli* DH5α-lux/βG, GL accompanied by *E. coli* DH5α and graded concentrations of cisplatin (10.41, 20.83, 41.66, 83.32, 166.65 and 333.3 μM) in media containing 25 μg ml^{-1} gentamycin, and subsequently incubated at 37 °C for 24 h. Cells were then washed three times with phosphate-buffered saline (PBS, Sigma, Germany) and incubated for an additional 24 h in fresh media containing 25 μg ml^{-1} gentamycin to kill residual bacteria (Chen et al., 2013; Cheng et al., 2013, 2008; Hsieh et al., 2015; Stritzker et al., 2008). 20 μl MTT solution (5 mg ml^{-1}, Sigma, Germany) was added to wells and incubated at 37 °C for 4 h. The contents of wells were then replaced with 150 μl dimethyl sulfoxide (DMSO, Merck, Germany) and the absorbance of each well was measured at 545 nm.

2.8. E. coli DH5α-lux/βG homing in tumors and normal organs

4 days after bacteria infusion and also on day 22 after start point of treatments, mice were sacrificed and tumor tissues (4 mice at each time point) and some of the normal organs (3 mice at each time point), including liver, spleen, kidneys, stomach, small intestine, and colon, were excised under aseptic conditions, weighed and homogenized in sterile LB medium (10% w/v).

2.8.1. Investigation of luminescent intensity

The luminescent emission rate of each homogenate suspension was measured by the luminometer with 5 replications.

2.8.2. Quantitation and comparison of E. coli DH5α-lux/βG accumulation in tumor and normal organs

Bacteria were counted by plating (in 10 μl volumes) serial dilutions of homogenates onto LB-agar culture medium containing 100 μg ml^{-1} ampicillin and incubated overnight at 37 °C. The colony forming units per mg of tissue were determined by counting bacterial colonies. A visibly detected single colony arising from a single plated bacteria was considered as a positive colony. The number of colonies was calculated according to the following formula (Wang et al., 2011):

$$\text{cfu/tissue} = \text{cfu}/0.01\ \text{ml} \times \text{dilution factor} \times \text{ml/tissue}$$

Finally, colonies were inoculated in LB and after overnight incubation at 37 °C, the bacterial luminescent emission was investigated.

2.9. In vivo anti-tumor therapy

CT26 cells (2×10^6 cells $mouse^{-1}$) were injected subcutaneously in the right flank of inbred male Balb/c mice. After 8–10 days, when tumor volumes reached 100–300 mm^3, mice were divided into six groups (n = 3–5) including: PBS, *E. coli* DH5α-lux/βG, GL (50 mg kg^{-1}), GL (100 mg kg^{-1}), *E. coli* DH5α-lux/βG and GL (50 mg kg^{-1}) and *E. coli* DH5α-lux/βG and GL (100 mg kg^{-1}). Bacteria were intravenously (tail vein) injected by a single dose of 4×10^7 cfu $mouse^{-1}$ at 4 days before starting treatments. PBS and GL (50 and 100 mg day^{-1}) were intraperitoneally administrated daily for 20 days. In order to investigate anti-cancer activity and side effects of the treatments, the tumor size and the weight of mice in different groups were measured using a digital caliper with a precision of 0.01 mm and a digital scale with a precision of 0.01 g, respectively at specified intervals (every 3 days, until day 22). The final volumes of tumor masses were calculated according to the following formula (Cheng et al., 2008; Hsieh et al., 2015):

$$\text{Tumor volume } (mm^3) = \text{Length (mm)} \times \text{width (mm)} \times \text{height (mm)} \times 0.5$$

2.10. Histopathological analysis of tumor tissues and normal organs

On day 22, animals were sacrificed and tumor, liver, and spleen were dissected and fixed in 10% paraformaldehyde. Fixed tissues were dehydrated in ethanol (Merck, Germany), embedded in paraffin (Merck, Germany), and sectioned using a microtome (Leitz, USA). 5 μm thickness sections were stained with hematoxylin and eosin (H&E) and investigated by light microscopy (Olympus, Japan).

2.11. Statistical analyses

All data were analyzed statistically by GraphPad Prism 7.00 using dose-response test and unpaired *t*-test. Values were expressed as mean ± SD or SEM and p values ≤ 0.05 were considered as statistically significant.

3. Results

3.1. Investigating GL toxicity on bacteria

The main question which needed to be considered prior to the main test was an evaluation of possible anti-bacterial effects of GL compound on *E. coli* DH5α-lux/βG. The broth microdilution assay was used to determine MIC_{50} values (minimum inhibitory concentration necessary to prevent the growth of 50% of the bacterial population). To this aim, after treatment of *E. coli* DH5α-lux/βG and *E. coli* DH5α with different concentrations of GL, the MIC_{50} values of this compound for bacteria were determined as 9500 and 90000 μM, respectively. Although use of GL in combination with lux/βG was significantly ($p \leq 0.0001$) more toxic on bacteria, our data indicate that it did not have any significant toxicity at concentrations less than 3000 μM on both bacteria (data not shown). Thus, 3000 μM was considered as the highest concentration of GL used for further *in vitro* experiments.

