Development of a theranostic prodrug for colon cancer therapy by combining ligand-targeted delivery and enzyme-stimulated activation

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Abstract
The high incidence of colorectal cancer worldwide is currently a major health concern. Although conventional chemotherapy and surgery are effective to some extent, there is always a risk of relapse due to associated side effects, including post-surgical complications and non-discrimination between cancer and normal cells. In this study, we developed a small molecule-based theranostic system, Gal-Dox, which is preferentially taken up by colon cancer cells through receptor-mediated endocytosis. After cancer-specific activation, the active drug Dox (doxorubicin) is released with a fluorescence turn-on response, allowing both drug localization and site of action to be monitored. The therapeutic potency of Gal-Dox was also evaluated, both in vivo and ex vivo, thus illustrating the potential of Gal-Dox as a colorectal cancer theranostic with great specificity.

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1. Introduction
Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide, with over one million new cases in Europe and the US every year [1,2]. It is the second most common cancer affecting women, after breast cancer, and the third most common in men, after prostate and lung cancers, with the overall risk for developing CRC being approximately 1 in 20 [3]. Typically, cancers confined to the colon are curable; however, if left untreated, they spread to the regional lymph nodes and metastasize to distant organs. Usually, the initial stages of the disease are curable with surgical excision and combined chemotherapy. However, an appreciable proportion of CRC patients in early stage treatment remain clinically remissive for a prolonged period of time, followed by approximately 50% chances of tumor recurrence with later metastasis [4–6]. Surgery is accompanied by various complications, including the formation of blood clots in the legs, bleeding at the surgical sites, and damage to nearby organs [7,8]. Hence, there is an urgent need for the development of new therapeutic strategies for CRC, with improved clinical outcomes.

Conventional therapeutic strategies, involving the systemic delivery of antitumor drugs, cannot distinguish between normal cells and proliferating cancerous cells, causing collateral damage to healthy tissue. To overcome such a formidable challenge, several targeted delivery systems have been developed, including small molecule-based drug delivery systems (DDS), liposomes, polymeric systems, aptamers, and inorganic nanoparticles [9–16]. Ideally, certain criteria must be fulfilled for the successful development of a drug delivery formulation. These include preferential targeting of tumors, maximal accumulation in tumors in vivo, and finally, an efficient drug release profile within the tumor, with minimal leakage to contiguous normal cells. Nanoformulations usually take advantage of leaky vasculature within the tumor mass for preferential accumulation, termed as enhanced permeability and retention effects (EPR). However, this significantly depends upon the state of angiogenesis and vascularization of solid tumors [17–20].

In past years, the design of nanomedicines in cancer therapeutics has been upgraded, by the incorporation of ligands that actively target overexpressed receptors on tumors. However, there are still
certain hurdles to be overcome to realize the final goal of precision medicine, including cost-effectiveness, pharmacokinetics, and disease-driven formulations [20]. This is where a small molecule-based combination diagnosis and therapy can play a significant role. In the past 20 years, several small molecule-based DDS have been designed and developed, for use in early diagnosis, bio-imaging, and therapeutics [10,11,15]. DDS are usually decorated with specific cancer targeting units, along with a drug activation trigger moiety, linked to the chemotherapy drug. For drug activation, several stimuli-responsive modes have been reported, including pH, elevated enzyme activity, redox status, light, and temperature [14,15,21,22]. From a pharmaco-economic perspective, suitable targeting units and drug activation modes can be chosen according to the cancer subtype, and synthetically designed. This requires the simultaneous development of new DDS with easier accessibility and promising pharmacokinetics that can also be translated to establish superior therapies for patients.

The lysosomal enzyme, β-galactosidase (β-gal) is known to be upregulated in various cancer subtypes, including liver, lung, and ovarian cancers [23–25]. Several efforts have been made to visualize the upregulated activity of β-gal real time in various preclinical cancer models [24–26]. β-gal activity has also been utilized to activate various cancer drug delivery formulations [27] and theranostic agents [28]. Considering cancer as a robust system, the preferential targeting and cellular uptake behavior of each designed DDS can be influenced by several factors, including complex structural construct, inherent molecular features, and variation in expression levels of both targeting and trigger modules [29–31]. Moreover, the overall safety and efficacy of complex DDS can be influenced by several parameters including biodistribution, potential immune toxicities and intended targeting that need to be addressed carefully in preclinical and clinical stages.

