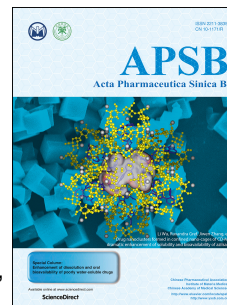


Journal Pre-proof

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ORIGINAL ARTICLE

A cyclodextrin-based nanoformulation achieves co-delivery of ginsenoside Rg3 and quercetin for chemo-immunotherapy in colorectal cancer

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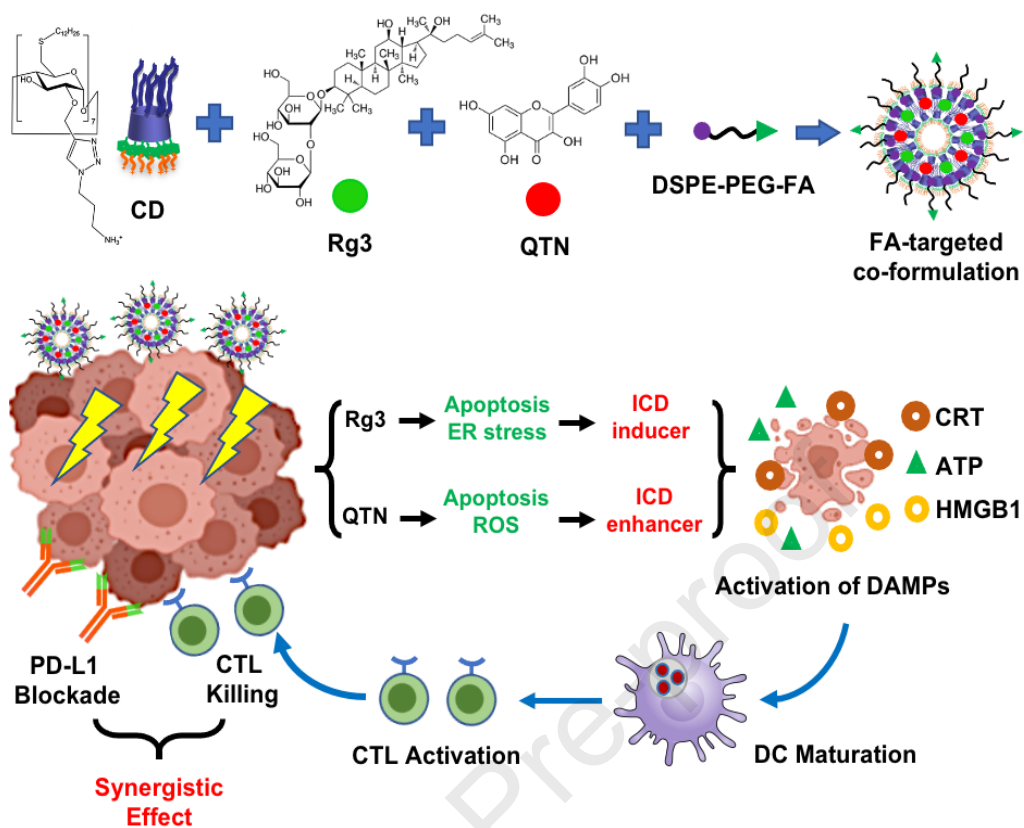
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Running title: A co-formulation achieves chemo-immunotherapy against colorectal cancer



A folate-targeted PEGylated cyclodextrin-based nanoparticle was developed for co-delivery of Rg3 and QTN. The combination of the resultant co-formulation and anti-PD-L1 antibody achieved chemo-immunotherapy for colorectal cancer.

Abbreviations: ATF6, activating transcription factor 6; ATP, adenosine triphosphate; CI, combination index; *CXCL9*, C-X-C motif chemokine 9; *CXCL10*, C-X-C motif chemokine 10; CTLA-4, cytotoxic T lymphocyte antigen 4; CRC, colorectal cancer; CRT, calreticulin; DAMPs, damage-associated molecular patterns; DCs, dendritic cells; ECL, enhanced chemiluminescence; EE, encapsulation efficiency; ER, endoplasmic reticulum; FA, folate; HMGB1, high-mobility group box 1; ICD, immunogenic cell death; *IFN- γ* , interferon-gamma; *IL-4*, interleukin-4; *IL-6*, interleukin-6; *IL-10*, interleukin-10; *IL-12*, interleukin-12; IRE1, inositol-requiring enzyme 1; LC, loading capacity; MDSCs, myeloid derived suppressor cells; MMR, mismatch repair; MR, molar ratio; NAC, *N*-acetyl-L-cysteine; NP, nanoparticle; p-IRE1, phosphorylation of IRE1; p-PERK, phosphorylation of PERK; PD-L1, programmed death-ligand 1; PEG, polyethylene glycol; PERK, PKR-like ER kinase; PFA, paraformaldehyde; PVDF, polyvinylidene fluoride; QTN, quercetin; ROS, reactive oxygen species; TAAs, tumor-associated antigens; TME, tumor microenvironment; UPR, unfolded protein response.

Abstract The immune checkpoint blockade therapy has profoundly revolutionized the field of cancer immunotherapy. However, despite great promise for a variety of cancers, the efficacy of immune checkpoint inhibitors is still low in colorectal cancer (CRC). This is mainly due to the immunosuppressive feature of the tumor microenvironment (TME). Emerging evidence reveals that certain chemotherapeutic drugs induce immunogenic cell death (ICD), demonstrating great potential for remodeling the immunosuppressive TME. In this study, the potential of ginsenoside Rg3 (Rg3) as an ICD inducer against CRC cells was confirmed using *in vitro* and *in vivo* experimental approaches. The ICD efficacy of Rg3 could be significantly enhanced by quercetin (QTN) that elicited reactive oxygen species (ROS). To ameliorate *in vivo* delivery barriers associated with chemotherapeutic drugs, a folate (FA)-targeted polyethylene glycol (PEG)-modified amphiphilic cyclodextrin nanoparticle (NP) was developed for co-encapsulation of Rg3 and QTN. The resultant nanoformulation (CD-PEG-FA.Rg3.QTN) significantly prolonged blood circulation and enhanced tumor targeting in an orthotopic CRC mouse model, resulting in the conversion of immunosuppressive TME. Furthermore, the CD-PEG-FA.Rg3.QTN achieved significantly longer survival of animals in combination with Anti-PD-L1. The study provides a promising strategy for the treatment of CRC.

KEY WORDS Nano drug delivery system; Chemotherapy; Immunotherapy; Combination therapy; Immunogenic cell death; Reactive oxygen species; Tumor microenvironment; Colorectal cancer

1. Introduction

As the third leading cause of cancer-related deaths worldwide (*e.g.*, 881,000 deaths estimated in 2018)¹, colorectal cancer (CRC, a cancer of the colon or rectum) is an obvious disease burden requiring effective, safe and widely-applicable treatments. Recent research in cancer immunology has led to the development of different immunotherapeutic strategies²⁻⁴. Among these, strategies that exert the blockade of immune checkpoint pathways [*e.g.*, cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death-ligand 1 (PD-L1)] have achieved favorable outcomes in a range of solid tumors⁵. However, it has been reported that only a minority of patients (up to 15% of the CRC population), who are identified with mismatch repair (MMR)-deficient CRC, respond positively to immune checkpoint blockade therapy⁶, while the response rate remains low in MMR-proficient CRC patients⁷. This failure is strongly attributed to the immunosuppressive feature of the tumor microenvironment (TME)⁸. Therefore, approaches designed to reprogram the TME may improve the therapeutic efficacy of immune checkpoint inhibitors⁹, potentially providing therapeutic benefit for the wide spectrum of CRC patients.

Immunogenic cell death (ICD) is characterized as immunogenic apoptosis that activates damage-associated molecular patterns (DAMPs) in dying or dead tumor cells in response to certain stimuli¹⁰. DAMPs as danger signals activate dendritic cells (DCs) for the presentation of tumor-associated antigens (TAAs), which subsequently induce T cell-mediated immunological responses against living tumor cells of the same kind¹⁰. The concept of ICD has revolutionized the traditional view of chemotherapeutic agents that are considered cytotoxic and poorly immunogenic. For example, chemotherapeutic drugs such as anthracyclines¹¹, oxaliplatin¹², bortezomib¹³ and cardiac glycosides¹⁴ have been identified as the putative ICD inducers. Recently, evaluation of chemotherapeutics as potential ICD inducers has gained increasing attention. In addition, ICD is often concomitant with the production of reactive oxygen species (ROS)¹⁰, and the efficacy of ICD may be enhanced by ROS-inducing strategies¹⁵⁻¹⁷. Therefore, it is hypothesized that a combination of ICD- and

ROS-inducing strategies will mediate the remodeling of immunosuppressive TME and achieve synergistic immunotherapeutic efficacy with immune checkpoint blockade.

Ginsenosides are a group of naturally occurring chemicals within the extract of ginseng (a traditional medicine with a long history of human use¹⁸). One of the well-studied ginsenosides, ginsenoside Rg3 has demonstrated different pharmacological effects¹⁹⁻²². The difference is the stereocenter on the C₂₀ of Rg3 generates two epimers namely 20(*S*)- and 20(*R*)-Rg3. They both exhibit cardio-protective functions, promote antitumor effects, and mediate immunological responses, albeit to different extents²³. In this study, the role of 20(*S*)-Rg3 (thereafter referred as Rg3, Fig. 1) as a potential ICD inducer was confirmed, to the best of our knowledge, for the first time using accepted and validated experimental approaches for the identification of ICD agents²⁴. In addition, quercetin (thereafter referred as QTN, Fig. 1) is a natural pigment (flavonoid), and has obtained wide attention in cancer treatment and prevention²⁵. In this study, ROS was effectively generated by QTN, which enhanced the activity of Rg3-mediated ICD. When Rg3 and QTN were co-formulated in a targeted amphiphilic cyclodextrin NP, they exerted chemo-immunotherapeutic effects in an orthotopic CRC mouse model, significantly improving the survival of animals when combined with Anti-PD-L1.

2. Materials and methods

2.1. Materials

20(*S*)-Ginsenoside-Rg3 (Cat. No. DST180521-028) and quercetin (Cat. No. DST180130-011) were purchased from DESITE Biotech (Chengdu, China). DSPE-mPEG₂₀₀₀ (Cat. No. C12251) was obtained from Xi'an Biological Technology Co., Ltd., and DSPE-PEG₂₀₀₀-Folate (Cat. No. C09235) were obtained from Xi'an Biological Technology Co., Ltd. (Xi'an, China). The other chemicals and reagents were purchased from Sigma–Aldrich unless mentioned otherwise. In addition, the amphiphilic cationic cyclodextrin (Fig. 5A) was produced as previously reported in our laboratories²⁶.

