Preclinical evaluation of fenretinide against primary and metastatic intestinal type-gastric cancer

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Abstract. In recent years there has been a decline in the incidence of gastric cancer, however the high mortality rate has remained constant. The present study evaluated the potential effects of the retinoid fenretinide on the viability and migration of two cell lines, AGS and NCI-N87, that represented primary and metastatic intestinal gastric cancer subtypes, respectively. It was determined that a similar2 dose of fenretinide reduced the viability of both the primary and metastatic cell lines. In addition, it was demonstrated that combined treatment with fenretinide and cisplatin may affect the viability of both primary and metastatic gastric cancer cells. Furthermore, a wound healing assay demonstrated an inhibitory effect for fenretinide on cell migration. As part of the characterization of the mechanism of action, the effect of fenretinide on reactive oxygen species production and lipid droplet content was evaluated, with the latter as an indirect means of assessing autophagy. These results support the hypothesis of combining using fenretinide with conventional therapies to improve survival rates in advanced or metastatic gastric cancer.

Introduction

Gastric cancer is the fifth most common tumor and the fourth leading cause of cancer related death worldwide. Although the incidence and mortality rates of gastric cancer have been declining in previous decades, 1,000,00 newly diagnosed cases are still reported each year, and close to 800,000 deaths from gastric cancer were estimated in 2020 (1). Moreover, ~10% of all cancer-related deaths are caused by gastric cancer (1,2). Gastric cancer may be classified into two main subtypes, the intestinal and diffuse type, using the Laurén classification, based on histological appearance (3). Intestinal gastric cancer represents 54% of all gastric cancers, and in general, has an higher prognostic value compared with diffuse gastric cancer (4). By contrast, the diffuse type presents in 32% of all gastric cancer, has a worse prognosis and is highly invasive and prone to metastasis (5).

Approximately one-third of all gastric cancer cases advance into metastatic sites, usually into the liver, peritoneum, lungs and bones (6,7). The clinical outcome for patients with advanced gastric cancer is poor, with a 5-year survival rate of 5-20% and a 10-month median overall survival time (8). The low survival time may be attributed to a lack of early diagnosis, as well as the high rate of recurrence and metastasis (9,10). Nonetheless, the majority of therapies such as surgical or endoscopical resection and peri-operative or adjuvant chemotherapies only target primary tumors and there is no standard regimen for metastatic or advanced gastric cancer. The drugs frequently used as first-line therapy are cisplatin or oxaliplatin, pyrimidine analogs such as 5-fluorouracil or capecitabine and anthracyclines, such as doxorubicin or epirubicin (11). However, when the cancer has metastasized, chemotherapy has only a palliative effect, and elevated concentrations of these drugs may produce toxic adverse effects in patients (12,13).

Due to the poor response of advanced gastric cancer to conventional chemotherapy, alternative therapeutics such as monoclonal antibodies, which include trastuzumab, ramucirumab, pembrolizumab and nivolumab, have been evaluated and approved by the United States Food and Drug Administration (8,14,15). Although positive results with the use of these drugs have been reported (16-19), there are a number of associated limitations. For instance, patients with HER2-positive tumors may potentially benefit from monoclonal antibody therapy using trastuzumab, hence there is a need to identify clinically useful predictive biomarkers (20,21). In addition, further studies are necessary to advance the current understanding of the adverse effects caused by monoclonal antibody therapies (22,23). Furthermore, clinical outcomes were most improved when these drugs were administered in combination with chemotherapy, despite an increased risk of adverse reactions (14,24-26).

Retinoids, which are natural and synthetic vitamin A derivatives, are ligands of retinoic acid receptors (RARs) and

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retinoic X receptors. In the nucleus, these receptors serve key roles in the regulation of cellular processes, including differentiation, cell growth and cell death. Due to their effects promoting cell differentiation, the naturally-occurring retinoids, all-trans retinoic acid (ATRA) and 13-cis-retinoic acid, are frequently used in pediatric oncology, primarily in acute myeloid leukemia and for the treatment of skin diseases such as psoriasis, acne vulgaris and keratinization disorder (27-29). However, treatments using these compounds on solid tumors have been less effective, mainly attributed to difficulties in effective delivery due to their limited solubility in aqueous solutions and reduced half-life in plasma (30,31). Fenretinide [N-(4-hydroxyphenyl) retinamide], also termed 4-HPR, is a synthetic amide of retinoic acid. Fenretinide has been used as a chemopreventive agent against breast cancer (32). *In vitro*, fenretinide has shown cytotoxic effects in neuroblastoma, melanoma and breast cancer cell lines (33-35), and it is well established that the mechanisms that mediate fenretinide cytotoxicity include p53-and caspase-independent apoptosis, activation of the cellular stress response and the induction of autophagy (36-38). In the previous 20 years, there have been several clinical trials involving fenretinide in patients with different solid tumors such as neuroblastoma, and breast, prostate and bladder cancer, however, the majority of the results from these trials have been less promising compared with those from *in vitro* studies (32,39-44). A predominant limitation is the low oral bioavailability of fenretinide, thus only a low concentration reaches the plasma. However, in the last five years, novel research has focused on developing improved oral formulations to achieve higher plasma drug concentrations (45,46).

The aim of the present study was to evaluate the effect of the retinoid fenretinide on the viability and migration of two cell lines that serve as models of primary and metastatic intestinal gastric cancer. To achieve this, the effect of several chemotherapeutic agents and the natural retinoid ATRA on the viability of these cell lines were compared, and the possible beneficial effect of combining fenretinide and cisplatin was evaluated.

Materials and methods

Cell culture. Human gastric cancer AGS (CRL-1739) and NCI-N87 (CRL-5822) cell lines were purchased from the American Type Culture Collection. The AGS cell line was derived from a gastric adenocarcinoma that was located in the stomach (47), whereas the NCI-N87 cell line was derived from a liver metastasis of a gastric carcinoma (48). Both cell lines were cultured in RPMI-1640 medium (cat. no. R8758; Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS; cat. no. F2442; Sigma-Aldrich; Merck KGaA) and 100 U/ml penicillin-streptomycin (cat. no. 15140122; Gibco; Thermo Fischer Scientific, Inc.) at 37˚C in a humidified incubator containing 5% CO_2.

Evaluation of cytotoxicity using the MTT assay. AGS ($1.5x10^4$ cells/well) and NCI-N87 ($3x10^4$ cells/well) cells were seeded in 96-well plates and incubated at 37˚C overnight. After which, cells were exposed to different concentrations of cisplatin (Pfizer, Inc.), doxorubicin (cat. no. 44583; Sigma-Aldrich; Merck KGaA), ATRA (cat. no. R2625; Sigma-Aldrich; Merck KGaA) or fenretinide (cat. no. H7779; Sigma-Aldrich; Merck KGaA) for 48 h at 37˚C. As all the drugs evaluated were light-sensitive, drugs and treated cells were protected from light. Doxorubicin and the retinoids, ATRA and fenretinide, were dissolved in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions. Cisplatin solution was directly dissolved in cell culture media.

After the treatments, the media was removed and the cells were incubated with MTT (final concentration, 0.5 mg/ml) solution dissolved in RPMI-1640 medium for 2 h at 37˚C. The media was then removed and 95% ethanol was added to the wells to dissolve the formazan crystals. Cells incubated in media with only the vehicle (DMSO<0.05%) were used as a control and were used to define 100% viability. Absorbance values were determined at a wavelength of 570 nm using a microplate reader (BioTek Cytation 3 Imaging Multi-Mode Reader; Biotek; Agilent Technologies, Inc.).

Evaluation of the combined effect of fenretinide and cisplatin. AGS ($1.5x10^4$ cells/well) and NCI-N87 ($3x10^4$ cells/well) cells were seeded in 96-well plates and incubated at 37˚C overnight. Fenretinide and cisplatin were added simultaneously for 48 h and the experiment was conducted with non-fixed ratio combinations. Two doses of cisplatin were selected, corresponding to values below the calculated IC_{50}. By contrast, three doses of fenretinide were chosen, one dose at the IC_{50} value, one below it and one above it. This strategy aimed to evaluate whether the combination with fenretinide could reduce the concentration of cisplatin required, thereby mitigating the adverse effects associated with cisplatin.

Combined drug action was studied through median effect analysis according to the Chou-Talalay method (49). The combination index (CI) for non-constant combination and drug reduction index (DRI) were calculated using the CompuSyn software (version 1.0; ComboSyn, Inc.). The DRI provided an estimation of the extent to which a dose of an agent in combination can be decreased to achieve effect levels similar to a single drug. The CI was defined as follows: CI<1, synergistic effect; CI>1, antagonistic effect; and CI=1, additive effect.