3.2. Activation of GL to GA by E. coli DH5α-lux/βG in vitro

To examine the effects of GL activation by *E. coli* DH5α-lux/βG on cell survival, CT26 cells were treated with different concentrations of GL, *E. coli* DH5α-lux/βG, GL accompanied by *E. coli* DH5α-lux/βG, GL accompanied by *E. coli* DH5α and also various concentrations of cisplatin. Results obtained from MTT assay showed a significant decrease in cell viability after treatment of CT26 cells with different concentrations of GL in combination with *E. coli* DH5α-lux/βG in comparison with control groups (Fig. 2). The IC_{50} value for GL accompanied by *E. coli* DH5α-lux/βG was determined as 210 μM which was comparable to IC_{50} value observed for cisplatin (100 μM). Comparing these data with the IC_{50} values obtained for GL in combination with *E. coli* DH5α (1181 μM) and GL (1305 μM) shows that *E. coli* DH5α-lux/βG could have efficiently converted GL to GA to inhibit the growth of CT26 tumor cells *in vitro* (Fig. 2). Treatment of cells with *E. coli* DH5α-lux/βG alone showed no obvious cytotoxic effects on CT26 cells (data not shown).

3.3. Homing of E. coli DH5α-lux/βG in tumors and normal organs

After intravenous injection of *E. coli* DH5α-lux/βG through the tail vein of CT26 tumor-bearing mice, bacterial colonization in different organs was measured at days 4 and 22. As shown in Fig. 3 luminescent bacteria were specifically accumulated in tumor tissues, however, when comparing their distribution in other organs, a much higher colonization was observed in colon, although it was significantly less than tumor tissue.

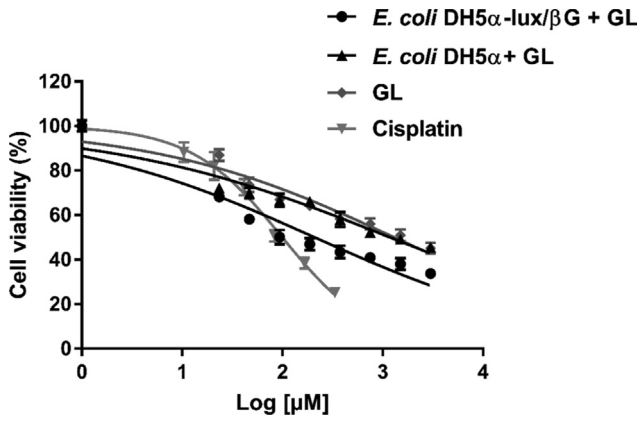

Fig. 2. *In vitro* activation of GL. Changes in survival rate of CT26 cells after treatment with different concentrations of GL, GL accompanied by *E. coli* DH5α-lux/βG, GL accompanied by *E. coli* DH5α and cisplatin. Data are mean ± SEM of triplicate experiments for each concentration.

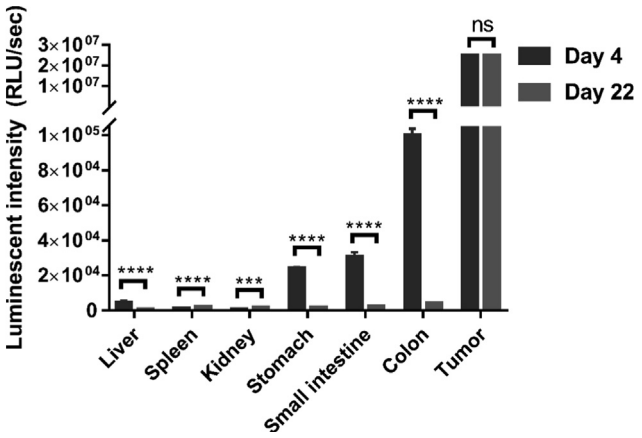

Fig. 3. The luminescent intensity of tumor and normal organs. Comparison of luminescent intensity due to accumulation of *E. coli* DH5α-lux/βG in tumor (4 mice at each time point) and normal organs (3 mice at each time point) of mice at days 4 and 22 after the start point of treatments. Data are mean ± SD of luminescent emission, and *** and **** indicate significant differences at $p \leq 0.001$ and $p \leq 0.0001$, respectively.

