Development of an oral nanotherapeutics using redox nanoparticles for treatment of colitis-associated colon cancer

Long Binh Vong a, Toru Yoshitomi a, Hirofumi Matsui b, c, Yukio Nagasaki a, b, d, *

a Department of Materials Science, Graduate School of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8573, Japan
b Master’s School of Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan
c Division of Gastroenterology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan
d Satellite Laboratory, International Center for Materials Nanoarchitectonics (WPI-MANA), National Institute for Materials Science (NIMS), University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8573, Japan

Abstract

Oral chemotherapy is the preferred treatment for colon cancer. However, this strategy faces many challenges, including instability in the gastrointestinal (GI) tract, insufficient bioavailability, low tumor targeting, and severe adverse effects. In this study, we designed a novel redox nanoparticle (RNPO) that is an ideal oral therapeutics for colitis-associated colon cancer treatment. RNPO possesses nitroxide radicals in the core, which act as reactive oxygen species (ROS) scavengers. Orally administered RNPO highly accumulated in colonic mucosa, and specifically internalized in cancer tissues, but less in normal tissues. Despite of long-term oral administration of RNPO, no noticeable toxicities were observed in major organs of mice. Because RNPO effectively scavenged ROS, it significantly suppressed tumor growth after accumulation at tumor sites. Combination of RNPO with the conventional chemotherapy, irinotecan, led to remarkably improved therapeutic efficacy and effectively suppressed its adverse effects on GI tract. Therefore, RNPO is promising oral nanotherapeutics for cancer therapies.

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1. Introduction

Inflammatory bowel disease (IBD), which includes chronic gastrointestinal (GI) disorders such as Crohn’s disease (CD) and ulcerative colitis (UC), affects millions of patients worldwide [1–4]. After 30 years of living with these diseases, 18–20% of UC and 8% of CD patients develop colitis-associated colon cancer (CAC), the third most common malignancy and one of the major causes of cancer-related death [5,6]. In IBD patients, the increasing of reactive oxygen species (ROS) causes oxidative stress and oxidative cellular damage promoting carcinogenesis [7,8]. It has been reported that antioxidants such as N-acetylcysteine and resveratrol inhibited CAC development [9,10]. While oral administration of drugs are preferred by patient due to its convenience and compliance, these low-molecular-weight (LMW) compounds are not always effective due to nonspecific drug distribution, low retention in the GI tract, and absorption in the bloodstream, causing undesired adverse effects in the entire body. On the other hand, chemotherapy using 5-fluorouracil (5-FU) or irinotecan (Iri) has been used alone or in combination with other drugs as the first-line therapeutic agents for colorectal cancer [11,12]. However, these anticancer drugs are insufficient bioavailability and low tumor targeting. Furthermore, patients treated with these chemotherapeutic agents suffer from severe adverse effects such as mucositis and diarrhea, which limits the dose intensification and compromises efficacy [13].

Nanotechnology has enabled significant advances in the areas of cancer diagnosis and therapy [14–16]. Though a number of nanoparticle-drug combinations are assessed in preclinical or clinical applications, most of delivery systems are intravenously injectable formulations and are incapable of oral administration [17,18]. On the other hand, it has been reported that nanocomposites such as silver nanoparticle for therapeutics itself exhibits the undesired toxicity on the GI tract after repeated oral administration [19,20]. Recently, we have developed an oral nanotherapy using a redox nanoparticle (RNPO) for suppressing
inflammation in mice with colitis [21] and indomethacin-induced small intestinal inflammation [22]. RNPO was prepared by self-assembly of methoxy-poly(ethylene glycol)-b-poly(4-[2,2,6,6-tetramethylpiperidine-1-oxyl]oxymethylstyrene)] (MeO-PEG-b-PMOT), which is an amphiphilic block copolymer with stable nitroxide radicals in a hydrophobic segment as a side chain via an ether linkage (Fig. 1A). The size of RNPO is approximately 40 nm in diameter, with a remarkably narrow distribution (Fig. 1B) and extremely high colloidal stability owing to the PEG shell layer. As shown in Fig. 1C, RNPO is stable and maintains micelle form under physiological conditions without aggregation. This stable character improves accumulation tendency of RNPO to colonic mucosa, but not commercially available polystyrene particles. Furthermore, these 40 nm particles prevent the uptake into bloodstream via mesentery. Along with these characteristics, we have confirmed that RNPO effectively scavenges ROS to result in significant suppression of inflammation in mice with colitis [21]. Suppression of inflammation in the tumor microenvironments is reported to work as suppressor of tumor progression and resistance against chemotherapy [23]. Notably, we have confirmed that RNPO did not cause any disturbance to the population of intestinal bacteria [24]. Based on these characteristics of RNPO, we proposed that it would be a suitable oral therapeutics for cancer. Thus, it is interesting to apply RNPO as a novel oral therapeutics for treatment of colon cancer.