Determination of reactive oxygen species (ROS) production. Intracellular generation of ROS was measured using 2',7'-dichlorofluorescein diacetate (DCFDA; cat. no. D6883; Sigma-Aldrich; Merck KGaA). DCFDA is a non-fluorescent probe that is internalized by the cell and when oxidized by ROS, is converted into the highly fluorescent 2',7'-dichlorofluorescein. AGS ($1.5x10^4$ cells/well) cells were seeded in black 96-well plates and left to adhere at 37˚C for 5 h, before treatment with fenretinide (3 μM) for 20 h at 37˚C. As a positive control of ROS production, cells were also treated with the oxidizing reagent tert-butyl hydroperoxide solution (TBHP; 1 mM; cat. no. B2633; Sigma-Aldrich; Merck KGaA) at 37˚C for 2 h. Before the conclusion of TBHP incubation, the cells were incubated with DCFDA (60 μM) at 37˚C for 30 min. Finally, the wells were rinsed twice with phosphate-buffered saline (PBS) and the intensity of the fluorescence was assessed using a BioTek Cytation 3 Imaging Multi-Mode Reader (Biotek; Agilent Technologies, Inc.), using 485 nm as the excitation wavelength and 528 nm as the emission wavelength. The

basal production of ROS was determined in cells treated with medium alone; this value was subtracted from the fluorescence detected when cells were incubated with fenretinide.

Role of ROS inhibition on cell viability. AGS ($8x10^3$ cells/well) and NCI-N87 ($1.6x10^4$ cells/well) cells were seeded in 96-well plates and incubated at 37˚C overnight. Cells were then treated with the antioxidant quercetin (80 μM) for 1 h before the addition of fenretinide (20-40 μM) or TBHP (0.6 μM, AGS; 4.8 μM, NCI-N87) as a positive control of ROS production for 4 h at 37˚C. For quercetin, a concentration of 80 μM was selected as it was the highest dose that did not show toxicity in either cell lines. In the case of fenretinide, concentrations >IC_{50} value that were reported from the MTT assay were used as the experiments were performed at 4 h instead of 48 h.

As various antioxidants interfere with the MTT assay, the sulforhodamine B assay was instead used to determine cell viability. Briefly, cells were fixed in 10% trichloroacetic acid for 1 h at 4˚C and stained with 0.04% sulforhodamine B solution (cat. no. S1402; Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. After which, the wells were rinsed three times, eachfor 30 sec, with 1% acetic acid at room temperature to remove the excess dye. Protein-bound dye was dissolved in 10 mM Tris base solution and the absorbance was determined at 530 nm using a microplate reader (BioTek Cytation 3 Imaging Multi-Mode Reader; Biotek; Agilent Technologies, Inc.).

Intracellular lipid droplet (LD) staining and fluorescence quantification. AGS ($8x10^3$ cells/well) and NCI-N87 ($1.6x10^4$ cells/well) cells were seeded in black 96-well plates and incubated at 37˚C overnight. After which, cells were treated with a moderately toxic dose of fenretinide (10 μM), which corresponded to the IC_{25} value or avasimibe (20-40 μM) for 48 h. A fenretinide concentration <IC_{50} value was selected as it allowed detection of a toxic effect while ensuring that most of the visual fields contained sufficient cells for meaningful quantification. This approach was particularly important as visual fields were randomly selected. Avasimibe (cat. no. PZ0190; Sigma-Aldrich; Merck KGaA), an inhibitor of acyl-CoA:cholesterol O-acyltransferases (ACATs), reduces LD synthesis and was therefore used as a control.

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature and incubated in a 0.5 μg/ml solution of Nile red (cat. no. 19123; Sigma-Aldrich; Merck KGaA) in PBS for 10 min at 37˚C. Images were captured using a fluorescence microscope with a red fluorescence protein filter (RFP; 531 and 593 nm) with a 20X objective under the same conditions of LED exposure, including integration time and gain, using a BioTek Cytation 3 Imaging Multi-Mode Reader (Biotek; Agilent Technologies, Inc.). For each treatment, images were captured from five independent wells. The Gen5 Image software (version 3.09; Agilent Technologies, Inc.) was used to quantify the fluorescence intensity. All images from each cell line were included in the experiment and an automatic setting was applied for preprocessing for RFP and bright-field channels. The cellular analysis tool was used to select the areas containing cells in the bright-field images, with threshold values for AGS and NCI-N87 of 500 and 4,000, respectively. The object size selection was set between 15-500 μm for both cell lines. The total signal fluorescence object sum intensity [Tsf (RFP 531,593)], total signal fluorescence object sum area [Tsf (bright field)] and the relative object sum intensity/object sum area were calculated for all images. The mean value and standard deviation were determined and statistical analyses were performed.

Wound healing assay. AGS ($1x10^6$ cells/well) cells were seeded on plastic 24-well plates and incubated at 37˚C overnight. Wounds were scraped in the middle of the well with a sterile pipette tip. To remove floating cells, wells were carefully rinsed twice with PBS. After which, cells were incubated with medium or fenretinide at a nontoxic concentration (6 μM) for 24 h. Each condition was performed in duplicate in media containing 5% FBS. AGS cell viability decreases considerably when FBS is completely depleted (50). It has been established that, when necessary, the medium can be supplemented with 2-5% FBS to maintain cell health during the assay (51). Since a low concentration of FBS was used in both the control and treated groups, cell proliferation was monitored through the entire wound-healing assay using the sulforhodamine B assay (data not shown). Light microscopy pictures were captured using a microplate reader (BioTek Cytation 3 Imaging Multi-Mode Reader; Biotek Instruments, Inc.) at 0 and 24 h time points. The percentage migration was calculated as the mean of three random measurements in each picture and expressed as a percentage of the control (time 0 h for each treatment) using the ImageJ software (version 1.49; National Institutes of Health).

Statistical analysis. For all the viability assays, dose-response curves were generated and the IC_{50} values were calculated using the Slide Write Plus software (version 6.10; Advanced Graphics Software, Inc.). All quantitative experiments were independently repeated three times and data were expressed as the mean ± standard deviation. To evaluate the significance of the results between two groups, the Mann-Whitney test or independent t-test was applied. For comparisons of ≥3 groups, one-way ANOVA followed by Bonferroni's post-hoc test was applied. All tests were performed using GraphPad Prism (version 5; Dotmatics) or SPSS (version 22; IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of cisplatin and doxorubicin on cell viability. The present study evaluated whether the AGS and NCI-N87 cell lines had a different response to cisplatin and doxorubicin, which are both frequently used to treat gastric cancer. The primary gastric cancer cell line AGS was sensitive to both cytotoxic agents, however, doxorubicin was more efficient, as the IC_{50} value was 0.39 μM, compared with cisplatin that had an IC_{50} value of ~20 μM (Table I). The metastatic cell line NCI-N87 demonstrated the opposite effect; cells exhibited a notably low response to doxorubicin, with doses as high as 90 μM being poorly toxic, since cell viability remained close to 80%. By contrast, both cell lines were affected in a similar manner by cisplatin treatment at concentrations <20 μM (Fig. S1). However, at concentrations >20 μM, AGS

cells presented lower viability compared with the NCI-N87 metastatic cell line.

Effect of fenretinide and ATRA on cell viability. The impact of the synthetic retinoid, fenretinide, was assessed using an MTT assay and its effects compared with those of its natural analog ATRA were recorded. ATRA was used to compare whether fenretinide-treated cells showed the same response observed with doxorubicin or cisplatin. Since ATRA has been shown to be ineffectively delivered in solid tumors (52), it was hypothesized in the present study that for fenretinide to be promising as a therapeutic drug in gastric cancer, the IC_{50} values should be significantly lower than those for ATRA.

The results of the present study showed not only that NCI-N87 cells were less sensitive to ATRA compared with AGS cells, but also that both cell lines had lower IC_{50} values when treated with fenretinide compared with ATRA. In fact, both cell lines had a similar response when treated with fenretinide, and the IC_{50} values were in a similar range as those obtained when using cisplatin (Table I; Fig. S1).

As MTT assays are primarily based on the activity of mitochondrial enzymes and cell metabolic health (53,54), a sulforhodamine assay was also performed to allow the determination of cell density based on the measurement of cellular protein content (55). The results obtained using the MTT and sulforhodamine assays were consistent with each other (Fig. S1C-F).

Potential synergism of fenretinide and cisplatin on cell viability. It has been suggested that retinoids could be used as co-adjuvants in combination with chemotherapy (56), therefore it was evaluated whether fenretinide increased the toxic effect of cisplatin on gastric cancer cells. Cisplatin was selected as it was the only chemotherapy drug that showed a toxic effect on both cell lines in the present study. Additionally, the current first-line treatment for advanced or metastatic unresectable gastric cancer is a dual chemotherapy regimen based on a platinum compound and fluoropyrimidine, which suggests that anthracyclines may not be needed for optimal results (57).

Following combined treatment with fenretinide and cisplatin for 48 h, CompuSyn software was used to calculate the CI and DRI for cisplatin. The combination of three non-fixed toxic doses of fenretinide (similar to IC_{50}) with two different doses of cisplatin was evaluated (Table II; Fig. 1). Based on the CI value, it was suggested that in both cell lines, there may be an additive or partial synergistic interaction between both drugs when a high fraction of cells is affected (fraction affected >0.7). Notably, the additive effect was stronger in the NCI-N87 metastatic cell line; combination index values were closer to 0.9 and the drug reduction index values for cisplatin were at least twice those observed in AGS cells. However, when a low fraction of cells were affected, it was possible to observe CI values that suggested antagonism. However, for anticancer agents, synergy is more relevant to therapy when a high fraction of cells are affected (49).

