Biochemical and Biophysical Research Communications 505 (2018) 787–793

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel derivative (GTN024) from a natural product, komaroviquinone, induced the apoptosis of high-risk myeloma cells via reactive oxygen production and ER stress

Mikio Okayama [a, b], Shotaro Kitabatake [a], Mariko Sato [a], Kota Fujimori [a], Daiju Ichikawa [a], Maiko Matsushita [a], Yutaka Suto [c], Genji Iwasaki [c], Taketo Yamada [d], Fumiyuki Kiuchi [e], Maki Hirao [f], Hisako Kunieda [f], Makoto Osada [f], Shinichiro Okamoto [b], Yutaka Hattori [a, f, *]

[a] Clinical Physiology and Therapeutics, Keio University Faculty of Pharmacy, Tokyo, Japan
[b] Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan
[c] Faculty of Pharmacy, Takasaki University of Health and Welfare, Gunma, Japan
[d] Department of Pathology, Saitama Medical University, Saitama, Japan
[e] Division of Natural Medicines, Keio University Faculty of Pharmacy, Tokyo, Japan
[f] Department of Hematology, Tokyo Saiseikai Central Hospital, Tokyo, Japan

ARTICLE INFO

Article history:
Received 19 September 2018
Accepted 28 September 2018
Available online 5 October 2018

Keywords:
Multiple myeloma
Natural product
Apoptosis
Reactive oxygen species
ER stress

ABSTRACT

New drugs have significantly improved the survival of patients with multiple myeloma (MM), but the prognosis of MM patients with high-risk cytogenetic changes such as t(4; 14), t(14; 16) or del17p remains very poor. A natural product, komaroviquinone (KQN), was originally isolated from the perennial semi-shrub *Dracocephalum komarovi* and has anti-protozoal activity against *Trypanosoma cruzi*, the organism causing Chagas' disease. Here we demonstrate that a novel KQN-derivative, GTN024, has an anti-MM effect both *in vitro* and *in vivo*. GTN024 induced the apoptosis of MM cell lines including those with high-risk cytogenetic changes. GTN024 produced reactive oxygen species (ROS) and increased phosphorylated eIF2α. The ROS production and subsequent endoplasmic reticulum (ER) stress are thought to play a key role in GTN024-induced apoptosis, as the apoptosis was completely abrogated by anti-oxidant treatment. In a mouse xenograft model, an intraperitoneal injection of 20 mg/kg of GTN024 significantly delayed tumor growth. Hematological toxicity and systemic toxicity as indicated by weight loss were not observed. These results suggest that the novel KQN-derivative GTN024 could become a candidate drug for treating high-risk MM.

1. Introduction

Multiple myeloma (MM) is a B-cell neoplasm that causes clonal plasma cell proliferation in bone marrow and bone lesions. The 5-year prevalence rate of MM incidence in Japan is reported to be 9.7 per 100,000 persons [1]. New agents such as proteasome inhibitors and immunomodulatory drugs (IMiDs) have significantly improved the overall survival of MM patients [2–5], drugs from different categories such as a histone-deacetylase inhibitor [6], an anti-SLAMF7 antibody [7], and an anti-CD38 antibody [8] have also been reported to be effective for refractory MM in combination therapy with IMiDs and proteasome inhibitors.

Despite these advances, the survival of certain groups of MM patients remains unsatisfactory [9–11]. Those patients are known as having 'high-risk MM,' and their MM cells frequently possess chromosomal abnormalities such as t(4; 14), t(14; 16), del17p, and 1q21 amplification. The revised International Staging System indicates that the overall survival of the patients with high-risk cytogenic abnormalities is significantly short [12]. Another limitation of newly developed drugs is their toxicities [13,14], which impede the optimal drug efficacy and result in unsatisfactory treatment outcomes, especially among elderly patients. New therapeutic modalities that are effective for high-risk MM with less side effects are thus currently an unmet clinical need in MM treatment.