3.3.1. Investigation of bacterial colonization by colony counting method

After homogenate preparation of tumor tissue and normal organs, each was serially diluted and cultured as triplicate on LB-agar plates containing 100 μg ml^{-1} ampicillin. Fig. 4A shows colonies formed from serial dilutions of the tumor, spleen, liver, and colon homogenates. The number of colonies formed at different dilutions were in agreement with luminescent results. As shown in Fig. 4B, results indicated specific accumulation and even proliferation of *E. coli* DH5α-lux/βG in the tumor tissue relative to liver, spleen, and colon normal organs.

3.4. In vivo anti-tumor therapy

CT26 tumor-bearing mice were randomly divided into 6 groups. *E. coli* DH5α-lux/βG was injected intravenously at 4 days before starting treatments, followed by daily administration of two doses of 50 mg kg^{-1} and 100 mg kg^{-1} of GL for 20 days. During the 20 days period of treatments, changes in tumor volumes were studied every 3 days, until day 22 (Fig. 5A). Our results indicated that tumor volumes significantly ($p \leq 0.001$) decreased after treatment with GL in combination with *E. coli* DH5α-lux/βG as compared to other groups treated with PBS, GL or *E. coli* DH5α-lux/βG (Fig. 5B). In order to evaluate the side effects of these treatments in different groups, the weight of mice was measured and recorded every 3 days and until day 22 (Fig. 5C). Results indicated that there was no significant difference between the PBS control group compared to other treated groups (Fig. 5D).

3.5. Histopathological analysis of tumor tissues and normal organs

H&E staining of tumor tissues obtained from mice treated with GL in combination with *E. coli* DH5α-lux/βG showed increased numbers of apoptotic cells as compared to tumor sections from other groups treated with PBS, GL or *E. coli* DH5α-lux/βG (Fig. 6). In order to evaluate the toxicity associated with the treated compounds, histopathological assessment of the spleen and liver was also carried out using H&E staining. The spleen and liver microstructure of PBS, GL or *E. coli* DH5α-lux/βG and GL in combination with *E. coli* DH5α-lux/βG treatment groups were normal.