Effect of fenretinide on ROS levels. Cancer cells exhibit increased ROS stress compared with normal cells, therefore targeting ROS levels may be useful as a therapeutic strategy (58). It was assessed whether fenretinide could induce an alteration in redox homeostasis. Following preincubation of both cell lines with the antioxidant quercetin and fenretinide, protection from cell death was only demonstrated in AGS cells (Fig. 2A and B). Nonetheless, the results of the present study suggested that other mechanisms besides ROS induction may also be implicated, as cell viability did not recover to levels similar to those in the control conditions. By contrast, for NCI-N87 metastatic cells, ROS production did not appear to be relevant for this effect as fenretinide did not enhance cell viability following TBHP exposure. The increase in ROS stress induced by TBHP was confirmed by the fact that the antioxidant quercetin was able to restore cell viability.

Notably, the TBHP concentration used to produce minimal toxic effect was increased ~8-fold compared with AGS cells.

To confirm the results observed in AGS cells, a DCFDA probe was used to detect intracellular ROS levels after treatment with fenretinide (Fig. 2C). After 20 h, a significant elevation in ROS was detected. However, this effect was smaller than the effect of TBHP, which was used as a positive control as it is a well-established ROS inducer (59).

Effect of fenretinide on LD content. Autophagy is implicated in fenretinide induced cell death (38). Xu and Fan (60) reported that LD content can be modified by autophagy. To assess this in the present study, AGS and NCI-N87 cells were stained with Nile red, a lipophilic agent that stains intracellular LDs. As a control, cells were incubated with avasimibe, an ACAT inhibitor that reduces LD formation (61).

In AGS cells, fenretinide significantly reduced Nile red fluorescence, which suggested a decrease in the number of LDs. By contrast, NCI-N87 cells treated with fenretinide showed no change in Nile red fluorescence in comparison with cells without treatment. Of note, when the NCI-N87 metastatic cell line was treated with avasimibe, LD content was significantly increased compared with the control cells (Fig. 3). A previous study reported that NCI-N87 cells regulate intracellular cholesterol differently from AGS cells. Furthermore, it was reported that NCI-N87 cells were more resistant to the effect of avasimibe when cell viability was assessed, suggesting the presence of a compensatory mechanism for cholesterol esterification in addition to ACAT-1 (50). This may partially explain why when treated with an inhibitor of LD formation, the opposite effect was observed. Since avasimibe changed LD content

Table I. IC_{50} values for retinoids and chemotherapy agents for AGS and NCI-N87 cells after 48 h.

Retinoids/chemotherapy agents	AGS (IC_{50}, μM)	NCI-N87 (IC_{50}, μM)
Cisplatin	18.49±4.54	27.25±2.74
Doxorubicin	0.39±0.02	>90.00[a]
All-trans retinoic acid	46.47±3.73	122.07±4.86[a]
Fenretinide	13.91±1.17	19.65±0.96

A comparison of IC_{50} values between cell lines, for each treatment at the same time point. Values are presented as the mean ± standard error of three independent experiments. [a]P<0.001.

Table II. CI and DRI values for non-constant combinations of fenretinide and cisplatin after 48 h.

Cell line	Cisplatin, μM	Fenretinide, μM	Fraction of affected cells (range, 0-1)	CI	DRI for cisplatin
AGS	5	10	0.46±0.01	1.37±0.02	2.15-2.70
		15	0.74±0.01	0.96±0.02	5.63-8.78
		20	0.82±0.01	0.99±0.01	5.63-8.78
	10	10	0.55±0.02	1.40±0.06	8.79-11.93
		15	0.78±0.01	0.95±0.02	3.54-5.34
		20	0.84±0.02	1.01±0.05	4.82-7.68
NCI-N87	5	15	0.45±0.02	1.21±0.05	2.66-3.83
		25	0.67±0.01	0.92±0.07	11.25-18.80
		30	0.73±0.02	0.88±0.07	14.84-30.83
	20	15	0.53±0.02	1.45±0.10	1.17-1.74
		25	0.67±0.01	1.14±0.12	2.63-4.70
		30	0.72±0.02	1.07±0.02	3.93-5.66

Values are presented as the mean ± standard error of three independent experiments performed. CI>1 indicates antagonism; CI=1 indicates an additive effect; and CI<1 indicates synergism. DRI is the dose reduction required for cisplatin when used in combination with fenretinide. CI, combination index; DRI, drug reduction index.

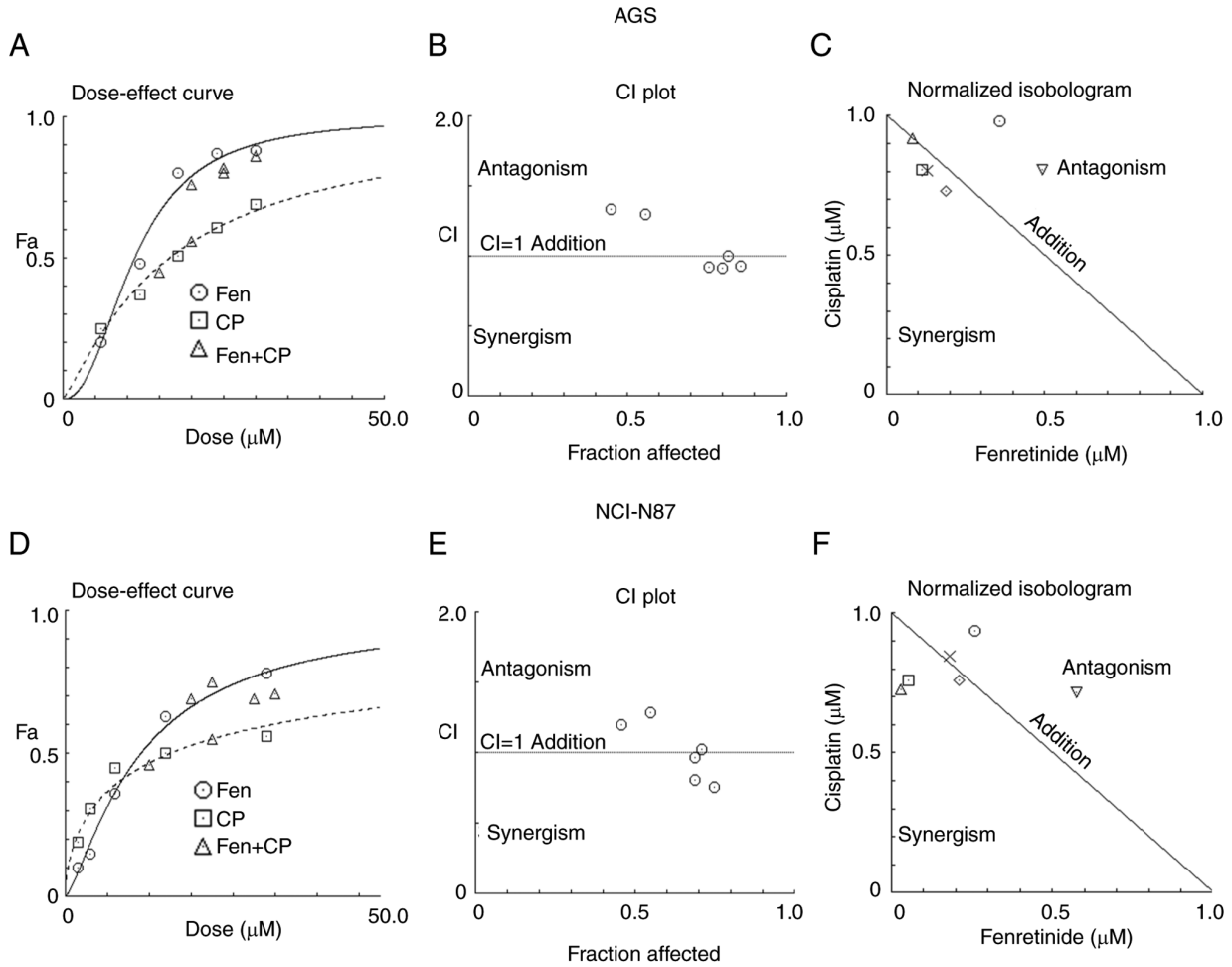

Figure 1. Effect of non-constant combinations of Fen with CP. Dose response curves, CI plots and normalized isobolograms were obtained through computer simulation using the Compusyn software. Representative graphs of the (A) dose-effect curve, (B) CI plot and (C) normalized isobologram for AGS cells are presented. Representative graphs of the (D) dose-effect curve, (E) CI plot and (F) normalized isobologram for NCI-N87 cells are presented. Each graph corresponds to a representative experiment of each cell line. For each cell line, three independent experiments were performed. In the dose-effect curves the solid and dashed lines correspond to the dose-effect curves for Fen and CP, respectively, each administered as a single therapy. Fen, fenretinide; CP, cisplatin; CI, combination index.

