Research Article

20 (S)-ginsenoside Rh2 inhibits colorectal cancer cell growth by suppressing the Axl signaling pathway in vitro and in vivo

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Background: Colorectal cancer (CRC) has a high morbidity and mortality worldwide. 20 (S)-ginsenoside Rh2 (G-Rh2) is a natural compound extracted from ginseng, which exhibits anticancer effects in many cancer types. In this study, we demonstrated the effect and underlying molecular mechanism of G-Rh2 in CRC cells in vitro and in vivo.

Methods: Cell proliferation, migration, invasion, apoptosis, cell cycle, and western blot assays were performed to evaluate the effect of G-Rh2 on CRC cells. In vitro pull-down assay was used to verify the interaction between G-Rh2 and Axl. Transfection and infection experiments were used to explore the function of Axl in CRC cells. CRC xenograft models were used to further investigate the effect of Axl knockdown and G-Rh2 on tumor growth in vivo.

Results: G-Rh2 significantly inhibited proliferation, migration, and invasion, and induced apoptosis and G0/G1 phase cell cycle arrest in CRC cell lines. G-Rh2 directly binds to Axl and inhibits the Axl signaling pathway in CRC cells. Knockdown of Axl suppressed the growth, migration and invasion ability of CRC cells in vitro and xenograft tumor growth in vivo, whereas overexpression of Axl promoted the growth, migration, and invasion ability of CRC cells. Moreover, G-Rh2 significantly suppressed CRC xenograft tumor growth by inhibiting Axl signaling with no obvious toxicity to nude mice.

Conclusion: Our results indicate that G-Rh2 exerts anticancer activity in vitro and in vivo by suppressing the Axl signaling pathway. G-Rh2 is a promising candidate for CRC prevention and treatment.

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1. Introduction

Colorectal cancer (CRC) was the third most common cancer diagnosed and the second leading cause of cancer-related deaths in the United States in 2020 [1]. Although significant progress has been made in multimodality therapy for CRC, the overall 5-yr survival rate remains poor [2]. Surgical resection and chemotherapy are common treatment options for patients with CRC; however, recurrence and distant metastases occurs frequently. Thus, new and more effective therapeutic targets and strategies are required.

Natural products have been used for disease treatment and prevention for thousands of years. Because of their relatively low toxicity, the anticancer activity of natural product compounds has attracted significant attention from researchers [3–5]. Ginseng is a well-known herbal medicine that exhibits various pharmacological and therapeutic activities. 20 (S)-ginsenoside Rh2 (G-Rh2) is one of
the major active components of ginseng and shows potent anti-
cancer effects in several cancer models [6–8]. G-Rh2 has been re-
ported to suppress cervical cancer cell proliferation by inhibiting
the AKT/GSK3β signaling pathway [9] and suppresses CRC cell
growth by blocking the PI3K/AKT signaling pathway [7]. Moreover,
G-Rh2 was reported to be effective at reversing drug resistance in
several cancer types [10,11]. However, the effects and molecular
mechanisms underlying G-Rh2 in CRC have yet to be fully
elucidated.

Axl (also referred to as Ark, Tyro7, or Ufo) is a receptor tyrosine
kinase that plays an important role in the metastatic potential and
overall prognosis of many solid cancers [12–17]. Axl appears to
function as an oncogene in various human malignancies, including
pancreatic [18], breast [19], and lung [12,20] cancers. Axl functions
through several downstream pathways including the MAPK/ERK,
pancreatic [18], breast [19], and lung [12,20] cancers. Axl functions
through several downstream pathways including the MAPK/ERK,

Evidence indicates that Axl is an attractive therapeutic target for
recently entered phase II clinical trials in multiple cancers [15,22].

2. Materials and methods

2.1. Reagents

G-Rh2 (>98% purity) was obtained from Chengdu Biopurify
Phytochemicals Ltd. (Chengdu, China). Primary antibodies for
detecting p-Axl (Tyr702), VEGFR2, Src (32G6), p-Axl (Tyr416), p-
PI3K (Tyr458), p-PI3K, p-ERK1/2, ERK1/2, p-p38, p-STAT3
expression in DLD1 cells. p-MTor (Ser 2448), mTOR, E-cadherin,
N-cadherin, and Bax were obtained from Cell Signaling Technology
Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture

Human CRC cell lines HCT15, HCT116, and DLD1 and the human
colon fibroblast cell line CCD-18Co were purchased from the
American Type Culture Collection (Manassas, VA, USA). HCT15 and
DLD1 cells were grown in RPMI-1640 medium. HCT116 cells were
grown in McCoy’s 5A medium. CCD-18Co cells were grown in MEM
medium. All media contained 10% FBS (Gibco) and 1% penicillin/
streptomycin. All the cells were maintained in a 37°C incubator
with 5% CO2.

2.3. Cell viability assay

The Cell Counting Kit-8 (CCK-8; Dojindo, Japan) assays were
used to measure cell viability. Briefly, cells were seeded into 96-
well plates (1 × 10⁴ cells/well) and exposed to varying concentra-
tions of G-Rh2 for 0, 24, 48, and 72 h. At the corresponding time
points, CCK-8 solution (10 μL) was added to each well of the plate
and incubated for an additional 1 h at 37°C. The absorbance of each
well was read at 450 nm using a spectrophotometer.

2.4. Anchorage-independent cell growth assay

CRC cells (8 × 10³) were suspended in complete growth medium
with 0.6% agar and various concentrations of G-Rh2 in the base
layer and 0.3% agar with the various G-Rh2 concentrations in the
top layer in 6-well plates. The plates were cultured in a cell culture
incubator for 2 weeks. The number of colonies was subsequently
counted using the ImageJ software.