2.2. Cell culture

CT26 (mouse CRC cell line) and HCT116 (human CRC cell line) were maintained within RPMI-1640 (Corning) with 10% fetal bovine serum (FBS; Corning) and 1%

Penicillin-Streptomycin (TransGen Biotech, Beijing, China). CT26-Luc (mouse CRC cell line, stably expressing luciferase) was maintained within RPMI-1640 with 10% FBS, 1% Penicillin-Streptomycin, and 1 $\mu\text{g}/\text{mL}$ puromycin (ThermoFisher). DC2.4 cells (mouse dendritic cell line) were maintained within RPMI-1640 with 10% FBS, 1 \times nonessential amino acids, 1 \times HEPES buffer, and 5.4 mmol/L β -mercaptoethanol. All cells were maintained at 37 $^{\circ}\text{C}$ with 5% CO_2 and 95% relative humidity.

2.3. Animals

Six to eight-week old female BALB/c and nude mice were obtained from Changchun Institute of Biological Products, China. The experiments have been approved by the Animal Ethics Committee of Jilin University. Mice were allowed to acclimate for at least 7 days in the housing facility before the experiment. Animals were group-housed (5 animals per cage) in standard conditions (room temperature of 22 ± 2 $^{\circ}\text{C}$ under a 12 h light/dark cycle: lights on at 08:00 and lights off at 20:00). All mice received food and water *ad libitum*.

2.4. Induction of immunogenic cell death by ginsenoside Rg3

The *in vitro* cytotoxicity of Rg3 was determined using MTT assay. CT26 and HCT116 cells (1×10^4 per well) were seeded within 96-well plates for one day, respectively. Subsequently, Rg3 ($[c] = 1, 5, 10, 20, 40, 80, 100, 120$ and 150 $\mu\text{mol}/\text{L}$) was added to cells for 24 h. Cells were then added with MTT reagent (5 mg/mL in PBS) at 37 $^{\circ}\text{C}$ for ~ 4 h, and the purple precipitate was dissolved by DMSO before measurement at 570 nm. IC_{50} was calculated using the GraphPad Prism software.

The *in vitro* apoptosis of Rg3 was assessed using flow cytometry (Becton Dickinson, FACSCalibur, NJ, USA). CT26 and HCT116 cells (2×10^5 per well) were seeded in 6-well plates for one day, respectively. After this, Rg3 ($[c] = 30$ $\mu\text{mol}/\text{L}$) was added to cells for 6, 12 and 24 h. Subsequently, apoptotic cells (%) were detected using Annexin V-FITC/propidium iodide assay (YEASEN Biotech, Shanghai, China) by BD FACSCalibur.

The activity of UPR signaling pathways was evaluated using Western blotting assay. CT26 and HCT116 cells (2×10^5 per well) were seeded in 6-well plates for one day, respectively. After this, Rg3 ($[c] = 30$ $\mu\text{mol}/\text{L}$) was added to cells for 6, 12 and 24 h. Subsequently, proteins were extracted from cells using ProteinExt[®] Mammalian Total

Protein Extraction Kit (TransGen Biotech) and qualified using BCA assay (Beyotime Biotech, Beijing, China). Proteins (35 to 50 μg per sample) were loaded onto the SDS-polyacrylamide gel and run at 80–100 V for 30–60 min. Proteins were then transferred to polyvinylidene fluoride (PVDF) membrane (Invitrogen) at 90 V for 1 h. The membrane was incubated overnight with antibodies (Supporting Information Table S1) at 4 °C. The secondary antibody (Table S1) was added to the membrane for 1.5 h. Proteins were detected using the enhanced chemiluminescence (ECL) solution (GE Healthcare). Quantification of protein bands was performed using densitometry (ImageJ), and all results were normalized to β -Actin.

The exposure of CRT was detected using immunofluorescent staining assay. CT26 and HCT116 cells (1×10^5 per well) were seeded in 24-well plates with glass bottoms for one day, respectively. Rg3 ($[c] = 30 \mu\text{mol/L}$) was added to cells for 6 h. After this, cells were incubated with 0.25% paraformaldehyde (PFA) for 5 min. Cells were then washed with PBS, which were followed by anti-CRT antibody (Table S1) for 1 h. After PBS washes, FITC-conjugated secondary antibody (Table S1) was added for 30 min. Subsequently, cells were treated by 4% PFA for 20 min and stained using DAPI (Beyotime Biotech) for confocal imaging (OLYMPUS, Olympus FV3000, Tokyo, Japan).

The secretion of ATP and release of HMGB1 were assessed using bioluminescent and ELISA assays. CT26 and HCT116 cells (5×10^5 per well) were seeded in 6-well plates for one day. Rg3 ($[c] = 30 \mu\text{mol/L}$) was added to cells for 12 h. Subsequently, the level of ATP and HMGB1 in the supernatants was measured using ENLITEN[®] ATP Assay System Bioluminescence Detection Kit (Promega) and ELISA kit (LS-F11641 and LS-F11642, LifeSpan BioSciences), respectively.

The maturation of DC2.4 cells was determined using flow cytometry (Becton Dickinson). CT26 (5×10^5 per well) were seeded in 6-well plates for one day. After this, Rg3 ($[c] = 30 \mu\text{mol/L}$) was added to cells for one day. Separately, DC2.4 cells (2×10^5 per well) were seeded in 6-well plates. Following 24 h, the supernatants were collected from Rg3-treated CT26 cells and transferred to DC2.4 cells for another 24 h. DC2.4 cells were treated with fluorophore-conjugated antibodies (Table S1), and the expression (%) of CD11c and CD86 was assessed using by BD FACSCalibur (Becton Dickinson).

The *in vivo* vaccination assay was performed as previously described²⁷. Briefly, 3×10^6 CT26 cells, either treated with DMSO, freeze-thawing three times on dry ice, or 30 $\mu\text{mol/L}$ Rg3 for 12 h, were s.c. implanted into the right flank of BALB/c mice or nude mice. One week later, 3×10^5 CT26 cells were s.c. implanted into the left flank. Tumor development in left flank was monitored to determine tumor-free mice.

2.5. Generation of reactive oxygen species by quercetin

The *in vitro* cytotoxicity of QTN was determined using MTT assay. CT26 and HCT116 cells (1×10^4 per well) were seeded within 96-well plates for one day, respectively. Subsequently, QTN ($[c] = 1, 5, 10, 20, 40, 80, 100, 120$ and $150 \mu\text{mol/L}$) was added to cells for 24 h. Cells were then added with MTT reagent at 37°C for ~ 4 h, and the purple precipitate was dissolved by DMSO before measurement at 570 nm. IC_{50} was calculated using the GraphPad Prism software. In a separate study, following seeding, cells were treated with or without *N*-acetylcysteine (NAC; 5 mmol/L) for 4 h. Subsequently, QTN ($[c] = 80 \mu\text{mol/L}$) was added into cells for 24 h, and cytotoxicity was measured as mentioned above.

The *in vitro* apoptosis of QTN was assessed using flow cytometry (Becton Dickinson). CT26 and HCT116 cells (2×10^5 per well) were seeded in 6-well plates for one day, respectively. After this, cells were treated with or without NAC (5 mmol/L) for 4 h. Subsequently, QTN ($[c] = 80 \mu\text{mol/L}$) was added to cells for 24 h, and apoptotic cells (%) were detected as mentioned above.

The activity of BCL-2/BAX/caspase 9/caspase 3 signaling pathways was evaluated using Western blotting assay. CT26 and HCT116 cells (2×10^5 per well) were seeded in 6-well plates for one day, respectively. After this, QTN ($[c] = 80 \mu\text{mol/L}$) was added to cells for 6, 12 and 24 h. The Western blotting assay was performed as mentioned above. Quantification of protein bands was performed using densitometry (ImageJ), and all results were normalized to β -actin.

To measure the formation of ROS, CT26 and HCT116 cells (2×10^5 per well) were seeded in 6-well plates for one day, respectively. After this, QTN ($[c] = 80 \mu\text{mol/L}$) was added to cells for 6, 12 and 24 h. The ROS level in cells was detected using 2',7'-dichlorodihydrofluorescein diacetate-based Reactive Oxygen Species Assay Kit (YEASEN Biotech) by BD FACSCalibur (Becton Dickinson) (488 nm/525 nm).

2.6. *In vitro* effects of ginsenoside Rg3 and quercetin

The *in vitro* cytotoxicity of “Rg3 + QTN” was determined using MTT assay. CT26 cells (1×10^4 per well) were seeded within 96-well plates for one day, respectively. Subsequently, “Rg3 + QTN” at different molar ratios (MR = 1:5, 1:2, 1:1, 2:1 and 5:1) was added to cells for 24 h, and IC_{50} was measured as mentioned above.

The *in vitro* apoptosis of “Rg3 + QTN” was assessed using flow cytometry (Becton Dickinson). CT26 (2×10^5 per well) were seeded in 6-well plates for one day, respectively. After this, cells were added with either single drugs or “Rg3 + QTN” ([c] = 12 and 12 $\mu\text{mol/L}$, respectively) for 24 h, and apoptotic cells (%) were detected as mentioned above.

The exposure of CRT was detected using immunofluorescent staining assay. CT26 (1×10^5 per well) were seeded in 24-well plates with glass bottoms for one day, respectively. Cells were then treated with or without NAC (5 mmol/L) for 4 h. Subsequently, cells were added with either single drugs or “Rg3 + QTN” ([c] = 12 and 12 $\mu\text{mol/L}$, respectively) for 6 h. The exposure of CRT was detected as mentioned above.

The secretion of ATP and release of HMGB1 were assessed using bioluminescent and ELISA assays. CT26 cells (5×10^5 per well) were seeded in 6-well plates for one day. Cells were then treated with or without NAC (5 mmol/L) for 4 h. Subsequently, cells were added with either single drugs or “Rg3 + QTN” ([c] = 12 and 12 $\mu\text{mol/L}$, respectively) for 12 h. The level of ATP and HMGB1 in the supernatants was measured as mentioned above.

The maturation of DC2.4 cells was determined using flow cytometry (Becton Dickinson). CT26 (5×10^5 per well) were seeded in 6-well plates for one day. After this, cells were then treated with or without NAC (5 mmol/L) for 4 h. Subsequently, “Rg3 + QTN” ([c] = 12 and 12 $\mu\text{mol/L}$, respectively) was added to cells for one day. Following 24 h, the supernatants were collected from Rg3-treated CT26 cells and transferred to DC2.4 cells for another 24 h. The expression (%) of CD11c and CD86 was assessed as mentioned above.