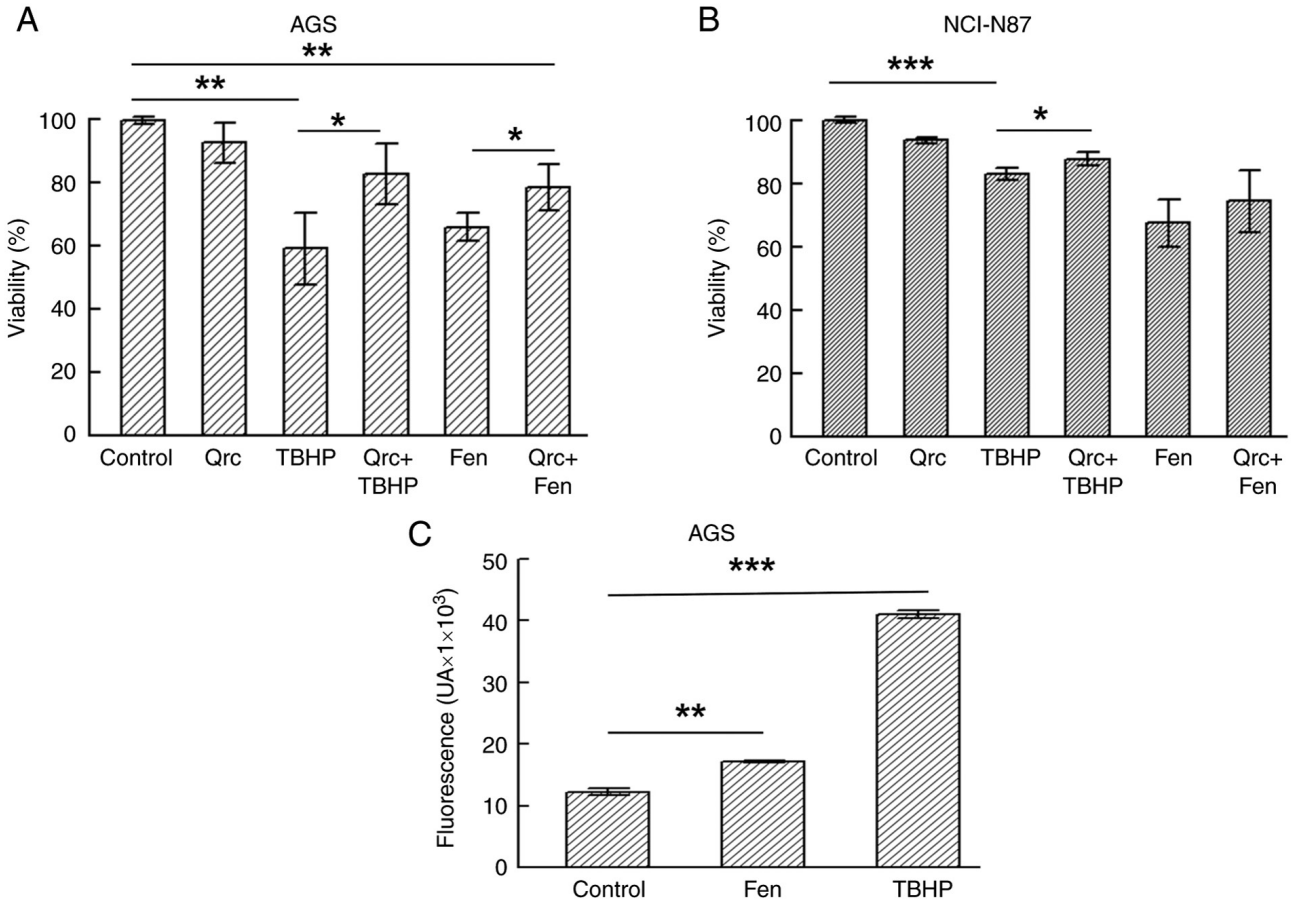

Figure 2. Effect of Fen on ROS levels and the role of ROS on the viability of gastric cancer cell lines treated with Fen. (A) Viability of AGS cells treated for 48 h with Fen after preincubating with Qrc. (B) Viability of NCI-N87 cells treated for 48 h with Fen after preincubating with Qrc. The oxidizing agent TBHP was used as a control for ROS induction. Values are expressed as the mean ± standard error of three independent experiments. (C) Fluorescence of the DCFDA probe after AGS cells were treated with Fen for 20 h. Values are presented as the mean ± standard error of three independent wells *P<0.05, **P<0.01 and ***P<0.001.UA, units of absorbance; ROS, reactive oxygen species; TBHP, tert-butyl hydroperoxide solution; Qrc, quercetin; DCFDA, 2',7'-dichlorofluorescein diacetate; Fen, fenretinide.

in these cells and fenretinide did not, these results suggest that fenretinide is not affecting the autophagy mechanisms that may break down LDs.

Effect of fenretinide on cell migration. Previous studies have demonstrated that, under basal conditions, NCI-N87 cells require a prolonged period, usually 48 h, to exhibit a migration effect in the wound healing assay (62-64). In comparison with other gastric cancer cell lines, including AGS cells, NCI-N87 cell line demonstrate a more differentiated epithelial phenotype characterized by high expression levels of E-cadherin and zonula occludens proteins (65). This phenotype facilitates the formation of highly cohesive monolayers, thereby hindering their migration. As NCI-N87 cells have a limited capacity to migrate in the wound healing assay, only the effect of fenretinide in the migration of AGS cells was evaluated. After 24 h incubation with a nontoxic concentration of fenretinide, a significant reduction in the capability of AGS cells to migrate and close the wound was observed (Fig. 4).

Discussion

A major limitation in gastric cancer treatment is the lack of early diagnosis. For advanced gastric cancer, surgery is not curative and conventional treatments such as radiotherapy and chemotherapy achieve very modest outcomes (57). Despite the use of several combinations of chemotherapeutics in advanced gastric cancer, the median overall survival time remains <1 year (66). There is consequently a need to investigate alternative options that overcome resistance to chemotherapy.

In the present preclinical study, AGS and NCI-N87 cell lines were used as they are well- characterized models of intestinal gastric cancer (48,67,68). AGS is a primary gastric cancer cell line that is considered to be highly invasive and moderately differentiated (69). The metastatic cell line NCI-N87 demonstrates upregulated levels of HER2, and thus has been extensively used as a model system for studying trastuzumab, and its mechanisms of acquired resistance over time in gastric cancer (70). Trastuzumab, a humanized monoclonal antibody that targets the extracellular domain of HER2, has shown significant therapeutic benefit in the treatment of patients with HER2-overexpressing gastric cancer (16). Simeone *et al* have demonstrated that HER2 overexpression reduced the sensitivity of breast cancer cells to fenretinide (71).

In the present study it was determined that the AGS and NCI-N87 cell lines respond differently to the chemotherapeutic agents, cisplatin and doxorubicin, demonstrating that the NCI-N87 cell line was markedly insensitive to

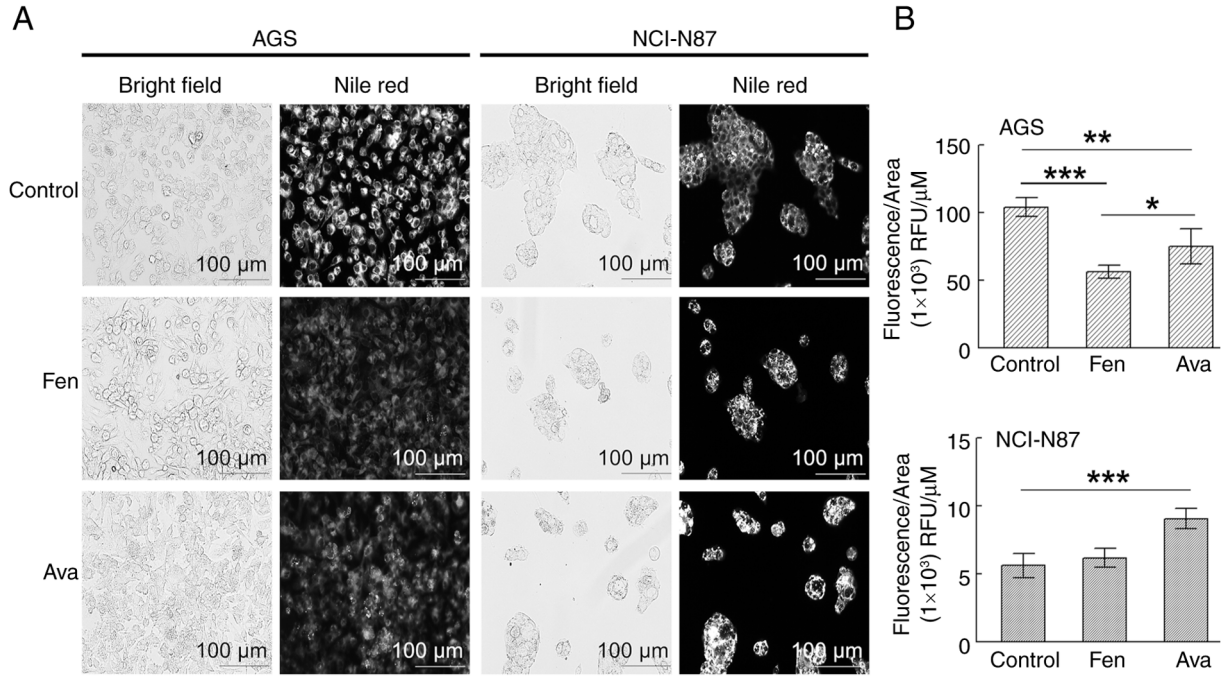

Figure 3. Effect of Fen on lipid droplet content. (A) Nile red staining of intracellular lipid droplets in gastric cancer cells. (B) Fluorescence quantification adjusted by area. Ava acts by inhibiting ACAT-1 and therefore reduces lipid droplet synthesis. Values are expressed as the mean ± standard error of at least five independent images. *P<0.05, **P<0.01 and ***P<0.001. Fen, fenretinide; Ava, avasimibe; ACAT1, acyl-CoA: cholesterol O-acyltransferase; RFU, relative fluorescence units.