In exploring a new direction for the advancement of antitumor chemotherapeutics, it is critical to understand the in vivo uptake and drug activation behavior of a novel DDS. This is because the optimal drug availability within a tumor is governed by DDS-associated features, which can alter the overall therapeutic efficacy and toxicity profile of the incorporated drug [30,31]. As detailed below, we focus on the β-gal enzyme as a target to both deliver and activate the DDS (Gal-Dox) for targeted drug delivery in vitro and in vivo, in CRC tumor models. The galactosiyte moiety of Gal-Dox serves as an excellent targeting ligand for asialoglyco-protein (ASGP) receptors, which play a significant role in transcriptional regulation at both cellular and molecular levels [32,33]. Doxorubicin (Dox) was chosen as a model anticancer drug. Gal-Dox showed a significant ability to target ASGP receptors on colon cancer cells, and enhanced activation behavior via lysosomal β-gal enzyme. We also validated the potential of Gal-Dox in a mouse tumor model and demonstrated its potential for effective cancer therapy.

2. Material and methods

2.1. UV/vis, fluorescence spectroscopy and high performance liquid chromatography methods

A stock solution of Gal-Dox was prepared in DMSO and for preliminary solution studies, 10 μM working solution in phosphate saline buffer (PBS) buffer solution (pH = 7.4, 37 °C) containing 1% DMSO was used. Excitation was done at 480 nm with all excitation and emission slit widths at 5 nm. The total volume for β-Galactosidase response and pH-dependent response tests was fixed at 3.0 mL. For HPLC analyses, a reverse-phase column (C18, 5 mm, Waters) equipped YL9101S (YL-Clarity) instrument was used. A UV–vis detector (480 nm) was used. The eluent used for each analysis was a water-acetonitrile gradient (0–30 min; acetonitrile from 5% to 85%) with a 1.0 mL/min flow rate.

2.2. Cellular uptake studies by fluorescence imaging and flow cytometry

To evaluate the intracellular delivery of Gal-Dox, confocal laser scanning microscopy (CLSM) and flow cytometry were both employed on HT-29, HepG2, and HeLa cells. Cells were seeded and incubated in μ-slide 8 well chamber (ibidi, Munich, Germany) at a density of 1 × 10⁴ cells per well. After overnight incubation, 10 μM Gal-Dox was treated into the culture media of well for various time points. All cells were fixed with 4% paraformaldehyde in PBS for 20 min at RT, following washing with phosphate-buffered saline (PBS) for three times. Nuclei and F-actin were counterstained with DAPI (Invitrogen, Molecular Probe, Eugene, OR, USA) and Alexa Fluor 488 phallolidin (Invitrogen, Molecular Probe) diluted in 3% BSA for 20 min, respectively. Furthermore, Lysotracker (Invitrogen, Molecular Probe) was added to the HT-29 cells at a concentration of 50 nM for 30 min for the examination of co-localization with Gal-Dox and lysosome. The fluorescence images were acquired using a CLSM (LSM 710, Carl Zeiss, and Germany). To further verify the Gal-Dox being targeted to ASGP receptor positive cell lines, the cellular uptake was analyzed by flow cytometry (FACS Calibur, BD Biosciences, USA). HT-29 and HeLa cells were incubated in 12-well plate at a density of 4 × 10⁵/well and pretreated with 1 mM of β-galactose (Sigma-Aldrich, St. Louis, MO, USA). After incubation for 4 h, the culture media were discarded, and the harvested cells were examined by a flow cytometry, following the treatment of 10 μM Gal-Dox for 2 h. The histogram plots were performed using FlowJo software (TreStar, Olten, Switzerland) and mean fluorescence intensity values were shown in the bar graph.