4. Discussion

Despite the great advancements in diagnosis and treatment of

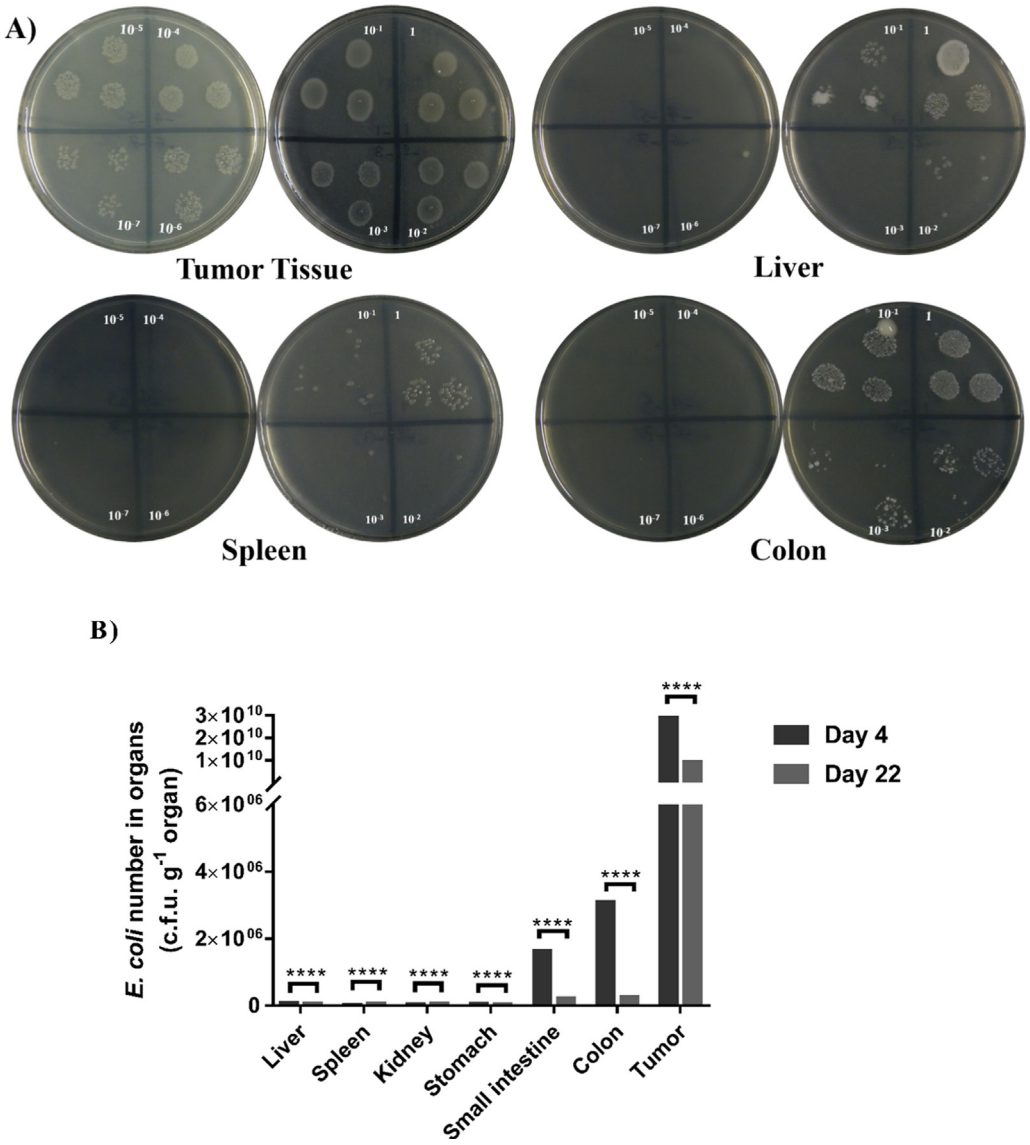

Fig. 4. Homing analysis of *E. coli* DH5α-lux/βG in tumors and normal organs. (A) Colonies formed from serial dilutions of the tumor, liver, spleen, and colon homogenates onto LB-agar plates. (B) Comparison of *E. coli* DH5α-lux/βG accumulation in tumor (4 mice at each time point) and normal organs (3 mice at each time point) of mice as measured at days 4 and 22 after injection. Data are the number of bacterial colonies per gram of organs ± SD. **** indicates significant differences at $p \leq 0.0001$.

cancer, the disease remains one of the leading causes of death in the world, and the number of people suffering from it, is steadily increasing (WHO, 2019). Colorectal cancer (CRC) ranks third in terms of incidence but second in terms of mortality (Bray et al., 2018). Conventional therapies including surgery, radiotherapy and chemotherapy currently used for colorectal cancer, suffer from some drawbacks including acquired resistance in patients with advanced tumors (Khan et al., 2013). Therefore, it is important to find new, targeted and alternative therapeutic methods that have the desired effects with the least side effects. Since William Coley, for the first time, successfully provided a document on bacterial efficacy in the treatment of highly advanced cancers (Nauts et al., 1953; Richardson et al., 1999), many studies have been conducted to increase bacterial efficacy for cancer treatment. With this approach, in recent decades many studies have been performed to produce non-toxic genetically modified bacteria to be used in combination with specific prodrugs for targeted cancer therapy (Zu & Wang, 2014). Bacterial therapy of cancer has unique advantages compared to other therapeutic methods; genetically engineered bacteria can specifically target tumor cells with less side effects (Chen et al., 2013). Among different bacterial therapies, one of the most trusted methods is Bacteria-directed enzyme prodrug therapy (BDEPT), which is an emerging form of treatment for cancer. BDEPT combines the innate ability of bacteria to selectively proliferate in tumors, with the capacity of prodrugs to be converted into active metabolites *in vivo*. Although BDEPT has undergone clinical trials, it still needs to achieve regulatory approvals before clinical use (Stritzker et al., 2008; Valencakova, 2016).

In present study, therapeutic efficacy of BDEPT approach based on the ability of *E. coli* DH5α-lux/βG in specific conversion of glycyrrhizic acid (GL) to glycyrrhetinic acid (GA) only in TME was evaluated. Antibacterial activity of GL was first investigated using broth microdilution assay. After determining the ineffectiveness of this compound in decreasing the growth rate of *E. coli* DH5α-lux/βG, the IC_{50} value related to the conversion of GL (prodrug) to GA (active drug) by *E. coli* DH5α-lux/βG was evaluated *in vitro* using MTT assay. The results indicated that growth of CT26 colon carcinoma cells was inhibited by GL accompanied by *E. coli* DH5α-lux/βG. In addition, after tumor induction in Balb/c mice through subcutaneous injection of CT26 cells, bacterial

