ApoG2 induces ER stress-dependent apoptosis in gastric cancer cells in vitro and its real-time evaluation by bioluminescence imaging in vivo

Jing Xin a,1, Yonghua Zhan a,1, Muhan Liu a, Hao Hu c, Limin Xia c, Yongzhan Nie c, Kaichun Wu c, Jimin Liang a,*, Jie Tian a,b,*

a School of Life Sciences and Technology, Xidian University, Xi’an 710071, China
b Institute of Automation, Chinese Academy of Sciences, Beijing 100190, China
c Institute of Digestive Diseases, Xijing Hospital, The Fourth Military Medical University, Xi’an 710032, China

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Abstract
Apogossypolone (ApoG2), a potent small molecular inhibitor of Bcl-2 family proteins, is reported to have a significant anti-cancer effect in several types of cancers, but it has not been investigated in gastric cancer. In this study, we demonstrate in vitro and in vivo that ApoG2 inhibits human gastric cancer. Gastric carcinoma cell growth and proliferation was significantly hampered in vitro, as measured by MTT and colony formation assays. Real-time bioluminescence imaging indicated that ApoG2 causes tumor growth delay in a murine xenograft model. Further studies revealed that the ApoG2 induced apoptosis in gastric cancer cells was associated with the endoplasmic reticulum stress-induced apoptosis pathway. Conclusively, our results indicate that ApoG2 may be a promising agent for gastric cancer therapy.

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1. Introduction
It is well known that gastric cancer is a common malignancy and the second leading cause of death associated with cancer [1,2]. Gastric cancer continues to be a major public health issue worldwide, especially in China, where the incidence and mortality rates of gastric cancer have been very high [3]. The predominant therapeutic strategy for advanced cancer is chemotherapy, but efficacy is plagued with poor prognosis and drug resistance [4–7]. Thus, discovering new chemotherapeutic drugs and effective treatment strategies is important to the management of gastric cancer.

Apoptosis or programmed cell death, being an important process for the maintenance of normal tissue homeostasis, plays an important role in the discovery of chemotherapeutics. Because defective apoptosis mediates pathological processes usually associated with tumorigenesis, the basic premise of anti-cancer drug design is to search for target molecules or target proteins related to tumor apoptosis [8]. B-cell lymphoma/leukemia-2 (Bcl-2) family proteins are regarded as central regulators of the apoptosis process. Some are considered pro-apoptotic proteins, such as Bak, Bax, Bad, Bik and Bim, while the others are anti-apoptotic proteins, such as Bcl-2, Bcl-xl and Mcl-1. All of these Bcl-2 family proteins are increasingly being investigated for novel drug development [9]. In particular, Bcl-2 and Bcl-xl proteins have a Bcl-2 homology domain 3 (BH-3), which creates a hydrophobic surface pocket potentially serving as a binding groove for pro-apoptotic factors [10]. As a result, newly designed and synthesized chemotherapeutic agents inhibit Bcl-2/Bcl-xl protein function by targeting the BH-3 domain to induce apoptosis [11,12].

In view of the above strategy, several non-peptide small molecule inhibitors of Bcl-2 family proteins have been synthesized and used for anti-cancer treatment studies in recent years [13–16]. One successful example is gossypol, an effective anti-tumor drug in clinical trials, which has been reported to have anti-proliferation activities in vitro against several types of cancer cells and anti-tumor effects in vivo in nude mice [17–21]. However, the drawback of gossypol is the two aldehyde groups in its chemical structure that are associated with toxicity and potential non-specific reactivity [22]. Hence, research has focused to optimize its chemical structure and improve its anti-cancer effect. Recently, apogossypolone (ApoG2), a derivative of gossypol, was synthesized by removing the two aldehyde groups and found to have superior anti-proliferation activity with less toxicity in various cancer cell lines: nasopharyngeal carcinoma, prostate cancer, human leukemic monocyte lymphoma, diffuse large-cell lymphoma, follicular lymphoma, pancreatic cancer, human hepatocellular carcinoma and so on [23–30]. Albeit ApoG2 has shown obvious anti-tumor effects in a variety of cancers, little is known about whether or
not ApoG2 can inhibit gastric cancer cell growth and proliferation nor its possible molecular mechanism for inducing apoptosis. Here we report the anti-cancer effects of ApoG2 on gastric cancer. The results demonstrate that ApoG2 could effectively inhibit the growth and proliferation of gastric cancer cell lines and delay tumor growth in vivo. To facilitate the translation of ApoG2 into clinical practice for gastric cancer therapy, we reveal in vivo drug therapy response and its anti-cancer mechanism.