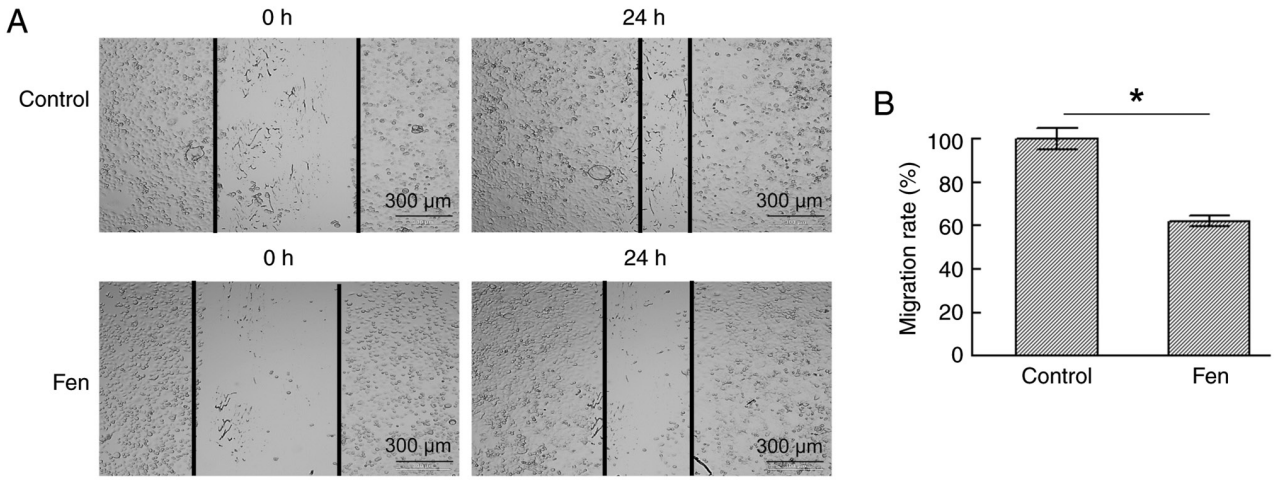

Figure 4. Migration of AGS cells treated fenretinide for 24 h. (A) Representative images from a wound healing assay of control cells and cells treated with fenretinide. (B) Degree of migration is presented as the percentage of the wound measurement from 0 h. Values are expressed as the mean ± standard error of at least three independent experiments performed in duplicate. *P<0.05. Fen, fenretinide.

doxorubicin,while AGS cells were affected at concentrations less than 1 μM. By contrast, it was demonstrated that both cell lines had similar sensitivity to cisplatin, especially at doses corresponding to the IC_{50} values.It has been hypothesized that the use of ATRA could be beneficial for the treatment of gastric cancer; however, its main limitations include its low availability and high resistance (30). Hence, the present study aimed to assess the cellular response of both cell lines to fenretinide, with the response to ATRA serving as a comparative benchmark. It was observed that NCI-N87 cells showed less sensitivity compared with AGS cells. However, in both cell lines, the IC_{50} values were more than twice as high as those observed for fenretinide. These results were consistent with those observed by Guarrera *et al* where, from a panel of gastric cancer cell lines, they classified AGS and NCI-N87 cells as low ATRA-sensitive (72).

The presence of a benzene ring instead of a cyclohexane ring allows fenretinide to be more hydrophilic, resulting in a higher bioavailability compared with ATRA (73). To the best of our knowledge, there is only one previous study evaluating the effect of fenretinide on gastric cancer cells. Liu *et al* reported that fenretinide inhibited the growth of the gastric cancer BGC 823 cell line and suggested that the effect was partly mediated by RARβ2 (74). In the present study, it was demonstrated that

fenretinide not only had a similar toxic effect on both AGS and NCI-N87 cell lines, but according to the IC_{50} values, this effect was comparable with those observed for cisplatin.

The idea combining naturally occurring agents with conventional chemotherapeutic agents has been gaining interest (75). Although cisplatin has been extensively used to treat gastric cancer, drug resistance and adverse side effects are its two main limitations. It has been previously suggested that combining cisplatin with certain natural products such as flavonoids, saponins and alkaloids may enable treatments to overcome the cisplatin resistance of cells and the adverse side effects of cisplatin use (76). The present study did not investigate the doxorubicin-fenretinide combination because a primary aim was to evaluate fenretinide as a potential co-adjuvant therapy for the treatment of metastatic tumors, as they present a poor response to current chemotherapy strategies. There are several chemotherapy regimens used for gastric cancer, and some of which involve simultaneous administration of three chemotherapy drugs that include doxorubicin or epirubicin in addition to cisplatin and 5-fluorouracil; however, according to the European Society of Medical Oncology, for metastatic gastric cancer the response rate is higher with dual therapy, combining only cisplatin and 5-fluorouracil (57).

The results of the present study indicated that combination of cisplatin with fenretinide had an additive effect in primary and metastatic gastric cancer cells, and according to the dose reduction index, this combination could enable cisplatin dose reduction up to 20-fold, which potentially benefits the survival of patients with gastric cancer by reducing the adverse effects of cisplatin. Fenretinide as an oral capsule has been reported to be well tolerated in clinical studies (35). However, achieving an optimal plasma drug concentration has been an obstacle due to the low bioavailability of fenretinide (35,73). Nonetheless, novel attempts to improve fenretinide solubility have been under development, including oral formulations with improved bioavailability and intravenous formulations that result in significantly higher plasma levels, alongside acceptable toxicity responses (73,77-80).

Multiple studies have suggested that fenretinide-induced cell death occurs through apoptosis, either through the production of ROS or the involvement of lipid secondary messengers such as dihydroceramide and dimethylsphingosine (38,81-86). In the present study it was observed that fenretinide induced ROS production in AGS cells. Furthermore, it was determined that ROS were relevant in the fenretinide-associated mechanism of cell death only in the primary gastric cancer cells. Lai and Wong (84) reported that in leukemia and cervical cancer cells, fenretinide caused a rapid increase in ROS levels, and this triggered the activation of the unfolded protein response. In addition, Darwiche *et al* (82) described in leukemia cells that fenretinide induced growth inhibition associated with ROS accumulation at early time points (<12 h), therefore it is possible that for the NCI-N87 cell line, the time evaluated (20 h) was not sufficient for this effect to be observed in the present study. However, it appears that NCI-N87 cells have different compensatory mechanisms to protect against oxidative stress as the response to the ROS-inducer TBHP was also low when compared with AGS cells.

Autophagy has a dual role in cancer by inhibiting tumor initiation but also supporting a pro-tumorigenic role in established disease and mediating treatment resistance (87). It has been previously reported that fenretinide is able to trigger an autophagic cell death pathway in breast cancer cells (38). As autophagy regulates the mobilization of fat from cellular deposits, such as LDs, Nile red staining was used as a means to indirectly study autophagy in fenretinide-treated cells. Notably, fenretinide treatment significantly reduced the number of LDs in the AGS cell line, but in NCI-N87 cells there was no significant effect when compared with the control. The effect of autophagy on the levels of LD formation has not been elucidated at present. However, Nguyen *et al* (88) reported that during autophagy, under starvation conditions, the amount of LDs increased (88). Although, this association between autophagy and LD content is complex, as in liver cells under starvation or induction by an acute stimulus, a selective autophagy process termed lipophagy degrades LDs in lysosomes (89). The crosstalk between LDs and autophagy still requires further study, but the results of the present study suggest that in AGS cells, fenretinide may affect autophagy pathways.

Retinoids have been reported to prevent the conversion of primary tumors to invasive malignancies by inhibiting invasion as well as angiogenesis (90,91). In the present study a significant reduction in AGS cell migration was observed when treated with fenretinide. In the liver cancer cell line HepG2, fenretinide inhibited its migration properties by regulating the p38-MAPK signaling pathway (92). There is also evidence of fenretinide acting on proteases involved in the degradation of the extracellular matrix. *In vivo* experiments have shown that fenretinide prevents the development of different metastases in mouse models of prostate and liver cancer (93). Due to fenretinide capacity to disrupt pro-invasive signaling pathways, its combination with chemotherapy may enhance treatment outcomes in patients with advanced gastric cancer.

Although fenretinide had a similar cytotoxic effect in the primary and metastatic cell lines in the present study, it is clear that there are different mechanisms involved in the cytotoxic effects that should be further investigated. The results of the present study suggest that in the metastatic cell line NCI-N87, neither ROS nor autophagy mediate the fenretinide mechanism of action. Apraiz *et al* (85) reported that in human leukemia cells, sphingolipid accumulation and ROS induction were not necessarily essential in fenretinide-mediated cytotoxicity.