In this study, we used azoxymethane (AOM) and dextran sodium sulfate (DSS) to chemically induced CAC in mice, and we confirmed the efficacy of oral RNPO as a nanomedicine and combination therapy. No blood absorption and non-toxicity of RNPO were observed despite of long-term oral administration, which improves accumulation in colon region and prevents undesired adverse effects to entire body. We also found that orally administered RNPO tends to internalize in colon cancer cells, but not normal colon cells, indicating the extremely low adverse effects of this oral nanotherapeutics. Oral administration of RNPO effectively suppressed inflammation in the colon region, resulting in both high protective and therapeutic effects against CAC development. It is interesting to note that when RNPO was combined with conventional chemotherapy, the therapeutic effect on CAC was significantly enhanced, retaining low adverse effects of the chemotherapy on the GI tract.

2. Materials and methods

2.1. Preparation of RNPO

RNPO was prepared by a self-assembling MeO-PEG-b-PMOT block copolymer, as previously reported [21,25]. Briefly, methoxy-poly(ethylene glycol)-b-poly(-chloromethylstyrene) (MeO-PEG-b-PCMS) was synthesized by the radical telomerization of chloromethylstyrene (CMS; Seimi Chemical Co., Ltd., Kanagawa, Japan) using methoxy-poly(ethylene glycol)-sulphanyl (MeO-PEG-SH; NOF Corporation Co., Ltd., Tokyo, Japan; Mn – 5000) as a telogen (the degree of polymerization of CMS – 16 units). The chloromethyl groups were converted to TEMPOs via a Williamsson ether synthesis of benzyl chloride in the MeO-PEG-b-PCMS block copolymer with the alkoxide of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), as previously reported (the extent of TEMPO modification – 85%). RNPO was prepared from MeO-PEG-b-PMOT using a dialysis method.

2.2. Cell lines and cultures

The mouse colorectal carcinoma cell line C-26 (RCB2657) was obtained from Riken BioResource Center (Riken Tsukuba Institute, Ibaraki, Japan). C-26 cells were grown in Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), and 1% antibiotics (penicillin/streptomycin/neomycin; Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5% CO2 at 37°C.

2.3. Cellular uptake of RNPO in vitro

The experiment was carried out using rhodamine-labeled RNPO (Rho-RNPO) to analyze the cellular uptake of these nanoparticles by fluorescent confocal microscope. Rho-RNPO was prepared via a thiourethane bond between MeO-PEG-b-PMOT and...
possessing reduced TEMPO moieties and rhodamine B isothiocyanate (Sigma—Aldrich, St. Louis, MO) in dimethylformamide–involved sodium hydride, as previously reported [21]. C-26 colon cancer cells were seeded in 12-well plates at a certain density (5 x 10^4 cells per well). After 2 d of culturing, the DMEM was replaced with fresh media, and the Rho-RNPO solution (100 μg/mL) was added. At a predetermined time intervals, the cells were washed 3 times with fresh media. Hoechst 33342 (Invitrogen) and LysoTracker (Green DND-26, Invitrogen) were added for 15 min at 37 °C before imaging in order to stain nuclei and lysosomes, respectively. Photos of cellular uptake were taken and analyzed using a fluorescence confocal microscope system (Zeiss LSM 700, Carl Zeiss Microscopy GmbH, Jena, Germany) under oil immersion at 63 x magnification.

2.4. Animal
All experiments were carried out using 7 to 8-week-old male ICR mice (32–35 g) purchased from Charles River Japan, Inc. (Yokohama, Japan). Mice were maintained in the experimental animal facilities at the University of Tsukuba under controlled temperature (23 ± 1 °C), humidity (50 ± 5%) and lighting (12 h light–dark cycles). The animals were given free access to food and water. All experiments were performed in accordance with the Regulation for Animal Experiments in the University of Tsukuba and the Fundamental Guideline for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology.