2.7. Preparation and characterization of co-formulations

The preparation has been optimized in terms of encapsulation efficiency (EE%) and

loading capacity (LC%) as follows: 2 mg of CD, 0.5 mg of Rg3 and 0.2 mg of QTN were dissolved in 5 mL chloroform (CHCl₃) in a round bottom flask and dried to form thin film using rotary evaporator. Subsequently, the thin film was rehydrated using 5 mL of ultrapure water, sonicated at 0 °C for 30 min, and incubated by slight shaking at 37 °C for 1–1.5 h, in order to obtain the CD.Rg3.QTN complex. In addition, a solution of DSPE-mPEG₂₀₀₀ and DSPE-mPEG₂₀₀₀-Folate (MR, ~4:1, 0.5 mg/mL) was prepared in 20 mmol/L HEPES buffer (pH = 7.4) at 60 °C with shaking for 15 min. As previously described^{28,29}, the “postinsertion” of DSPE-mPEG₂₀₀₀/DSPE-mPEG₂₀₀₀-folate into preformed CD. Rg3.QTN complex was carried out at 60 °C with shaking for 1 h. As a result, ~1.5% (mol/mol) of FA on the outer surface per formulation was achieved. Non-targeted co-formulation was produced as mentioned above without the use of DSPE-mPEG₂₀₀₀-folate. In addition, the rhodamine-labelled formulations were prepared as described above containing ~0.02% (w/w) of rhodamine.

The EE% and LC% were assessed using HPLC (Shimadzu, SPD-20A, Kyoto, Japan) [C18 column; UV at 203 nm for Rg3, mobile phase = acetonitrile and water (0.05% phosphoric acid), 50:50; UV at 370 nm for QTN, mobile phase = methanol and water (0.2% phosphoric acid), 70:30]. As EE% was > 95% for both drugs, co-formulations were used for *in vitro* and *in vivo* experiments without further purification as shown in Eqs. (1) and (2).

$$EE (\%) = (\text{Weight of encapsulated drug} / \text{Weight of added drug}) \times 100 \quad (1)$$

$$LC (\%) = (\text{Weight of encapsulated drugs} / \text{Weight of nanoparticles}) \times 100 \quad (2)$$

The particle size and zeta potential were measured using Malvern Nano-ZS as described previously³⁰. The morphology of NPs was observed using TEM (Jeol, JEOL JEM1230, Tokyo, Japan) as described previously³¹. Briefly, ~5 μL of samples were added to a 400-mesh carbon-filmed copper grid and stained using 2% (w/w) uranyl acetate before TEM analysis. Moreover, co-formulations containing 250 μg and 100 μg QTN (MR = 1:1) in 0.01 mol/L PBS (pH = 5.5 and 7.4) was placed within the dialysis bag (MWCO = 2 kDa; Solarbio®, Beijing, China). The dialysis bag was incubated at 37 °C within the release medium (0.5% Tween-80 0.01 mol/L PBS solution). Samples were collected at different time points (the release medium with

the same volume was supplemented), and the concentration of free drugs was determined using HPLC (Shimadzu) as mentioned above.

2.8. *In vitro* studies of co-formulations

The cellular uptake of co-formulations (containing 0.05% Rhodamine, *w/w*) was assessed using confocal microscopy (Olympus) and flow cytometry (Becton Dickinson). CT26 and HCT116 cells (1×10^5 per well) were seeded in 24-well plates with glass bottoms for one day, respectively. Rhodamine-containing co-formulations (12 $\mu\text{mol/L}$ Rg3 and 12 $\mu\text{mol/L}$ QTN) were added to cells for 6 h. After this, cells were treated by 4% PFA for 20 min and stained using DAPI (Beyotime Biotech) for confocal imaging (Olympus). In addition, CT26 and HCT116 cells (1×10^5 per well) were seeded in 24-well plates for one day, respectively. Rhodamine-containing co-formulations (12 $\mu\text{mol/L}$ Rg3 and 12 $\mu\text{mol/L}$ QTN) were added to cells for 4 h. Subsequently, rhodamine-positive cells (%) were detected using BD FACSCalibur.

The *in vitro* cytotoxic, antiproliferative and antimetastatic activities of co-formulations were determined using MTT, scratch and colony formation assays, respectively. CT26 and HCT116 cells (1×10^4 per well) were seeded within 96-well plates for one day, respectively. Subsequently, co-formulations (12 $\mu\text{mol/L}$ Rg3 and 12 $\mu\text{mol/L}$ QTN) were added to cells for 24 h, and IC_{50} was measured as mentioned above. In addition, the *in vitro* scratch assay was carried out as previously described³². Briefly, when CT26 and HCT116 cells reached confluence, the cell monolayer was washed thoroughly with PBS, scraped with a p200 pipette tip to create a “scratch”, and washed again with PBS. Cells were replaced with serum-free growth medium and added with co-formulations (12 $\mu\text{mol/L}$ Rg3 and 12 $\mu\text{mol/L}$ QTN) for 12 h. The cell-free areas before and after the incubation of co-formulations were imaged under the microscope and measured using ImageJ. Furthermore, the *in vitro* colony formation assay was performed as previously described³³. Briefly, CT26 and HCT116 cells seeded in 6-well plates with 30-50% confluence were treated with co-formulations (12 $\mu\text{mol/L}$ Rg3 and 12 $\mu\text{mol/L}$ QTN) for 4 weeks. The colonies were stained with 0.2% crystal violet and counted under the microscope (OLYMPUS, Olympus CK2, Tokyo, Japan).

2.9. *In vivo* toxicity, pharmacokinetics and biodistribution of co-formulations

Healthy BALB/c mice were treated with co-formulations as described in Fig. 7A ($n =$

5). Body weight was regularly recorded. In addition, major organs, the whole blood and the serum were obtained on Day 30 to analyze histopathology, myelosuppression, and hepatic/renal functions as previously described (Fig. 7B–D)^{33,34}.

Following the procedures as previously described³⁴, the cecum wall of BALB/c mice was injected with 5×10^5 CT26-Luc cells to establish orthotopic CRC mouse model. After the inoculation (Day 0), 100 μ L of luciferin (10 mg/mL; Pierce) were intraperitoneally (i.p.) administrated to animals, and tumor progression was monitored using IVIS[®] *In Vivo* Optical System (Perkin Elmer). When tumor was developed to ~ 5 to 10×10^8 p/s/cm²/sr, mice were used for pharmacokinetics and tissue distribution: 1) Co-formulations containing 10 mg/kg of Rg3 and 4 mg/kg of QTN were i.v. administrated, and the blood (~ 50 μ L) was collected at different time points (Fig. 7E, $n = 4$). Drugs in the plasma were extracted with ethyl acetate, dried with nitrogen, and reconstituted in the mobile phase for HPLC (Shimadzu). Half-life was evaluated using DAS 2.0 software. 2) Co-formulations containing $\sim 0.05\%$ (w/w) of DiD (ThermoFisher), 10 mg/kg of Rg3 and 4 mg/kg of QTN were i.v. injected to animals, and biodistribution was detected (640 nm/670 nm) using IVIS[®] *In Vivo* Optical System (PerkinElmer, IVIS Kinetic, MA, USA) ($n = 4$).

2.10. Combination of targeted co-formulation and anti-PD-L1 for CRC therapy

When tumor was developed to ~ 5 to 10×10^8 p/s/cm²/sr, as described in Fig. 8A, mice ($n = 5$) were treated with either Anti-PD-L1 (Bioxcell, clone 10F.9G2, 100 μ g per mouse, i.p.), targeted co-formulation (10 mg/kg of Rg3 and 4 mg/kg of QTN per mouse, i.v.), or the combination. Tumor progression was monitored using IVIS[®] *In Vivo* Optical System (PerkinElmer).

Separately, 2 days after two injections (Day 20; the timepoint chosen to analyze immunological effects was generally within one week following treatment)³⁵⁻³⁹, tumors were collected for following studies: 1) Apoptosis. Tumors ($n = 3$) were fixed with 4% PFA, conducted on paraffin-embedded slides, and permeabilized^{33,34}. DNA fragments were detected using the TransDetect[®] Fluorescein TUNEL Cell Apoptosis Kit (TransGen Biotech), and nuclei were stained with DAPI (Beyotime Biotech), for confocal microscopic analysis (Olympus); 2) Measurement of immune cells. Single cells from tumors ($n = 4$) were generated using collagenase A (1 mg/mL; Sigma) and DNase (200 μ g/mL; Invitrogen)^{33,34}. After removal of red blood cells using the ACK

buffer (Gibco), cells were incubated with fluorophore-labeled antibodies (Table S1), fixed using 4% PFA, and analyzed using BD FACSCalibur. 3) Measurement of cytokines and chemokines. Tumors ($n = 4$) were homogenized within TriZol Up reagent (TransGen Biotech) using the tissue grinder (Scientz, Zhengjiang, China). The homogenates collect the supernatant for RT-PCR. First-strand cDNA was generated using the TransScript[®] First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech). Quantitative real-time RT-PCR was performed using TransStart[®] Top Green qPCR SuperMix kit (TransGen Biotech) by StepOnePlus[™] Real-Time PCR System (ThermoFisher, QuantStudio 3, MA, USA). The reaction was carried out: 94 °C for 30 s, 45 cycles of 5 s at 94 °C, and 30 s at 60 °C. The primers were listed in Supporting Information Table S2.

The depletion study of T cells was carried out as previously reported^{33,34}. Briefly, 100 µg of either anti-CD4 (clone GK1.5, Bioxcell), anti-CD8 (clone 53-6.72, Bioxcell) or IgG (polyclonal, Bioxcell) antibodies were i.p. given per mouse (Fig. 8H) before the treatment of targeted co-formulation (10 mg/kg of Rg3 and 4 mg/kg of QTN). Tumor progression was monitored using IVIS[®] *In Vivo* Optical System (PerkinElmer) ($n = 4$).