To evaluate the anti-tumor effects of drugs in vivo, the primary monitoring method for superficial tumors is the measurement of tumor volume periodically by a caliper or for internal tumors by the endpoint measurement of tumor weight and volume. Although it is rapid and easy to perform, the drawback of these methods cannot be disregarded because the volume or weight of the total tumor measured by a caliper or a balance include regions of necrosis and edema [31]. Thus, these methods cannot assess the treatment response precisely. Recently, novel monitoring methods to evaluate the anti-tumor effects of new drugs in real time and noninvasively are being developed according to advanced imaging technologies [32,33]. Molecular imaging is a newly established research area that offers a noninvasive method for studying biological processes at the cellular and sub-cellular levels in living subjects [34]. It reveals the therapy response noninvasively, so it has significant contributions in developing new drugs in the pre-clinical stage [35]. In this study, we evaluate the in vivo anti-cancer effect of ApoG2 by bioluminescence imaging, as a promising imaging technique having the advantages of being low-cost and high-throughput with fine temporal resolution, ease of use, minimal background signal and requiring neither ionizing radiation nor radioactive materials [36,37].

Furthermore, the exact molecular mechanism of ApoG2 for inducing apoptosis in the different cancer models is unclear to date. Molecular understanding of ApoG2 anti-cancer effects can provide greater insight to future drug development, especially in the Bcl-2 family proteins. In this report, we investigate the underlying intracellular signal transduction pathway involved in ApoG2-induced apoptosis in gastric cancer by Western blot analysis and measurement of intracellular reactive oxygen species (ROS) and calcium ion ([Ca2+]i) concentration.

2. Materials and methods

2.1. Cell culture and reagents

The human moderately-differentiated gastric carcinoma cell line SGC-7901 and SGC-7901-LUC were obtained from the State Key Laboratory of Cancer Biology, Digestion Department of Xijing Hospital, Fourth Military Medical University. The cells were cultured in RPMI-1640 medium (HyClone) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin in a humidified incubator at 37 °C with 5% CO2. ApoG2 and gossypol were synthesized in our laboratory and dissolved in pure dimethylsulfoxide (DMSO) at the stock concentration of 10, 20, 30, 40, and 50 μmol/L ApoG2 or gossypol or 1% DMSO. About 48 h later, the cells were fixed in mounting medium for 10 min at room temperature, washed with PBS twice, and then stained with Hoechst 33,258 for 5 min. Cover slips were then washed and mounted on slides with glycerol and imaged with a fluorescence microscope (Olympus).

ApoG2-induced apoptosis was also quantified using flow cytometry (BD FACSCanto) by labeling phosphatidylserine exposure on apoptotic cell membranes using FITC-labeled Annexin-V and propidium iodide (PI) stain. The cells were treated with either ApoG2, gossypol or 1% DMSO for 48 h at the same concentrations as described previously. Then, the cells were harvested, centrifuged at 800 rpm for 5 min, washed with PBS, mixed again, re-suspended in PBS, stained with Annexin-V FITC for 1 h and PI for 30 min, and finally analyzed by FACSc

