

Piperlongumine combined with vitamin C as a new adjuvant therapy against gastric cancer regulates the ROS–STAT3 pathway

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Abstract

Objective: To investigate the effects of piperlongumine (PL) and vitamin C (VC) on signal transducer and activator of transcription 3 (STAT3) signalling in gastric cancer cell lines.

Methods: *In vivo* tumour xenograft anticancer assays were undertaken to confirm the anticancer activity of PL. Cell viability, flow cytometry and Western blot assays were undertaken to evaluate the anticancer effects of PL, VC and combinations of PL and VC in AGS and KATO III cells.

Results: Both PL and VC induced apoptosis and inhibited cell proliferation in AGS and KATO III cells. These effects were dependent on reactive oxygen species (ROS). PL effectively suppressed STAT3 activation while VC caused abnormal activation of STAT3. The combination of PL and VC exhibited a stronger apoptotic effect compared with either agent alone. PL reversed the abnormal activation of STAT3 by VC, which could be a key to their synergistic effect.

Conclusions: PL combined with VC exhibited a stronger anticancer effect by regulating the ROS–STAT3 pathway, suggesting that this combination might be a potential adjuvant therapy for gastric cancer.

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Keywords

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Introduction

Gastric cancer (GC) has become a serious cancer with high rates of morbidity and mortality.¹ Surgical resection is a common option for early GC, while advanced GC requires radiotherapy, chemotherapy and targeted therapy.² Increasing numbers of patients are diagnosed with advanced GC, but because targeted drugs are expensive, chemotherapy remains a routine treatment for GC. Unfortunately, nonselective killing and drug resistance have become the major problems associated with current chemotherapy, leading to severe side-effects and adverse reactions.³ Hence, improving the current chemotherapy status through combined application of several drugs has become a promising strategy. Fluorouracil (5-FU), oxaliplatin, irinotecan and paclitaxel have been used as first-line chemotherapy drugs against GC.^{4,5} Although the combined application of these drugs is a routine treatment strategy, multidrug resistance has become a serious obstacle.⁶

Vitamin C (VC) has gradually shown its anticancer potential over the past few decades.^{7,8} Recent research has demonstrated that VC targets three vulnerabilities of cancer cells, including the redox imbalance, epigenetic reprogramming and oxygen-sensing regulation.⁸ Current clinical studies showed that intravenous injection of high-dose VC significantly enhanced the efficacy of several GC chemotherapy drugs, such as 5-FU, oxaliplatin, irinotecan and paclitaxel.^{9,10} These exciting findings indicate that VC has the potential to be used as an adjuvant agent in future chemotherapy

regimens. Although VC cannot fulfil the anticancer requirements when used alone, this unique vitamin exhibits good adjunctive effects when combined with other anticancer agents.^{11,12} Considering the safety and economic advantages of VC, finding a solution to improve its application in cancer therapy could be a valuable approach.

Piperlongumine (PL), a natural alkaloid, was originally isolated from *Piper longum* L.¹³ Previous research has demonstrated that PL is a potent anticancer compound with reliable selectivity.¹⁴ PL promotes reactive oxygen species (ROS) production and induces apoptosis of different types of cancer cells.¹⁵ Its mechanism of action for killing cells involves diverse signalling pathways, including mitogen-activated protein kinase, phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin, nuclear factor kappa B, glutathione S-transferase P1 and thioredoxin reductase-1.^{15,16} However, poor water solubility and organ toxicity limit the prospects of PL as a single anticancer agent.^{13,17} Recent studies demonstrated that PL enhanced the sensitivity of a variety of chemotherapeutic drugs, including cisplatin, paclitaxel and doxorubicin (DOX) in different types of cancer cells.^{18,19} Meanwhile, PL has been shown to be a natural inhibitor of STAT3,²⁰ indicating that PL may improve drug resistance when combined with other chemotherapeutic drugs. Today, adjuvant chemotherapy has become a critical strategy for clinical cancer therapy.⁵ Given the good anti-cancer prospects of PL and the good safety of VC, it is important to

study whether the combination of PL and VC could produce a stronger anticancer effect and even improve drug resistance.

This present study aimed to confirm the anticancer activity of PL *in vivo* and then to evaluate the effects of using PL and VC alone or in combination on cell proliferation and apoptosis in two GC cell lines in order to determine if the combination of VC and PL has the potential to be useful in future clinical chemotherapy.

Materials and methods

Reagent preparation

Piperlongumine was purchased from ApexBio (Houston, TX, USA) and was dissolved in dimethyl sulfoxide (DMSO). VC and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St Louis, MO, USA) and were dissolved in deionized H₂O.

Cell lines and culture

Human gastric cancer cell lines AGS and KATO III were purchased from the American Type Culture Collection (Manassas, VA, USA). Both cell lines were cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 100 U/ml penicillin and streptomycin (Thermo Fisher Scientific). Both cell lines were cultured in a 37°C incubator with 5% CO₂.

Cell viability assay

Both cell lines were inoculated into 96-well plates at a density of 3×10^3 cells/well. After overnight incubation, cells were exposed to a concentration of the agent, either alone or in combination, for 24 h, 48 h and 72 h. Then the cells were treated with a working solution of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT;

Sigma-Aldrich) for 4 h. The absorbance of each well was measured at 570 nm using an Epoch microplate reader (BioTek, Winooski, VT, USA).

Cell apoptosis assay

An annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining kit (BD Biosciences, San Jose, CA, USA) was used to identify apoptotic and necrotic cells. Both cell lines were exposed to different concentrations of PL and VC, either alone or in combination, for 48 h. After incubation, cells were collected and resuspended in 500 µl binding buffer and then stained with 5 µl of annexin V-FITC and 5 µl of PI in the dark for 15 min. The proportions of apoptotic and necrotic cells were detected using an Accuri C6 flow cytometer (BD Biosciences). Cells showing up as annexin V⁻/PI⁻ were identified as healthy cells, annexin V⁻/PI⁺ were identified as necrotic cells, annexin V⁺/PI⁻ were identified as early apoptotic cells and annexin V⁺/PI⁺ were identified as late apoptotic cells.