2.5. Cellular internalization of RNPO in vivo
Cellular isolation procedure was based on a previous report with modifications [26]. Colon tissues were collected from normal mice and mice with AOM/DSS-induced CAC at 5 h after oral administration of RNPO (300 mg/kg); then, gently removed feces with PBS, followed by a mechanical fragmentation. Colonics tissues were treated with collagenase (10 mg/mL, Wako Pure Chemical Industries, Osaka, Japan) for 30 min at 37 °C with slow agitation, followed by a centrifugation at 10,000 rpm at 4 °C for 5 min. Cell pellets were gently resuspended in acetic acid (0.1 M, pH 3). Samples were centrifuged to separate extracellular RNPO and intracellular internalized RNPO. Supernatants and cell pellets were oxidized by potassium ferricyanide (10 mM; Kanto Chemical Co., Inc, Tokyo, Japan) for electron spin resonance (ESR) measurement under conditions described in the Supplementary materials.

2.6. Induction of colitis and CAC by AOM and DDS
Colitis was induced in mice by 3% (wt/vol) DSS (5000 Da; Wako Pure Chemical Industries, Osaka, Japan) supplemented in the drinking water for 7 d. For the CAC model, mice were injected intra-peritoneally with 10 mg/kg body weight of AOM (Sigma—Aldrich, St. Louis, MO) followed by 2 cycles of 7-d of 3% DSS in the drinking water for 70 d.

2.7. Endoscopic imaging and tumor scoring
To continuously observe the tumor development in CAC mice, we used a video endoscopy system (TESALA AVS, Olympus, Tokyo, Japan) for mouse according to the manufacturer’s instructions. The experimental endoscope setup consisted of a probe (2.7 mm outer diameter) with a rod lens containing a light-emitting diode light source, a camera unit connected to a laptop monitor, and an air supply to facilitate regulated inflation of the mouse colon. After setting up the endoscope system, the mice were anesthetized by inhalation of isofluorane (Intervet, Inc., Tokyo, Japan). The endoscopic procedure was viewed on a color monitor and real time video was recorded via firewire connected to a laptop with Light Capture software (I-O Data device, Inc., Kanazawa, Japan).

A previously described tumor scoring system was used to evaluate tumor development in mouse colons [27]. Tumors observed during endoscopies were counted to obtain the overall number of tumors. The sizes of all tumors in a given mouse were also scored to yield the tumor score. Tumor size was graded as follows: grade 1 (very small but detectable tumor), grade 2 (tumor covering up to 1/8 of the colonic circumference), grade 3 (tumor covering up to 1/4 of the colonic circumference), grade 4 (tumor covering up to 1/2 of the colonic circumference), and grade 5 (tumor covering more than 1/2 of the colonic circumference). The endoscopy analysis was performed weekly or every 2 weeks starting after the second cycle of DDS administration until the end of the experiment.

2.8. Iri-induced intestinal mucositis in mice
Intestinal mucositis was induced in mice by daily intraperitoneal injection of Iri (50 mg/kg) for 4 d [28]. To confirm the effect of RNPO, mice were given RNPO (300 mg/kg) by oral gavage daily during Iri treatment. Diarrhea assessment was performed after the treatment. The severity of diarrhea was scored as previously described [29] (0 [normal—normal stools or absent], 1 [slight—wet and soft stools], 2 [moderate—wet and unformed stools], 3 [severe—watery stools]). At day 5, mice were sacrificed, and blood and intestinal samples were collected for hematological and histological assessments, respectively.

2.9. Statistical analysis
All values are expressed as mean ± standard error of the mean (SEM). Differences between groups were examined for statistical significance using 1-way analysis of variance, followed by Bonferroni or Turkey’s post hoc test (SPSS software; IBM Corp, Armonk, NY). A value of P < 0.05 was considered significant for all statistical analyses.