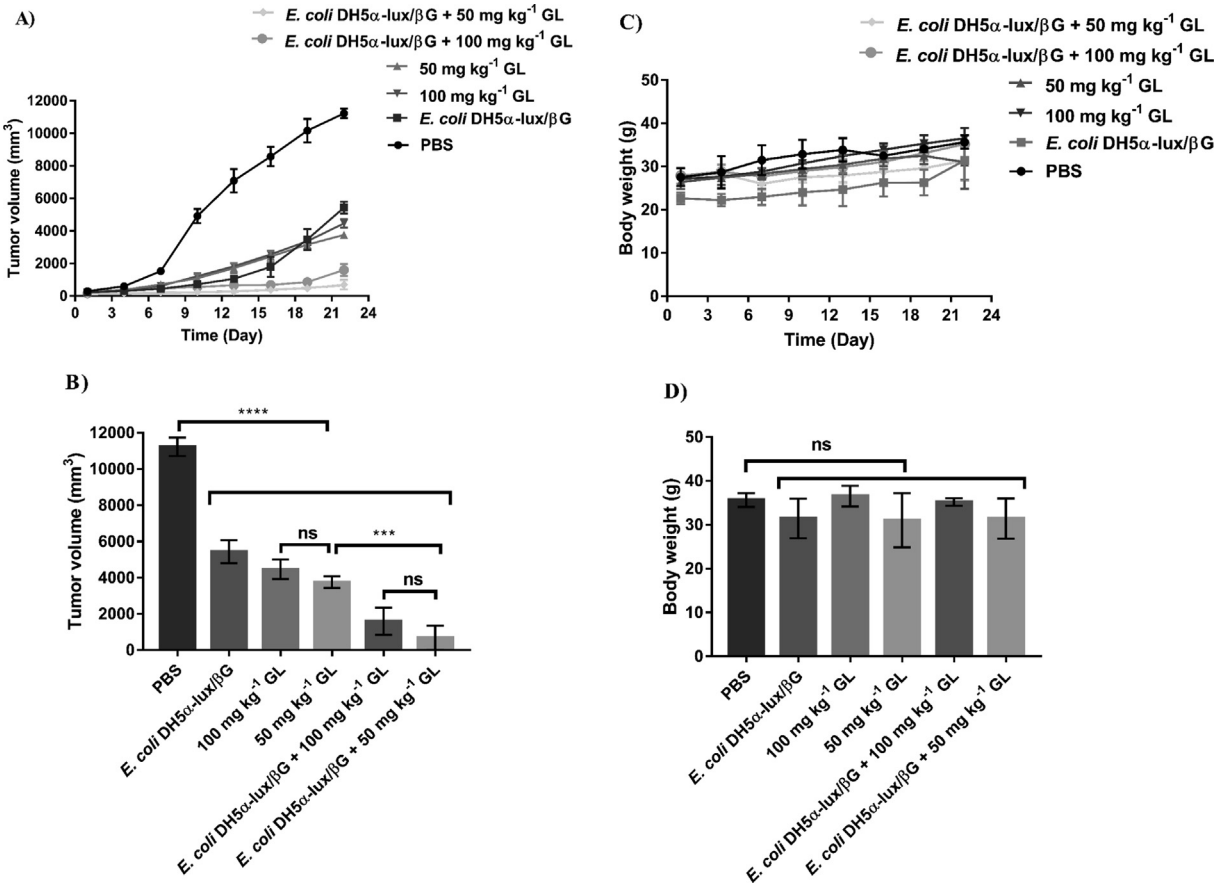

Fig. 5. *In vivo* anti-tumor therapy. (A) Tumor volume changes in different groups during the 20-day treatment course. The measurements were carried out every 3 days, until day 22. Data represent mean ± SEM. (B) Comparison of tumor volumes in different groups of mice at day 22 of treatments. Data are mean ± SD. ns indicates non-significant, and *** and **** indicate significant differences at $p \leq 0.001$ and $p \leq 0.0001$, respectively. (C) Weight changes in mice after 20 days of various treatments. Animals were weighed every 3 days, until day 22. Data are shown as mean ± SEM. (D) Comparison of the mean weight of the mice in different groups at day 22. Data are mean ± SD and ns indicates non-significant.

colonization in mouse and anti-cancer effects of various treatments were investigated in control and test groups. The results of this section indicate the specific accumulation and even reproduction of the bacteria in the tumor tissue and their effective clearance from normal organs. *In vivo* imaging of tumor-bearing mice after i.v. injection of *E. coli* DH5α-lux/βG also demonstrated specific localization of bacteria in tumors irrelative of tumor type and immune status of the mice (Hsieh et al., 2015). The luminescent intensity as a marker for bacterial abundance decreased significantly in all organs at day 22 after injection which confirms the bacterial clearance by immune system in normal organs. However, the intensity of luminescent at days 4 and 22 after injection of bacteria remained overloaded, indicating the sustained presence of bacteria in the tumor tissue. The results of colony counting confirmed the luminescent results. Meanwhile, in both spleen and kidney organs, the number of bacteria at day 22 increased compared to day 4. Given the fact that these organs are responsible for cleansing blood infections, justifies this observation (Abbas et al., 2018). This prolonged accumulation of bacteria in the tumor tissue versus clearance from the normal organs allows repeated administration of prodrugs following only one initial injection of the bacteria. The *in vivo* results indicated the efficacy of BDEPT approach in mice bearing colon carcinoma as compared to the control PBS treated group. This suggests the therapeutic effects of *E. coli* DH5α-lux/βG and GL compound in BDEPT. In explaining the anti-cancer effects observed for bacteria in monotherapy, it should be noted that some bacteria can also kill cancer cells by competing for food or secreting certain toxic metabolites (Cheng et al., 2008; Hsieh et al., 2015). The anti-cancer effects of GL monotherapy were also reported by Zhang *et al.*, in CRC. In their study, the anti-proliferative effects of GL were induced through inhibition of 11 β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme (Zhang et al., 2009). Nevertheless, when the bacteria and GL compound were co-administrated, tumor growth was more significantly reduced compared to monotherapy groups. However, increasing GL concentration did not improve the therapeutic index. Considering the optimal accumulation of *E. coli* DH5α-lux/βG in the tumor tissue, this may be due to two reasons; first, angiogenesis heterogeneity within the tumor can affect GL accumulation, the second reason might be saturation of βG enzyme which is produced in periplasmic space. Accordingly, in the study of Cheng *et al.*, when βG enzyme was expressed on the surface of *E. coli* by fusion to the bacterial autotransporter protein Adhesin, it increased enzyme activity and the prodrug conversion rate about 2.6-fold higher than that of the βG enzyme in the periplasmic space of the bacteria (Cheng et al., 2013). Moreover, the side effects of these treatments were assessed by analyzing weight loss and histopathological studies. Comparing body weight in different groups indicated no significant differences compared to control groups, which is in agreement with previous reports (Cheng et al., 2008; Hsieh et al., 2015).