2.5. Cell cycle distribution and apoptosis

Cells (2 × 10⁵) were seeded in 60-mm dishes and exposed to
different concentrations of G-Rh2 for 48 h. For cell cycle analysis,
G-Rh2-treated cells were harvested and subsequently fixed in 70%
edthanol overnight. The cells were then stained with propidium
iodide (PI, 20 μg/mL). For apoptosis analysis, G-Rh2-treated cells
were harvested and stained with annexin V (BioLegend, California,
USA) and PI and analyzed by FACS Verso flow cytometry (BD Sci-
ence, California, USA).

2.6. In vitro pull-down assay

CRC cell lysates (500 μg) were incubated with G-Rh2-Sepharose
4B (or Sepharose 4B only for the control) beads (50 μl, 50% slurry)
in a reaction buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl,
1 mM DTT, 0.01% NP40, and 2 μg/mL bovine serum albumin)
overnight at 4°C with gentle rocking. They were then washed five
times with wash buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM
NaCl, 1 mM DTT, and 0.01% NP40). Finally, protein binding was
visualized by western blotting.

2.7. Immunofluorescence staining

CRC cells (1 × 10⁶) were seeded into Lab-Tek II Chamber Slides
(Thermo Fisher) and treated with different concentrations of G-Rh2
for 24 h. Cells were then fixed in 4% formaldehyde for 15 min.
Following permeabilization with 0.3% Triton X-100, the cells were
incubated with blocking buffer (5% bovine serum albumin in PBS)
for 1 h followed by Axl antibody (1:200) overnight at 4°C. Subse-
sequently, the cells were incubated with Alexa fluor 488-conjugated
secondary antibody (Jackson Immunoresearch Laboratories, Inc.
West Grove, PA, USA) for 2 h at room temperature. After washing,
the coverslips were mounted using a fluorescent mounting me-
dium with 4’6-diamidino-2-phenylindole (DAPI). Representative
images were captured using a fluorescence microscope (Leica).

2.8. Western blotting analysis

CRC cells (1 × 10⁶) were seeded into 100-mm dishes and
exposed to 0, 20, and 40 μM G-Rh2 at 37°C. After 48 h, cells were
harvested and washed with ice-cold PBS on ice. Tumor tissues (50
mg) were crushed and ground into a powder with a liquid nitrogen-
cooled mortar and pestle. Cells or tissues were lysed using PRO-
PREP™ lysis buffer on ice for 40 min. The mixture was centrifuged
and supernatants was collected. A total of 30 μg of protein was
loaded and separated on SDS-PAGE gels (8%–12%) and transferred
onto PVDF membranes (0.22 μm, Merck Millipore). The membranes
were blocked using 5% BSA for 1 h and incubated with primary
antibodies at 4°C overnight. The following day, the membranes
were incubated with an HRP-conjugated secondary antibody for 1 h
at room temperature. Signals were developed with an ECL detec-
tion kit and visualized with the Da Vinci Fluorescence Imaging
System (Da Vinci-K, Seoul, Korea).
2.9. Transfection

The pcDNA Axl plasmid was a gift from Rosa Marina Melillo (Addgene plasmid #105932) [24]. The transfection experiments were carried out using FuGENE® HD transfection reagent (Promega) following the manufacturer’s instructions. To establish stable Axl-overexpressing cell lines, the transfected cell lines were exposed to 600 μg/mL of G418 (Invitrogen) for 2 wk. The selected cells were used in subsequent experiments.

2.10. Lentiviral production and infection

For Axl knockdown in CRC cells, five lentiviral human Axl shRNA vectors were purchased from Sigma-Aldrich company (SHCLND-NM_021913; among them sh-Axl#2 sequence: 5’-CCGGCGGCTGTCATGAAGGAATTTCTCGAGAAATTCCTTCATGCAGA-3’; sh-Axl#5 sequence: 5’-CCGGCGGTGAAGACGATGAAGATTTCTCGAGAAATTCCTTCATGCAGA-3’). Next, 293T cells were cotransfected with pLKO.1-scrambled, sh-Axl#2, and sh-Axl#5 vectors and packaging plasmids (pMD2.G, pMDLg/p RRE, and pRSV-Rev) using the FuGENE® HD transfection reagent. After 48 h, the lentiviruses were harvested by filtration using a 0.45 μm filter. The cultured HCT116 cells were infected with lentiviruses using 8 μg/mL of polybrene (Sigma). After 24 h of infection, the medium was replaced with fresh complete growth medium containing 2 μg/mL of puromycin for 2 days. The selected cells were used for subsequent experiments.

2.11. In vivo xenograft model

BALB/c-nu mice (male, aged 5–6 weeks) were purchased from Orient Bio Inc. (Seongnam, Gyeonggi, Korea). All animal experiments were performed under protocols approved by and accordance with the guidelines of the Kyungpook National University Animal Use and Care Committee (2015-0135). HCT116 cells were collected in PBS at a final concentration of 1 × 10^7 cells/mL. The cells (200 μL) were subcutaneously injected into the flanks of mice. Twelve days after injection, the mice were randomly divided into three groups (n = 6 mice/group) and treated with an intraperitoneal injection of G-Rh2 (10 mg/kg and 50 mg/kg) or vehicle (PBS) three times a week for 21 days. For Axl knockdown xenograft assay, the mice were randomly divided into two groups (n = 4 mice/group) and sh-Mock or sh-Axl#2 HCT116 cells (5 × 10^6 cells in 0.2 mL PBS) were subcutaneously injected into the flanks of each mouse. Tumor volumes and body weights were recorded every 4 days. Tumor volumes were assessed using the following formula: (length × width × height × 0.52).