A series of anti-neoplastic drugs were developed from natural

* Corresponding author. Clinical Physiology and Therapeutics, Keio University Faculty of Pharmacy, Tokyo, Japan.
E-mail address: hattori-yt@pha.keio.ac.jp (Y. Hattori).

https://doi.org/10.1016/j.bbrc.2018.09.177
0006-291X/

products, and there are some reports describing anti-neoplastic activities of anti-protozoal agents. For example, nifurtimox, a drug for Chagas' disease, showed anti-tumor effects against neural tumor cells. Nifurtimox induced the apoptosis of neuroblastomas by inhibiting extracellular signal-regulated kinase (ERK) phosphorylation [15]. Artesunate, an anti-malaria drug, also showed anti-MM effects by inhibiting nuclear factor-kappa B (NFκB) function [16]. With these drugs, the anti-tumor activities were discovered as off-target effects in drug repositioning studies.

Komaroviquinone (KQN) is one of the natural products isolated from the perennial semi-shrub *Dracocephalum komarovi* (family Laminaceae), which shows anti-protozoal activities. Suto et al. reported the asymmetric synthesis of KQN [17]. In a study of the structure activity relationship study of KQN [17], a series of low-molecular-weight compounds were discovered to exhibit promising anti-protozoal activities against *Trypanosoma cruzi*, which is the causative pathogen of Chagas' disease [18]. It is also reported that KQN was reduced by *T. cruzi* old yellow enzyme (TcOYE) to form its semiquinone and produced reactive oxygen species (ROS), which showed trypanocidal activities [19]. A biomedical assay of both KQN and its derivatives demonstrated that the new KQN-derivative GTN024 had high anti-proliferation activities against MM cells [20]. In addition, in the above-cited structure-activity relationship study, GTN024 was shown to be readily accessible and a valuable compound for the further pharmacodynamic study of MM cells [20].

With this background, we carried out the present study to determine the anti-tumor effects of GTN024 on MM cell lines including those with high-risk cytogenetic changes, and we clarified this promising new drug's mode of action and safety.

2. Materials and methods

2.1. Cells

The human myeloma cell lines KMM1, KMS11, KMS21, KMS26, KMS27, KMS28 and KMS34 were kindly provided by Dr. T. Otsuki (Kawasaki Medical School, Kurashiki, Japan) [21]. The cell line MUM24 was established in our laboratory from a patient with thalidomide-resistant MM [22]. These cell lines were maintained in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (Pen Strep, Gibco). Chronosomal abnormalities were detected by the fluorescence *in situ* hybridization (FISH) analysis. (LSI medience, Tokyo).

2.2. Reagents

GTN024 (Fig. 1A) was prepared as described by Suto et al. [18]. In the present *in vitro* study, GTN024 was diluted in phosphate-buffered saline (PBS, Sigma-Aldrich) containing 1% Tween®80 (Otsuka Pharmaceutical, Tokyo) and 10% DMSO.

2.3. Patient's samples

Bone marrow samples were collected from MM patients treated at Tokyo Saiseikai Central Hospital. The collection of clinical samples was approved by ethical committee of Saiseikai Central Hospital (No. 28–66) and the Faculty of Pharmacy, Keio University (No. 170616–5, 180615–5). Written informed consent for their samples to be used was obtained from all patients. Cells were isolated by centrifugation with Lymphoprep™ (Axis-Shield, Oslo, Norway). Cells were labeled with CD138 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The magnetically labeled CD138-positive cells were purified by MACS Columns (Miltenyi Biotec).

2.4. Trypan blue exclusion assay

Cells (2×10^5 cells/mL) were seeded on six-well plates and cultured in various concentrations of GTN024 (0–20 μM) with or without 6 mM of *N*-acetyl cysteine (NAC, Sigma-Aldrich) or 3 mM of glutathione (GSH, Sigma-Aldrich) at 37 °C in 5% CO_2. The cells were stained with Trypan Blue Stain 0.4% (Gibco) and viable cells were counted by an automatic cell counter TC20™ (Bio-Rad, Hercules, CA). Viable cells were counted three times, and the average was calculated. The IC_{50} of GTN024 was calculated by approximation.