2.2. Cell viability assay

Cell viability in vitro was determined by an MTT assay. SGC-7901 (1 × 105) cells were seeded in sterile 96-well flat-bottomed plates and incubated overnight. Then, the diluted ApoG2 or gossypol was added to the wells at final concentrations of 1, 5, 10, 20, 30, 40, and 50 μmol/L. Every concentration was tested in triplicate. The plates were then incubated in a humidified incubator at 37 °C with 5% CO2 for 24, 48, and 72 h respectively. After the desired time points, 20 μL of 5 mg/mL MTT solution was added to each well and the plates were incubated for 4 h. Formazan crystals formed at the bottom of the well, so culture supernatants from the wells were removed carefully. Next, 150 μL of DMSO was added to each well. After shaking the wells for 15 min, the formazan crystals dissolved completely. The plates were then read for optical density (OD) at 490 nm on a microplate reader. The cell viability at different drug concentrations was calculated as follows: ([OD of drug treated-OD of control-OD of blank treated]) × 100%. The 50% inhibitory concentration of ApoG2 was defined as the concentration that caused 50% growth inhibition of cells. Cell viability in vitro was also evaluated by bioluminescence imaging technology. Briefly, 1 × 107 SGC-7901-LUC cells were seeded in black, clear bottom 96-well plates and incubated for 24 h. Then, the cells were treated with 1% DMSO or the desired concentration of ApoG2 for 24, 48 and 72 h. At the end of each time point, the bioluminescence intensity emitted from the cells was obtained from the IVIS imaging system (Xenogen Corporation, Alameda, California) and analyzed using the IVIS Living Image 4.3.1 software (Caliper Life Sciences). Before measuring, 150 μg/mL D-luciferin was added to each well. Imaging parameters were as follows: exposure time: 1 min; Binning: 8; lens aperture ([f/stop]: 2; field of view: 12.5.

2.3. Apoptosis analysis

ApoG2-induced apoptosis and morphological changes were visualized by Hoechst 33,258 nuclear staining kit according to the manufacturer’s instructions. Briefly, SGC-7901 cells were seeded on bio-clean cover slips in 6-well plates at a density of 2 × 105 and allowed to attach overnight. Then, the cells were treated with 10, 20, 30, 40, and 50 μmol/L ApoG2 or gossypol or 15% DMSO. About 48 h later, the cells were fixed in mounting medium for 10 min at room temperature, washed with PBS twice, and then stained with Hoechst 33,258 for 5 min. Cover slips were then washed and mounted on slides with glycerol and imaged with a fluorescence microscope (Olympus).

To determine the anti-tumor effect in vivo, the animal tumor xenograft model was constructed. The animal study protocol was done in accordance with the Fourth Military Medical University animal protocol. Female athymic nude (nu/nu) mice (4–6 weeks old, weighing 20–25 g) were purchased from the animal center of the Fourth Military University. SGC-7901-LUC cells (1 × 107) that stably express luciferase were inoculated into the mice by subcutaneous injection. When subcutaneous tumor masses developed to approximately 70–100 mm3, mice were divided into two groups randomly. The treatment group received ApoG2 at 100 mg/kg every 3 d for a total of 18 d. Bioluminescence imaging in vivo was performed before drug injection. D-luciferin at 150 mg/kg was injected into the abdominal cavity of the mice 10 min prior to bioluminescence imaging. Animals were then anesthetized (2% isoflurane, 0.3 L/min oxygen) and placed onto the warmed stage inside the light-tight camera box with continuous exposure while under 2% isoflurane. Imaging parameters were as follows: exposure time 1 min; Binning: 8; lens aperture ([f/stop]: 2; field of view: 12.5. Tumor volume was measured by calipers every 3 d and estimated by the equation \( V = (A \times B^2)/2 \), where \( V \) is the tumor volume, \( A \) is the length of the tumor and \( B \) is the width of the tumor. At the treatment endpoint, mice were injected with \( \alpha \)-luciferin in the same way as described before, anesthetized and finally euthanized. Organs were excised, weighed, and imaged to collect ex vivo bioluminescence images. After imaging, the excised organs were fixed in a 10% formalin solution and paraffin embedded to prepare tissue sample sections. All samples were stained with H&E and microscopically examined.