2.12. Immunohistochemical analysis

Tissue samples from mice were fixed with formalin and embedded in a paraffin, and cut into 4-μm sections. Next, they were hybridized with primary antibodies Ki-67 (1:500), p-AKT (1:200), p-p38 (1:800), and Bax (1:200). Finally, the sections were mounted with DPX Mountant for histological analysis.

2.13. Statistical analysis

Statistical analysis was performed using the GraphPad Prism® software (GraphPad Software Inc. CA, USA). Statistically significant differences were tested using Student’s t-test. All data represent means ± SD from at least three individual experiments. P < 0.05 was considered statistically significant.

3. Results

3.1. G-Rh2 suppresses CRC cell growth without toxicity to normal colon fibroblast cells

To explore the anticancer activity of G-Rh2 in CRC cells, we investigated the effect of G-Rh2 on cellular response. We treated HCT15, HCT116, and DLD1 cells with 0, 10, 20, 40, or 60 μM G-Rh2 for 24 h in 6-well plates. After 24 h of treatment, we observed that CRC cell density significantly decreased compared with control cells at 40 μM G-Rh2 and most of the cells were dead at 60 μM (Fig. S1). Next, we used a CCK-8 assay to evaluate the cell viability of HCT15, HCT116, and DLD1 cells after treatment with various concentrations of G-Rh2 for 24 h. The results showed that G-Rh2 inhibited cell viability in a concentration-dependent manner and the IC50 values for G-Rh2 on HCT15, HCT116, and DLD1 cells were 39.50, 40.81, and 46.16 μM, respectively (Fig. 1B). The viability of the CCD-18Co normal human colon fibroblast cell line was not affected by G-Rh2 treatment, even at 80 μM (Fig. 1B). Therefore, 0, 10, 20, and 40 μM of G-Rh2 were selected for additional in vitro studies. The CCK-8 assay results revealed that G-Rh2 significantly inhibited the proliferation of CRC cells in a dose- and time-dependent manner (Fig. 1C). Besides, G-Rh2 markedly reduced the number of colonies in an anchorage-independent cell growth assay (Fig. 1D and E). Collectively, these results indicate that G-Rh2 exhibits potent antitumor activity in CRC cells with minimal cytotoxic effects on normal colon fibroblast cells.

3.2. G-Rh2 inhibits the migration and invasion of CRC cells

To explore the effect of G-Rh2 on the migration and invasion capacity of CRC cells, wound-healing and transwell assays were conducted. The wound-healing assay results indicated that the migration of HCT15, HCT116, and DLD1 cells was significantly inhibited following treatment with G-Rh2 for 12 and 24 h compared with the control (Fig. 2A and B). Next, we performed transwell assays to further verify the effect of G-Rh2 on the cell migration and invasion ability. The transwell assay results indicated that G-Rh2 inhibited the migration and invasion capacity of CRC cells in a dose-dependent manner (Fig. 2C–F). Epithelial-mesenchymal transition (EMT) is known to be closely associated with cancer cell invasion and metastasis [25], and we detected the expression of EMT markers in CRC cells after G-Rh2 treatment by western blot analysis. Specifically, N-cadherin and vimentin were downregulated and E-cadherin was upregulated following treatment with G-Rh2 in HCT15, HCT116, and DLD1 cells (Fig. 2E). These results indicate that G-Rh2 plays a role in preventing CRC metastasis.

3.3. G-Rh2 induces G0/G1 phase arrest and apoptosis of CRC cells

To further explore the molecular mechanisms of G-Rh2 in CRC cell growth, we used a flow cytometry assay to determine cell cycle distribution and apoptosis in CRC cell lines following G-Rh2 treatment. As shown in Fig. 3A and B, compared with the control, G-Rh2 treatment significantly increased the ratio of G0/G1 phase cells. Then we explored how G-Rh2 regulated cell cycle-related proteins. We found that the G0/G1-phase cell cycle regulatory proteins, cyclin E1, cyclin D1, CDK 4, and CDK 6, were downregulated by G-Rh2 treatment in these three cell lines (Fig. 3E). In addition, the percentage of apoptotic HCT15, HCT116, and DLD1 cells significantly increased following treatment with G-Rh2 for 48 h (Fig. 3C and D). Moreover, G-Rh2 upregulated the protein levels of the proapoptotic proteins p53, cleaved caspase3, and cleaved PARP, whereas the
expression of the antiapoptotic protein survivin was down-regulated in CRC cells (Fig. 3F).