2.5. MTT assay

Collected clinical samples (6×10^4 cells/mL) were seeded on 96-well plates and cultured with various concentrations of GTN024 (0–30 μM) at 37 °C in 5% CO_2 for 48 h. The viability of the cells was calculated by MTT dye absorbance (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

2.6. Apoptosis detection assay

Cells (2×10^5 cells/mL) were seeded on six-well plates and cultured at 37 °C in 10 μM GTN024 for 72 h. Apoptotic cells were detected by an annexin V-FITC Apoptosis Detection Kit (BioVision, San Francisco, CA) following the manufacturer's protocol. Briefly, cells were collected and resuspended in 500 μL of 1 × Binding Buffer and stained with annexin V –FITC and propidium iodide (PI) for 5 min. The cells were analyzed using a BD™ LSRII flow cytometer (Becton Dickinson, Lincoln Park, NJ).

2.7. Detection of reactive oxygen species

Cells (2×10^5 cells/mL) were seeded on six-well plates and cultured at 37 °C with or without 6 mM NAC or 3 mM GSH for 2 h. Then, 20 μM GTN024 was added and incubated in 5% CO_2. After 72 h, 1 μM chloromethyl-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen, Carlsbad, CA) was added and incubated for 30 min. The stained cells were analyzed using the BD LSRII flow cytometer.

2.8. Western blotting

Cells were cultured with GTN024 and lysed in 1% NP-40 buffer containing 1 mM PMSF, 1 mM Na_2PO_4, 20 mM NaF, 2 mM Na_2PO_7, and protease inhibitors (Complete Protease Inhibitor Mixture, Roche Diagnostics, Mannheim, Germany). After incubation for 10 min on ice, the lysates were centrifuged at 15,000 rpm for 10 min at 4 °C, and the supernatants were collected. The amount of protein was evaluated by BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

The lysates were mixed with Laemmli's buffer (1.33% SDS, 10% glycerol, 0.083 M Tris-HCl, 0.04% bromphenol blue, 2% 2-ME) and boiled for 5 min. The lysates were subjected onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a PVDF membrane. The membranes were blocked with 5% skim milk and then immunoblotted. Antibodies against eIF2α (Cell Signaling Technology, Danvers, MA), *p*-eIF2α (Cell Signaling Technology), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), and cleaved caspase-3 (Cell Signaling Technology) (diluted at 1:1000) was used. The second antigen-antibodies was a horseradish peroxidase (HRP)-coupled anti-rabbit, anti-mouse Ig antibody (diluted at 1:500). Antigen-antibody complexes were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