Western blot analysis

Human gastric cancer cell lines AGS and KATO III (3×10^6 cells) were homogenized under the action of protein lysate buffer and then centrifuged at 13 800 g for 10 min at 4°C to collect the protein pellet (Sorvall™ Legend™ Micro 17R Microcentrifuge; Thermo Fisher Scientific). A BCA protein assay kit (Beyotime, Shanghai, China) was used to determine the protein concentration. The proteins (30 µg per lane) were then separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis at 100 V for 90 min. The proteins were then transferred to polyvinylidene difluoride membranes (Roche, Basel, Switzerland) using electroblot apparatus at 25 V for 0.5 h

(Trans-Blot Turbo; Bio-Rad, Hercules, CA, USA). The membranes were incubated in a blocking solution of 5% skimmed milk at room temperature for 1 h. The membranes were then incubated with the following primary antibodies at 4°C overnight: β -actin (mouse antihuman antibody; dilution 1:1000; Beyotime); and p-STAT3 (rabbit antihuman antibody), STAT3 (mouse antihuman antibody) and survivin (rabbit antihuman antibody) (dilution 1:1000 for all three; Cell Signal Technology, Danvers, MA, USA). β -actin served as an internal control. The membranes were washed with $1 \times$ phosphate-buffered saline (PBS) Tween-20 (PBST, pH 7.4) five times. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig)G (H + L) (dilution 1:1000; Beyotime) and HRP-conjugated goat antirabbit IgG (H + L) (dilution 1:1000; Beyotime) at 25°C for 1 h. The membranes were washed with $1 \times$ PBST (pH 7.4) five times. The membranes were developed using an enhanced chemiluminescence reagent kit (BeyoECL Star; Beyotime) and scanned using a ChemiDoc MP Imaging System (Bio-Rad) to obtain visual immunoreactivity signals.

In vivo antitumour study

Five-week-old male nude mice (weight range: 16–18 g; HuaFuKang Bioscience, Beijing, China) were housed in an animal barrier facility at Xi'an Medical University. All animals were housed in individually ventilated cages under a 12-h light/12-h dark cycle with free access to food and water. AGS cells (5×10^6) were injected subcutaneously into the right side of the nude mice. When the tumours reached a volume of 50 mm^3 , the nude mice were randomly divided into two groups and injected intraperitoneally (i.p.) with the vehicle (10 mM PBS pH 7.4) or 4 mg/kg PL dissolved in 10 mM PBS (pH 7.4) once every

day for 9 days. Tumours were measured daily with a calliper and the volume was determined using the formula of $(\text{length} \times \text{width}^2)/2$. At the end of the therapy, all nude mice were executed and each tumour was excised and weighed. All animal study procedures were performed in accordance with protocols approved by Xi'an Medical University Medical Ethics Review Committee. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, and associated guidelines from the EU Directive 2010/63/EU for animal experiments.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 5.0 (Graphpad Software Inc., San Diego, CA, USA). Data are expressed as the mean \pm SD. One-way analysis of variance was used to analyse the significance of any differences between multiple treatment groups. A P -value < 0.05 was considered statistically significant.

Results

In vivo antitumour studies were undertaken in nude mice to determine the effectiveness of PL. Twelve male nude mice with AGS tumour xenografts were treated with i.p. injections of vehicle or 4 mg/kg PL. After 9 days, all nude mice were sacrificed and each tumour was removed and weighed. PL significantly decreased the tumour growth rates as demonstrated by tumour volume compared with the vehicle control group ($P < 0.001$) (Figures 1a and 1b). The tumour weight on day 9 of the vehicle control group was significantly higher than that of the PL group ($P < 0.001$) (Figure 1c). There were no significant differences in the changes in body weight between the