4.1. The rationale of the present study

In this study, *E. coli* was selected as a carrier for delivering βG into tumor tissue. As compared with *Salmonella typhimurium*, which was studied in a human clinical trial (Nemunaitis et al., 2003), *E. coli* has a much higher ability to home into tumors. *E. coli* can selectively colonize in solid tumors, providing high tumor/normal tissue colonization ratios (Hsieh et al., 2015; Stritzker et al., 2008; Westphal et al., 2008; Zhang

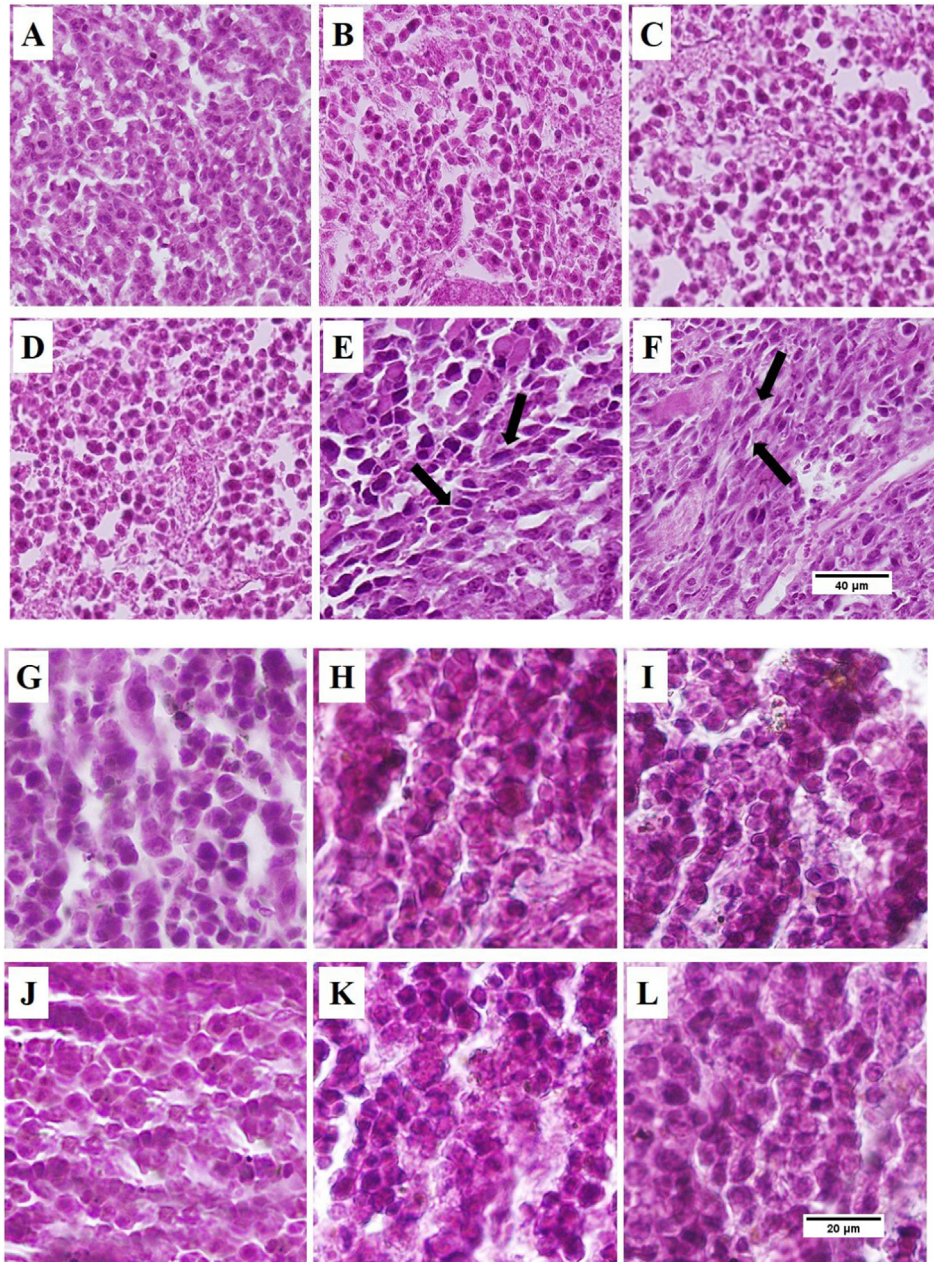

Fig. 6. Histopathological analysis of tumor tissues and normal organs by H&E staining. Photomicrographs of tumor cross sections in different groups treated with (A) PBS, (B) *E. coli* DH5α-lux/βG, (C) 50 mg kg^{-1} GL, (D) 100 mg kg^{-1} GL, (E) *E. coli* DH5α-lux/βG and 50 mg kg^{-1} GL and (F) *E. coli* DH5α-lux/βG and 100 mg kg^{-1} GL. Apoptotic cells with pyknotic nuclei (Hou et al., 2016; Arghiani et al., 2014) are observed in *E. coli* DH5α-lux/βG and 50 mg kg^{-1} GL and *E. coli* DH5α-lux/βG and 100 mg kg^{-1} GL treated groups. H&E stained photomicrographs of spleen sections in (G) PBS group, after administration of (H) *E. coli* DH5α-lux/βG, (I) 50 mg kg^{-1} GL, (J) 100 mg kg^{-1} GL, (K) *E. coli* DH5α-lux/βG and 50 mg kg^{-1} GL, (L) *E. coli* DH5α-lux/βG and 100 mg kg^{-1} GL. No obvious pathological damage could be observed in the spleens of treated groups. Histopathological changes in cross sections of livers after administration of various compounds in (M) PBS group, after administration of (N) *E. coli* DH5α-lux/βG, (O) 50 mg kg^{-1} GL, (P) 100 mg kg^{-1} GL, (Q) *E. coli* DH5α-lux/βG and 50 mg kg^{-1} GL, (R) *E. coli* DH5α-lux/βG and 100 mg kg^{-1} GL. Normal hepatocytes indicate the safety of various treatments in the livers of mice in different groups.