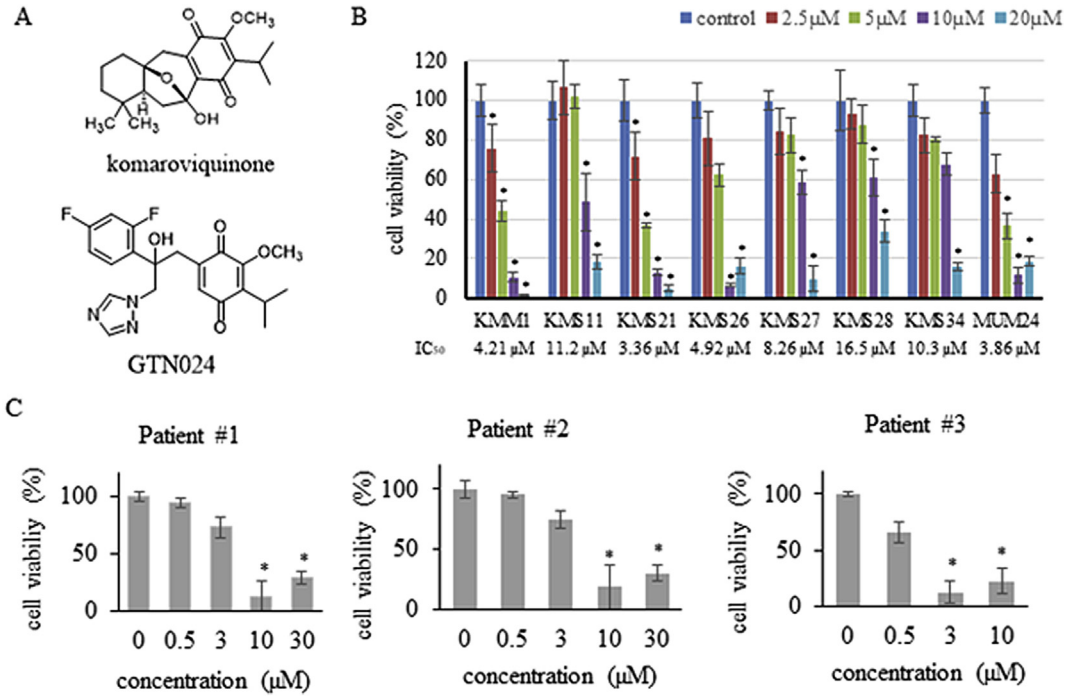

Fig. 1. GTN024 induced cell death in MM cell lines and MM patients. (A) The chemical structures of komaroviquinone and GTN024. (B) The inhibitory effects of GTN024 on MM cells with chromosomal abnormalities [22,35]. Cells were cultured with GTN024 for 48 h. The number of viable cells was counted by staining with trypan blue. Bars: means ± SD, n = 3. *p < 0.05 vs. control. (C) The inhibitory effects of GTN024 on MM clinical samples. Cells were collected from clinical bone marrow samples obtained from three MM patients. The viability of $CD138^+$ cells treated with GTN024 was measured by MTT assay. Bars: indicate means ± SD, n = 3. *p < 0.05 vs. control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.9. Toxicity assessment

To evaluate the toxicity of GTN024 *in vivo*, we intraperitoneally injected 0, 10, 20, or 40 mg/kg of GTN024 in 1% Tween®80 and 10% DMSO in saline to 5-wk-old male ICR mice (Clea, Tokyo) daily for three consecutive days. The body weights of the mice were measured every 3 days. We took peripheral blood samples with a heparinized hematocrit tube (Terumo, Tokyo) from the tail veins of the mice every 3 days. Blood samples were diluted 10 times by Türk's solution (Merck, Darmstadt, Germany), and the numbers of leukocytes and neutrophils were counted under a light microscope. All animal experiments were approved by the Ethics Committee for Animal Experiments at Keio University (Approval no. 12067-(2)).

2.10. In vivo tumor growth assay

KMS11 cells (3×10^7 cells) were inoculated into 5-wk-old male ICR/SCID mice (Clea) subcutaneously in the flank. When the resulting tumors reached 100 mm^3, 20 mg/kg of GTN024 (1% Tween®80, 10% DMSO in saline) was injected intraperitoneally daily for three consecutive days. The tumor volume was calculated by length × $width^2$ × 0.52 [23].

2.11. Histopathologic examination

Xenografted mice were sacrificed, and isolated tumors were fixed with 10% formalin and embedded in 5-μm pieces of paraffin. Sliced sections were stained with hematoxylin and eosin (H&E). Anti-human cleaved PARP polyclonal antibody (Cell Signaling Technology Japan, Tokyo), anti-human cleaved caspase-3 (Asp175) polyclonal antibody (Cell Signaling Technology Japan), anti-human Ki-67 monoclonal antibody (clone MIB-1) (Dako Japan, Tokyo), and anti-human PCNA polyclonal antibody (Atlas Antibodies, Stockholm, Sweden) were used for immunohistochemistry.

2.12. Statistical analysis

The significance of differences was determined using Student's *t*-test. The level of significance was set at $p < 0.05$.

3. Results

3.1. GTN024 inhibited the growth of MM cells

We examined the tumoricidal effects of GTN024 against various MM cell lines. GTN024 induced the cell death of all of the MM cell lines tested (KMM1, KMS11, KMS21, KMS26, KMS27, KMS28, KMS34, and MUM24) (Fig. 1B). IC_{50} varied from 3.36 μM (KMS21) to 16.5 μM (KMS28). One arm of chromosome 17 is deleted in KMM1, KMS11, KMS26, KMS34 and MUM24 cells, and KMS11, KMS26, KMS28, KMS34 and MUM24 also show t(4; 14) (our unpublished data).