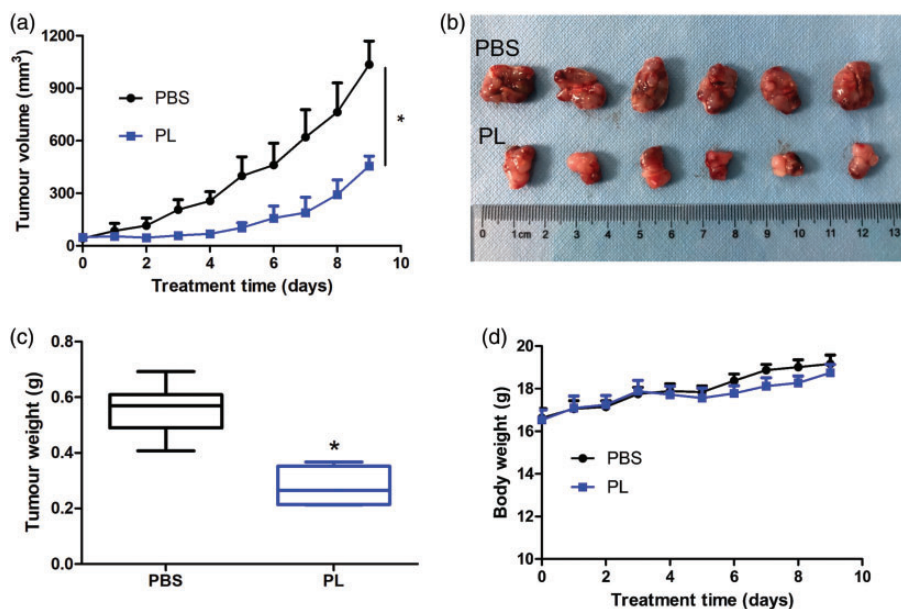


Figure 1. Evidence that piperlongumine (PL) suppressed AGS xenograft tumour growth *in vivo*. A total of 5×10^6 AGS cells were injected in the right flank of male nude mice and when the tumour volume reached 50 mm³, the nude mice were randomly divided into two groups ($n = 6$ per group). The nude mice were treated with 10 mM phosphate-buffered saline (PBS; pH 7.4) or PL dissolved in 10 mM PBS (pH 7.4) via intraperitoneal injections once every day for 9 days. (a) Tumours were measured using callipers each day and the volumes were calculated using the formula of $(\text{length} \times \text{width}^2)/2$. Data presented as mean \pm SD. (b) On day 9 after treatment, all nude mice were sacrificed and the tumours excised. (c) Tumour weights on day 9. The central black horizontal line for each group is the median. The extremities of the boxes are the 25th and 75th percentiles and the error bars represent the minimum and maximum outliers. (d) Body weight of the treated nude mice over time. Data presented as mean \pm SD. * $P < 0.001$ compared with the control group; analysis of variance. The colour version of this figure is available at: <http://imr.sagepub.com>.

two groups during treatment (Figure 1d), which indicated that PL had no significant toxicity.

The inhibitory effects of PL and VC were measured in two human GC cell lines, AGS and KATO III, over three treatment durations and a range of concentrations. Both cancer cell lines were inhibited by PL in a dose- and time-dependent manner, with IC₅₀ values of 6.49 μ M (24 h), 4.44 μ M (48 h) and 4.09 μ M (72 h) in AGS cells; and IC₅₀ values of 8.58 μ M (24 h), 6.25 μ M (48 h) and 6.29 μ M (72 h) in KATO III cells (Figure 2a). VC inhibited the growth of both cancer cell lines in a dose-dependent manner, with IC₅₀ values of 1.75 mM (24 h),

1.91 mM (48 h) and 1.83 mM (72 h) in AGS cells; and IC₅₀ values of 1.41 mM (24 h), 1.40 mM (48 h) and 1.30 mM (72 h) in KATO III cells (Figure 2b).

As shown in Figure 3, both PL and VC induced the apoptosis of AGS and KATO III cells in a dose-dependent manner. AGS cells appeared to be more sensitive than KATO III cells to PL, while the sensitivity of both cell lines to VC was similar. The data demonstrated that PL induced apoptosis more strongly than VC, which was consistent with the MTT results.

In order to determine the mechanism by which PL and VC induced apoptosis, Western blot analyses were undertaken to

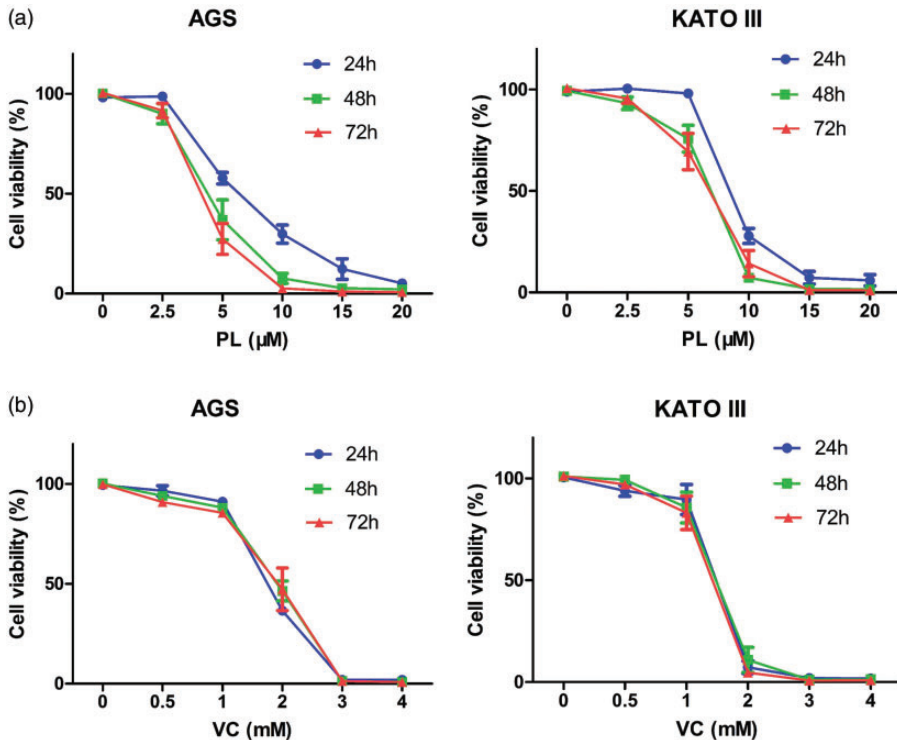


Figure 2. Piperlongumine (PL) and vitamin C (VC) inhibited the proliferation of two gastric cancer cell lines *in vitro*: (a) AGS and KATO III cells were treated with a range of concentrations of PL for 24 h, 48 h and 72 h. The cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; (b) AGS and KATO III cells were treated with a range of concentrations of VC for 24 h, 48 h and 72 h. The cell viability was determined using an MTT assay. A control group (0) was treated with the vehicle dimethyl sulfoxide. Data presented as the mean of triplicate experiments. The colour version of this figure is available at: <http://imr.sagepub.com>.

measure the influence of PL and VC on the STAT3 pathway. As shown in Figure 4, PL decreased STAT3 phosphorylation at different doses in both GC cell lines, while VC showed the opposite effect. PL inhibited the levels of survivin in KATO III cells, but upregulated the levels of survivin in AGS cells. VC increased survivin levels in AGS cells, but decreased survivin levels in KATO III cells.

The role of ROS in the apoptosis-inducing effects of PL and VC was determined using preincubation of AGS and KATO III cells with 5 mM NAC. As shown in Figure 5, high doses of PL and

VC induced apoptosis, but preincubation with NAC inhibited this apoptosis-inducing effect.