3.4. G-Rh2 directly binds to Axl and inhibits the Axl signaling pathway in CRC cells

Axl is a poor prognostic marker of colorectal cancer [26], and aberrant expression of Axl is related to cancer cell metastasis [27] and acquired drug resistance [20,28]. We found that the expression of p-Axl and Axl was higher in CRC cells (HCT15, HCT116, and DLD1) compared with CCD-18Co normal colon fibroblast cells (Fig. 4A). Some evidence indicated that G-Rh2 has a role in reversing drug resistance, such as Adriamycin-resistant breast cancer cells [29] and 5-FU resistance colorectal cancer cells [11]. Besides, G-Rh2 could regulate Axl downstream PI3K/Akt signaling pathways expression in several cancer types [30,31]. Thus, we considered whether G-Rh2 influences the Axl signaling pathway. To test this hypothesis, we performed an in vitro pull-down assay to explore whether G-Rh2 could bind with Axl proteins. The results indicated that G-Rh2 could directly bind to Axl (Fig. 4B). Besides, we found that G-Rh2 failed to bind with the receptor tyrosine kinases Flt-1 and VEGFR-2 (Fig. 4B). Then we detected the influence of G-Rh2 on Axl and its downstream protein expression by western blotting assay. We found that G-Rh2 inhibited the phosphorylation of Axl, Src, ERK, PI3K, AKT, mTOR, and GSK-3β in HCT15 and HCT116 cells (Fig. 4C). The immunofluorescence assays results showed that Axl expression was inhibited by G-Rh2 treatment in HCT15 and HCT116 cells (Fig. 4C). The immunofluorescence assays results showed that Axl expression was inhibited by G-Rh2 treatment in HCT15 and HCT116 cells (Fig. 4C). The immunofluorescence assays results showed that Axl expression was inhibited by G-Rh2 treatment in HCT15 and HCT116 cells (Fig. 4C). The immunofluorescence assays results showed that Axl expression was inhibited by G-Rh2 treatment in HCT15 and HCT116 cells (Fig. 4C). The immunofluorescence assays results showed that Axl expression was inhibited by G-Rh2 treatment in HCT15 and HCT116 cells (Fig. 4C). 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Fig. 2. G-Rh2 suppresses the migration and invasion of CRC cells. (A) and (B) Wound-healing assay in HCT15, HCT116, and DLD1 cells following treatment with G-Rh2 for 12 and 24 h, respectively (scale bar, 250 μm). (C) and (D) The migration abilities of HCT15, HCT116, and DLD1 cells were determined by transwell assay after treatment with different doses of G-Rh2 (0, 10, 20, 40 μM). (E) and (F) Invasion assay in HCT15, HCT116, and DLD1 cells following treatment with G-Rh2. (G) Western blot analysis of E-Cadherin, N-Cadherin, Vimentin, and β-actin expression in HCT15, HCT116, and DLD1 cells treated with different doses of G-Rh2 (0, 20, 40 μM).
3.5. Axl promotes the proliferation, migration, and invasion of CRC cells

To further investigate the functions of Axl in CRC cells, we knocked down Axl in HCT116 cells using lentiviral shRNA. Immunoblotting results showed that the Axl expression was silenced following infection with Axl shRNAs (especially sh-Axl#2 and -Axl#5) (Fig. 5A). Then, we explored the effect of Axl on CRC cell growth by various experiments. We found the cell viability was significantly inhibited in Axl knockdown cells (Fig. 5B). In addition, Axl knockdown inhibited the cell migration and invasion ability (Fig. 5C and D) and induced G0/G1 phase cell cycle arrest (Fig. 5E and F). Knockdown of Axl decreased the colony number in HCT116 cells, whereas G-Rh2 failed to further inhibit colony formation in Axl knockdown cells compared with the control (Fig. 5G and H), indicating that Axl was the primary target of G-Rh2 during CRC cell growth. Our in vivo study showed that Axl knockdown significantly suppressed tumor growth and decreased tumor weight in xenograft mice (Fig. S1–K). In addition, to further verify the role of Axl in CRC cells, we established two stable Axl-overexpressing CRC cell lines (Fig. S3A). CCK-8 assays revealed that Axl overexpression significantly promoted CRC cell viability (Fig. S3B). Axl overexpression also increased the colony number in HCT15 and HCT116 cells (Figs S3C, S3D) and promoted the migration and invasion capacity of HCT15 and HCT116 cells (Figs S3E, S3F). Moreover, the overexpression of Axl diminished the effect of G-Rh2 in inhibiting the colony formation ability in HCT15 and HCT116 cells (Figs S3C, S3D). Collectively, these results indicate that Axl promotes the proliferation, migration, and invasion of CRC cells and that it is the main target of G-Rh2 to inhibit the growth of CRC cells.

3.6. G-Rh2 suppresses HCT116 xenograft tumor growth in nude mice

Based on our in vitro results, we further examined the antitumor effects of G-Rh2 in tumor-bearing mice. The xenograft model was established by subcutaneously injecting of HCT116 cells into the flanks of nude mice. After 12 days, the mice were divided into three groups, and treated with different doses of G-Rh2 for 3 weeks. The results indicated that 10 and 50 mg/kg of G-Rh2 significantly suppressed tumor growth compared with that in the vehicle-treated group (Fig. 6A and B). Meanwhile, G-Rh2 treatment reduced the protein levels of p-Axl, p-Src, p-ERK, and p-p38 in tumor tissues (Fig. 6D), which is consistent with our in vitro results. Additionally, there were no obvious differences in the histological structure of the liver and lung in these three groups, indicating that G-Rh2 was well-tolerated (Fig. 6C). Immunohistochemistry results indicated that the expression of Ki67, p-AKT, and p-p38 were significantly suppressed by G-Rh2 treatment, whereas the Bax expression was increased (Fig. 6E and F). These results indicate the HCT116 cell proliferation was inhibited and apoptosis was induced by G-Rh2 treatment in vivo. Collectively, G-Rh2 apparently inhibits HCT116 xenograft tumor growth in vivo by suppressing the Axl signaling pathway with no significant toxicity to mice. A schematic diagram for the underlying mechanism of the effects of G-Rh2 in CRC is shown in Fig. 6G.