3. Results
3.1. Accumulation of free drinking RNPO in the GI tract and its non-toxicity
The accumulation of nanoparticles in the colon region is one of the most important features of an effective nanomedicine for colon diseases including cancer. RNPO was given to mice in free drinking water for a week and ESR assays were used to assess the distribution of RNPO in the GI tract. ESR assays were performed for the blood and the main GI tract organs (stomach, small intestine, cecum, and colon) at a predetermined time. As shown in Fig. 2A, RNPO accumulation in the GI tract gradually increased over the administration time, particularly in the small intestine, cecum and colon regions, indicating high accumulation and long retention of RNPO in these areas. Conversely, we did not observe RNPO uptake in the bloodstream of mice, even for long-term administration. Lack of bloodstream uptake prevented potential adverse effects of the nitroxide radicals to the entire body.

To investigate the toxicity of RNPO in the GI tract, healthy mice were treated long-term with free drinking RNPO for one month; hematology was analyzed and histology was assessed for tissues from the GI tract as well as other organs. There were no remarkable differences in the hematological analysis of RNPO-treated mice as compared to healthy mice (Fig. 2B). Additionally, there were no noticeable toxicities in tissues from the GI tract and other organs, even in mice treated with a high concentration of RNPO (5 mg/mL) for a month (Fig. 2C and Supplementary Fig. S1). These results indicate that, during long-term oral administration, RNPO highly accumulates in the GI tract without any observed toxicity to healthy organs.

3.2. Specific cellular internalization of RNPO in cancer tissues
Specific cellular internalization of RNPO in colon tissues was analyzed in vivo using mice with AOM/DSS-induced CAC and compared to healthy mice. After oral gavage of RNPO, colon tissues were collected from these mice and isolated cells were oxidized for ESR assays. It is interesting to note that the total ESR intensity of RNPO is significantly higher in cancer tissues compared to normal tissues (Fig. 3A). Alternatively, RNPO remarkably surrounded mucosa of tumor sites due to the defective structure of mucus layer in these sites [30] and exhibition of abnormal tight junction [31], resulting in the facile penetration of the nanoparticles to mucosa in cancer tissues. Additionally, clear ESR signals were detected inside cancer cells, but not inside normal cells (Fig. 3A), indicating that RNPO did not internalize in healthy cells. This result demonstrates that RNPO tends to accumulate in cancer cells, where large amounts of ROS and pro-inflammatory cytokines are produced. The ESR spectra of RNPO also give the information about its morphology. Basically, the ESR signal of LMW nitroxide radical TEMPO has a sharp triplet due to an interaction between the 14N nuclei and the unpaired electron in the dilute solution. After the nitroxide radicals are introduced into the hydrophobic core of RNPO, the ESR spectrum of RNPO becomes broader (Supplementary Fig. S2). The broad ESR signals of RNPO were observed outside of both normal cells and cancer cells (Fig. 3B and C), indicating that RNPO remains in a core—shell-type micelle form when it exists outside of cells. Notably,
no ESR signals were detected inside normal cells (Fig. 3D), which is in sharp contrast to the signals observed in cancer cells (Fig. 3E). Interestingly, a triplet peak on ESR spectrum was observed inside cancer cells (Fig. 3E), indicating exposure of the nitroxide radicals after disintegration of RNPO inside cancer cells. In order to investigate the intracellular internalization mechanism of RNPO in cancer cells, we confirmed the uptake of RNPO in C-26 colon cancer cells in vitro. Here, RNPO was labeled using Rhodamine, making its red under a fluorescent microscope. Nuclei were blue and lysosomes were green when stained with Hoechst 33342 and Lyso-tracker Green DND-26, respectively. The merged image in Fig. 3F shows yellow and red fluorescence in the cytoplasm, suggesting uptake of RNPO into C-26 cancer cells via both endocytosis pathway and simply diffusion due to the leaky cellular membranes of damaged cancer cells. Higher accumulation in colonic mucosa, specific internalization in cancer cells, and low uptake in normal cells are the most important characteristics of RNPO, which are anticipated for high therapeutic efficiency with extremely low adverse effects.