To get a better understanding of the regulatory role of ROS in STAT3 signalling, protein levels of p-STAT3 and STAT3 were measured using Western blot analyses. As shown in Figure 6, a high dose of PL significantly inhibited STAT3 phosphorylation compared with the control group ($P < 0.001$). However, this inhibitory effect disappeared after NAC preincubation. The presence or absence of NAC did not affect the regulatory effect of VC on STAT3 phosphorylation.

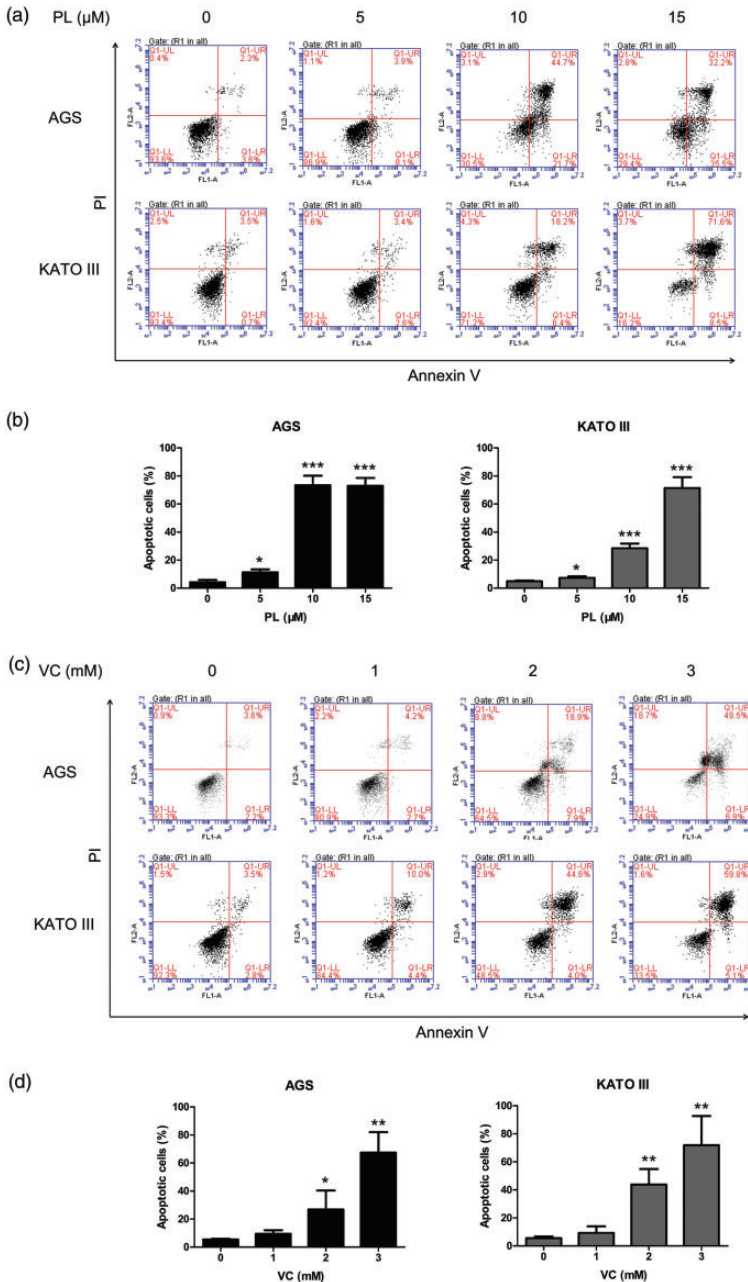


Figure 3. Piperlongumine (PL) and vitamin C (VC) induced apoptosis in two gastric cancer cell lines *in vitro*. (a) AGS and KATO III cells were treated with a range of concentrations of PL for 48 h and then stained with annexin V/propidium iodide (PI) for the flow cytometric analysis of the levels of apoptosis; (b) the proportion of cells that were apoptotic after treatment with PL. Data presented as mean \pm SD; (c) AGS and KATO III cells were treated with a range of concentrations of VC for 48 h and then stained with annexin V/PI for the flow cytometric analysis of the levels of apoptosis; (d) the proportion of cells that were apoptotic after treatment with VC. Data presented as mean \pm SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control group (treated with the vehicle dimethyl sulfoxide); analysis of variance. The colour version of this figure is available at: <http://imr.sagepub.com>.