2.3. In vitro anti-cancer assay

HT-29 and HeLa cells were plated to 96 well plates at a density of 1 × 10⁵ cells per well for 12 h, and then treated with Dox and Gal-Dox at various concentrations for 24 h. Then, the culture media were replaced with fresh medium and the anti-cancer effect was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Roche Diagnostics GmbH, Mannheim, Germany) colorimetric assay. MTT was added and incubated for an additional 2 h at 37 °C. The MTT absorbance was measured at 570 nm using a microplate reader (EL800, Bio-Tek Instruments, Winooski, VT, USA) after the treatment of solubilization buffer (Roche Diagnostics) to dissolve formazan crystals. All experiments were carried out with three replicates.

2.4. In vivo tumor targeting and anti-cancer effects

For in vivo tumor-targeted imaging and anti-tumor activity, colorectal cancer xenografts were established by subcutaneously inoculating six-week-old male BALB/c nude mice (Orient Bio, Sungnam, Korea) with HT-29 cells. All in vivo experimental procedures involving animals were approved by the Korea University Institutional Animal Care and Use Committee (IACUC). A total of 1 × 10⁵ viable HT-29 cells were injected into the flanks of nude mice after anesthetization. When the tumors reached about 5–10 mm in diameter, Dox or Gal-Dox was injected intravenously into the tail vein in a single dose of 5 mg/kg. Whole-body fluorescence images were continuously monitored and the dissected major organs (i.e., liver, kidney, heart, lung, tumor) were visualized or analyzed after sacrificing at 6 h, 24 h, 48 h, using an in vivo imaging system (Maestro, CRI Inc., Woburn, MA, USA). Moreover, tumors were fixed in 4% paraformaldehyde solution, embedded...
routinely in paraffin, sectioned at 5 μm thickness, mounted using with DAPI containing Vecta shield (Vector Laboratories, CA, USA). To validate the therapeutic efficacy of Gal-Dox, HT-29-inoculated xenograft mice were subcutaneously injected with Saline, and 3 mg/kg dose of free Dox and Gal-Dox (xenografts, n = 5) four times every other day. Tumor volumes and body weights were measured every 3 day using a caliper and terminated at 36 days. For in vivo toxicity study, blood samples were collected from all the mice for the measurement of serum biochemical parameters including blood urea nitrogen (BUN), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) at day 30 after tumor inoculation. Also, one mouse from each group was sacrificed, and main organs such as heart, lung, liver, spleen, and kidney, were submitted for routine haematoxylin and eosin (H&E) staining.

3. Results and discussion

3.1. Synthesis, fluorescence response, and mechanism of Gal-Dox activation

Gal-Dox was synthesized by following a series of steps in good to excellent yield (Fig. 1A and S1, detailed in the supporting information). Following a reported procedure, galactosidase linker intermediate 2 was synthesized. Further alcohol activation by p-nitrobenzyl chloroformate and base-mediated substitution with free Dox resulted in the formation of intermediate 4. The acetyl groups were de-protected with sodium methoxide in anhydrous methanol and treated with cation exchange resin, resulting in the formation of the final product, Gal-Dox. All synthesized intermediates and products were well characterized by $^1$H/$^{13}$C nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (Figs. S14–25).

To obtain a deeper insight into binding sites at the atomic level, docking and molecular dynamics (MD) simulations were studied, including β-gal and Gal-Dox interactions. The molecular binding site between Gal-Dox and β-gal, and the decomposed binding free energies, are shown in Fig. 1B and S2, respectively. The calculated binding free energy between Gal-Dox and β-gal was found to be −20.22 kcal/mol. According to the binding interaction calculation, four significant interacting residues in β-galactosidase were observed, specified as N90 (red), P501 (orange), H528 (blue) and W987 (pink). Considering the calculated binding free energies among whole residues, W987 showed the strongest interaction with the Gal-Dox ligand. Details of the simulation are provided in the supporting information (Figs. S2–3).