We then examined whether GTN024 could induce cell death in $CD138^+$ cells obtained from three MM patients. Patient #1 and #2 had untreated MM and both of their MM cells had 1q21 amplification. Patient #3 had been treated with and showed resistance to bortezomib, lenalidomide, pomalidomide, ixazomib, and elotuzumab. Based on the results shown in Fig. 1C, their IC_{50} values in response to GTN024 were calculated as 5.09 μM (patient #1), 5.94 μM (patient #2), and 0.84 μM (patient #3).

3.2. Anti-MM effect via the ROS production of GTN024

We examined the ROS production in GTN024-treated MM cells. The flow cytometric analysis using CM-H_2DCFDA showed that GTN024 significantly induced ROS production in MM cells (Fig. 2A).

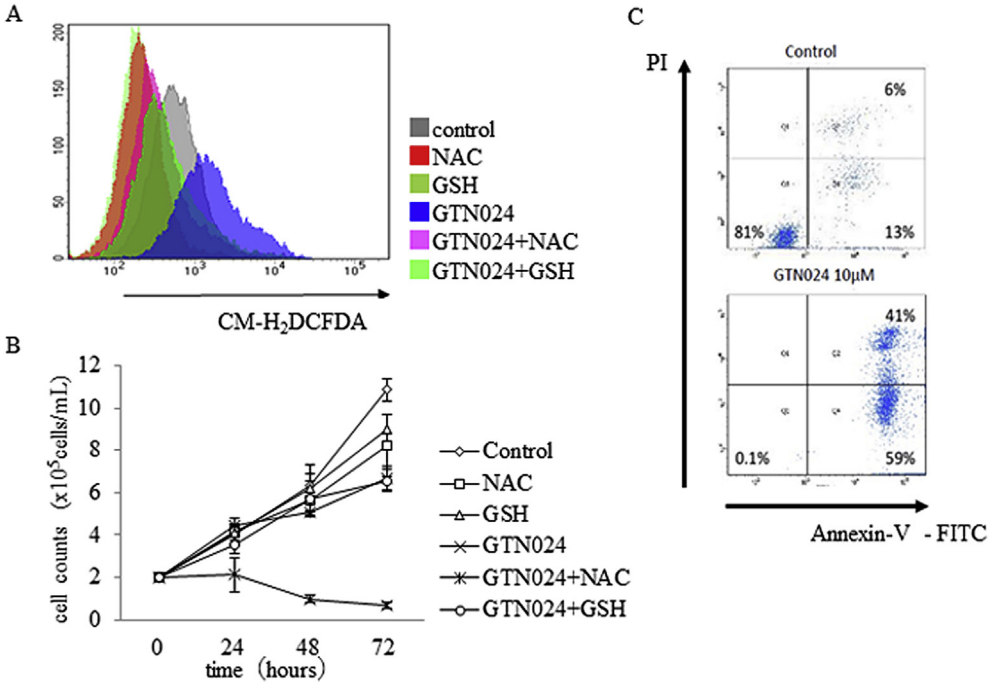

Fig. 2. GTN024 treatment resulted in ROS-mediated apoptotic cell death. (A) MUM24 cells (2×10^5 cells/mL) were incubated with or without NAC or GSH for 2 h and GTN024 was added. ROS levels were determined by staining with CM-H_2DCFDA. Stained cells were analyzed using a FACS BD LSRII. (B) MUM24 cells (2×10^5 cells/mL) were cultured with NAC or GSH for 2 h, and GTN024 was added. The number of viable cells was counted by staining with trypan blue. Bars: mean ± SD, n = 3. *$p < 0.05$ vs. control. (C) MUM24 cells (2×10^5 cells/mL) were cultured with 10 μM GTN024 for 72 h stained with Annexin V-FITC and propidium iodide (PI) followed by an analysis with the BD LSRII system.

We next examined whether the growth inhibitory effect of GTN024 depends on ROS production. As shown in Fig. 2B, the growth inhibition by GTN024 treatment was mostly abrogated when the MM cells were preincubated with anti-oxidants. The flow cytometric analysis also showed that the GTN024 treatment of MM cells increased the number of annexin V^+ cells, indicating that GTN024 induced apoptosis (Fig. 2C).