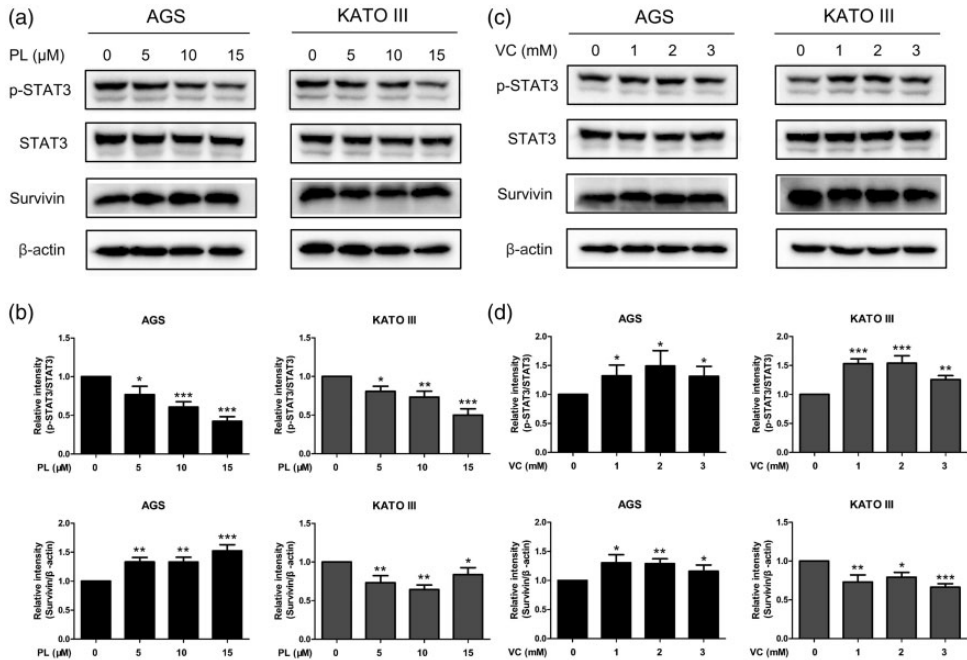


Figure 4. Piperlongumine (PL) and vitamin C (VC) regulated signal transducer and activator of transcription 3 (STAT3) signalling in opposite ways in two gastric cancer cell lines *in vitro*: (a) AGS and KATO III cells were treated with a range of concentrations of PL for 24 h and then they were subjected to Western blot analysis to measure the protein levels of p-STAT3, STAT3 and survivin. β -actin served as an internal control; (b) the relative levels of protein compared with β -actin after treatment with PL. Data presented as mean \pm SD; (c) AGS and KATO III cells were treated with a range of concentrations of VC for 24 h and then they were subjected to Western blot analysis to measure the protein levels of p-STAT3, STAT3 and survivin. β -actin served as an internal control; (d) the relative levels of protein compared with β -actin after treatment with VC. Data presented as mean \pm SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control group (treated with the vehicle dimethyl sulfoxide); analysis of variance.

To investigate whether PL and VC exhibited a synergistic effect on AGS and KATO III cell apoptosis, the cells were treated with a combination of doses of PL (5 μ M, 10 μ M) and VC (1 mM) and then subjected to flow cytometry to measure the rate of apoptosis. As shown in Figure 7, the combined use of 5 μ M PL and 1 mM VC in both cell lines significantly increased the rate of apoptosis compared with either agent used alone ($P < 0.05$). In KATO III cells, the combination of 10 μ M PL and 1 mM VC significantly increased the rate of apoptosis compared with

10 μ M PL alone ($P < 0.01$). However, no such effect was observed in AGS cells.

Western blot analysis was used to measure the levels of STAT3 pathway-related proteins, p-STAT3, STAT3 and survivin, in AGS and KATO III cells treated with PL (5 μ M, 10 μ M) and VC (1 mM) alone or in combination for 24 h. As shown in Figure 8, the combination of the two agents had a synergistic inhibitory effect on the levels of p-STAT3 in both cancer cell lines compared with either agent used alone. Similar to the previous apoptosis results, the combination of 10 μ M PL and

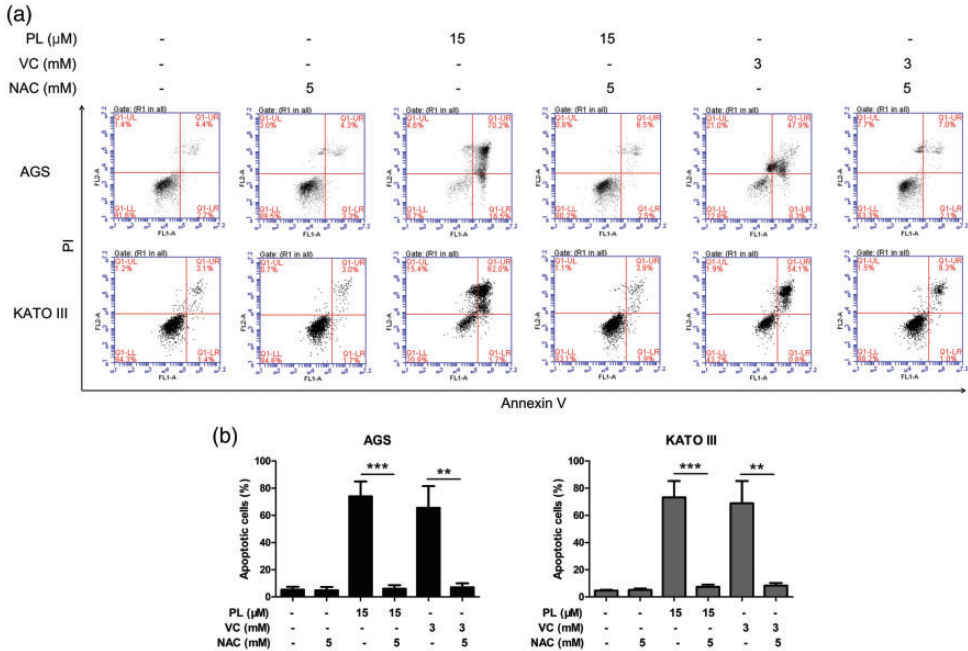


Figure 5. Piperlongumine (PL) and vitamin C (VC) induced reactive oxygen species-dependent apoptosis in two gastric cancer cell lines *in vitro*: (a) AGS and KATO III cells were treated with 15 μ M PL or 3 mM VC with/without 5 mM N-acetyl-L-cysteine (NAC) preincubation for 48 h and then stained with annexin V/propidium iodide (PI) for the flow cytometric analysis of the levels of apoptosis; (b) the proportion of cells that were apoptotic after treatment with PL, VC and NAC. Data presented as mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; analysis of variance. The colour version of this figure is available at: <http://imr.sagepub.com>.

1 mM VC in AGS cells did not show a greater inhibitory effect than the combination of 5 μ M PL and 1 mM VC. The regulation of survivin by PL and VC was not exactly the same as that of p-STAT3. In AGS cells, only 10 μ M PL and 1 mM VC synergistically inhibited the levels of survivin. In KATO III cells, the two combination groups showed a stronger inhibitory effect than PL alone, but no significant effect was observed compared with VC alone.