2.11. Statistical analysis

GraphPad prism software was applied for statistical analysis. Results were exhibited as mean \pm standard deviation (SD). The significance between two groups was assessed using unpaired Student's *t*-test. The significance between three or more groups was assessed using the one-way ANOVA (Bonferroni's correction). Kaplan-Meier survival analysis with log-rank Mantel-Cox test was used to determine the overall survival rate and tumor free rate. The combination index (CI) was determined as previously described⁴⁰, $CI < 1$ suggests the synergistic effect. In this work, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Ginsenoside Rg3 induces immunogenic cell death in CRC cells

The cytotoxicity (IC_{50}) of Rg3 was assessed in mouse CT26 and human HCT116 CRC cell lines using the MTT assay (Fig. 2A). The antiproliferative effect of Rg3 was similar in two cell lines, with $IC_{50} \sim 32$ µmol/L for CT26 (24 h incubation) and IC_{50}

~30 $\mu\text{mol/L}$ for HCT116 (24 h incubation) (Fig. 2A). Flow cytometry (Becton Dickinson) results show that Rg3 induced apoptosis in a time-dependent manner, causing ~40% and ~35% apoptosis (24 h incubation) in CT26 and HCT116 cell lines, respectively (Fig. 2B). These results indicate that Rg3-elicited apoptosis is the mechanism for inhibition of CRC cell growth.

The potential of Rg3 as the ICD inducer was assessed in CT26 and HCT116 cell lines. The induction of ICD is critically linked to two types of stress, namely endoplasmic reticulum (ER) stress and autophagy^{41,42}. When cancer cells undergo the ER stress, the unfolded protein response (UPR) signaling pathways including inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6) are activated⁴³. As shown in Fig. 2C, the phosphorylation of IRE1 protein (p-IRE1) was upregulated by Rg3, and the expression of p-IRE1 relative to total IRE1 protein was significantly increased ($P<0.001$) in two cell lines (Supporting Information Fig. S1). Rg3 also mediated two downstream effects associated with the IRE1 signaling pathway, namely the downregulation of BCL-2 protein and the upregulation of BAX protein (Figs. 2C and Fig. S1). In addition, the phosphorylation of PERK protein (p-PERK) was induced by Rg3, and the expression of p-PERK relative to total PERK protein was significantly enhanced ($P<0.05$ and $P<0.01$) in CT26 and HCT116 cells (Figs. 2C and Fig. S1). Accordingly, the cleavage of Caspase 9 and Caspase 3 (c-Caspase 9 and c-Caspase 3, two downstream proteins of the PERK signaling pathway) was significantly upregulated ($P<0.01$ and $P<0.001$; Fig. 2C and Fig. S1). Furthermore, the expression of ATF6 protein and the cleavage of Caspase 4 (c-Caspase 4, one downstream protein of the ATF6 signaling pathway) were significantly ($P<0.05$, $P<0.01$ and $P<0.001$) enhanced by Rg3 in CT26 and HCT116 cells (Figs. 2C and Fig. S1). When autophagy (or autophagocytosis, a natural process for removal of cytoplasmic components⁴⁴) proceeds in cancer cells, autophagosomes, a double-membrane vesicle, transport unnecessary or dysfunctional components into lysosomes for degradation⁴⁵. The microtubule-associated protein light chain 3 (LC3) is an essential protein associated with autophagosome biogenesis, and has been confirmed as the most widely used marker for the autophagy pathway⁴⁶. The formation of autophagosomes is evident with the transformation of LC3-I (a cytosolic form of LC3) into LC3-II (a membrane-bound form of LC3)⁴⁷. As shown in Figs. 2C and Fig. S1, the ratio of LC3-II/LC3-I was

significantly ($P<0.05$) increased by Rg3. Therefore, results in Fig. 2C indicate that ER stress and autophagy, two types of stress necessary for the ICD induction, were effectively induced by Rg3 in CRC cells.

Endogenous DAMPs are activated in cancer cells during ICD in response to ER stress and autophagy. The exposure of calreticulin (CRT, a protein mainly residing in the endoplasmic reticulum), secretion of adenosine triphosphate (ATP) and release of high-mobility group box 1 (HMGB1, a nuclear protein) are three essential hallmarks that can be used to accurately predict the ICD potential of candidate drugs⁴⁸. In response to the ER stress, CRT, a protein located inside the ER lumen, is translocated onto the plasma membrane of pre-apoptotic cancer cells⁴⁹. During apoptosis, autophagy is required for ATP secretion from dying cells into the extracellular milieu⁴². At the late stage of apoptosis, HMGB1, a non-histone chromatin protein, is released from the nucleus into the extracellular environment⁵⁰. The capacity of Rg3 to activate these ICD hallmarks was assessed in CT26 and HCT116 cells (Fig. 2D). Results indicate that the exposure of CRT, secretion of ATP and release of HMGB1 were significantly ($P<0.05$) activated following treatment with Rg3 (Fig. 2D), suggesting that Rg3 could elicit the apoptosis, ER stress and autophagy.

DAMPs can activate the transition of immature DCs to a mature phenotype⁵¹. When DC2.4 cells (mouse DCs) were challenged with PBS and the growth medium used for DMSO-treated or Rg3-treated CT26 cells, the number of CD11c⁺ and CD86⁺ population (defined as mature DCs⁵²⁻⁵⁴) was measured using flow cytometry (Becton Dickinson). Results show that no significant difference in the number of CD11c⁺ and CD86⁺ cells was found between the DMSO-treated group (Fig. 2E) and the PBS group (data not shown). In contrast, DC maturation was significantly ($P<0.01$) increased in the Rg3-treated group (Fig. 2E), indicating that Rg3-induced cell death was immunogenic, and could promote the mature status of DCs.

Tumor cells undergoing ICD *in vitro* can be used as the vaccine to induce T cell-mediated antitumor immunity against living tumor cells of the same kind *in vivo*²⁴. Thus, the *in vivo* vaccination assay was performed to further identify the ICD potential of Rg3 (Fig. 2F). CT26 cells were pre-treated with either DMSO, freeze-thawing or Rg3. Subsequently, these cells were subcutaneously (s.c.) injected into the right flank of immunocompetent mice. The same animals were s.c. injected with living CT26 cells at the left flank one week later. Consequently, mice injected with DMSO-treated or freeze-thawed cells succumbed to the rechallenging of living CT26 cells, and tumor

growth was found in all animals within 15 days (Fig. 2F). In contrast, inoculation of mice with CT26 cells treated by Rg3 significantly prevented subsequent growth of living CT26 cells ($P < 0.001$) (Fig. 2F). As a confirmatory study, nude mice (immunodeficient), which are characterized with a lack of functional T cells, were used for the vaccination assay. As a result, the antitumor effect arising from Rg3-treated CT26 cells was significantly abolished in nude mice (Fig. 2F). These results suggest that Rg3 was able to convert the CRC cells into the endogenous vaccine, which may mediate the maturation of DCs to activate T cells against live cells of the same type. Taken together, results in Fig. 2 confirmed the potential of Rg3 as the ICD inducer against CRC.

3.2. Quercetin causes reactive oxygen species in CRC cells

The cytotoxicity (IC_{50}) of QTN was assessed in CT26 and HCT116 cell lines using the MTT assay (Fig. 3A). QTN led to similar antiproliferative effect in the two cell lines, with $IC_{50} \sim 80 \mu\text{mol/L}$ for CT26 (24 h incubation) and $IC_{50} \sim 81 \mu\text{mol/L}$ for HCT116 (24 h incubation) (Fig. 3A). QTN also caused $\sim 70\%$ and $\sim 50\%$ apoptosis (24 h incubation) in CT26 and HCT116 cells, respectively (Fig. 3B). The apoptotic effects of QTN were confirmed by the downregulation of BCL-2 (an apoptosis-suppressing protein) and the upregulation of BAX (an apoptosis-regulating protein), caspase 9, and caspase 3 (Figs. 3C and Supporting Information Fig. S2). Thus, QTN-induced apoptosis is the main mechanism for the inhibition of CRC cell growth.

The BCL-2/BAX/caspase 9/caspase 3 signaling pathways are closely linked with the formation of ROS (one of apoptotic stimuli)⁵⁵. The level of ROS in two CRC cell lines was measured using a bioluminescent assay, showing that QTN generated ROS in a time-dependent manner (Fig. 3D). To investigate the role of QTN-mediated ROS in antitumor effects, CRC cells were treated with *N*-acetyl-L-cysteine (NAC, a chemical used for the neutralization of ROS^{56,57}) prior to the treatment of QTN (Fig. 3E). Results show that QTN-mediated cytotoxicity and apoptosis were significantly ($P < 0.05$ and $P < 0.01$) impeded by the pretreatment of NAC (Fig. 3E and F), suggesting that QTN could induce the formation of ROS for antiproliferative and apoptotic effects in CRC cells.

3.3. Combination of quercetin and ginsenoside Rg3 shows a synergistic effect in CRC cells

The concomitant activation of ER stress and ROS production is critical for the activity of DAMPs⁵⁸, and the efficacy of ICD may be effectively improved by ROS-inducing strategies¹⁵⁻¹⁷. MTT results show that combining the two drugs achieved a synergistic antiproliferative effect (Fig. 4A). When the molar ratio (MR) of the two drugs was optimized at 1:1 (Figs. 4A and Supporting Information Fig. S3), the IC₅₀ of the combined QTN and Rg3 regimen was reduced to ~12 µmol/L and (Fig. 4A), which was significantly ($P<0.05$) lower compared to results following exposure to the individual drugs (IC₅₀ of QTN ~80 µmol/L and IC₅₀ of Rg3 ~32 µmol/L) (Figs. 2A and 3A). The combined regimen also significantly ($P<0.01$) enhanced the apoptotic effects (~80%, 24 h incubation) in CT26 cells as compared to either QTN (~20%) or Rg3 (~30%) (Fig. 4B). Similar cytotoxic and apoptotic effects in HCT116 cells, were also recorded in the CT26 cells. These results indicate that a synergistic antitumor effect was achieved by the combination of QTN and Rg3.

The synergistic ICD effects of QTN and Rg3 were assessed in CT26 cells, as this cell line was chosen to establish the tumor-bearing mouse model for analysis of the *in vivo* immunological response. As shown in Fig. 4C, the exposure of CRT onto the cell membrane was not achieved by treatment with either DMSO or QTN, in contrast Rg3 significantly ($P<0.05$) triggered the translocation of CRT. Notably, the combination further ($P<0.01$) activated the CRT exposure (Fig. 4C). In addition, the combined regimen significantly ($P<0.01$ and $P<0.001$) elicited the secretion of ATP and the release of HMGB1 in CT26 cells (Fig. 4D and E) and triggered the maturation (CD11c⁺ and CD86⁺) of DCs (Fig. 4F) relative to single drugs. It is worth noting that the pretreatment of NAC significantly ($P<0.05$ and $P<0.01$) dampened the activity of ICD hallmarks and the maturation of DCs (Fig. 4C–F), suggesting that the ICD efficacy of Rg3 in the CRC cells was significantly enhanced by QTN and that this synergy was, at least, in part due to the production of ROS.