3.3. Excessive ER stress pathway appears to be the cause of GTN024's anti-MM activity

Since GTN024 showed tumoricidal effects in an ROS-dependent manner, we hypothesized that GTN024 would cause excessive endoplasmic reticulum (ER) stress. We observed that the phosphorylation of eIF2α was increased in GTN024-treated MM cells (Fig. 3A). We also observed an increased amount of cleaved caspase-3 following treatment with GTN024 (Fig. 3B).

3.4. Safety and the anti-MM effects of GTN024 in vivo

To determine the optimal dosage of GTN024 in the KMS11-xenografted mice model, we administered 10, 20, or 40 mg/kg day of GTN024 to ICR mice by intraperitoneal injection for three consecutive days (days 1–3). When mice were treated with 40 mg/kg day of GTN024, significant weight loss and bowel obstructions were observed. However, when 10 mg/kg day or 20 mg/kg day were given to mice, no loss of body weight was observed (Fig. 4A). No hematological toxicities were detected at any dosage levels (Fig. 4A). Given these results, we considered 20 mg/kg for three consecutive days as the maximal tolerated dose for mouse experiments.

To examine the growth-inhibitory effects of GTN024 *in vivo*, we intraperitoneally injected 20 mg/kg of GTN024 in KMS11-xenografted mice for three consecutive days. As shown in Fig. 4B, GTN024 significantly delayed tumor growth in xenografted mice. At 14 days after the first injection, the average tumor volume was 383 mm^3 in the GTN024-treated mice, and 843 mm^3 in the control mice ($p < 0.05$).

The H&E staining showed an agglutination of chromatin in the tumors of GTN024-treated mice (Fig. 4C). Cleaved-caspase-3-positive and PARP-positive cells were also significantly increased in the GTN024-treated tumors. In contrast, staining with MIB-1, a strong marker of cell proliferation, was weaker in the tumors of the GTN024-treated mice compared to those of the control mice (Fig. 4C).

4. Discussion

A number of novel drugs have been derived from natural products. For example, irinotecan, a derivative of camptothecin that was originally isolated from the deciduous tree *Camptotheca acuminata*, is now widely used as an anti-cancer agent for lung cancer and colorectal cancer [24]. Paclitaxel, which is derived from the tree *Taxus brevifolia*, induces the apoptosis of cancer cells via binding to β-tubulin, inducing depolymerization and stabilizing microtubules [25]. A variety of molecular-targeting agents have been developed, but their success is limited to a few malignant diseases such as chronic myelogenous leukemia.

Multiple myeloma is a genetically heterogenenous disease, and target molecules (such as a driver gene mutation) have not been discovered. More therapeutic options including novel compounds are needed for treating high-risk MM patients to improve their prognoses and quality of life. The exploitation of clinically effective drugs is time-consuming and involves significant costs. Therefore, natural products remain an important resource for drug development. In this study, we focused on a novel natural compound, KQN, and its derivative, GTN024 analogues.