Discussion

The current status of chemotherapy for GC is not optimistic. Drug resistance leads to recurrence and a low 5-year survival

rate,²¹ which needs to be improved urgently. Although recent clinical research has found that VC cooperates with a variety of first-line drugs against cancer, it is hard to avoid drug resistance.²² Therefore, a specific agent, such as PL, might be needed to overcome this problem and help VC become a more effective anticancer treatment. This current study focused on the anticancer effects and mechanisms of action of PL combined with VC in AGS and KATO III cells. The current data suggest that these two agents synergistically inhibited GC cell proliferation. The regulation of the ROS-STAT3 pathway by PL could be the key mechanism of action of this combination.

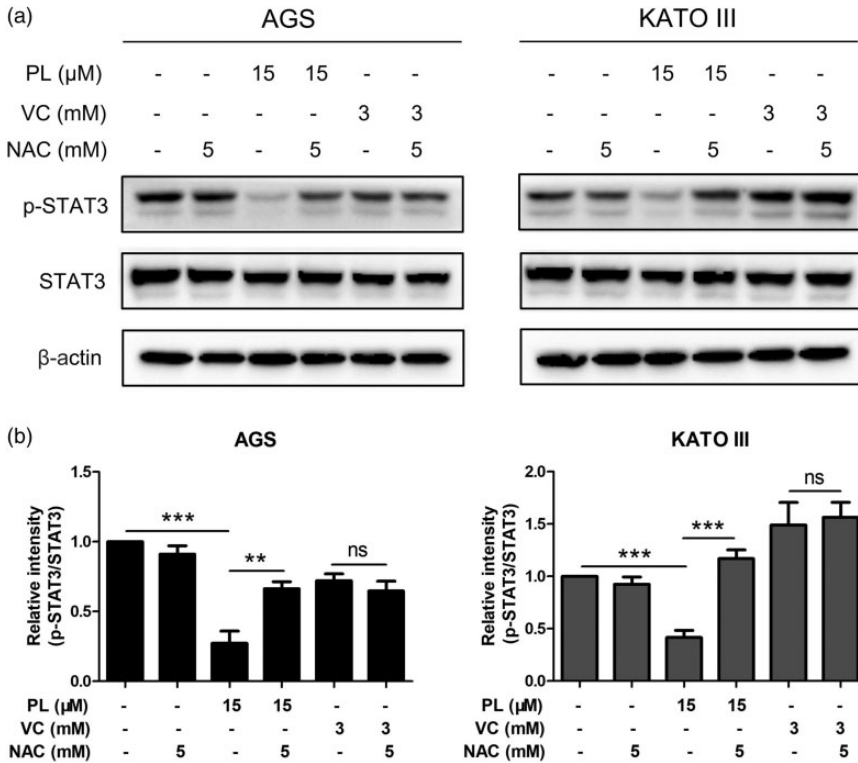


Figure 6. The regulation of signal transducer and activator of transcription 3 (STAT3) signalling by piperlongumine (PL) was dependent upon reactive oxygen species but that of vitamin C (VC) was not in two gastric cancer cell lines *in vitro*: (a) AGS and KATO III cells were treated with 15 μM PL or 3 mM VC with/without 5 mM N-acetyl-L-cysteine (NAC) preincubation for 48 h and then they were subjected to Western blot analysis to measure the protein levels of p-STAT3 and STAT3. β-actin served as an internal control; (b) the relative levels of protein compared with β-actin after treatment. Data presented as mean ± SD; **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, not significant; analysis of variance.

Reactive oxygen species in cancer cells play a central role in regulating and inducing apoptosis, thereby modulating cancer cell proliferation, survival and drug resistance.²³ Recent studies reported that both PL and VC generate large amounts of ROS that cancer cells cannot tolerate, thereby inducing cancer cell apoptosis.^{24,25} In the present study, PL and VC effectively induced apoptosis of GC cells through oxidative stress. Both 15 μM PL and 3 mM VC caused more than 60% apoptosis in two GC cell lines. However, these apoptosis-inducing effects were completely inhibited

after being preincubated with 5 mM NAC, which is an effective antioxidant. These current findings suggest that the apoptotic effect induced by PL and VC both depended on ROS, which was consistent with previous reports.^{25,26} In addition, Western blot analyses showed that PL and VC exhibited different mechanisms of action to induce apoptosis of GC cells. As shown in Figure 4, 15 μM PL effectively inhibited the phosphorylation of STAT3 in both GC cell lines. This effect was abrogated by preincubation with 5 mM NAC (Figure 6). Preincubation with NAC did