Overall, the present study provided preclinical evidence that fenretinide exhibits anti-tumoral activity on primary gastric cancer cells through the induction of ROS, possibly due to cell death by autophagy. Moreover, fenretinide may be a potential therapeutic option for metastatic gastric cancer as it may inhibit cell migration and act together with cisplatin. Currently, there is an imperative need to improve the poor prognosis resulting from conventional chemotherapy in patients with advanced gastric cancer, and further preclinical research evaluating the potential benefits of fenretinide alone or as a co-adjuvant for the treatment of gastric cancer, especially for metastatic tumors, should be performed.

The main limitation of the present study was that only two cancer cell lines were evaluated. However, these cell lines are well-characterized and have been widely used in previous literature (15,48,65,94-96). Future studies should examine the

effect of fenretinide in other gastric cancer cell lines derived from metastatic sites. Additionally, given the complex role of autophagy in cancer, further studies are required to determine the causative mechanisms of the effect of fenretinide on primary cell lines, and whether the targeting of autophagy could be a promising therapeutic strategy for gastric cancer.

Although improving fenretinide low drug solubility remains a challenge, as aforementioned, in recent years there has been a growing interest in developing improved oral formulations and using alternative routes of administration that may overcome the low plasma concentration seen in earlier clinical trials. The results of the present study provide a basis for further research that could aid the understanding of the molecular mechanisms responsible for the cytotoxic effect and inhibition of migration caused by fenretinide, either alone or in combination with cisplatin, particularly those observed with the metastatic cell line. It is important to continue the investigation of novel therapeutic alternatives for primary and metastatic gastric cancer that may improve the survival of patients with this disease and reduce the adverse effects of the use of chemotherapy at high doses.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

NO and CD conceived and designed the study, analyzed the results and wrote and reviewed the manuscript. NO and CD confirm the authenticity of all the raw data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
2. Guggenheim DE and Shah MA: Gastric cancer epidemiology and risk factors. J Surg Oncol 107: 230-236, 2013.
3. Lauren P: The two histological main types of gastric carcinoma: Diffuse and so-called intestinal-type carcinoma. An attempt at a histoclinical classification. Acta Pathol Microb Scand 64: 31-49, 1965.
4. Cisło M, Filip AA, Offerhaus GJA, Ciseł B, Rawicz-Pruszyński K, Skierucha M and Polkowski WP: Distinct molecular subtypes of gastric cancer: From Laurén to molecular pathology. Oncotarget 9: 19427-19442, 2018.
5. Ma J, Shen H, Kapesa L and Zeng S: Lauren classification and individualized chemotherapy in gastric cancer. Oncol Lett 11: 2959-2964, 2016.
6. Silvestris N, Pantano F, Ibrahim T, Gamucci T, De Vita F, Di Palma T, Pedrazzoli P, Barni S, Bernardo A, Febbraro A, *et al*: Natural history of malignant bone disease in gastric cancer: Final results of a multicenter bone metastasis survey. PLoS One 8: e74402, 2013.
7. Riihimäki M, Hemminki A, Sundquist K, Sundquist J and Hemminki K: Metastatic spread in patients with gastric cancer. Oncotarget 7: 52307-52316, 2016.
8. Apicella M, Corso S and Giordano S: Targeted therapies for gastric cancer: Failures and hopes from clinical trials. Oncotarget 8: 57654-57669, 2017.
9. Drebber U, Baldus SE, Nolden B, Grass G, Bollschweiler E, Dienes HP, Hölscher AH and Mönig SP: The overexpression of c-met as a prognostic indicator for gastric carcinoma compared to p53 and p21 nuclear accumulation. Oncol Rep 19: 1477-1483, 2008.
10. Morgan E, Arnold M, Camargo MC, Gini A, Kunzmann AT, Matsuda T, Meheus F, Verhoeven RHA, Vignat J, Laversanne M, *et al*: The current and future incidence and mortality of gastric cancer in 185 countries, 2020-40: A population-based modelling study. EClinicalMedicine 47: 101404, 2022.
11. Marin JJGG, Perez-Silva L, Macias RIRR, Asensio M, Peleteiro-Vigil A, Sanchez-Martin A, Cives-Losada C, Sanchon-Sanchez P, De Blas BS and Herraez E: Molecular bases of mechanisms accounting for drug resistance in gastric adenocarcinoma. Cancers (Basel) 12: 2116, 2020.
12. Okines A, Verheij M, Allum W, Cunningham D and Cervantes A; ESMO Guidelines Working Group: Gastric cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 21 (Suppl 5): v50-v54, 2010.
13. Leite de Oliveira R, Deschoemaeker S, Henze AT, Debackere K, Finisguerra V, Takeda Y, Roncal C, Dettori D, Tack E, Jönsson Y, *et al*: Gene-targeting of Phd2 improves tumor response to chemotherapy and prevents side-toxicity. Cancer Cell 22: 263-277, 2012.
14. Yamaguchi K, Boku N, Muro K, Yoshida K, Baba H, Tanaka S, Akamatsu A and Sano T: Real-world safety and effectiveness of nivolumab in Japanese patients with unresectable advanced or recurrent gastric/gastroesophageal junction cancer that has progressed after chemotherapy: A postmarketing surveillance study. Gastric Cancer 25: 245-253, 2022.
15. Araújo D, Ribeiro E, Amorim I and Vale N: Repurposed drugs in gastric cancer. Molecules 28: 319, 2023.
16. Shitara K, Bang YJ, Iwasa S, Sugimoto N, Ryu MH, Sakai D, Chung HC, Kawakami H, Yabusaki H, Lee J, *et al*: Trastuzumab deruxtecan in previously treated HER2-positive gastric cancer. N Engl J Med 382: 2419-2430, 2020.
17. Janjigian YY, Kawazoe A, Yañez P, Li N, Lonardi S, Kolesnik O, Barajas O, Bai Y, Shen L, Tang Y, *et al*: The KEYNOTE-811 trial of dual PD-1 and HER2 blockade in HER2-positive gastric cancer. Nature 600: 727-730, 2021.
18. Shitara K, Rha SY, Wyrwicz LS, Oshima T, Karaseva N, Osipov M, Yasui H, Yabusaki H, Afanasyev S, Park YK, *et al*: Neoadjuvant and adjuvant pembrolizumab plus chemotherapy in locally advanced gastric or gastro-oesophageal cancer (KEYNOTE-585): An interim analysis of the multicentre, double-blind, randomised phase 3 study. Lancet Oncol 25: 212-224, 2024.
19. Masetti M, Al-Batran SE, Goetze TO, Thuss-Patience P, Knorrenschild JR, Goekkurt E, Folprecht G, Ettrich TJ, Lindig U, Luley KB, *et al*: Efficacy of ramucirumab combination chemotherapy as second-line treatment in patients with advanced adenocarcinoma of the stomach or gastroesophageal junction after exposure to checkpoint inhibitors and chemotherapy as first-line therapy. Int J Cancer 154: 2142-2150, 2024.
20. Guven DC, Sahin TK, Erul E, Rizzo A, Ricci AD, Aksoy S and Yalcin S: The association between albumin levels and survival in patients treated with immune checkpoint inhibitors: A systematic review and meta-analysis. Front Mol Biosci 9: 1039121, 2022.