et al., 2010). In agreement with these studies, we observed tumor: liver and tumor: spleen bacterial ratios of 3.7×10^5: 1 and 1.8×10^6: 1, respectively at four days after i.v. injection of *E. coli* into mice. While, the ratio of accumulation in the tumor: liver and tumor: spleen of *Salmonella* was shown to be between 100 and 1000: 1 in mice (Clairmont et al., 2002; Pawelek et al., 1997). This makes *E. coli* a preferred candidate for subsequent clinical trials. In addition, unlike other delivery systems such as liposomes, antibodies, or defective viruses, bacteria can replicate in tumor tissues. These two main advantages of using *E. coli* as a delivery system, along with the fact that DH5α strain does not produce endotoxins, as well as the presence of specific transporters for glucuronide compounds in this bacterium (Cheng et al., 2008; Hsieh et al., 2015), were the reasons for harnessing *E. coli* DH5α-lux/βG in the present study.

On the other hand, the optimum activity of human β-glucuronidase (βG) is at pH = 4.5, which is why the enzyme is located in lysosomes and cellular microsomes in general. Contrary to this, the optimal activity of the bacterial βG is at pH = 7 and the enzyme is located in the periplasmic space of the bacterium. Since the pH in the interstitial space of the tumor cells (the TME) is about 7, makes it an optimal microenvironment for the activity of bacterial enzyme rather than its human type (Zhang et al., 2010; Engin et al., 1995; Ho et al., 1985).