3.3. RNPO prevents AOM/DSS-induced CAC by suppressing inflammation

Since we confirmed that RNPO preferentially accumulates at the site of colon tumor, we became interested in how RNPO works in the CAC model mice. In this model, colon cancer is driven by the combination of a carcinogenic agent (AOM) and an inflammatory agent (DSS). We previously confirmed that orally administered RNPO strongly scavenges ROS in DSS-induced colitis mice and almost completely cures [21]. Separately, we found that intravenously administered RNPO suppresses an activation of nuclear factor kappa B (NF-κB) in cancer cells of mice with subcutaneously transplanted tumors [32]. If oral administration of RNPO works similarly in CAC mice without any adverse effects to the entire body, it will be an ideal cancer chemotherapeutics. Fig. 4 shows a protective effect of orally administered RNPO to the CAC mice. Here, RNPO (200 mg/kg) was administered daily by oral gavage for 1 week for the first and fourth weeks, which are the same terms of DSS treatment (Fig. 4A). In contrast to the CAC mice, which experience a significantly reduction in body weight during DSS treatment, no body weight loss was observed during RNPO administration (Fig. 4B). As anticipated, a disease activity index significantly decreased (Fig. 4C) in RNPO-treated mice. The pro-inflammatory cytokine interferon-gamma (IFN-γ) was also significantly reduced in RNPO-treated mice (Fig. 4D), suggesting that oral administration of RNPO effectively suppressed inflammation in the colon, even in CAC mice. During 70 d of treatment, we used an endoscope system to confirm colon tumor development in mouse and evaluated the tumor score, which significantly increased in mice treated with
AOM/DSS (Supplementary Movie S1). In contrast to the AOM/DSS-treated mice, mice given RNPO did not show the increase in tumor scores (Fig. 4E and F). Furthermore, histologically, the colons of AOM/DSS-treated mice possessed high levels of adenoma-carcinoma, which were not observed in mice treated with RNPO (Fig. 4G). This result indicates that oral gavage of RNPO during DSS administration effectively suppressed inflammation, completely preventing colon tumor development.

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3.4. Therapeutic effect of free drinking RNPO against AOM/DSS-induced CAC

Simultaneous administration of RNPO and the inflammation agent DSS led to complete suppression of inflammation and colon tumor progression. The next challenge was to test the ability of RNPO to work after DSS treatment. Here, drinking water was supplemented with different concentrations of RNPO in drinking water (1 mg/mL, 2.5 mg/mL, or 5 mg/mL) starting after the DSS administration and continuing until the end of the experiment, as shown in Fig. 5A. Fig. 5B and C shows a profile of tumor progression during this treatment. As shown in the figure, no significant differences in tumor score were observed in mice treated with 1 mg/mL or 2.5 mg/mL RNPO compared to AOM/DSS-treated mice. In contrast, mice given 5 mg/mL RNPO had significantly reduced tumor scores compared to AOM/DSS-treated mice (Fig. 5B and C). Histological assessments (Fig. 5D) revealed carcinoma tissues in mice treated with 1 mg/mL or 2.5 mg/mL RNPO; however, only adenomas, but not carcinomas, were observed in mice given 5 mg/mL RNPO (Fig. 5D). These results indicate that free drinking RNPO also works effectively to retard tumor growth, even after tumor generation in CAC mice.

3.5. RNPO improves the anticancer efficacy of Iri and reduces its adverse effects

On the basis of above investigation, it is confirmed that our antioxidative strategy based on polymer nanotherapeutics is a robust colon cancer treatment. However, treatment with a single antioxidative agent may not completely cure colon cancer. There are a large number of anticancer drugs that function by versatile mechanisms to eliminate cancer cells. Combination of these anticancer drugs with RNPO is a promising strategy. Iri is used to treat lung, esophageal, gastric, and colon cancers. Iri interacts with cellular DNA topoisomerase I, causing the apoptosis and death of cancer cells. However, Iri efficiency is strongly suppressed by the cancer microenvironment. In particular, oxidative stress in the tumor environment, such as overproduction of ROS and activation of NF-κB increases cancer cell resistance to Iri treatment [33]. Several other groups reported that the use of ROS scavengers to inhibit NF-κB expression enhance anti-tumor effects of Iri in vitro [34–36]. We were interested in examining whether the ROS scavenging effect of RNPO enhanced chemotheraphy of Iri in CAC mice. Here, Iri (0.25 mg/kg, 2.5 mg/kg or 5 mg/kg) was given by oral gavage 5 times per week for 4 weeks, while RNPO (2.5 mg/mL) was given to mice in free drinking water (Fig. 6A). It should be noted that 0.25 mg/kg of Iri is a very low dose treatment that did not suppress tumor growth on its own. A slight (but not significant) combination effect was observed when Iri (0.25 mg/kg) was combined with free drinking
RNPO (2.5 mg/mL) (Fig. 6B). Interestingly, when higher dose of Iri (2.5 mg/kg) was administered in combination with free drinking RNPO, a remarkable suppression of tumor growth was observed in mice treated with combination compared to mice treated with Iri alone (Fig. 6C). Furthermore, combination with Iri (5 mg/kg) and RNPO completely inhibited tumor growth in CAC mice (Fig. 6D and Supplementary Movie 2). This result demonstrates that combination with free drinking RNPO effectively improves the anticancer efficacy of Iri.