21. Sahin TK, Rizzo A, Aksoy S and Guven DC: prognostic significance of the royal marsden hospital (RMH) score in patients with cancer: A systematic review and meta-analysis. Cancers (Basel) 16: 1835, 2024.
22. Dall'Olio FG, Rizzo A, Mollica V, Massucci M, Maggio I and Massari F: Immortal time bias in the association between toxicity and response for immune checkpoint inhibitors: A meta-analysis. Immunotherapy 13: 257-270, 2021.
23. Rizzo A, Mollica V, Tateo V, Tassinari E, Marchetti A, Rosellini M, De Luca R, Santoni M and Massari F: Hypertransaminasemia in cancer patients receiving immunotherapy and immune-based combinations: The MOUSEION-05 study. Cancer Immunol Immunother 72: 1381-1394, 2023.
24. Ricci AD, Rizzo A and Brandi G: DNA damage response alterations in gastric cancer: Knocking down a new wall. Future Oncol 17: 865-868, 2021.
25. Chung HC, Bang YJ, Fuchs CS, Qin SK, Satoh T, Shitara K, Tabernero J, Van Cutsem E, Alsina M, Cao ZA, *et al*: First-line pembrolizumab/placebo plus trastuzumab and chemotherapy in HER2-positive advanced gastric cancer: KEYNOTE-811. Future Oncol 17: 491-501, 2021.
26. Boku N, Satoh T, Ryu MH, Chao Y, Kato K, Chung HC, Chen JS, Muro K, Kang WK, Yeh KH, *et al*: Nivolumab in previously treated advanced gastric cancer (ATTRACTION-2): 3-year update and outcome of treatment beyond progression with nivolumab. Gastric Cancer 24: 946-958, 2021.
27. Jabbar N, Khayyam N, Arshad U, Maqsood S, Hamid SA and Mansoor N: An outcome analysis of childhood acute promyelocytic leukemia treated with ATRA and arsenic trioxide, and limited dose anthracycline. Indian J Hematol Blood Transfus 37: 569-575, 2021.
28. Kutny MA, Alonzo TA, Abla O, Rajpurkar M, Gerbing RB, Wang YC, Hirsch BA, Raimondi S, Kahwash S, Hardy KK, *et al*: Assessment of arsenic trioxide and all-trans retinoic acid for the treatment of pediatric acute promyelocytic leukemia: A report from the Children's oncology group AAML1331 trial. JAMA Oncol 8: 79-87, 2022.
29. Ramchatesingh B, Villarreal AM, Arcuri D, Lagacé F, Setah SA, Touma F, Al-Badarin F and Litvinov IV: The use of retinoids for the prevention and treatment of skin cancers: An updated review. Int J Mol Sci 23: 12622, 2022.
30. Giuli MV, Hanieh PN, Giuliani E, Rinaldi F, Marianecci C, Screpanti I, Checquolo S and Carafa M: Current trends in ATRA delivery for cancer therapy. Pharmaceutics 12: 707, 2020.
31. Ferreira R, Napoli J, Enver T, Bernardino L and Ferreira L: Advances and challenges in retinoid delivery systems in regenerative and therapeutic medicine. Nat Commun 11: 4265, 2020.
32. Veronesi U, Mariani L, Decensi A, Formelli F, Camerini T, Miceli R, Di Mauro MG, Costa A, Marubini E, Sporn MB and De Palo G: Fifteen-year results of a randomized phase III trial of fenretinide to prevent second breast cancer. Ann Oncol 17: 1065-1071, 2006.
33. Xie H, Zhu F, Huang Z, Lee MH, Kim DJ, Li X, Lim DY, Jung SK, Kang S, Li H, *et al*: Identification of mammalian target of rapamycin as a direct target of fenretinide both in vitro and in vivo. Carcinogenesis 33: 1814-1821, 2012.
34. Mittal N, Malpani S, Dyson M, Ono M, Coon JS, Kim JJ, Schink JC, Bulun SE and Pavone ME: Fenretinide: A novel treatment for endometrial cancer. PLoS One 9: e110410, 2014.
35. Cooper JP, Reynolds CP, Cho H and Kang MH: Clinical development of fenretinide as an antineoplastic drug: Pharmacology perspectives. Exp Biol Med (Maywood) 242: 1178-1184, 2017.
36. Hail N, Kim HJ and Lotan R: Mechanisms of fenretinide-induced apoptosis. Apoptosis 11: 1677-1694, 2006.
37. Corazzari M, Lovat PE, Armstrong JL, Fimia GM, Hill DS, Birch-Machin M, Redfern CP and Piacentini M: Targeting homeostatic mechanisms of endoplasmic reticulum stress to increase susceptibility of cancer cells to fenretinide-induced apoptosis: The role of stress proteins ERdj5 and ERp57. Br J Cancer 96: 1062-1071, 2007.
38. Fazi B, Bursch W, Fimia GM, Nardacci R, Piacentini M, Di Sano F and Piredda L: Fenretinide induces autophagic cell death in caspase-defective breast cancer cells. Autophagy 4: 435-441, 2008.
39. Garaventa A, Luksch R, Lo Piccolo MS, Cavadini E, Montaldo PG, Pizzitola MR, Boni L, Ponzoni M, Decensi A, De Bernardi B, *et al*: Phase I trial and pharmacokinetics of fenretinide in children with neuroblastoma. Clin Cancer Res 9: 2032-2039, 2003.
40. Sabichi AL, Lerner SP, Atkinson EN, Grossman HB, Caraway NP, Dinney CP, Penson DF, Matin S, Kamat A, Pisters LL, *et al*: Phase III prevention trial of fenretinide in patients with resected non-muscle-invasive bladder cancer. Clin Cancer Res 14: 224-229, 2008.
41. Schneider BJ, Worden FP, Gadgeel SM, Parchment RE, Hodges CM, Zwiebel J, Dunn RL, Wozniak AJ, Kraut MJ and Kalemkerian GP: Phase II trial of fenretinide (NSC 374551) in patients with recurrent small cell lung cancer. Invest New Drugs 27: 571-578, 2009.
42. Moore MM, Stockler M, Lim R, Mok TSK, Millward M and Boyer MJ: A phase II study of fenretinide in patients with hormone refractory prostate cancer: A trial of the cancer therapeutics research group. Cancer Chemother Pharmacol 66: 845-850, 2010.
43. Villabanca JG, London WB, Naranjo A, McGrady P, Ames MM, Reid JM, McGovern RM, Buhrow SA, Jackson H, Stranzinger E, *et al*: Phase II study of oral capsular 4-hydroxyphenylretinamide (4-HPR/Fenretinide) in pediatric patients with refractory or recurrent neuroblastoma: A report from the Children's oncology group. Clin Cancer Res 17: 6858-6866, 2011.
44. Aristarco V, Serrano D, Maisonneuve P, Guerrieri-Gonzaga A, Lazzeroni M, Feroce I, Macis D, Cavadini E, Albertazzi E, Jemos C, *et al*: Fenretinide in young women at genetic or familial risk of breast cancer: A placebo-controlled biomarker trial. Cancer Prev Res (Phila) 17: 255-263, 2024.
45. Orienti I, Salvati V, Sette G, Zucchetti M, Bongiorno-Borbone L, Peschiaroli A, Zolla L, Francescangeli F, Ferrari M, Matteo C, *et al*: A novel oral micellar fenretinide formulation with enhanced bioavailability and antitumour activity against multiple tumours from cancer stem cells. J Exp Clin Cancer Res 38: 373, 2019.
46. Matteo C, Orienti I, Eramo A, Zeuner A, Ferrari M, Passoni A, Bagnati R, Ponzo M, Bello E, Zucchetti M and Frapolli R: Validated LC-MS/MS assay for the quantitative determination of fenretinide in plasma and tumor and its application in a pharmacokinetic study in mice of a novel oral nanoformulation of fenretinide. Pharmaceutics 16: 387, 2024.
47. Barranco SC, Townsend CM, Casartelli C, Macik BG, Burger NL, Boerwinkle WR and Gourley WK: Establishment and characterization of an in vitro model system for human adenocarcinoma of the stomach. Cancer Res 43: 1703-1709, 1983.
48. Park JG, Frucht H, LaRocca RV, Bliss DP, Kurita Y, Chen TR, Henslee JG, Trepel JB, Jensen RT and Johnson BE: Characteristics of cell lines established from human gastric carcinoma. Cancer Res 50: 2773-2780, 1990.
49. Chou TC: Drug combination studies and their synergy quantification using the chou-talalay method. Cancer Res 70: 440-446, 2010.
50. Ortiz N, Delgado-carazo JC and Díaz C: Importance of mevalonate pathway lipids on the growth and survival of primary and metastatic gastric carcinoma cells. Clin Exp Gastroenterol 14: 217-228, 2021.
51. Main KA, Mikelis CM and Doçi CL: In vitro wound healing assays to investigate epidermal migration. Methods Mol Biol 2109: 147-154, 2020.
52. Trump DL, Smith DC, Stiff D, Adedoyin A, Day R, Bahnson RR, Hofacker J and Branch RA: A phase II trial of all-trans-retinoic acid in hormone-refractory prostate cancer: A clinical trial with detailed pharmacokinetic analysis. Cancer Chemother Pharmacol 39: 349-356, 1997.
53. Adan A, Kiraz Y and Baran Y: Cell proliferation and cytotoxicity assays. Curr Pharm Biotechnol 17: 1213-1221, 2016.
54. Ghasemi M, Turnbull T, Sebastian S and Kempson I: The mtt assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. Int J Mol Sci 22: 12827, 2021.
55. Vichai V and Kirtikara K: Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc 1: 1112-1116, 2006.
56. Koay DC, Zerillo C, Narayan M, Harris LN and Digiovanna MP: Anti-tumor effects of retinoids combined with trastuzumab or tamoxifen in breast cancer cells : Induction of apoptosis by retinoid/trastuzumab combinations. Breast Cancer Res 12: R62, 2010.
57. Lordick F, Carneiro F, Cascinu S, Fleitas T, Haustermans K, Piessen G, Vogel A and Smyth EC; ESMO Guidelines Committee. Electronic address: clinicalguidelines@esmo.org: Gastric cancer: ESMO clinical practice guideline for diagnosis, treatment and follow-up. Ann Oncol 33: 1005-1020, 2022.