4. Discussion

Colorectal cancer is still one of the cancers with a higher mortality rate, and it is necessary to find new treatment drugs. Accumulating evidence has indicated that many natural compounds can play a role in cancer prevention and treatment. G-Rh2 is an active component extracted from P. ginseng [32] that exerts potent anti-cancer activity in various cancers [7,33]. Previous studies indicated that G-Rh2 could inhibit CRC cell growth by activating p53 [34] and inhibiting TOPK activity [7]. In the present study, we found that G-Rh2 inhibits CRC cell growth by targeting and inhibiting the Axl signaling pathway in vitro and in vivo.

Axl has been reported to be highly expressed in several human cancers and associated with poor prognosis and drug resistance [15,35–38]. Axl is involved in cancer cell proliferation, migration, and invasion, rendering it a promising therapeutic target for cancer treatment [19,36]. Indeed, Axl has been shown to be a potential therapeutic target in HER2+ breast cancer [39] and lung cancer [40]. Suppressing Axl expression effectively inhibits tumor growth in various cancer models [41–43]. In this study, we found that Axl was highly expressed in CRC cells and G-Rh2 could directly bind to Axl, resulting in the inhibition of Axl signaling pathway. Axl knockdown suppressed CRC cell growth in vitro and in vivo, indicating that Axl plays an essential role in CRC development and progression. To verify the role of Axl in the process of G-Rh2 inhibiting the growth of CRC cells, we treated Axl overexpression and Axl knockdown cells with G-Rh2. We found that Axl overexpression can attenuate the effect of G-Rh2, whereas the growth inhibition effect of G-Rh2 in Axl knockdown cells was not further enhanced. This indicates that Axl is a key target of G-Rh2, which inhibits the growth of CRC cells.

PI3K/AKT is one of the downstream pathways of Axl, which plays a considerable role in many biological processes (including cell growth [44], cell cycle regulation [45], apoptosis [46]). Axl promotes tumor metastasis and decreases chemosensitivity through the activation of the PI3K/Akt/GSK3β signaling pathway [47]. Abnormal expressions of the PI3K/AKT pathway proteins are common in cancers including CRC [48]. Several PI3K/AKT signaling pathway inhibitors have exhibited antitumor effects in cancer treatment [49–51]. In this study, G-Rh2 suppressed the p-AKT, p-mTOR, and p-GSK-3β expressions by inhibiting Axl in CRC cells. Src, which is another downstream protein of Axl [52], was reported to mediate cell growth, migration, and angiogenesis through the MAPK, PI3K, and STAT3 signaling pathways [53,54]. Src can regulate cell proliferation and cell cycle arrest by activating ERK1/2 and AKT [55,56]. In our study, G-Rh2 significantly downregulated p-Src and p-ERK1/2 expressions in CRC cells both in vitro and in vivo.

EMT is a key process that influences tumor invasion and metastasis. A direct link between EMT and metastasis has been confirmed in various tumor cell lines [57]. Upregulation of N-cadherin and downregulation of E-cadherin is the hallmark of EMT in cancer cells [58]. We found that G-Rh2 significantly inhibited CRC cell migration and invasion in vitro by reducing the expression of vimentin and N-cadherin and increasing E-cadherin in CRC cells. This demonstrates that G-Rh2 suppresses EMT in CRC cells. A role for Axl in EMT has also been reported in various cancers. For example, one study reported that Axl upregulation controls the motility of breast cancer cells driven by EMT [59]. Axl is highly expressed in advanced CRC and was significantly related to the expression of cell migration genes [16]. Our results also indicate
that Axl is involved in the EMT process and that Axl overexpression promoted the migration and invasion ability of CRC cells, whereas its knockdown resulted in the opposite effect.

Animal models are widely regarded as essential for the study of the efficacy of antitumor drugs. G-Rh2 has been reported to inhibit tumors in several cancer xenograft models including H1299 lung...
Fig. 5. Axl knockdown inhibits CRC cell growth in vitro and in vivo. (A) Axl knockdown in HCT116 cells was detected by the western blotting. (B) Cell viability in Axl knockdown HCT116 cells was determined by the CCK-8 assay. (C) and (D) Migration and invasion ability of Axl knockdown HCT116 cells (scale bar, 128 μm). (E) and (F) Flow cytometry analysis of
the cell cycle in Axl knockdown HCT116 cells. (G) and (H) Colony formation assay in Axl knockdown HCT116 cells treated with 0, 20, and 40 μM of G-Rh2 (scale bar, 250 μm). (J) Tumor volume. (K) Tumor weight. (L) Images of xenograft tumors. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.
cancer cells [8] and A375 melanoma cells [60]. In this study, we investigated the antitumor effects of G-Rh2 in an HCT116-derived xenograft model and demonstrated that G-Rh2 significantly suppressed HCT116 xenograft tumor growth by inhibiting the Axl signaling pathway without obvious toxicity in nude mice.

In summary, our results indicate that G-Rh2 clearly inhibits CRC cell growth in vitro and in vivo by inhibiting the Axl signaling pathway. Our results indicate that G-Rh2 is a potential therapeutic candidate that should be further tested for use against CRC and other solid tumors.

Declarations of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.07.004.