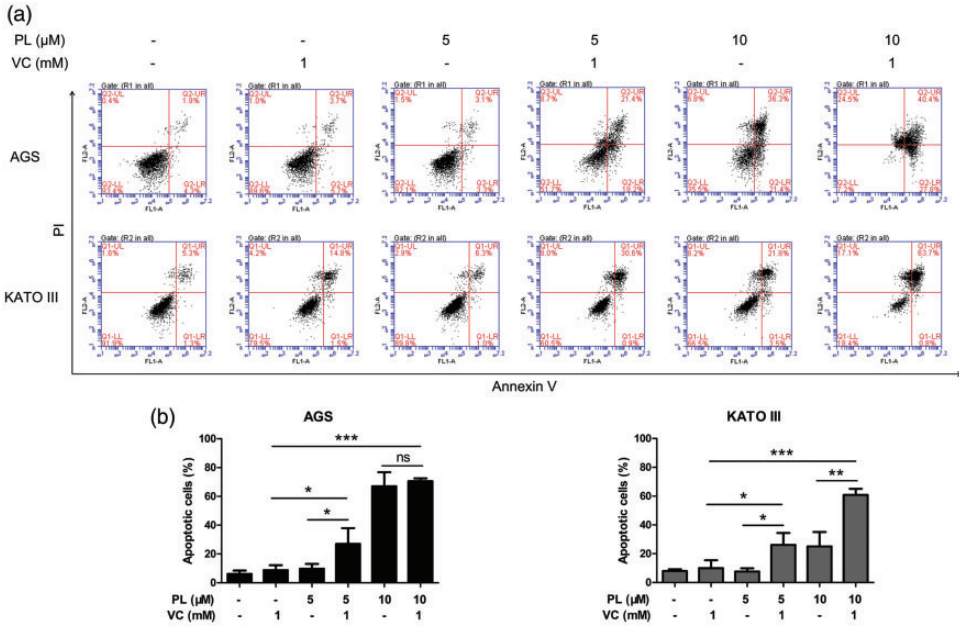


Figure 7. Piperlongumine (PL) and vitamin C (VC) synergistically induced apoptosis in two gastric cancer cell lines *in vitro*: (a) AGS and KATO III cells were treated with PL (5, 10 μM) and VC (1 mM) alone or in combination for 48 h and then stained with annexin V/propidium iodide (PI) for the flow cytometric analysis of the levels of apoptosis; (b) the proportion of cells that were apoptotic after treatment. Data presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant; analysis of variance. The colour version of this figure is available at: <http://imr.sagepub.com>.

not change the effects of VC on the phosphorylation of STAT3. Therefore, these current results demonstrated that ROS induced by PL could further inhibit the activation of STAT3, while VC showed no such effects.

It was reported that activated ROS production played a key role in the suppression of JAK2/STAT3 signalling.²⁷ In this current study, the effect of PL in inhibiting the activation of STAT3 was dependent on ROS, which was consistent with previous studies.²⁰ However, the abnormal activation of STAT3 by VC did not depend on ROS, which suggested that PL and VC regulated the STAT3 signalling pathway in very different ways. Taken together, both PL and VC induced apoptosis of GC cells by generating ROS, however, only the

PL-induced inhibition of STAT3 was beneficial for the anticancer effects.

The JAK/STAT signalling pathway plays an important role in the proliferation, survival and drug resistance of cancer cells.²⁸ STAT3 is a key member of the STAT family and has become an important target due to its important role in cancer regulation.²⁹ STAT3 is abnormally activated in a variety of solid tumours and non-solid tumours thereby leading to cancer deterioration.^{30,31} STAT3 is constitutively activated in many cancer types and such hyperactivation is associated with a poor clinical prognosis.³² Previous research has reported that constitutive activation of STAT3 leads to cisplatin resistance in GC cells and disruption of STAT3 could re-sensitize GC cells to chemotherapy

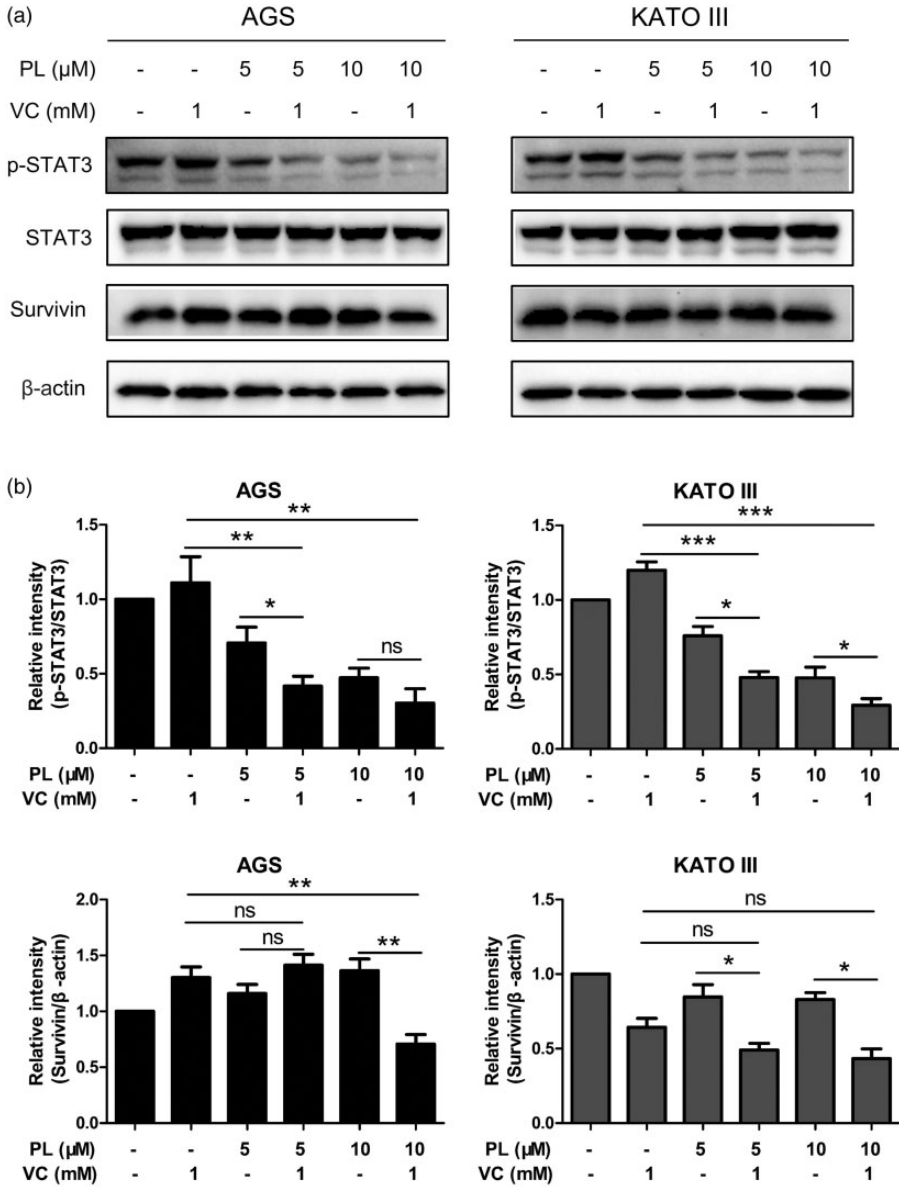


Figure 8. Piperlongumine (PL) and vitamin C (VC) synergistically inhibited signal transducer and activator of transcription 3 (STAT3) signalling in two gastric cancer cell lines *in vitro*: (a) AGS and KATO III cells were treated with PL (5, 10 μM) and VC (1 mM) alone or in combination for 24 h and then they were subjected to Western blot analysis to measure the protein levels of p-STAT3, STAT3 and survivin. β-actin served as an internal control; (b) the relative levels of protein compared with β-actin after treatment. Data presented as mean ± SD; **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, not significant; analysis of variance.