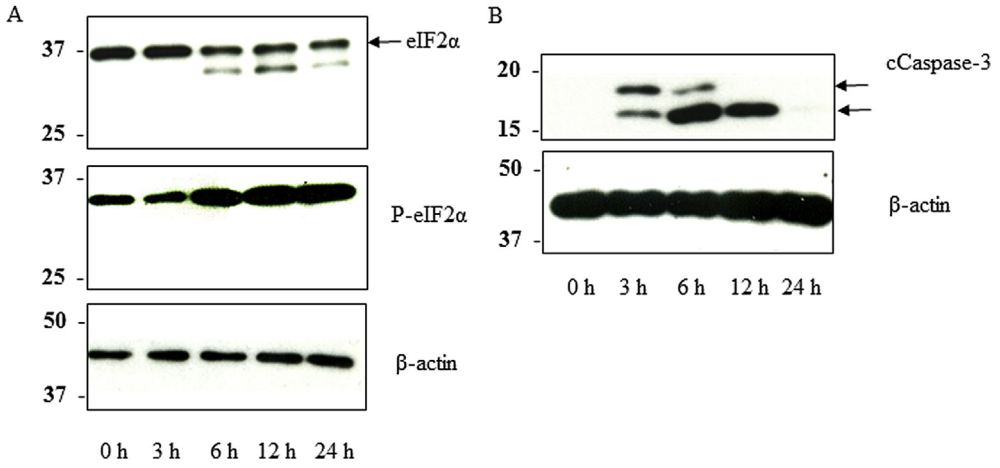

Fig. 3. GTN024 induced ER stress-mediated cell death in MUM24 cells. MUM24 cells were treated with GTN024 for 3, 6, 12, or 24 h. The cells were lysed and analyzed by immunoblotting against eIF2α, *p*-eIF2α, β-actin, and cleaved caspase-3. The lower band of eIF2α was considered as the degradation of eIF2α [36].

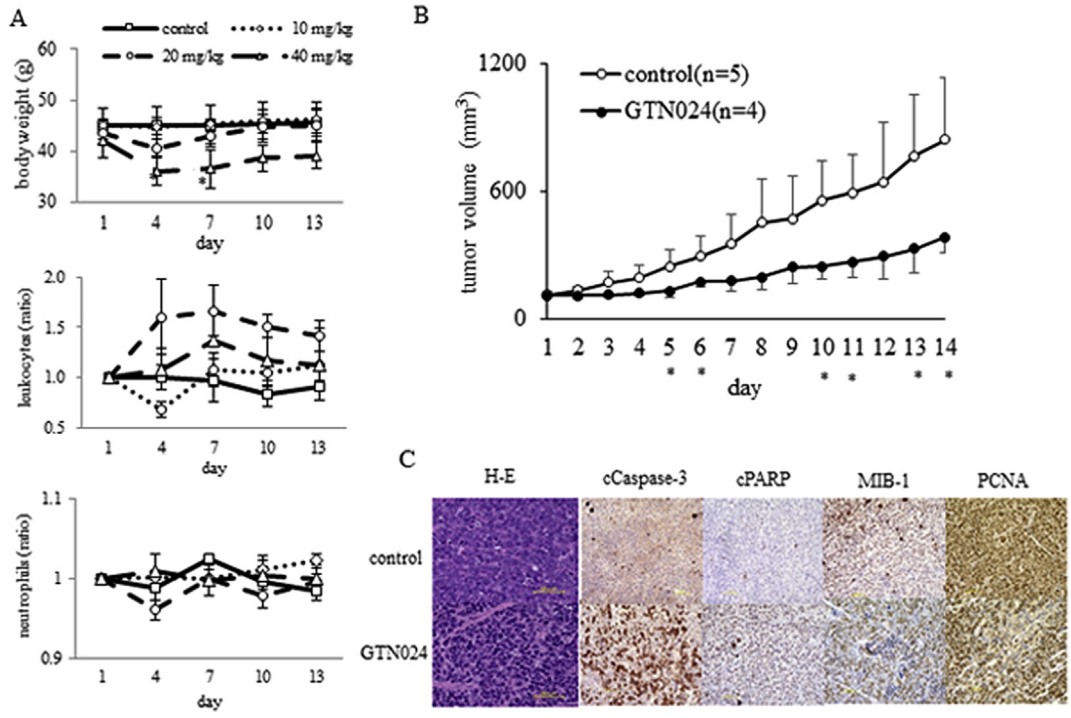

Fig. 4. Toxicity and anti-MM effects of GTN024 in the *in vivo* model. (A) ICR mice were treated with 10, 20, or 40 mg/kg of GTN024 for three consecutive days (days 1–3). Blood samples were collected from tail veins. Leukocyte and neutrophil numbers were counted after Türk's solution staining. Bars: mean ± SD, n = 3. *p < 0.05 vs. control. (B) Suppressed growth of MM cells in the xenograft mouse model by GTN024 20 mg/kg. GTN024 was given when xenografted tumor exceeded 100 mm^3. GTN024 20 mg/kg was given intraperitoneally on days 1–3. Bars: mean ± SD. *p < 0.05 (control vs. GTN024). (C) Stained xenografted tumor of a GTN024-treated mouse. Xenograft tumors in mice after treatment with or without GTN024 were collected and stained with H&E, anti-cleaved caspase-3, anti-cleaved PARP, anti-MIB-1, and anti-PCNA.