References

[1] Siegel RL, Miller KD, Goding Sauer A, Fedewa SA, Butterly LF, Anderson JC, Cercek A, Smith RA, Jemal A, Sauer Goding, et al. Colorectal cancer statistics, 2020. CA Cancer J Clin 2020;70:74–105.
[2] Moghimi-Dehkordi B, Safari A. An overview of colorectal cancer survival rates and prognosis in Asia. World J Gastrointest Oncol 2012;4:71–5.
[3] Zhao R, Choi BY, Wei L, Fredimoses M, Yin F, Fu X, Chen H, Liu K, Kundu JK, Dong Z, et al. Acetylshikonin suppressed growth of colorectal tumour tissue and cells-originated protein kinase. Br J Pharmacol 2020;177:2303–19.
[4] Hong P, Liu QW, Xie Y, Zhang QH, Liao L, He QY, Li B, Xu WW. Echinatin suppresses esophageal cancer tumor growth and invasion through inducing Akt/mTOR-dependent autophagy and apoptosis. Cell Death Dis 2020;11:524.
[5] Li Y, Xi Z, Chen X, Cai S, Liang C, Wang Z, Li Y, Tan H, Lao Y, Xu H. Natural compound oblongifolin C confers gemcitabine resistance in pancreatic cancer by downregulating Sph/MAPK/ERK pathways. Cell Death Dis 2018;9:538.
[6] Tong-Lin Wu T, Tong YC, Chen IH, Niu HS, Li Y, Cheng JT. Induction of apoptosis in prostate cancer by ginsenoside Rh2. Oncotarget 2019;10:11369–18.
[7] Yang J, Yuan D, Xing T, Su H, Zhang S, Wen J, Bai Q, Dang D. Ginsenoside Rh2 inhibiting HCT116 colon cancer cell proliferation through blocking PDZ-binding kinase/T-LAK cell-originated protein kinase. J Ginseng Res 2016;40:300–8.
[8] Ge C, Gan Y, Cai H. Ginsenoside Rh2 inhibited proliferation by inducing ROS Mediated ER stress dependent apoptosis in Lung cancer cells. Biol Pharm Bull 2017;40:2117–24.
[9] Shi X, Yang J, Wei G. Ginsenoside 20(S)-Rh2 exerts anti-cancer activity through the Akt/GSKbeta signaling pathway in human cervical cancer cells. Mol Med Rep 2018;17:4811–6.
[10] Ma J, Gao L, Lu H, Cao D, Liu G, Wei G, Chen A, Yang Y, Zhang H, Huo J. Reversal effect of ginsenoside Rh2 on oxaliplatin-resistant colon cancer cells and its mechanism. Exp Ther Med 2019;18:630–6.
[11] Liu GW, Liu YH, Jiang GS, Ren WD. The reversal effect of ginsenoside Rh2 on drug resistance in human colorectal carcinoma cells and its mechanism. Hum Cell 2018;31:189–98.
[12] Kim D, Bach DH, Fan YH, Luu TT, Hong JY, Park HJ, Lee SK. AXL degradation in combination with EGFR-TKI can delay and overcome acquired resistance in non-small cell lung cancer cells. Cell Death Dis 2019;10:361.
[13] Axelrod HD, Valkenbg K, Amend SR, Hicks JL, Parsana P, Torga G, DeMarzo AM. Pienta KJ. AXL is a putative tumor suppressor and dormancy regulator in prostate cancer. Mol Cancer Res 2019;17:356–69.
[14] Zhang G, Wang M, Zhao H, Cui W. Function of Axl receptor tyrosine kinase in non-small cell lung cancer. Oncol Lett 2019;17:2465–71.
[15] Ludwig KF, Du W, Sorrelle NR, Wnuk-Lipinska K, Topalovski M, Toombs JE, Cruz YH, Yabuuchi S, Rajeshkumar NV, Maitra A, et al. Small-molecule inhibition of Axl targets tumor suppressor and enhances chemotherapy in pancreatic cancer. Cancer Res 2018;78:2460.
[16] Uribe DJ, Mandel EK, Watson A, Martinez JD, Leighton JA, Ghosh S, Rothlin CV. The receptor tyrosine kinase Axl promotes migration and invasion in colorectal cancer. PLoS One 2017;12:e0179795.
[17] Divine LM, Nguyen MR, Meller E, Desai RA, Arief B, Rankin EB, Bligh KD, Meyerson C, Hagemann IS, Massad M, et al. AXL modulates extracellular matrix protein expression and is essential for invasion and metastasis in endometrial cancer. Oncotarget 2016;7:77291–305.
[18] Song X, Akaska H, Wang H, Abbaahsaliadeh R, Shin JH, Zang F, Chen J, Logodon CD, Maitra A, Bean AJ, et al. Hematopoietic progenitor kinase 1 down-regulates the oncogenic receptor tyrosine kinase AXL in pancreatic cancer. J Biol Chem 2020;295:2348–58.
[19] Zajac O, Leclerc K, Nicolas A, Meseure D, Marchio C, Vincent-Salomon A, Roman-Roman S, Schoumacher M, Dubois T. AXL controls directed migration of mesenchymal triple-negative breast cancer cells. Cells 2020;9:247.
[20] Okura N, Nishioka N, Yamada T, Taniguchi H, Tanimura K, Katayama Y, Yoshimura A, Watanabe S, Kikutani T, Shiotu S, et al. ONO-7475, a novel AXL inhibitor, suppresses the adaptive resistance to initial EGFR-TKI treatment in EGFR-mutated non-small lung cancer. Clin Cancer Res 2020;26:2244–56.
[21] Melarangno MG, Friddell YW, Berk BC. The Gαs/AXL system: a novel regulator of vascular cell function. Trends Cardiovasc Med 1999;9:250–3.
[22] Tian Y, Zhang Z, Miao L, Yang Z, Yang W, Qian D, Cai H, Wang Y. Anexelektro (AXL) increases resistance to EGFR-TKI and activation of AXL and ERK2 in non-small cell lung cancer cells. Oncol Res 2016;24:295–303.
[23] Li J, Shi C, Zhou R, Han Y, Xu S, Ma H, Zhang Z. The crosstalk between AXL and YAP promotes tumor progression through STAT3 activation in head and neck squamous cell carcinoma. Cancer Sci 2020;111:3222–35.
[24] Krishnamoorthy GP, Guida T, Alfano L, Avilla E, Santoro M, Carlomagno F, Merillo RM. Molecular mechanism of 17-allylamo-17demethoxygeldanamycin (17-AAG)-induced AXL receptor tyrosine kinase degradation. J Biol Chem 2013;288:17481–94.
[25] Crichton CJ, Gibbons DL, Kurei JM. The role of epithelial-mesenchymal transition programming in invasion and metastasis: a clinical perspective. Cancer Manag Res 2013;5:187–95.
[26] Dunne PD, McArt DG, Blayney JK, Kalimutho M, Greer S, Wang T, Srivastava S, Ong R, Arthur K, Liu Z, Kim K, et al. AXL is a regulator of inherent and chemotherapy-induced invasion and predicts a poor clinical outcome in early-stage colon cancer. Clin Cancer Res 2014;20:164–75.
[27] Abu-Thuraia A, Goyette MA, Boulais J, Delliaux C, Apcher C, Schott C, Chichat D, Baggiolini M, Thibault R, Goyette G, et al. AXL targeter human lung cancer cell proliferation and apoptosis. Cell Death Dis 2015;6:1–6.
[28] Lü JM, Yao Q, Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. Curr Vasc Pharmacol 2009;7:293–302.
[29] Zajac O, Leclerc K, Nicolas A, Meseure D, Marchio C, Vincent-Salomon A, Roman-Roman S, Schoumacher M, Dubois T. AXL controls directed migration of mesenchymal triple-negative breast cancer cells. Cells 2020;9:247.
[30] Okura N, Nishioka N, Yamada T, Taniguchi H, Tanimura K, Katayama Y, Yoshimura A, Watanabe S, Kikutani T, Shiotu S, et al. ONO-7475, a novel AXL inhibitor, suppresses the adaptive resistance to initial EGFR-TKI treatment in EGFR-mutated non-small lung cancer. Clin Cancer Res 2020;26:2244–56.
[31] Melarangno MG, Friddell YW, Berk BC. The Gαs/AXL system: a novel regulator of vascular cell function. Trends Cardiovasc Med 1999;9:250–3.
Li Y, Wei X, AXL receptor tyrosine kinase as a promising anti-cancer approach: functions, molecular mechanisms and clinical applications. Mol Cancer 2019;18:153.