2.6. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

To examine apoptosis in the tumors, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was carried out using the in situ Cell Death Detection Kit, POD (Roche). Briefly, paraaffin-embedded tissue sections were treated with protease K (20 μg/mL) in 10 mM Tris–HCl (pH 7.5) for 30 min at room temperature after being dehydrated and rehydrated. The slides were rinsed with PBS for 5 min, two times. The sections were then incubated with 50 μL TUNEL reaction mixture per sample at 37 °C for 1 h in a humidified chamber. At the end
of incubation, the slides were rinsed again with PBS for 5 min, three times. The apoptotic cells were analyzed by a fluorescence microscope at 475–490 nm wavelengths. Then, the sections were incubated with 50 μL Converter-POD per sample in a humidified chamber for 30 min at 37°C. The slides were washed again with PBS for 5 min, three times. At last, the TUNEL labels were visualized using diamino benzidine (DAB) as peroxidase substrates and analyzed by light microscopy.

2.7. Intracellular reactive oxygen species (ROS) level measurement

Intracellular reactive oxygen species (ROS) content of ApoG2-treated SGC-7901 cells was measured by a DCFH-DA fluorescence probe (Beyotime). SGC-7901 cells were treated with or without ApoG2 at 10, 20, 30, 40, and 50 μmol/L for 48 h. Then, the cells were harvested, washed twice with PBS, and incubated with 10 μmol/L DCFH-DA for 20 min at 37°C in complete darkness. The cells were washed twice with PBS and analyzed with flow cytometry.

2.8. Intracellular calcium ion ([Ca^{2+}]_i) concentration measurement

[Ca^{2+}]_i concentration was measured by a Fluor-3/AM fluorescence probe (Beyotime). Pre-treated ApoG2 SGC-7901 cells were harvested, washed twice with PBS, and incubated with 50 μmol/L Fluor-3/AM for 45 min at 37°C in complete darkness. The cells were then washed twice with PBS and analyzed with flow cytometry.

2.9. Western blotting analysis

SGC-7901 cells were treated with 10, 20, 30, 40, and 50 μmol/L ApoG2 or 1% DMSO for 48 h. The cells were then harvested, washed with PBS and lyed in a lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF) for 10 min at 4°C. The proteins were extracted and fractionated in 12% SDS–PAGE gels and subsequently semi-dry transferred to a nitrocellulose membrane. The membranes were blocked for 1 h at room temperature with TBS-Tween-20 containing 5% non-fat dry milk and then incubated with the primary antibodies for Bcl-2 (1:1000), Bax (1:1000), ADD1 (1:500), Calpain1 (1:500), Calpain2 (1:500), eIF2α (1:1000), p-eIF2α (1:1000), Caspase-12 (1:500), and β-actin (1:2000) overnight at 4°C. Following incubation with the appropriate primary antibody, membranes were washed with TBS-Tween-20 and reacted with peroxidase-conjugated secondary antibody corresponding to the primary antibody for 1 h at room temperature. The reactive bands were visualized by enhanced chemiluminescence reagents (Pierce).

2.10. Statistical analysis

Data are presented as the mean ± S.D. of three replicate experiments. All statistical analysis was performed on SPSS16.0 software (Chicago, IL). The statistical difference between the means was analyzed by a Student’s t test. Significance was set at the 5% level.

3. Results

3.1. Anti-growth effect by ApoG2 on SGC-7901 cells in vitro

To investigate the cancer cell anti-growth activity of ApoG2, the MTT assay and in vitro bioluminescence imaging were performed. The cell viability of SGC-7901 after being treated with ApoG2 significantly decreased in a time- and dose-dependent manner (top of Fig. 1A). The IC_{50} concentration of ApoG2 was 18.7 μM at 72 h. ApoG2 and gossypol anti-growth effects at 72 h were also compared in this study. The inhibition of cell survival by gossypol was more significant (bottom of Fig. 1A). In vitro bioluminescence imaging was measured using the IVIS imaging system and analyzed with the IVIS Living Image 4.3.1 software. First, we observed the stable bioluminescent signal of SGC-7901-LUC cell lines expressing luciferase. The bioluminescence intensity was highly correlated to the cell number (R^2 = 0.9988), as shown in Fig. 1B. Subsequently, SGC-7901-LUC cells were treated with or without ApoG2 at different drug doses and times and then imaged on the IVIS imaging system. Bioluminescence intensity emitted from pre-treated SGC-7901-LUC cells decreased significantly in a time- and dose-dependent manner compared with the control (Fig. 1C). According to the correlation between cell number and bioluminescence intensity, the results show that ApoG2 inhibited the growth of SGC-7901 cells in vitro.