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Since that high dose of Iri administration is known to cause severe adverse effects in the GI tract such as diarrhea and intestinal inflammation, we also investigated the efficacy of RNPO against Iri-induced mucositis. Mice receiving Iri alone (50 mg/kg, daily intra-peritoneally injection for 4 d) exhibited severe diarrhea scores, weight loss, and neutropenia. These adverse effects were remarkably reduced in RNPO-treated mice (Fig. 6E and Supplementary Fig. S3). Histological investigation of GI tract organs (duodenum, jejunum, ileum, and colon) showed a remarkable recovery of these tissues in RNPO-treated mice compared to mice treated Iri alone (Fig. 6F). Notably, superoxide levels were also suppressed in mice.
treated with Iri and RNPO compared to mice treated with Iri alone, once again confirming that suitable ROS scavenging at inflammation sites is a robust strategy for tumor treatment (Supplementary Fig. S4). These results indicate that oral administration of RNPO not only significantly enhances the anticancer efficacy of Iri against CAC development, but also effectively suppresses the severe adverse effects of Iri.

4. Discussion

Despite important advances in detection, surgery, and chemotherapy, colon cancer is difficult to treat and has a high mortality rate [37]. Current clinical trials and treatment strategies use single agents and combination strategies, but many of these regimens have severe adverse effects and complicated administration processes [38,39]. For many years, a number of anticancer drugs for colon cancer treatment have been developed and used alone and combination in clinical such as 5–FU, oxaliplatin, leucovorin, bevacizumab and Iri [40,41]. Although they are effective in suppressing development of carcinoma to some extent, they have severe adverse effects that raise significant concerns among both physicians and patients, limiting their use [42].

High doses of drugs are required to achieve sufficient delivery of anticancer drugs to treat CAC. However, high doses are associated with undesirable adverse effects, because almost all LMW drugs tend to metabolize in the upper GI tract or be absorbed into the bloodstream. LMW TEMPO is well-known as a stable nitroxide radical with ability to scavenge ROS. It has been used for therapeutic applications, including antioxidative stress and cancer therapies [43–45]. However, LMW TEMPO spreads to entire body after administration, especially internalizes in healthy cells and even in mitochondria, disturbing normal respiratory system, which causes severe adverse effects to healthy cells. Most of antioxidants investigated so far have the similar issue limiting their clinical application. Our strategy is to covalently install ROS scavenging moiety to large molecular weight chains in order to avoid the possible internalization in healthy cells and mitochondria. For this objective, we have recently developed 2 types of nitroxide radical
containing nanoparticles (RNPs): pH-sensitive RNPN and pH-insensitive RNPO, viz., RNPN disintegrates under acidic environments, while RNPO does not disintegrate regardless of changes in pH. Nitroxide radicals are covalently conjugated to the matrix and confined in the core of these nanoparticles, which shows high biocompatibility, including long-term blood circulation via intravenous administration and low toxicity [46]. In addition, RNPs have been studied as therapies for oxidative stress injuries such as cerebral and renal ischemia reperfusion injuries, hemorrhage [25,47,48] and cancer [32]. Since the pH-disintegrative character of RNPN is not suitable for CAC treatment via oral administration, we have developed an oral nanotherapeutics using RNPO with therapeutic effects against CAC model mice in this study.