Collins F, Lee JT, Navolanic PM, Steelman LS, Shelton JG, Blalock WL, Lotsberg ML, Wnuk-Lipinska K, Terry S, Tan TZ, Lu N, Trachsel-Moncho L, Koopman LA, Terp MG, Zom GG, Janmaat ML, Jacobsen K, Gresnigt-van den Abeelen L, Miricescu D, Totan A, Stanescu-Spinu II, Badoiu SC, Stefani C, Greabu M, PI3K/akt/mTOR signaling pathway in breast cancer: from molecular landscape to clinical applications. Mol Oncol 2011;5:128195.

Bae CA, Ham IH, Oh HJ, Lee D, Woo J, Son SY, Yoon JH, Lorens JB, Brekken RA, Kim TM, et al. Inhibiting the GAS6/AXL axis suppresses tumor progression by blocking the interaction between cancer-associated fibroblasts and cancer cells in gastric carcinoma. Gastric Cancer 2020;23:824–36.

Lotsberg ML, Wnuk-Lipinska K, Terry S, Tan TZ, Lu N, Trachsel-Moncho L, Røsland GV, Siraji MI, Jacobsen K, Gresnigt-van den Abeelen L, Miricescu D, Totan A, Stanescu-Spinu II, Badoiu SC, Stefani C, Greabu M, PI3K/akt/mTOR signaling pathway in breast cancer: from molecular landscape to clinical applications. Mol Oncol 2011;5:128195.

Kong L, Lu X, Chen X, Wu Y, Zhang Y, Shi H, Li J. Qigesan inhibits esophageal cancer growth and apoptosis via PI3K/AKT signaling. J Cell Mol Med 2020;24:1450–60.

Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JC, Blalock WL, Franklin RA, McCubrey JA. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. Leukemia 2003;17:590–603.

Zhu C, Wei Y, Wei X. AXL receptor tyrosine kinase as a promising anti-cancer approach: functions, molecular mechanisms and clinical applications. Mol Cancer 2019;18:153.

Cardone C, Blauensteiner B, Moreno-Viedma V, Martini G, Simeon V, Goyette MA, Duhamel S, Aubert L, Pelletier A, Savage P, Thibault MP, Collina F, La Sala L, Liotti F, Prevete N, de Bree F, Pencheva N, et al. Enaportamab vedotin, an AXL-specific antibody-drug conjugate, shows preclinical antitumor activity in non-small cell lung cancer. JCI Insight 2019;4:e128195.

Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, Tiron C, Vitiello PP, Ciardiello V, Matrone N, Troiani T, et al. AXL is a predictor of poor survival and of resistance to anti-EGFR therapy in RAS wild-type metastatic colorectal cancer. Eur J Cancer 2020;138:1–10.