To obtain insight into the preliminary stability and activation mode of Gal-Dox, the compound was incubated in phosphate buffered saline (PBS, pH 7.4, 37°C) for 24 h. No significant decomposition was observed during this period (determined by fluorescence microscopy and high performance liquid chromatography, HPLC), suggesting sufficient chemical stability (Fig. 1 and S4). Gal-Dox exhibited a weak fluorescence emission at 590 nm (excitation wavelength λexc. = 480 nm). Interestingly, exposure of Gal-Dox (10 μM) to β-gal (1 U/mL) resulted in cleavage of the galactosidase moiety, followed by the 1,6-elimination route to release free Dox with simultaneous fluorescence enhancement at 590 nm (Fig. 1C and D). Consequently, we used the fluorescence enhancement at 590 nm as an activation response, for the observation of direct Dox release in further studies. The Gal-Dox activation and
drug release mechanisms were also determined using mass spectroscopy (MS) and HPLC (Fig. 1E and S7). The HPLC profile showed a peak with an elution time of 20.4 min, corresponding to Gal-Dox. Upon treatment with β-gal, a new peak appeared at 15.3 min, corresponding to free Dox. Altogether, treatment of Gal-Dox with β-gal resulted in the complete release of active Dox, over a time period of 2 h. Given the disparities in pH and the presence of other bio-analytes between cancerous and normal healthy cells, we next evaluated the fluorescence behavior of Gal-Dox, in the presence of various bio-analytes, and at a range of pHs. No appreciable change in the fluorescence signal of Gal-Dox was observed at different pHs, or in the presence of various bio-analytes, including reductase, GSH, H2O2, and amino acids (Figs. 5S–6). These results suggested that Gal-Dox should exhibit high stability under biological conditions. Gal-Dox exhibited distinct fluorescence enhancement (590 nm) only upon treatment with the β-gal enzyme.

3.2. ASGP receptor-targeted delivery of Gal-Dox into colorectal cancer cells

The specific interaction between the galactose residue and ASGP receptors has been used for hepatocyte-targeted DDS via receptor-mediated endocytosis [34]. In this study, Gal-Dox was employed as a theranostic prodrug against the intestinal ASGP receptors in colon cancer [35]. To confirm the selective delivery of Gal-Dox to colon cancer cells overexpressing ASGP receptors, we evaluated its targeting ability both in HT-29 cells, a colon adenocarcinoma cell line, and in HepG2 cells, a well-known cancer cell line bearing the ASGP receptor. As shown in Fig. 2, HT-29 and HepG2 cells showed pronounced intracellular fluorescence, while relatively low fluorescence signals were detected in HeLa cells, an ASGP receptor-negative cell line (control) [36,37], even after 2 h incubation. Co-localization experiments confirmed that Gal-Dox was specifically localized with lysosomes of HT-29 cells, in which β-gal activity occurs (Figs. S8–9). Especially, intracellular fluorescence was clearly observed 30 min after treatment because of its targeting ability for ASGP receptors, while free Dox was not detectable at the same time. Fluorescence in Gal-Dox-treated cells was reached a plateau after 6 h (Fig. S8). To further verify the ASGP receptor-targeted cellular uptake of Gal-Dox, a competition assay was conducted by flow cytometry analysis. Initially, HT-29 cells were pretreated 100 times with free galactose as a competitor, followed by the addition of Gal-Dox at a concentration of 10 μM with incubation for 2 h. There was no significant difference in fluorescence intensity between HeLa cells treated with excess galactose and control untreated cells, whereas the fluorescence intensity in HT-29 cells pretreated with galactose remarkably decreased (Fig. S10). Altogether, these results support that the cellular uptake of Gal-Dox can be specifically achieved by ASGP receptor-mediated endocytosis. To the best of our knowledge, this is the first report citing the specific targeting properties of a small molecule-based prodrug system to ASGP receptor-overexpressing colon cancer cells, through cellular uptake via receptor-mediated endocytosis.