58. Raza MH, Siraj S, Arshad A, Waheed U, Aldakheel F, Alduraywish S and Arshad M: ROS-modulated therapeutic approaches in cancer treatment. J Cancer Res Clin Oncol 143: 1789-1809, 2017.
59. Parveen SM, Reddy KR and Ummanni R: Dimethylarginine Dimethylaminohydrolase-1 expression is increased under tBHP-induced oxidative stress regulates nitric oxide production in PCa cells attenuates mitochondrial ROS-mediated apoptosis. Nitric Oxide 138-139: 70-84, 2023.
60. Xu C and Fan J: Links between autophagy and lipid droplet dynamics. J Exp Bot 73: 2848-2858, 2022.
61. Zhu Y, Chen CY, Li J, Cheng JX, Jang M and Kim KH: In vitro exploration of ACAT contributions to lipid droplet formation during adipogenesis. J Lipid Res 59: 820-829, 2018.
62. Seo JH, Jeong ES and Choi YK: Therapeutic effects of lentivirus-mediated shRNA targeting of cyclin D1 in human gastric cancer. BMC Cancer 14: 175, 2014.
63. Park JH, Seo JH, Jeon HY, Seo SM, Lee HK, Park J, Kim JY and Choi YK: Lentivirus-mediated VEGF knockdown suppresses gastric cancer cell proliferation and tumor growth in vitro and in vivo lentivirus-mediated VEGF knockdown suppresses gastric cancer cell proliferation and tumor growth in vitro and in vivo. Onco Targets Ther 13: 1331-1341, 2020.
64. Xia P, Liang J, Jin D and Jin Z: Reversine inhibits proliferation, invasion and migration and induces cell apoptosis in gastric cancer cells by downregulating TTK. Exp Ther Med 22: 929, 2021.
65. Basque JRÂ, Chénard M, Chailler P and Ménard D: Gastric cancer cell lines as models to study human digestive functions. J Cell Biochem 81: 241-251, 2001.
66. Patel TH and Cecchini M: Targeted therapies in advanced gastric cancer. Curr Treat Options Oncol 21: 70, 2020.
67. Katuri V, Tang Y, Marshall B, Rashid A, Jogunoori W, Volpe EA, Sidawy AN, Evans S, Blay J, Gallicano GI, *et al*: Inactivation of ELF/TGF-b signaling in human gastrointestinal cancer. Oncogene 24: 8012-8024, 2005.
68. Jang M, Koh I, Lee SJ, Cheong JH and Kim P: Droplet-based microtumor model to assess cell-ECM interactions and drug resistance of gastric cancer cells. Sci Rep 7: 41541, 2017.
69. Wang YG, Xu L, Jia RR, Wu Q, Wang T, Wei J, Ma JL, Shi M and Li ZS: DDR2 induces gastric cancer cell activities via activating mTORC2 signaling and is associated with clinicopathological characteristics of gastric cancer. Dig Dis Sci 61: 2272-2283, 2016.
70. Espelin CW, Leonard SC, Geretti E, Wickham TJ and Hendriks BS: Dual HER2 targeting with trastuzumab and demonstrates synergistic antitumor activity in breast and gastric cancer. Cancer Res 76: 1517-1527, 2016.
71. Simeone A, Broemeling L, Rosenblum J and Tari AM: HER2/neu reduces the apoptotic effects of N-(4-hydroxyphenyl)retinamide (4-HPR) in breast cancer cells by decreasing nitric oxide production. Onocogene 22: 6739-6747, 2003.
72. Guarrera L, Kurosaki M, Garattini SK, Gianni M, Fasola G, Rossit L, Prisciandaro M, Di Bartolomeo M, Bolis M, Rizzo P, *et al*: Anti-tumor activity of all-trans retinoic acid in gastric-cancer: Gene-networks and molecular mechanisms. J Exp Clin Cancer Res 42: 298, 2023.
73. Alfei S and Zuccari G: Attempts to improve lipophilic drugs' solubility and bioavailability: A focus on fenretinide. Pharmaceutics 16: 579, 2024.
74. Liu G, Wu M, Levi G and Ferrari N: Inhibition of cancer cell growth by all-trans retinoic acid and its analog N-(4-hydroxyphenyl) retinamide: A possible mechanism of action via regulation of retinoid receptors expression. Int J Cancer 78: 248-254, 1998.
75. Lin SR, Chang CH, Hsu CF, Tsai MJ, Cheng H, Leong MK, Sung PJ, Chen JC and Weng CF: Natural compounds as potential adjuvants to cancer therapy: Preclinical evidence. Br J Pharmacol 177: 1409-1423, 2020.
76. Dasari S, Njiki S, Mbemi A, Yedjou CG and Tchounwou PB: Pharmacological effects of cisplatin combination with natural products in cancer chemotherapy. Int J Mol Sci 23: 1532, 2022.
77. Mohrbacher AM, Yang AS, Groshen S, Kummar S, Martin E, Kang MH, Tsao-Wei D, Reynolds CP, Newman EM and Maurer BJ: Phase I study of fenretinide deliverded intravenously in patients with relapsed or refractory hematologic malignancies: A California cancer consortium trial. Clin Cancer Res 23: 4550-4555, 2017.
78. Orienti I, Francescangeli F, De Angelis ML, Fecchi K, Bongiorno-Borbone L, Signore M, Peschiaroli A, Boe A, Bruselles A, Costantino A, *et al*: A new bioavailable fenretinide formulation with antiproliferative, antimetabolic, and cytotoxic effects on solid tumors. Cell Death Dis 10: 529, 2019.
79. Bensa V, Calarco E, Giusto E, Perri P, Corrias MV, Ponzoni M, Brignole C and Pastorino F: Retinoids delivery systems in cancer: Liposomal fenretinide for neuroectodermal-derived tumors. Pharmaceuticals (Basel) 14: 854, 2021.
80. Thomas JS, El-khoueiry AB, Maurer BJ, Groshen S, Jacek K, Cobos E, Gandara DR, Lenz HJ, Kang MH, Reynolds CP and Newman EM: A phase I study of intravenous fenretinide (4-HPR) for patients with malignant solid tumors. Cancer Chemother Pharmacol 87: 525-532, 2022.
81. Brack E, Wachtel M, Wolf A, Kaech A, Ziegler U and Schäfer BW: Fenretinide induces a new form of dynamin-dependent cell death in pediatric sarcoma. Cell Death Differ 27: 2500-2516, 2020.
82. Darwiche N, Abou-Lteif G and Bazarbachi A: Reactive oxygen species mediate N-(4-hydroxyphenyl)retinamide-induced cell death in malignant T cells and are inhibited by the HTLV-I oncoprotein tax. Leukemia 21: 261-269, 2007.
83. Wang H, Maurer BJ, Liu YY, Wang E, Allegood JC, Kelly S, Symolon H, Liu Y, Merrill AH Jr, Gouazé-Andersson V, *et al*: N-(4-Hydroxyphenyl)retinamide increases dihydroceramide and synergizes with dimethylsphingosine to enhance cancer cell killing. Mol Cancer Ther 7: 2967-2976, 2008.
84. Lai WL and Wong NS: The PERK/eIF2 alpha signaling pathway of unfolded protein response is essential for N-(4-hydroxyphenyl) retinamide (4HPR)-induced cytotoxicity in cancer cells. Exp Cell Res 314: 1667-1682, 2008.
85. Apraiz A, Idkowiak-baldys J, Nieto-rementería N, Boyano MD, Hannun YA and Asumendi A: Dihydroceramide accumulation and reactive oxygen species are distinct and nonessential events in 4-HPR-mediated leukemia cell death. Biochem Cell Biol 90: 209-223, 2012.
86. Kraveka JM, Li L, Szulc ZM, Bielawski J, Ogretmen B, Hannun YA, Obeid LM and Bielawska A: Involvement of dihydroceramide desaturase in cell cycle progression in human neuroblastoma cells. J Biol Chem 282: 16718-16728, 2007.
87. Lim J and Murthy A: Targeting autophagy to treat cancer: Challenges and opportunities. Front Pharmacol 11: 590344, 2020.
88. Nguyen TB, Louie SM, Daniele JR, Tran Q, Dillin A, Zoncu R, Nomura DK and Olzmann JA: DGAT1-dependent lipid droplet biogenesis protects mitochondrial function during starvation-induced autophagy. Dev Cell 42: 9-21, 2017.
89. Martinez-Lopez N and Singh R: Autophagy and lipid droplets in the liver. Ann Rev Nutr 35: 215-237, 2015.
90. Bouriez D, Giraud J, Gronnier C and Varon C: Efficiency of all-trans retinoic acid on gastric cancer: A narrative literature review. Int J Mol Sci 19: 3388, 2018.
91. Yücel EI and Sahin M: Fenretinide reduces angiogenesis by downregulating CDH5, FOXM1 and eNOS genes and suppressing microRNA-10b. Mol Biol Rep 47: 1649-1658, 2020.
92. Zeng J, Zhang H, Tan Y, Sun C, Liang Y, Yu J and Zou H: Aggregation of lipid rafts activates c-met and c-Src in non-small cell lung cancer cells. BMC Cancer 18: 611, 2018.
93. Sogno I, Venè R, Ferrari N, De Censi A, Imperatori A, Noonan DM, Tosetti F and Albini A: Angioprevention with fenretinide: Targeting angiogenesis in prevention and therapeutic strategies. Crit Rev Oncol Hematol 75: 2-14, 2010.
94. Mayer B, Klement G, Kaneko M, Man S, Jothy S, Rak J and Kerbel RS: Multicellular gastric cancer spheroids recapitulate growth pattern and differentiation phenotype of human gastric carcinomas. Gastroenterology 121: 839-852, 2001.
95. Carl-McGrath S, Ebert MPA, Lendeckel U and Röcken C: Expression of the local angiotensin II system in gastric cancer may facilitate lymphatic invasion and nodal spread. Cancer Biol Ther 6: 1218-1226, 2007.
96. Takaishi S, Okumura T, Tu S, Wang SSW, Shibata W, Vigneshwaran R, Gordon SA, Shimada Y and Wang TC: Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells 27: 1006-1020, 2009.