3.4. Preparation and physicochemical characterization of co-formulations

The *in vivo* application of chemotherapeutic agents is seriously impeded by low solubility, poor pharmacokinetics, and non-specific tissue distribution. Recently, development of nano delivery systems has achieved high bioavailability, controlled drug release, prolonged systematic circulation, and improved tumor distribution for chemotherapeutic agents^{33,34,59,60}. In addition, Rg3 and QTN possess distinctive physicochemical characteristics (*e.g.*, the log P of Rg3 and QTN \approx 2.63 and 1.81, and

the molecular weight of Rg3 = 785 and 302), therefore, a nano delivery system is desirable for co-delivery of two drugs in order to achieve synergistic effects. However, to our best knowledge, no study has been reported for co-encapsulation of Rg3 and QTN in a nanoformulation for CRC.

Previously, a range of cyclodextrin-based NPs have been generated and characterized using a variety of *in vitro* and *in vivo* models^{28,61-65}. Among these functionalized cyclodextrins, an amphiphilic cationic β -cyclodextrin (thereafter referred as CD, Fig. 5A) has demonstrated high gene delivery efficacy^{28,63-65} and low levels of cytotoxicity and immunotoxicity⁶⁶. In this study, a FA-targeted PEG-modified CD-based nanoformulation was developed for co-encapsulation of Rg3 and QTN (Fig. 5A). The FA-targeted co-formulation (CD-PEG-FA.Rg3.QTN) demonstrated loading capacity (LC%, ~12% (w/w) for Rg3 and ~6% (w/w) for QTN, MR of Rg3 and QTN ~ 1:1), a low particular size (~110 nm) and a near neutral zeta potential (Fig. 5B), which were similar to those achieved by the non-targeted counterpart (CD-PEG.Rg3.QTN). In addition, the targeted co-formulation displayed a spherical structure (Fig. 5C), which was similar to that observed by non-targeted counterpart.

As shown in Fig. 5D, ~30% of both drugs were released from the targeted co-formulation at 8 h in neutral PBS (pH 7.4), while drug release was remarkably increased (~70%) at 8 h in acidic PBS (pH 5.5). At 48 h, ~70% and ~90% of drug release was observed in the targeted co-formulation at the neutral and acidic PBS, respectively. This data indicates that the CD formulation may achieve a higher drug release at acidic pH environment, which is also similar to other amine-functionalized NPs that can also facilitate pH-sensitive drug release^{67,68}. The mechanism underlying this phenomenon is likely due to the protonation of amine groups in response to the external acidic conditions (see review in⁶⁹). It is worth noting that the release of both Rg3 and QTN from the targeted co-formulation was similar at either pH, suggesting that two drugs may be simultaneously delivered inside the bloodstream and released at the tumor site. Following storage at 4 °C the targeted co-formulation remained stable for up to one week when no significant aggregation of was detected (Fig. 5E). In addition, the non-targeted co-formulation demonstrated similar drug release and stability to that observed for the targeted counterpart.

3.5. *In vitro* anticancer effects of co-formulations

To confirm the active targeting delivery, cellular uptake of non-targeted and targeted co-formulations containing rhodamine was assessed using confocal microscopy (Olympus) (Fig. 6A) and flow cytometry (Becton Dickinson) (Fig. 6B) in CT26 and HCT116 cells (they both express the folate receptor^{70,71}). Results show that targeted co-formulation achieved significantly higher uptake of rhodamine ($P<0.05$ and $P<0.01$) than non-targeted counterpart in two cell lines (Figs. 6A and B, and Supporting Information Figs. S4), confirming the FA-mediated delivery effect.

As shown in Fig. 6C, targeted co-formulation significantly reduced cell viability ($P<0.05$, ~25% at 24 h incubation) relative to non-targeted counterpart (~40% at 24 h incubation) and combination of free drugs (~55% at 24 h incubation) in CT26 and HCT116 cells. Notably, antiproliferative effect achieved by co-formulations was not due to nanotoxicity, as blank co-formulations could not inhibit cell growth (Supporting Information Fig. S5). Furthermore, antimetastatic activity of targeted co-formulation was assessed using scratch assay (Fig. 6D) and colony formation assay (Fig. 6E). Results show that targeted co-formulation significantly ($P<0.01$) slowed down the migration of cancer cells (Fig. 6D, ~35%) and reduced the number of colonies (Fig. 6E, ~5) as compared to non-targeted counterpart (~20% and ~12) and combination of free drugs (~5% and ~20). Results in Fig. 6 indicate that targeted co-formulation achieved FA-mediated antiproliferative and antimetastatic effects in CRC cells.

3.6. *In vivo* toxicity, pharmacokinetics and biodistribution of co-formulation

The *in vivo* toxicity of free drugs and co-formulations was assessed in healthy mice (Fig. 7A and Supporting Information Figs. S6). As shown in Fig. S6, no significant body weight loss was found in animals intravenously (i.v.) injected with free Rg3 up to 50 mg/kg or free QTN up to 25 mg/kg relative to PBS. In addition, the body weight was not significantly reduced in animals i.v. injected with targeted co-formulation containing two drugs at different doses (Rg3 up to 50 mg/kg and QTN up to 20 mg/kg, MR ~1:1) as compared to PBS (Fig. 7A). The H&E staining results show that no significant histological changes were found in major organs of mice following i.v. injection of targeted co-formulation (Rg3 = 50 mg/kg and QTN = 20 mg/kg) as compared to PBS (Fig. 7B). Moreover, no significant hematological toxicity (Fig. 7C) and liver/kidney injuries (Fig. 7D) were caused by targeted co-formulation (Rg3 = 50

mg/kg and QTN = 20 mg/kg) as compared to PBS. These indicate that no systemic toxicity was caused by targeted co-formulation under the doses tested.

In general, following systemic administration chemotherapeutic drugs display short half-lives and rapid clearance from the body, which will significantly lessen therapeutic efficacy. It is well established that modification with PEG sterically prevents NPs from non-specific absorption of serum proteins, improving the blood circulation of NPs⁷². In this study, the half-lives of the free drugs and drugs encapsulated within the targeted co-formulation was evaluated using an orthotopic CT26-Luc derived CRC mouse model (Fig. 7E). Results showed that the concentration of two drugs in the plasma decreased quickly, and the minimum level was detected at 8 h post injection ($t_{1/2}$ ~20 min for QTN and 10 min for Rg3; Fig. 7E). By contrast, the two drugs in targeted co-formulation were significantly more slowly removed from the plasma ($t_{1/2}$ ~1.4 h for QTN and 1.3 h for Rg3; Fig. 7E), indicating prolonged blood circulation of the drugs. Of note, free drugs demonstrated distinct half-life; in contrast, they presented similar half-life when co-delivered by targeted co-formulation. The data indicate that the two drugs may be simultaneously delivered into the blood. In addition, non-targeted formulation demonstrated similar half-life observed by targeted counterpart.

Tissue distribution of targeted co-formulation was also determined using an orthotopic CRC mouse model. Twelve h following i.v. injection of DiD-labeled co-formulations, tumors and major organs were *ex vivo* imaged using the IVIS[®] *In Vivo* Imaging System (Fig. 7F). Results show that targeted co-formulation achieved significantly higher tumor accumulation (~3.5 folds; $P<0.05$) but significantly less liver accumulation (~1.5 folds; $P<0.05$) than non-targeted counterpart (Fig. 7F). The results indicate that the targeted co-formulation significantly improved tumor retention and reduced non-specific tissue distribution.

It is known that chemotherapeutic drugs, due to poor pharmacokinetics and non-specific tumor delivery, have to be used in large doses for therapeutic outcome, but such excessive treatment will cause serious side effects. In this study, the targeted co-formulation significantly extended the circulation time and enhanced delivery to the tumor (Fig. 7E and F), suggesting that the targeted co-formulation potentially provides a low-dosage strategy that is sufficient for treating CRC as compared with

free unformulated drugs. The chemo-immunotherapeutic efficacy of the targeted co-formulation in combination with anti-PD-L1 was investigated in the following *in vivo* studies.

3.7. Combination therapy of targeted co-formulation and anti-PD-L1 in an orthotopic CRC model

Chemo-immunotherapeutic efficacy was assessed using an orthotopic CT26-Luc derived CRC mouse model. To investigate the potential of a low-dosage strategy, co-formulations containing Rg3 (10 mg/kg) and QTN (4 mg/kg) (MR ~1:1) were chosen in this study. Results of Supporting Information Fig. S7 show that no significant tumor growth was achieved by the combination of free drugs at higher doses (25 mg/kg Rg3 and 10 mg/kg QTN; toxic signs were observed when doses of Rg3 and QTN of 50 and 20 mg/kg, respectively, were used as compared to PBS. In contrast, tumor growth was significantly ($P<0.05$) slowed down by the non-targeted co-formulation relative to the combination of free drugs, while therapeutic efficacy was further ($P<0.05$) improved by targeted co-formulation (Fig. S7). These results confirm that the targeted co-formulation achieved FA-mediated delivery effect, and could significantly improve the therapeutic efficacy at lower doses as compared to the free drugs at higher doses. Based on these results, targeted co-formulation containing Rg3 (10 mg/kg) and QTN (4 mg/kg) was chosen for combination therapy with anti-PD-L1.

The response rate of immune checkpoint blockade remains low in patients diagnosed with MMR-proficient CRC⁷. Indeed, anti-PD-L1 could not generate antitumor efficacy compared to PBS in an orthotopic CRC mouse model (established with CT26 cells, characterized as an MSS CRC cell line^{73,74} (Fig. 8A and B), which was similar to results previously observed⁷⁵. In contrast, the targeted co-formulation achieved significantly ($P<0.01$) better antitumor efficacy than Anti-PD-L1 alone (Fig. 8A and B). Notably, a combination of the “targeted co-formulation + anti-PD-L1” further ($P<0.05$) enhanced therapeutic outcome relative to targeted co-formulation alone (Fig. 8A and B). Consequently, the combined strategy significantly prolonged the survival of diseased mice (median survival ~96 days) as compared to either PBS (~38 days), anti-PD-L1 (~40 days) or targeted co-formulation (~62 days) (Fig. 8C).

Immunofluorescent staining results showed that no difference was detected in

apoptotic tumor cells between anti-PD-L1 and PBS, while the targeted co-formulation significantly induced the apoptosis in tumor cells ($P<0.05$; ~11%) (Fig. 8D). The combined strategy further ($P<0.05$) enhanced the apoptosis in tumor cells (~27%) (Fig. 8D).