3.3. β-Galactosidase mediated activity of Gal-Dox

As Gal-Dox exhibited β-gal-triggered Dox release, we next confirmed enzyme-specific Gal-Dox activation via the reduction of β-gal activity within the cells. To knock down the expression of β-gal, an siRNA strategy was attempted to investigate the activity of Gal-Dox in the presence of the low level of endogenous β-gal within HT-29 cells. For this, HT-29 cells were treated with various concentrations of β-gal siRNA for 48 h, and the optimal siRNA concentration (50 nM) was determined by western blot analysis for further studies (Fig. 3A). After β-gal expression interference by pretreated siRNA (50 nM) for 48 h, HT-29 cells were incubated with 10 μM of Gal-Dox for 0.5 h and 12 h. While fluorescence decreased in HT-29 cells with low β-gal expression, we observed relatively strong fluorescence in control siRNA-untreated cells (Fig. 3B and Fig. S11). Enzyme-specific activation of Gal-Dox was further confirmed by flow cytometric analysis, which provided a comparison between the mean fluorescence intensity of Gal-Dox-treated HT-29 cells, with and without siRNA knockout (Fig. 3C). Collectively, these siRNA silencing results suggest that the activation of

Fig. 2. Targeted cellular uptake of Gal-Dox through ASGP receptor-mediated endocytosis. HT-29, HepG2 and HeLa cells were incubated with 10 μM Gal-Dox for 2 h, fixed with 4% paraformaldehyde, and counterstained with DAPI and Alexa Fluor 488-Phalloidin to visualize nuclei (blue) and F-actin (green), respectively. Confocal microscopy images of Gal-Dox (red) were obtained with a laser using an excitation wavelength of 480 nm and an emission wavelength of 560–590 nm. Scale bar: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Gal-Dox could be achieved by upregulated β-gal-responsive triggering. This sophisticated system permits simultaneous Dox release and fluorescence activation-based imaging, for greater precision in monitoring the drug activation event and its location.

3.4. Anticancer effects of Gal-Dox with cell selectivity

The cytotoxicity of Gal-Dox was evaluated in HT-29 and HeLa cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as its anti-proliferative activity can be determined by selective cellular uptake via ASGP receptor-mediated endocytosis. Both cells were treated with free Dox and Gal-Dox at concentrations ranging from 1 to 200 μM, for 24 h. Although Gal-Dox did not significantly reduce viability even at the maximum dose in both cells, similar behavior was observed for both free Dox- and Gal-Dox-treated HT-29 cells (Fig. 4A). However, a remarkable difference in cytotoxicity was detected between free Dox- and Gal-Dox-treated HeLa cells (Fig. 4B). The comparison of IC50 values indicated that Gal-Dox-treated HT-29 cells possessed approximately 3-fold more potent therapeutic effects than HeLa cell (Table S1). These results demonstrate that targeted Gal-Dox delivery via the overexpressed ASGP receptors in colon cancer cells not only improved cellular uptake, but also enhanced its anticancer effect.

Fig. 3. Gal-Dox activation through β-galactosidase-mediated cleavage. (A) Western blot analysis of β-gal knock-down by siRNA. HT-29 cells were transfected with β-gal siRNA (25 nM and 50 nM) for 48 h. The silencing effect of siRNA was analyzed by western blot, using an anti-β-gal antibody and β-actin antibody as a loading control. (B) After transfection of siRNA (50 nM) directed against β-gal for 48 h, HT-29 cells were treated with 10 μM Gal-Dox for 0.5 h, and examined with confocal microscopy. Filter sets: Doxorubicin (λex: 480/λem: 560–590), F-actin (λex: 495/λem: 518 and DAPI (λex: 358/λem: 461) Scale bar: 20 μm. (C) Flow cytometry analysis was conducted with HT-29 cells treated with 10 μM Gal-Dox after transfection of 50 nM of β-gal siRNA.