3.2. Apoptosis induced by ApoG2 in SGC-7901 cells

To determine whether ApoG2 induces apoptosis, Hoechst 33,258 staining and flow cytometry were performed. As shown in Fig. 2A, apoptotic characteristics of SGC-7901 cells, such as cell shrinkage and nuclear fragmentation, were clearly exhibited after treatment with ApoG2. Apoptotic bodies increased in a dose-dependent manner. The number of apoptotic cells were counted among randomly selected 200 cells on a high power field and analyzed statistically. Compared with the DMSO-treated control group, the ability to induce apoptosis by ApoG2 showed a significant difference above 20 μM (Fig. 2B). The flow cytometry assay also illustrated that ApoG2 can lead to apoptosis (Fig. 2C). The percentage of apoptosis induced by ApoG2 was 7.5%, 11.4%, 32.5%, 55.3%, and 46.1% for 10, 20, 30, 40, and 50 μmol/L ApoG2, respectively. In this study, we also compared the apoptotic activities of ApoG2 with gossypol. From the Hoechst 33,258 and flow cytometry, ApoG2 led to apoptosis but to a lesser extent than gossypol. As shown in Fig. 2C, ApoG2-induced apoptotic cells were mainly in an early apoptotic phase, and gossypol-induced apoptotic cells were mainly in a late apoptotic phase.

3.3. Anti-proliferation activity of ApoG2

To test the anti-proliferation ability of ApoG2, the colony formation assay was performed. As shown in Fig. 3, ApoG2 resulted in an obvious decrease in colony numbers with various doses compared with the DMSO-treated control group, and the percentage of colony formation of SGC-7901 cells treated with ApoG2 was higher than gossypol at the same concentration. Fourteen days later, SGC-7901 proliferation ability was nearly inhibited completely when treated with 1 μmol/L of ApoG2. This reveals that ApoG2 has a stronger anti-proliferation effect on gastric cancer cells than gossypol.

3.4. Anti-tumor effect of ApoG2 in vivo

Before evaluating the anti-tumor effects of ApoG2 in vivo, we compared the toxicities of ApoG2 and gossypol in mice using detailed methods as shown in Text S1. The maximal tolerable dose (MTD) of ApoG2 and gossypol was evaluated in normal nude mice by intraperitoneal injection. The MTD of ApoG2 and gossypol was 800 mg/kg and 100 mg/kg, respectively. Animals exhibited no weight loss or lethargic behavior after being injected with ApoG2. In comparison, animals displayed toxicity with gossypol, such as scurfy and rough hair as well as lethargic behavior, in spite of no apparent weight loss after injection. The lethal dose of gossypol was 50 mg/kg daily via intraperitoneal injection for 2 d or 25 mg/kg intraperitoneal injection for 4 d. The effects of ApoG2 and gossypol on renal toxicity and hepatotoxicity were assessed by monitoring blood urea nitrogen (BUN), creatinine (CRE), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The data are shown in Table 1. Gossypol led to an approximately 3.5-fold increase in ALT and an approximately 3-fold increase in AST, while ApoG2-treated mice showed no increase in ALT or AST. Unlike the results for liver toxicity, neither gossypol nor ApoG2 significantly affected indicators of renal function: BUN and CRE. This reveals that ApoG2 is less toxic than gossypol.