In addition, the TME was significantly reprogrammed by the combined strategy as compared to PBS, anti-PD-L1 or the targeted co-formulation alone (Fig. 8E and F). Immunostimulatory cells such as CD8⁺ T cells, CD4⁺ T cells and activated DCs were significantly ($P<0.05$) upregulated within the tumor by the combined strategy (Fig. 8E), which were accompanied by the increment of *IFN- γ* , *IL-12*, *CXCL9* and *CXCL10* (*IFN- γ* and *IL-12* are responsible for the activation of antitumor immunity⁷⁶), and *CXCL9* and *CXCL10* function positively for T cell infiltration at the tumor site⁷⁷ (Fig. 8F). In addition, the immunosuppressive cells such as regulatory T cells (Tregs), myeloid derived suppressor cells (MDSCs) and tumor-associated macrophages (M2) were significantly downregulated within the tumor by the combined strategy (Fig. 8E), and were accompanied by the reduction of *IL-4*, *IL-6* and *IL-10* (they promote the activation of immune suppressive cells⁷⁸) (Fig. 8F).

It has been reported that the ICD-mediated antitumor immunity relies on the activation of effector T cells¹⁰. When orthotopic CRC mice were injected with targeted co-formulation following the removal of CD4⁺ or CD8⁺ T cells, the anticancer outcome was significantly abolished (Fig. 8G). In contrast, the therapeutic efficacy of targeted co-formulation was not affected by pretreatment with the isotype IgG antibody (Fig. 8G), confirming the role of targeted co-formulation in the induction of T cell-mediated immunological responses against CRC.

Taken together, results in Fig. 8 indicate that the targeted co-formulation was able to induce ICD (T cell-mediated immunogenic responses) for reprogramming the immunosuppressive TME, which significantly improved the therapeutic efficacy when combined with PD-L1, providing a promising strategy for CRC patients.

4. Discussion

Increasing evidence demonstrates that certain chemotherapeutic drugs can induce ICD, which profoundly modulate the immunosuppressive TME, remodeling “cold” (non-T cell-inflamed) tumors into “hot” (T cell-inflamed) ones¹⁰. The combination of ICD-inducing chemotherapeutics with other immunotherapies has demonstrated great

promise for improving the survival of cancer patients⁷⁹. Therefore, investigation of chemotherapeutic drugs (considered cytotoxic and poorly immunogenic) as potential ICD agents has received increasing attention. In this study, the potential of Rg3 [20(S)-Rg3] as an ICD inducer against CRC cells was confirmed for the first time using *in vitro* and *in vivo* experimental approaches (Fig. 2) that have been accepted and validated for the identification of ICD agents²⁴. When Rg3 was combined with QTN (a ROS inducer, Fig. 3), the ICD efficacy was significantly improved (Fig. 4).

Recent developments of nano delivery systems have significantly facilitated *in vivo* delivery of chemotherapeutic agents for cancer therapy^{35-39,80-84}. However, to the best of our knowledge, no study has previously reported the co-encapsulation of Rg3 and QTN in a nanoformulation for CRC. In this study, a FA-targeted PEGylated amphiphilic cyclodextrin NP was developed for co-encapsulation of Rg3 and QTN at an optimal molar ratio (1:1) with favorable physicochemical properties (Fig. 5). The targeted co-formulation (CD-PEG-FA.Rg3.QTN) achieved synergistic *in vitro* anticancer effects (Fig. 6), and improved the pharmacokinetics and biodistribution in an orthotopic CRC mouse model (Fig. 7). Consequently, the CD-PEG-FA.Rg3.QTN altered the immunosuppressive nature of the TME, significantly prolonging the survival of orthotopic CRC mice when combined with anti-PD-L1 (Fig. 8). It is known that only patients with MMR-deficient CRC respond to anti-PD-L1 as monotherapy⁶, while the response rate is poor in MMR-proficient CRC⁷. Therefore, our combined strategy potentially provides therapeutic benefit for a wider spectrum of CRC patients.

It must be borne in mind that the identified features of ICD are only “thin end of the wedge”, therefore, future investigation of ICD hallmarks is of critical importance for progress in the validation of emerging ICD inducers. In addition, the hepatic metastasis is known as the commonest form of distant metastasis in CRC, therefore, therapeutic efficacy of “targeted co-formulation + PD-L1 blockade” strategy will be assessed using mice with experimental CRC liver metastases⁸⁵, in order to confirm the potential for the treatment of CRC liver metastasis.

5. Conclusions

Rg3 is demonstrate to be an inducer of ICD, and QTN enhances chemo-immunotherapeutic effects in an orthotopic CRC mouse model by increasing

ROS. Therefore, identification of emerging ICD inducers has received increasing attention, which will advance ICD-based cancer immunotherapy and synergize with immune checkpoint blockade therapy.

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Author contributions

Jianfeng Guo supervised the project. Zhuo Yu and Jianfeng Guo designed the research. Dandan Sun carried out the experiments and performed data analysis. Yifang Zou, Liu Song, Shulan Han, Hao Yang and Di Chu participated part of the experiments. Yun Dai, Jie Ma, Cairiona M O'Driscoll, Zhuo Yu and Jianfeng Guo provided resources. Dandan Sun, Zhuo Yu and Jianfeng Guo wrote the manuscript. Yun Dai, Jie Ma, Cairiona M O'Driscoll and Jianfeng Guo revised the manuscript. All authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Figure 1 A combination of folate-targeted Rg3/QTN cyclodextrin-based co-formulation and anti-PD-L1 for chemo-immunotherapy in CRC.

Figure 2 Ginsenoside Rg3 induced immunogenic cell death in CRC cells. (A) IC_{50} of Rg3 for CT26 and HCT116 cells at 24 h. Data are presented as mean \pm SD ($n = 3$). (B) Apoptosis (%) in CT26 and HCT116 cells following treatment of Rg3 at 6, 12 and 24 h. Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$ relative to DMSO. (C) The activity of UPR signaling pathways following treatment of Rg3 ($[c] = 30 \mu\text{mol/L}$) at 6, 12 and 24 h. The quantification was demonstrated in Fig. S1. (D) The characterization of ICD in CRC cells following treatment of Rg3 ($[c] = 30 \mu\text{mol/L}$), including CRT exposure (6 h), ATP secretion (12 h) and HMGB1 release (12 h). Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ relative to DMSO; scale bar = 20 μm . (E) The expression of CD11c and CD86 in DCs stimulated with the supernatant from Rg3 ($[c] = 30 \mu\text{mol/L}$) -treated cells (24 h). Data are presented as mean \pm SD ($n = 3$). $**P < 0.01$ relative to DMSO. (F) The *in vivo* vaccination assay using BALB/C and nude mice. Data are presented as mean \pm SD ($n = 4$). $***P < 0.001$.

Figure 3 Quercetin caused reactive oxygen species in CRC cells. (A) IC_{50} of QTN for CT26 and HCT116 cells at 24 h. Data are presented as mean \pm SD ($n = 3$). (B) Apoptosis (%) in CT26 and HCT116 cells following treatment of QTN ($[c] = 80 \mu\text{mol/L}$) at 24 h. Data are presented as mean \pm SD ($n = 3$). $**P < 0.01$ relative to DMSO. (C) The activity of Bcl-2/BAX/Caspase9/Caspase3 signaling pathways following treatment of QTN ($[c] = 80 \mu\text{mol/L}$) at 6, 12 and 24 h. The quantification was demonstrated in Fig. S2. (D) The ROS level in CT26 and HCT116 cells following treatment of QTN ($[c] = 80 \mu\text{mol/L}$) at 6, 12 and 24 h. Data are presented as mean \pm SD ($n = 3$). $**P < 0.01$ and $***P < 0.001$ relative to DMSO. (E) Cell viability (%) of CT26 and HCT116 cells with or without NAC prior to treatment of QTN ($[c] = 80 \mu\text{mol/L}$) (24 h). Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$ relative to untreated control. (F) Apoptosis (%) in CT26 and HCT116 cells with or without NAC prior to treatment of QTN ($[c] = 80 \mu\text{mol/L}$) (24 h). Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$ relative to untreated control.

Figure 4 Synergistic effects of Rg3 and QTN in CT26 cells. (A) IC_{50} of drug combination at 24 h. Data are presented as mean \pm SD ($n = 3$). CI values at IC_{50} were

shown in Fig. S3. (B) Apoptosis (%) caused by drug combination at 24 h. Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$ relative to DMSO. (C) The CRT exposure with or without NAC before treatment of drug combination (6 h). Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$ relative to DMSO; scale bar = 20 μm . (D) The ATP secretion with or without NAC before treatment of drug combination at 12 h. Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$, between NAC and No NAC. (E) The HMGB1 release with or without NAC before treatment of drug combination at 12 h. Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$, between NAC and No NAC. (F) The expression of CD11c and CD86 in DCs stimulated (24 h) by the supernatant from Rg3-treated cells with or without pretreatment of NAC. Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, relative to DMSO.