Fig. 4. Anticancer effects of Gal-Dox in HT-29 cancer cells (A) and HeLa (B) cells. Both cells were treated with Gal-Dox and Dox as a positive control, at various concentrations for 24 h. Cell viability was then determined using the MTT assay.
3.5. In vivo/ex vivo diagnosis and chemotherapeutic in xenograft mouse models

To investigate the antitumor activity of Gal-Dox, in vivo tumor-targeting ability was initially confirmed in HT-29 tumor-bearing mice. For bio-distribution studies using an in vivo fluorescence imaging system, saline, free Dox and Gal-Dox were intravenously injected into mice at a single dose of 3 mg/kg, and real-time whole-body fluorescence imaging was performed 2d after administration. While the tumor-targeted accumulation of Gal-Dox was continuously visualized by enhanced fluorescence signal up to 48 h in the HT-29 tumor, free Dox was observed to slightly clear from the tumor site in 24 h post injection (Fig. 5 and S12A). After sacrificing the mice 48 h later, the tumor tissues were harvested and subsequently subjected to ex vivo fluorescence imaging (Fig. S12B). Collectively, whole-body fluorescence imaging results were comparable to those for the dissected tumor tissues (Fig. 5B) and biodistribution studies (Fig. S12C and D) from saline-, free Dox-, and Gal-Dox-treated mice. The in vivo therapeutic efficacy of Gal-Dox was also evaluated in nude mice bearing HT-29 cell-inoculated tumors, following intravenous administration of Gal-Dox (5 mg/kg, four times every second day), and controls including saline and free Dox. As shown in Fig. 5C, tumor growth was significantly retarded in Dox- and Gal-Dox-injected xenograft mice, compared to saline-injected mice. However, administration of Gal-Dox showed remarkable tumor growth inhibition (53.1%) compared to free Dox treatment (34.9%, see Fig. 5D). Although Dox has been known to have several adverse effects such as cardiotoxicity, no significant in vivo toxicities were observed during this study (Fig. S13). We believe that this remarkable therapeutic response of Gal-Dox reflects its efficient tumor uptake behavior by the galactosidase unit, which not only serves to improve cancer targeting, but also as an efficient prodrug activation mode to deliver the active Dox.

4. Conclusions

In conclusion, a small molecule-based theranostic prodrug, Gal-Dox, was successfully developed for colon cancer chemotherapy, using receptor-mediated targeting and enzyme-responsive activation strategies. The imaging properties and therapeutic efficacy of Gal-Dox in colorectal cancer models have been successfully demonstrated both in vitro and in vivo. Gal-Dox is preferentially taken up by HT-29 cancer cells through ASGP receptor-mediated endocytosis and was activated by elevated lysosomal β-gal enzyme. In particular, the theranostic activation event is accompanied by a simultaneous fluorescence turn-on response. This also supports the additional possibility of monitoring both the site of drug activation, and the therapeutic response towards the tumor, during early stages of treatment. Compared to the pre-reported multi-component DDS systems incorporating Galactosidase as trigger, drugs and cancer-specific targeting units altogether in a complex design, Gal-Dox with simple design possesses remarkable tumor specific targeting and therapeutic potential in ASGP receptor positive cell lines. Additionally, complexity of pre-designed DDS as multi-component requires detailed design and orthogonal analysis with reproducible scale-up process for achieving a consistent therapeutic response.

Fig. 5. Tumor-targeted accumulation and in vivo antitumor activity of Gal-Dox in nude mice bearing HT-29 cancer xenografts. (A) In vivo fluorescence imaging of Gal-Dox-treated HT-29 xenograft nude mice. Mice bearing HT29 subcutaneous xenografts were intravenously injected with 5 mg/kg of Gal-Dox and in vivo fluorescent images were obtained 48 h after treatment using the Maestro in vivo imaging system. (B) Fluorescence images in paraffin-embedded tumor tissue. Scale bar: 20 μm (C) Tumor growth inhibition in the various groups. HT-29-bearing mice were intravenously injected with saline, Dox, or Gal-Dox at a dose of 3 mg/kg, four times every second day. Tumor volume was measured twice per week. Data are shown as mean ± SE. **: P < 0.05 versus Dox; ***: P < 0.001 versus saline, determined using Student’s t-test. (D) Tumor growth inhibition after treatment with Dox and Gal-Dox at 36 days.
commercial product for intended physiological properties, pharmacological profiles, and biological response. Gal-Dox with a simple design, can be accessed with ease synthetically from pharmacological point of view also. Hence Gal-Dox shows the potential as an ideal theranostic system in future personalized cancer chemotherapeutics.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.biomaterials.2017.11.019.

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