Suto et al. synthesized a series of KQN-related compounds. The above-cited structure-activity relationship study revealed that the hydroquinone moiety is necessary to potent anti-tumor effects of these compounds [20]. Among them, GTN024, which has a benzoquinone moiety, showed significant anti-MM effects, and was readily accessible because it has only a single chiral carbon [20]. We therefore conducted further research regarding GTN024.

Our present findings demonstrated that GTN024 had *in vitro* anti-MM effects against MM cell lines and clinical samples obtained from MM patients including those with high-risk chromosomal abnormalities, indicating that GTN024 is a promising candidate for treating MM patients with high-risk cytogenic changes.

As described in results, we concluded that 20 mg/kg for 3 days is the maximum tolerated dose for our mouse model. In our mouse xenograft model, significant anti-MM effects were produced by the same schedule of drug administration without severe toxicities. The results of the histopathological examination confirmed that GTN024 caused the apoptosis of xenografted tumors.

KQN, the mother compound of GTN024, was reduced by TcOYE to its semiquinone form, and the production of ROS is the key mechanism of anti-trypanocidal effects [18,19]. In the present study, we observed that GTN024 showed ROS production and

induced the apoptosis of MM cells, which were abrogated by antioxidants. We therefore speculate that the anti-MM effects of GTN024 are due mainly to the cytotoxicity by ROS. Several ROS-mediated compounds have also shown significant cytotoxicity against MM cells, via various pathways such as the inhibition of thioredoxin 1 by PX-12 [26], DNA damage by an ATR inhibitor [27], and the activation of p53 by CP-31398 [28]. It is thus apparent that ROS-mediated cytotoxicity plays an important role in treatments for MM.

MM cells are characterized by the excessive accumulation of unfolded M-protein. In this study, we focused on endoplasmic reticulum (ER) stress, because ROS cause the apoptosis of cells by an excessive ER response, which could be a therapeutic target in MM [29–32]. ROS induced ER stress via many signals including PERK and mitochondria pathway [33,34]. Here we focused on eIF2α, a key molecule of ER stress, and our findings showed an increased phosphorylation of eIF2α in MM cells by GTN024. We also observed increased level of cleaved caspase-3. These results suggested that ROS-mediated ER stress is a putative target pathway of GTN024-induced apoptosis.

In conclusion, we developed GTN024 from a natural product, KQN, and our present results demonstrated the induction of the apoptosis of MM cells with high-risk cytogenic abnormalities *in vitro* and *in vivo*. The major merits of using GTN024 in MM treatments are as follows. First, GTN024 showed cytotoxicity to MM cells with high-risk chromosomal changes that are resistant to currently available drugs. Some of the cell lines used in this study are resistant to lenalidomide or dexamethasone [22,23]. MUM24 cells were established from a thalidomide-resistant patient [22]. Second, in our mouse xenograft model, GTN024 significantly inhibited tumor growth without eminent hematological or systemic side effects when the mice were treated with 10–20 mg/kg of GTN024. Third, GTN024 induced apoptosis via ROS-mediated excessive ER stress, to which MM cells were highly vulnerable. We therefore propose that GTN024 is a promising candidate compound for the treatment of high-risk MM.

Acknowledgements

We thank for Prof. Otsuki for the kind gift of MM cell lines. We also thank Shogo Mori, Koya Kakimoto, and Sayaka Motonaga for doing the growth-inhibition assay. This work was supported in part by a Grant-in-Aid for Scientific Research and a grant from the Private University Strategic Research Base Development Program of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (to Y.H.); grants-in-aid from the Translational Research Network Program, Japan Agency for Medical Research and Development (AMED), #15lm0103010j0002 and #16lm0103010j0003 (Y.H.); and International Myeloma Foundation Japan's grants (M.M.), and Japanese Society of Myeloma (JSM) Research Award (D.I.).

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.09.177.

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