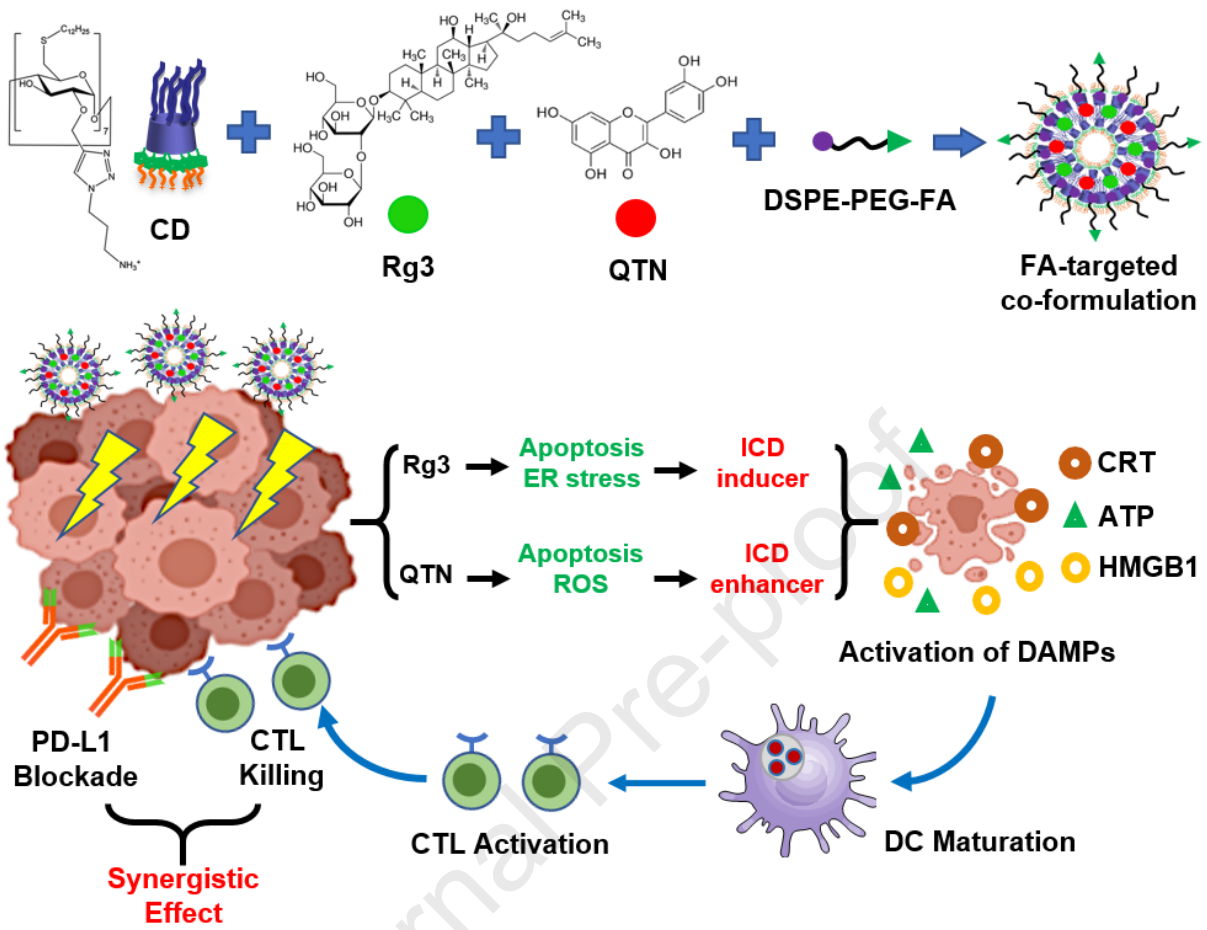
Figure 5 Preparation and physicochemical characterization of FA-targeted co-formulation. (A) Formulation schematic. (B) The EE%, LC%, particle size and surface charge of targeted co-formulation. Data are presented as mean \pm SD ($n = 3$). (C) TEM image of targeted co-formulation (scale bar = 100 nm). (D) The *in vitro* release of drugs from targeted co-formulation in 0.01 M PBS (pH = 5.5 and 7.4). Data are presented as mean \pm SD ($n = 4$). (E) Particle size of targeted co-formulation following storage at 4 $^{\circ}\text{C}$ in aqueous solution. Data are presented as mean \pm SD ($n = 4$). $*P < 0.05$ and $**P < 0.01$ relative to Day 0; NS, no significance. Non-targeted co-formulation also demonstrated similar physicochemical results observed by targeted counterpart.

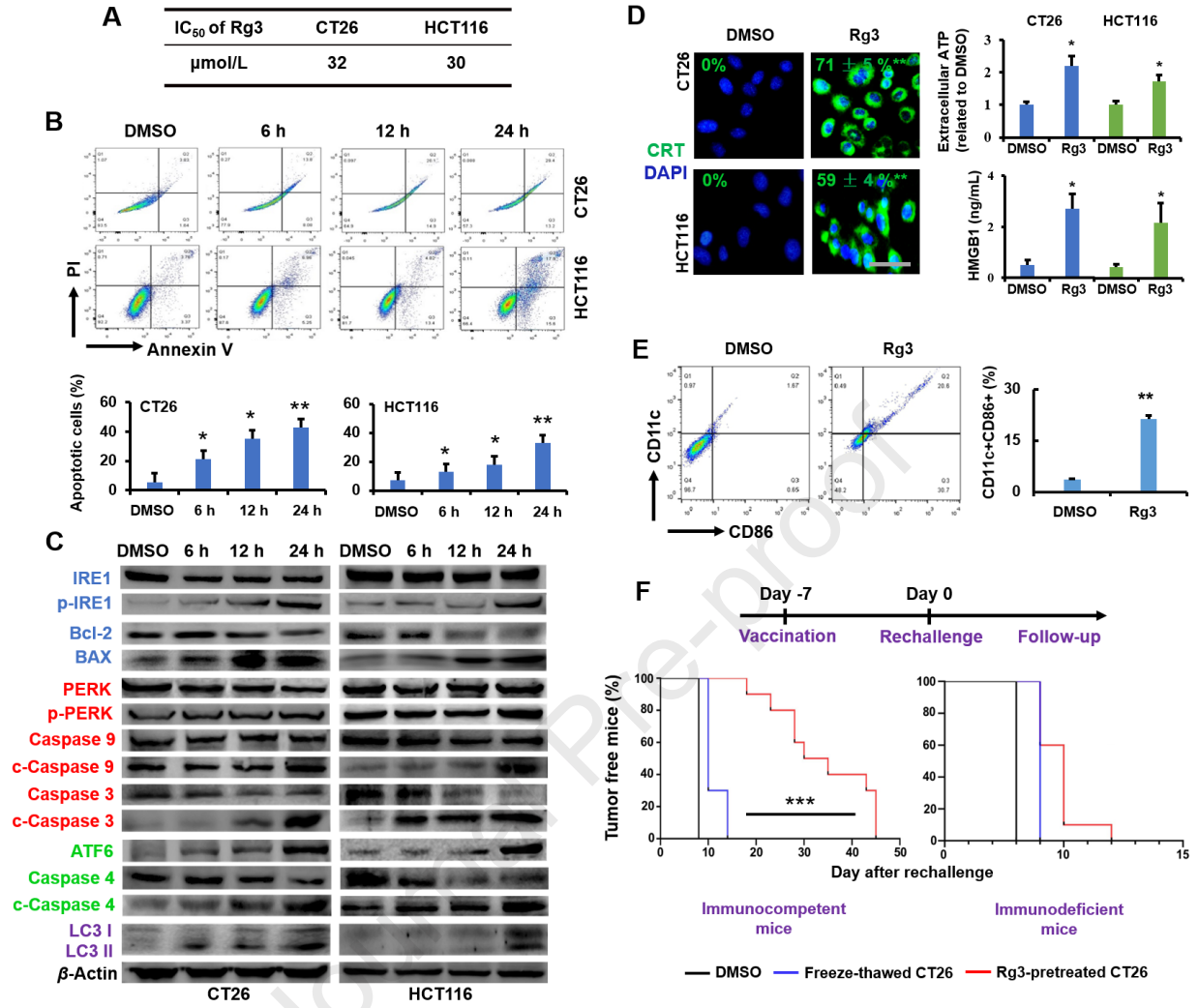
Figure 6 *In vitro* studies of co-formulations. (A) Cellular uptake of co-formulations (NT co-formulation = non-targeted co-formulation; co-formulation = targeted co-formulation) containing Rhodamine was assessed at 6 h using confocal microscopy (scale bar = 5 μm). The quantification was shown in Fig. S4. (B) Cellular uptake of co-formulations containing Rhodamine was assessed at 4 h using flow cytometry (BD). Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$, relative to PBS. (C) Cell viability (%) of co-formulations at 24 h. Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$, relative to free drugs. (D) Cell-free areas before and after treatment of co-formulations (12 h) were imaged and measured for the relative scratch area (%) (scale bar = 50 μm). Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$, relative to free drugs. (E) The colony formation following treatment of co-formulations (4 weeks) (scale bar = 50 μm). Data are

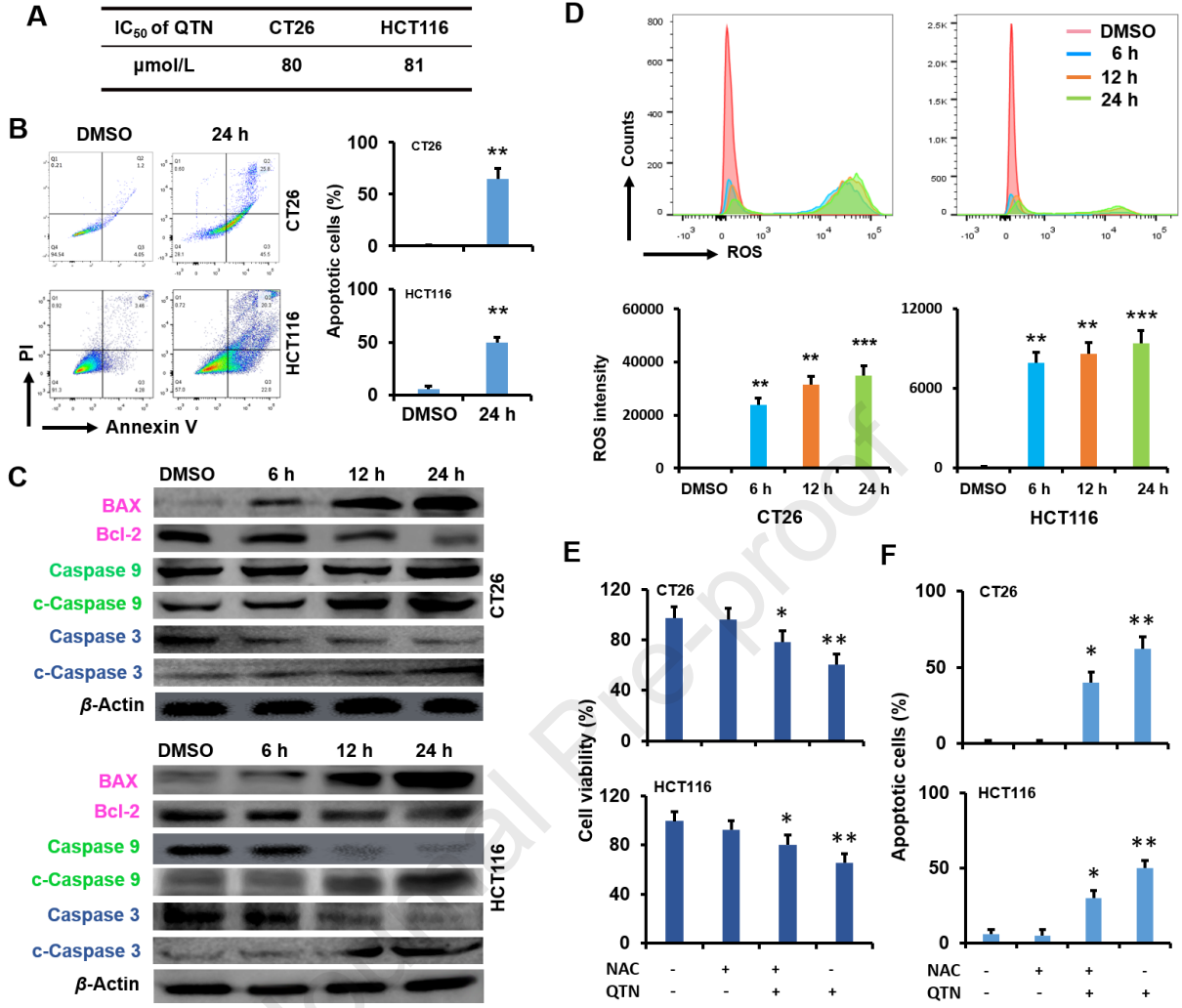
presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$, relative to free drugs.