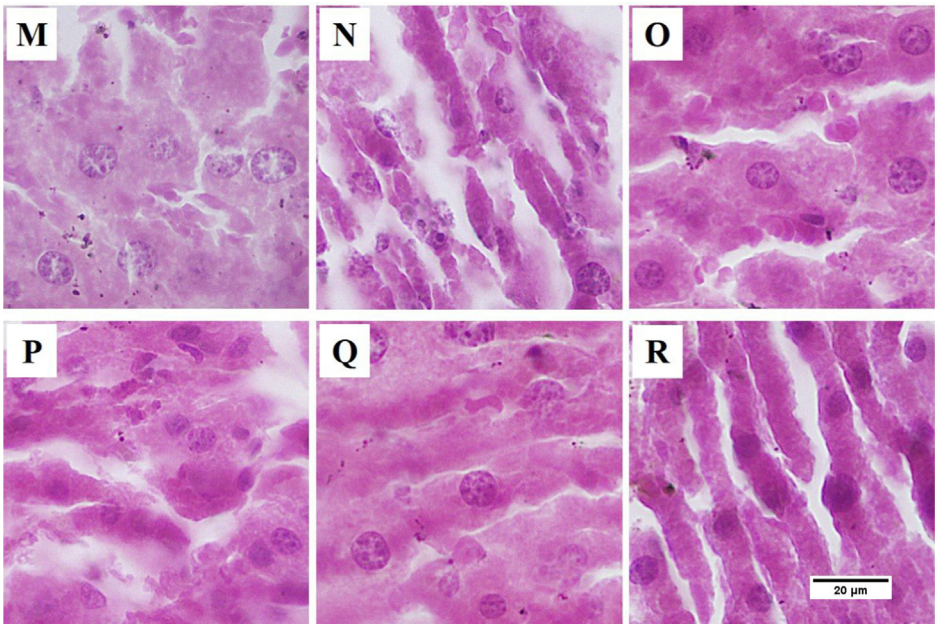

Fig. 6. (*continued*)

Glucuronide precursors such as GL, are charged at physiological pH. This prevents these compounds from passing through the membrane of mammalian cells and thus prevents them from activating by cytoplasmic or lysosomal enzymes within host cells (Cheng et al., 2013, 2008; Aldridge et al., 1985). Given that GL compound is a natural substance and its anti-inflammatory and anti-cancer properties have been proven already (Roohbakhsh et al., 2016; Zhang et al., 2009) and, on the other hand *E. coli*, has specific transporters for glucuronide compounds on its membrane, it was hypothesized that simultaneous application of GL as a prodrug and *E. coli* as a concomitant delivery and converting system would be beneficial in BDEPT.

Most colorectal cancers are adenocarcinoma and often begin with lesions called polyps (National Cancer Institute, 2019). Studies have shown that prostaglandin E2 (PGE2) derived from cyclooxygenase 2 (COX-2) enzyme pathway plays a very important role in the progression of colorectal cancer, and both selective and non-selective COX-2 inhibitors reduce the number and size of polyps (Stewart and Prescott, 2009). The expression of the 11β-HSD2 enzyme which converts activated glucocorticoids to inactive ketone forms increased in human and mouse colon adenomas, and this is correlated with the COX-2 gene overexpression and the increased activity of the COX-2 enzyme (Zhang et al., 2009). Pharmacologic inhibition or gene silencing of 11β-HSD2 lead to reduced production of PGE2 in tumor tissues and prevents the formation of adenomas, tumor growth and metastasis in mice (Stewart and Prescott, 2009). Therefore, inhibiting the 11β-HSD2 enzyme could be the same achievement as a new approach to chemoprevention and targeted treatment of colorectal cancer, through increasing the glucocorticoid activity of the tumor and controlling the local activity of COX-2.

GL blocks the conversion of active cortisol to inactive cortisone via suppression of the COX-2–mediated signaling pathway through inhibition of 11β-HSD2, reduces tumor COX-2 activity, tumor growth, and metastasis. GA is 200–1000 times more active than GL, which means it is a more potent inhibitor for 11β-HSD2 enzyme. Controlling this enzyme by GA, as already mentioned, inhibits the expression of COX-2 in a regulated manner and suppresses colon carcinogenesis (Stewart and Prescott, 2009; Zhang et al., 2009). Overall, our results suggest that this BDEPT system can specifically target tumor cells, significantly delay tumor growth and exert anti-tumor effects, while it has no toxicity on the normal spleen and liver organs. However, future studies measuring activated drug concentrations in tumors and serum will be important to confirm these results.

5. Conclusion

In summary, we demonstrated that the light-emitting and βG-expressing *E. coli* DH5α-lux/βG could specifically activate GL to GA *in vitro* and replicate and accumulate in tumors *in vivo*. Combination treatment with *E. coli* DH5α-lux/βG and GL significantly suppressed tumor growth with minimal toxicity in comparison to therapy with bacteria or prodrug monotherapy. Our data suggest new criteria for expressing prodrug-activating enzymes from conditionally replicating bacteria to allow the development of new tumor-specific herbal pharmacotherapy protocols.

6. Ethics approval and consent to participate

This study was authorized by the Animal Care and Ethics Committee of Ferdowsi University of Mashhad (Code of ethics: IR.UM.REC.1398.146).

7. Availability of data and materials

All data analyzed during current study are included in this published article. Further details could be available upon reasonable request.

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CRediT authorship contribution statement

Amin Afkhami-Poostchi: Conceptualization, Investigation, Visualization, Methodology, Writing - original draft. **Mansour Mashreghi:** Methodology, Validation. **Mehrdad Iranshahi:** Methodology, Validation. **Maryam M. Matin:** Conceptualization, Methodology, Validation, Resources, Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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