Fig. 6. Oral administration of RNPO enhances the anticancer effect of Iri and reduces its side effect on the GI tract. (A) The scheme of AOM/DSS-induced CAC and administration of Iri and RNPO. RNPN (2.5 mg/mL) was given to mice in drinking water, while different doses of Iri (0.25 mg/kg, 2.5 mg/kg, or 5 mg/kg) were given daily by oral gavage 5 times per week for 4 weeks. (B) to (D) combination effect of Iri and RNPO against CAC development was evaluated by assessment of tumor scores. (B) The mice were given 0.25 mg/kg Iri; (C) 2.5 mg/kg Iri; or (D) 5 mg/kg Iri. The data are expressed as mean ± SEM, *P < 0.05, n = 6 mice. (E) and (F) The effect of co-treatment with RNPO to reduce Iri-induced GI toxicity. (E) The diarrhea score and (F) histological assessment of small intestine and colon sections by H&E staining. The data are expressed as mean ± SEM, *P < 0.05 and ***P < 0.001, n = 5 mice. Representative sections are shown for n = 3 mice. Scale bars = 100 μm.
confirmed the safety characteristics of orally administered RNPO\(^\text{G}\) via gavage daily for 1 week, but further investigation is required for the long-term applications. Here, we found that free drinking RNPO\(^\text{G}\) significantly accumulates in GI tract after 1 week administration, especially in the small intestine, cecum and colon regions, while no blood uptake is observed, which prevents undesired adverse effects of nitrooxide radicals to entire body even for the long-term administration (Fig. 2A). After oral administration, RNPO\(^\text{G}\) highly internalizes in cancer tissues compared to healthy colon tissues, resulting in a high therapeutic effect and extremely low GI toxicity with this nanotherapeutics. In fact, even when a high dose of RNPO\(^\text{G}\) was given orally for 1 month, no toxicities were observed in the GI tract or other organs (Fig. 2B and C).

We have previously reported that oral administration of RNPO\(^\text{G}\) highly accumulates in the colonic mucosa and effectively scavenges ROS, leading to suppression of inflammation in mice with DSS-induced colitis without damaging the intestinal microflora\([21,24]\). If RNPO\(^\text{G}\) works against inflammation in CAC via the same mechanism, RNPO\(^\text{G}\) may be an ideal nanomedicine for these types of diseases. As anticipated, we found that oral administration of RNPO\(^\text{G}\) along with DSS treatment clearly suppressed inflammation in the colon, significantly preventing carcinoma progression in the CAC mouse model. It is not surprising because inflammation is an important factor to promote cancer development. Simultaneous administration of RNPO\(^\text{G}\) with DSS protected again the generation of inflammation, resulting in suppression of carcinoma propagation (Fig. 4). It should be rather noted that administration of RNPO\(^\text{G}\) after DSS treatment also effectively suppressed tumor progression in mice given free drinking water with 5 mg/ml RNPO\(^\text{G}\) (Fig. 5). We confirmed that the stability of RNPO\(^\text{G}\) in the GI tract and exposure of nitrooxide radicals inside cancer cells are critical factors for achievement of an effective oral drug for colon cancer therapy (Fig. 3). It has been reported that the decrease of inflammation in the tumor microenvironments prevents activation of NF-kB to result in suppression of resistance of cancer cells from apoptotic tendency\([51]\). Because RNPO\(^\text{G}\) clearly suppressed inflammation around tumor microenvironment, a combination treatment with RNPO\(^\text{G}\) and conventional cancer drugs is a robust strategy. In this study, we investigated the combination of oral RNPO\(^\text{G}\) with Iri and found that the anticancer efficacy was significantly enhanced with the combination compared to treatment with Iri alone (Fig. 6). It is also interesting to note that co-treatment with RNPO\(^\text{G}\) significantly suppressed the adverse effects in GI tract caused by Iri treatment, indicating that a synergistic effect was successfully achieved.

5. Conclusions

In summary, we have developed a novel antioxidative redox nanoparticle, RNPO\(^\text{G}\), possessing capacity to highly accumulate in colon area and selectively internalize in cancer cells. Long-term oral administration of RNPO\(^\text{G}\) exhibited extremely low toxicity due to a lack of RNPO\(^\text{G}\) absorption into the bloodstream and lower uptake by healthy intestinal cells. Oral administration of RNPO\(^\text{G}\) protected against tumor progression and displayed an anticancer therapeutic effect to prevent tumor development in a CAC mouse model. In addition, co-treatment with RNPO\(^\text{G}\) and Iri achieved a significantly enhanced anticancer effect with suppression of the severe adverse effects of Iri on the GI tract. Taken together, our results indicate that the combination of novel antioxidative nanoantitumortherapeutics with conventional anticancer drugs is a strategy for patients-friendly anticancer therapy.

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Appendix A. Supplementary data

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References