Kooyman LA, Brandhorst M, Forssmann U, de Bree F, Pencheva N, et al. Enaportamab vedotin, an AXL-specific antibody-drug conjugate, shows preclinical antitumor activity in non-small cell lung cancer. JCI Insight 2019;4:e128195.

Franklin RA, McCubrey JA. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. Leukemia 2003;17:590–603.

Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JC, Blalock WL, Franklin RA, McCubrey JA. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. Leukemia 2003;17:590–603.

Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JC, Blalock WL, Franklin RA, McCubrey JA. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. Leukemia 2003;17:590–603.

Han Y, Peng Y, Fu Y, Cai C, Guo Y, Liu S, Li Y, Chen Y, Shen E, Long K, et al. MLI1 deficiency induces cetuximab resistance in colon cancer via Her-2/PI3K/AKT signaling. Adv Sci 2020;7:2000112.

Nunnery SE, Mayer IA. Targeting the PI3K/AKT/mTOR pathway in hormone-positive breast cancer. Drugs 2020;80:1683–97.

Akbari Dilmaghani N, Safaroghli-Azar A, Pourbagheri-Sigaroodi A, Bashash D, Lotsberg ML, Wnuk-Lipinska K, Terry S, Tan TZ, Lu N, Trachsel-Moncho L, Koopman LA, Terp MG, Zom GG, Janmaat ML, Jacobsen K, Gresnigt-van den Abeelen L, Miricescu D, Totan A, Stanescu-Spinu II, Badoiu SC, Stefani C, Greabu M, PI3K/akt/mTOR signaling pathway in breast cancer: from molecular landscape to clinical applications. Mol Oncol 2021;13:618--42.

Brooks CM, Jerrard DM, Davis ME, Summerlin SA, et al. AXL targeting abrogates blocking the interaction between cancer-associated fibroblasts and cancer cells in gastric carcinoma. Gastric Cancer 2020;23:824–36.

Liu H, Wu Y, Zhu S, Liang W, Wang Z, Wang Y, Lv T, Yao Y, Yuan D, Song Y. PTPIP71B promotes cell proliferation and metastasis through activating src and ERK1/2 in non-small cell lung cancer. Cancer Lett 2015;359:218–25.

Song RX, Zhang Z, Santen RJ. Estrogen rapid action via protein complex formation involving ERalpha and Src. Trends Endocrinol Metab 2005;16:473–53.

Huang H, Li X, Wang Y, Zhao G, Wang Y, He Y, et al. PI3K/AKT/mTOR signaling pathway in breast cancer: from molecular landscape to clinical applications. Mol Oncol 2017;11:1430–47.

Kim H, Lee HS, Kim TH, Lee JS, Lee ST, Lee SJ. Growth-stimulatory activity of TIMP-2 is mediated through c-Src activation followed by activation of FAK, PI3-kinase/AKT, and ERK1/2 independent of MMP inhibition in lung adenocarcinoma cells. Oncotarget 2015;6;42905–22.

Lei J, Ingbar DH. Src kinase integrates PI3K/Akt and MAPK/ERK1/2 pathways in T3-induced Na-K-ATPase activity in rat alveolar cells. Am J Physiol Lung Cell Mol Physiol 2011;301:1765–71.

Liu H, Wu Y, Zhu S, Liang W, Wang Z, Wang Y, Lv T, Yao Y, Yuan D, Song Y. PTPIP71B promotes cell proliferation and metastasis through activating src and ERK1/2 in non-small cell lung cancer. Cancer Lett 2015;359:218–25.

Yeung KT, Yang J. Epithelial-mesenchymal transition in tumor metastasis. Nat Rev Cancer 2013;13:97–110.

Kim H, Lee HS, Kim TH, Lee JS, Lee ST, Lee SJ. Growth-stimulatory activity of TIMP-2 is mediated through c-Src activation followed by activation of FAK, PI3-kinase/AKT, and ERK1/2 independent of MMP inhibition in lung adenocarcinoma cells. Oncotarget 2015;6;42905–22.

Baumann C, Ullrich A, Toka R, Toka GAS6-expressing and self-sustaining cancer cells in 3D spheroids activate the PDK-RSK-mTOR pathway for survival and drug resistance. Mol Oncol 2017;11:1430–47.

Huang H, Li X, Wang Y, Zhao G, Wang Y, He Y, et al. PI3K/AKT/mTOR signaling pathway in breast cancer: from molecular landscape to clinical applications. Mol Oncol 2017;11:1430–47.

Heuvel E, Brandhorst M, Forssmann U, de Bree F, Pencheva N, et al. Enaportamab vedotin, an AXL-specific antibody-drug conjugate, shows preclinical antitumor activity in non-small cell lung cancer. JCI Insight 2019;4:e128195.

Miricescu D, Totan A, Stanescu-Spinu II, Badoiu SC, Stefani C, Greabu M, PI3K/akt/mTOR signaling pathway in breast cancer: from molecular landscape to clinical applications. Mol Oncol 2021;13:618--42.

Kim H, Lee HS, Kim TH, Lee JS, Lee ST, Lee SJ. Growth-stimulatory activity of TIMP-2 is mediated through c-Src activation followed by activation of FAK, PI3-kinase/AKT, and ERK1/2 independent of MMP inhibition in lung adenocarcinoma cells. Oncotarget 2015;6;42905–22.

Huang H, Li X, Wang Y, Zhao G, Wang Y, He Y, et al. PI3K/AKT/mTOR signaling pathway in breast cancer: from molecular landscape to clinical applications. Mol Oncol 2017;11:1430–47.