Based on the ApoG2 results, we further evaluated the anti-cancer effect of ApoG2 in subcutaneous SGC-7901-LUC cell xenografts in mice. The tumor burden was measured in real-time with sequential whole-body imaging using the IVIS imaging system. As established in Fig. 1B which verifies that the number of SGC-7901-LUC cells is correlated with bioluminescence signal intensity, the bioluminescent signals from mice reflected the tumor growth status, and imaging data showed that the bioluminescent signals...
were relatively lower in the treatment group than the control group (Fig. 4A). The comparison of total bioluminescence intensity between the treatment group and control group shows that ApoG2 resulted in tumor growth delay (Fig. 4B). Next, we quantified the bioluminescent signals by regions of interest (ROI) that encompassed the tumor tissue. Excised organs were subjected to bioluminescence imaging at the end of the drug treatment. The results illustrate that the bioluminescent signals were completely emitted from the tumor (Fig. 4C). Tumor growth was also monitored by caliper and balance measurements. The results show that ApoG2 has significant anti-cancer activity in gastric cancer, consistent with bioluminescence imaging analysis at the end of the drug treatment. The results illustrate that the bioluminescent signals were completely emitted from the tumor (Fig. 4C). Tumor growth was also monitored by caliper and balance measurements. The results show that ApoG2 has significant anti-cancer activity in gastric cancer, consistent with bioluminescence imaging analysis (Fig. 4D–F). Finally, pathological examination was carried out by hematoxylin and eosin (H&E) staining along with a TUNEL assay to determine if ApoG2 induces apoptosis of gastric cancer in vivo. As shown in Fig. 5A and B, the cells in tumor tissues of the ApoG2 treatment group exhibited necrotic or pyknotic nuclei. However, there were no significant lesions in other normal tissues. The results demonstrate that ApoG2 has an excellent anti-cancer therapy response while having low toxicity to normal tissues. As shown in Fig. 5C, the number of TUNEL-positive cells, stained brown in the ApoG2 treatment group, was significantly more than that of the control group. The results show that treatment with ApoG2 could significantly induce apoptosis and furthermore inhibit tumor growth.

3.5. ROS production by ApoG2 in SGC-7901 cells

It has been reported that ApoG2-mediated cell death in nasopharyngeal carcinoma is partly ROS-dependent [41,42]. Here, we tested the accumulation of ROS in ApoG2 treated gastric cancer cells by flow cytometry (Fig. 6A). The relative fluorescence intensity of DCFH-DA (% of the control) is shown in Fig. 6B. After pretreatment with the highest concentration of ApoG2, DCFH-DA fluorescence intensity increased by 738% of the normal conditions. Compared with the control group, treatment with ApoG2 resulted in significantly increased ROS generation in a dose-dependent manner.
3.6. Effect of ApoG2 on \([\text{Ca}^2+]\) in SGC-7901 cells

\([\text{Ca}^2+]\) concentration is a key intracellular signal in the apoptotic pathway [43]. Therefore, whether ApoG2 leads to \([\text{Ca}^2+]\) elevation in SGC-7901 cells was investigated using the fluorescent probe Fluo-3/AM. As shown in Fig. 6C, after treating cells with 10–50 \(\mu\text{mol/L}\) ApoG2 at 10 \(\mu\text{mol/L}\) increments, the level of \([\text{Ca}^2+]\) correspondingly increased by 4.4%, 5.1%, 8.6%, 20.6% and 28.8% and only by 0.6% in the control samples. The relative fluorescence intensity of Fluo-3/AM (% of the control) reveals that \([\text{Ca}^2+]\)
increased as the concentration of ApoG2 rose, compared with the control group (Fig. 6D).

3.7. Activation of ER stress pathway by ApoG2

One recent study indicates that high levels of ROS and an elevation of \([\text{Ca}^{2+}]\) could elicit ER stress-induced apoptosis. Therefore, in this study we monitored expression levels of a series of proteins involved in the ER-stress pathway via western blot analysis after cell treatment with different concentrations of ApoG2. ApoG2 caused higher expression of calpain-1, calpain-2 and further activated and cleaved caspase-12 in SGC-7901 cells (Fig. 7A). In addition, elf2x and p-elf2x levels also increased (Fig. 7B). GADD153 (growth-arrest-and-DNA-damage-inducible-gene 153), regarded as a marker of ER stress-mediated apoptosis [44], was increased in ApoG2-treated cells (Fig. 7B). The Bcl-2 and Bax proteins are two downstream targets of GADD153 and therefore also play an important role in apoptotic signal pathways. ApoG2 induced down-regulation of Bcl-2 protein expression and up-regulation of Bax protein expression in SGC-7901 cells (Fig. 7C). This type of relationship increases the pro-apoptotic/anti-apoptotic Bcl-2 family protein ratio, which can be used to determine whether cancer cells are progressing towards apoptosis.