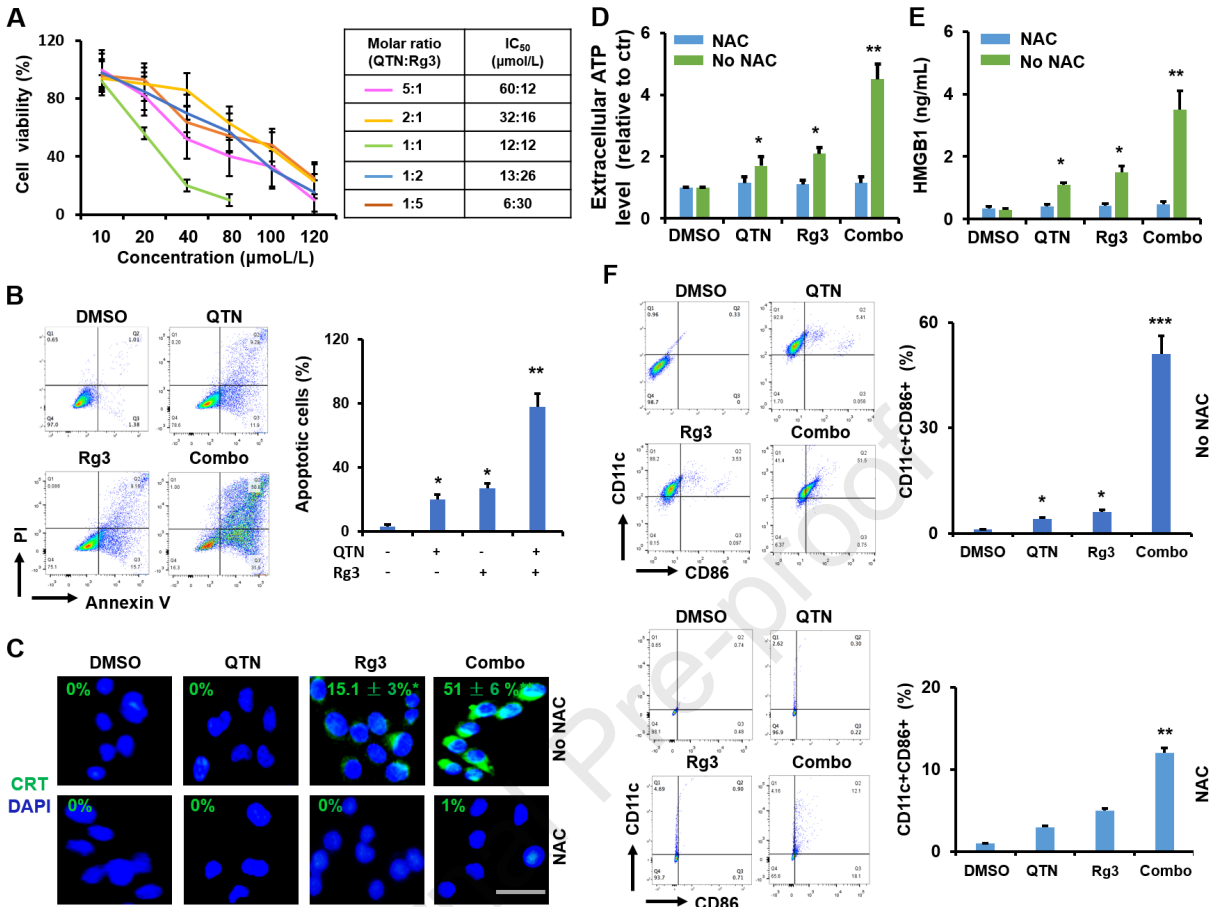
Figure 7 *In vivo* toxicity, pharmacokinetics and biodistribution of targeted co-formulation. (A) The body weight over a 30-day period following i.v. treatment of PBS and targeted co-formulation on Days 1, 3, 5, and 7. Data are presented as mean \pm SD ($n = 5$). (B) Major organs were collected on Day 30 and assessed using H&E staining assay. No significant toxic sign was found in targeted co-formulation as compared to PBS (scale bar = 50 μ m) (C) Hematological analysis including red blood cells (RBCs), white blood cells (WBCs), platelets (PLTs) and hemoglobin (HGB) was carried out on Day 30. Data are presented as mean \pm SD ($n = 4$). (D) The liver/kidney functions including alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (CRE) were determined on Day 30. Data are presented as mean \pm SD ($n = 4$). (E) The concentration of drugs in the plasma was plotted at different time points. Data are presented as mean \pm SD ($n = 4$). (F) Biodistribution of DiD-labeled co-formulations was detected (640 nm/670 nm) using IVIS[®] *In Vivo* Optical System. Data are presented as mean \pm SD ($n = 4$). $*P < 0.05$ in orthotopic CRC mouse model.

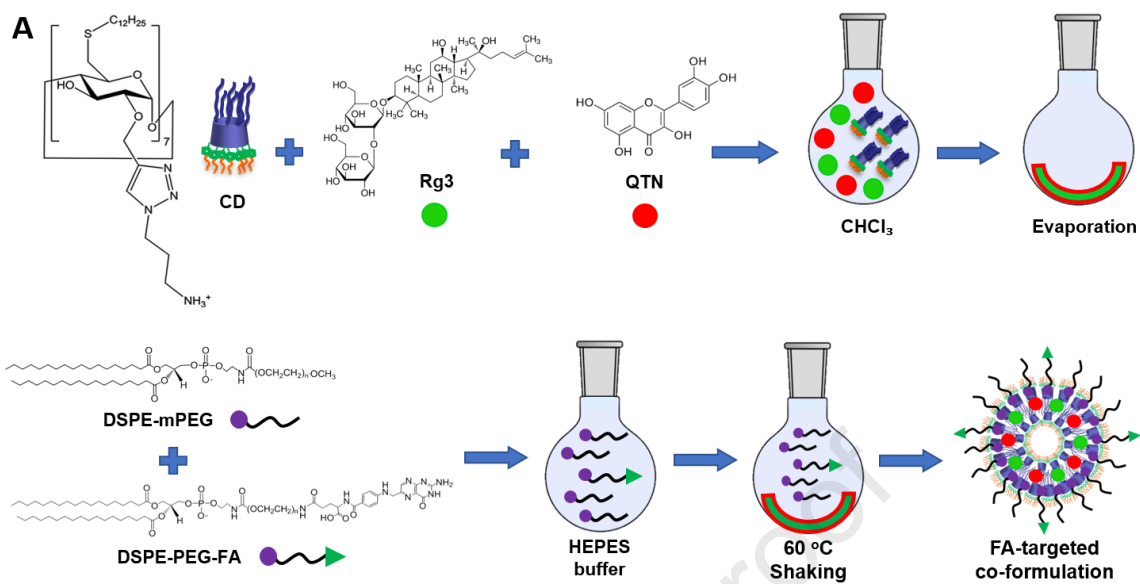
Figure 8 Combination therapy of targeted co-formulation and Anti-PD-L1 for CRC. (A) Treatment schedule and IVIS images. (B) The CRC progression over a 35-day period. Data are presented as mean \pm SD ($n = 5$). $*P < 0.05$ and $**P < 0.01$; ns, no significance. (C) Animal survival (median survival: PBS \sim 38 days, Anti-PD-L1 \sim 40 days, targeted co-formulation \sim 62 days, and combination \approx 96 days). Data are presented as mean \pm SD ($n = 5$). $**P < 0.01$ and $***P < 0.001$. (D) Immunofluorescent staining assay (green = DNA fragments and blue = nuclei) on Day 20 to assess apoptosis in the tumor (scale bar = 50 μ m). Data are presented as mean \pm SD ($n = 3$). $*P < 0.05$ and $**P < 0.01$, relative to PBS. (E) Level of immune cells in the tumor on Day 20 was analyzed using flow cytometry (BD). Data are presented as mean \pm SD ($n = 4$). $*P < 0.05$ and $**P < 0.01$; NS, no significance. (F) The mRNA expression of cytokines and chemokines in the tumor on Day 20 was analyzed using real time RT-PCR. Data are presented as mean \pm SD ($n = 4$). $*P < 0.05$ and $**P < 0.01$; NS, no significance. (G) Orthotopic CRC mice treated with targeted co-formulation following the removal of CD4⁺ or CD8⁺ T cells. Data are presented as mean \pm SD ($n = 4$). $*P < 0.05$ and $**P < 0.01$; NS, no significance.



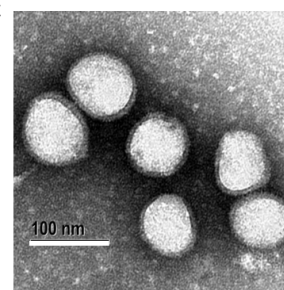
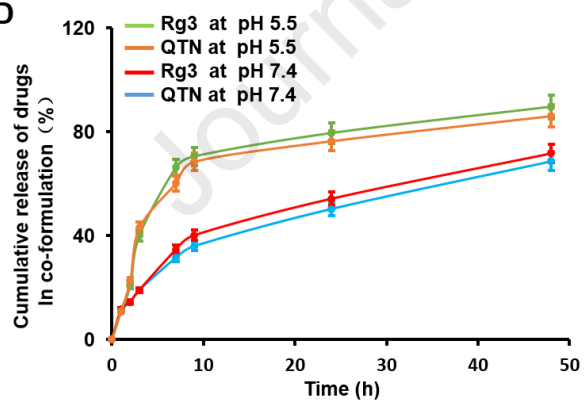






**B**

NP	EE (%)	LC (%)	Particle size (PDI)	Surface charge
CD-PEG-FA.Rg3.QTN	Rg3 = 96 ± 4 QTN = 90 ± 2	Rg3 = 12 ± 2 QTN = 6 ± 1	108 ± 6 nm (0.3)	6 ± 1 mV

C**D****E**