4. Discussion

Chemotherapy, inhibition or reversion of the process of carcinogenesis using non-toxic substances, is now considered an essential
method to prevent and/or treat gastric cancer. Resistance to chemotherapy is not uncommon and usually leads to the failure of chemotherapy against gastric cancer [45]. Therefore, it is necessary to develop novel therapeutic strategies and drugs for the...
management of gastric cancer. Recently, the strategy of blocking the activities of anti-apoptotic proteins has gained more and more attention. Several non-peptide small molecular inhibitors of Bcl-2 family proteins have been synthesized and used in anti-cancer therapy studies for various types of cancers; particularly, ApoG2 has been reported to have significant anti-cancer activity in vitro or in vivo for several types of cancers. However, little research has focused on ApoG2 for gastric cancer therapy. As we know, proliferation and apoptosis are extensively used biomarkers employed for diagnosis and measurement of tumor aggressiveness and are therefore used to evaluate tumor responses of new anti-cancer drugs [46]. Hence, we evaluated the anti-proliferation and induction of apoptosis effects of ApoG2 in gastric cancer in vitro and in vivo. The MTT assay and colony formation assay showed that ApoG2 effectively inhibited growth and induced apoptosis in gastric cancer in vitro. In this present study, we found the IC50 concentration of ApoG2 for the SGC-7901 gastric cancer cell line was relatively high compared with similar studies in different types of cancers, such as human lymphoma U937 cells (9.26 μM), nasopharyngeal carcinoma C666-1 cells (1.7 μM), CNE-1 cells (2.06 μM) and CNE-2 cells (4.92 μM); on the other hand, it was relatively low compared with some other types of cancers, such as human hepatocellular carcinoma Bel-7402 cells (21.61 μM) and HepG2 cells (30.63 μM) [23,27,30]. Therefore, the treatment efficacy of ApoG2 may depend on the types of cancers and the degree of tumor differentiation. It is necessary to investigate the anti-cancer effect on other gastric cancer cell lines, and it is actively underway in our laboratory. In addition, the colony formation assay demonstrated that a smaller dose of ApoG2 can inhibit the growth of gastric cancer cells with a long treatment time. Thus, the anti-cancer effect of ApoG2 on gastric cancer may improve by increasing the treatment time. Furthermore, we compared ApoG2 and gossypol for their anti-growth effect and ability to induce apoptosis in gastric cancer cells. The inhibition of cell survival and the ability of apoptosis induction of gossypol were more significant than ApoG2 in the short term. However, the colony formation assay revealed that ApoG2 had a higher anti-proliferation effect than gossypol. Based on the above, it is reasonable to speculate that this may be caused by higher toxicity of gossypol.

Gossypol is a natural product isolated from cotton seeds and roots and has two aldehyde groups in its chemical structure that are associated with toxicity. Thus, ApoG2 was synthesized by removing the two aldehyde groups and found to maintain anti-cancer effects with less toxicity in several types of cancers [26,30]. In this study, we compared the systemic toxicities of ApoG2 and gossypol in mice by measuring renal and liver function, observing toxic signs, such as scruffy and rough hair and lethargic behavior, and performing H&E staining. The results indicate that ApoG2 has lower toxicity than gossypol, consistent with previous reports [26,47]. Therefore, ApoG2 may be a useful new anti-cancer agent for gastric cancer therapy. In addition, several previous studies have reported that ApoG2 combined with adriamycin or cisplatin has a synergistic effect that enhances anti-cancer activities in human hepatocellular carcinoma therapy and in nasopharyngeal carcinoma therapy [23,30]. It reminds us that ApoG2 may be used as a safe and effective agent combined with targeted or conventional drugs for anti-gastric cancer therapy. This work is now actively underway in our laboratory.

To facilitate the translation of ApoG2 into clinical practice for gastric cancer therapy, in vivo drug therapy response was examined. Measuring the tumor volume or tumor weight are the traditional monitoring methods to evaluate anti-tumor effects of drugs in vivo. However, this leads to misguided results of drug therapy response because necrosis and edema are disregarded in the measurement. Bioluminescence imaging is a novel promising real-time, fast, noninvasive, high throughput and sensitive in vivo visualization method for estimating drug therapy response. We evaluated the anti-cancer effect of ApoG2 in vivo by real-time, whole-body bioluminescence imaging and traditional monitoring methods. The ultimate results show that ApoG2 led to obvious tumor growth delay; even though, the anti-cancer effect of ApoG2 on gastric cancer in the treatment group was not so impressive. Because of the lower toxicity, the treatment efficacy of ApoG2 for gastric cancer in preclinical studies may be enhanced by increasing dosage and/or treatment time and combining with targeted or conventional drugs. Compared with traditional monitoring methods, bioluminescence imaging improves the sensitivity of detection and provides a novel non-invasive and effective evaluation tool of anti-cancer effects, thus promoting drug development.

The death receptor pathway, mitochondrial pathway and endoplasmic reticulum stress-induced apoptosis pathway are three principal ways to induce apoptosis [48]. However, the possible mechanism of ApoG2 in gastric cancer is unclear. Previous studies demonstrated that ApoG2-mediated cell death in nasopharyngeal carcinoma is partly ROS-dependent [41]. Therefore, in this study, we tested the concentration of ROS and [Ca2+]i by flow cytometry in SGC-7901 cells treated with ApoG2. ApoG2 resulted in the generation of ROS and an increase in the [Ca2+]i level, both of which could trigger ER stress. Such ER stress can trigger several specific signaling pathways, such as ER-associated protein degradation and the unfolded protein response (URP). Among them, the URP can elicit paradoxical outputs, which induce cytoprotective and destructive functions that trigger apoptosis when the stress is prolonged or such adaptive responses fail [49,50]. To our knowledge, URP is mediated via three ER transmembrane sensors, IRE1α (inositol-requiring transmembrane kinase and endonuclease), PERK (protein kinase-like ER kinase) and ATF6 (activation of transcription factor 6) [51]. After self-rescue is triggered, combined activation of IRE1α, PERK and ATF6 initiates the cell’s apoptotic response via activating eIF2α, triggering eIF2α phosphorylation and activating proapoptotic molecules such as GADD153/CHOP [52,53]. In this study, our data show that ApoG2 upregulated eIF2α, p-eIF2α and GADD153 expression in SGC-7901 cells. When ER stress occurs, calpains are activated, which further activate caspase-12 located on the ER membrane. Caspase-12 is then dissociated from the ER membrane and cleaved, initiating downstream apoptotic pathways [54]. Our data indicate that after treatment of ApoG2, the expression of calpain-1, calpain-2 and cleaved caspase-12 is significantly increased in SGC-7901 cells. In
addition, it is noteworthy that Bcl-2 and Bax proteins, as two downstream targets of GADD153, play an important role in apoptotic signal pathways. Bcl-2 family proteins are usually considered key regulators of mitochondrial-mediated cell death and are also involved in the regulation of ER stress-mediated apoptosis [44]. In this study, SGC-7901 cells treated with ApoG2 demonstrated down-regulated Bcl-2 protein expression and up-regulated Bax protein expression, thus resulting in an increase in the Bcl-2/Bax ratio. Certainly, other ER stress-related genes, such as ATF6, GRP78 and GRP94, should be studied in future work to advance understanding of ApoG2-induced ER stress-mediated apoptosis.

In summary, we demonstrated a novel small-molecule inhibitor of anti-apoptotic Bcl-2 family protein, ApoG2, with significant anti-tumor activity in vitro and in vivo in gastric cancer. It can elevate intracellular ROS and calcium ion concentration, up-regulate expression of calpain-1, calpain-2, p-eIF2z, GADD153 and Bax protein and activate cleavage of caspase-12, while down regulating the expression of Bcl-2 protein. Our findings suggest that ApoG2 can effectively inhibit the growth and proliferation of gastric cancer cells, induce apoptosis associated with ER stress and provoke a strong tumor treatment response in vivo. ApoG2 may be a promising new agent for gastric cancer therapy.

Conflict of interest

All authors declare that there are no conflicts of interest concerning this article.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2013.03.019.

References