drugs.³³ Since activation of STAT3 is an essential feature of chemotherapeutic drug resistance, blocking the STAT3 pathway to improve the efficacy of chemotherapy has become a promising strategy.³⁴

Among the many small molecules that can inhibit the activation of STAT3, PL has attracted the attention of researchers due to its good effectiveness and safety.¹⁶ PL has broad-spectrum anticancer activity while showing less toxicity to normal cells.³⁵ In addition, the effective inhibition of STAT3 activation by PL suggests that it has the potential to improve chemotherapy resistance.³⁶ In the present study, PL and VC showed opposite regulatory effects on p-STAT3. PL effectively inhibited the activation of STAT3 in both GC cells, which was consistent with previous studies.^{37,38}

However, VC causing activation of STAT3 indicates that it is likely to cause drug resistance when combined with other chemotherapy drugs.³⁵ Although having the opposite regulatory effects on p-STAT3, the combination of the two agents showed a synergistic inhibitory effect. Flow cytometric analysis demonstrated that the combination of the two agents induced a stronger apoptotic effect compared with either of the single-agent groups. Western blot analysis demonstrated that PL reversed the adverse effect of VC and together the two agents showed a stronger inhibitory effect on p-STAT3 in both GC cell lines.

Previous research reported that PL could reverse the abnormal activation of STAT3 by DOX,¹⁹ which was consistent with these current findings. One theory is that the combined application of PL and VC creates some synergistic effects in which PL inactivates STAT3 through direct binding while VC enhances the bioavailability of PL.³⁹ Then these two agents work together to inhibit the STAT3 pathway, which in turn

causes more ROS production thereby achieving a stronger anticancer effect. These current data indicated that PL and VC may be used together as a promising adjuvant to enhance the anticancer effects of chemotherapeutics, and more importantly, to overcome their drug resistance.

Unexpectedly, the regulation of survivin by the two agents was not similar to that of p-STAT3. PL increased the levels of survivin in AGS cells, while VC reduced the levels of survivin in KATO III cells. Nevertheless, 10 μ M PL and 1 mM VC showed a synergistic inhibitory effect on survivin in both cell lines. These data indicated that although survivin can be regulated by other pathways, high-dose PL combined with VC could effectively reduce survivin levels.

In conclusion, PL and VC showed anti-proliferative properties against GC cells and this effect was significantly enhanced when the two agents were combined. PL promoted ROS as well as reversing the abnormal activation of STAT3 caused by VC, which could be the key mechanism of action of this combination. This would be expected to overcome chemotherapy resistance. Therefore, the combination of PL and VC as an adjuvant provides a new strategy in future GC chemotherapy.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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References

1. Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Akinyemiju TF, Al Lami FH, et al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2016: A systematic analysis for the global burden of disease study. *JAMA Oncol* 2018; 4: 1553–1568.
2. Catanese S and Lordick F. Targeted and immunotherapy in the era of personalised gastric cancer treatment. *Best Pract Res Clin Gastroenterol* 2021; 50-51: 101738.
3. Wein L and Loi S. Mechanisms of resistance of chemotherapy in early-stage triple negative breast cancer (TNBC). *Breast* 2017; 34: S27–S30.
4. Ashwanikumar N, Kumar NA, Nair SA, et al. Dual drug delivery of 5-fluorouracil (5-FU) and methotrexate (MTX) through random copolymeric nanomicelles of PLGA and polyethylenimine demonstrating enhanced cell uptake and cytotoxicity. *Colloids Surf B Biointerfaces* 2014; 122: 520–528.
5. Kang YK, Yook JH, Park YK, et al. PRODIGY: A phase III study of neoadjuvant docetaxel, oxaliplatin, and S-1 plus surgery and adjuvant S-1 versus surgery and adjuvant S-1 for resectable advanced gastric cancer. *J Clin Oncol* 2021; 39: 2903–2913.
6. Zhang C, Ma Q, Shi Y, et al. A novel 5-fluorouracil-resistant human esophageal squamous cell carcinoma cell line Eca-109/5-FU with significant drug resistance-related characteristics. *Oncol Rep* 2017; 37: 2942–2954.
7. Fiorillo M, Tóth F, Sotgia F, et al. Doxycycline, azithromycin and vitamin C (DAV): A potent combination therapy for targeting mitochondria and eradicating cancer stem cells (CSCs). *Aging (Albany NY)* 2019; 11: 2202–216.
8. Ngo B, Van Riper JM, Cantley LC, et al. Targeting cancer vulnerabilities with high-dose vitamin C. *Nat Rev Cancer* 2019; 19: 271–282.
9. Nakayama N, Ishido K, Chin K, et al. A phase I study of S-1 in combination with nab-paclitaxel in patients with unresectable or recurrent gastric cancer. *Gastric Cancer* 2017; 20: 350–357.
10. Shinkai M, Imano M, Chiba Y, et al. Phase II trial of neoadjuvant chemotherapy with intraperitoneal paclitaxel, S-1, and intravenous cisplatin and paclitaxel for stage IIIA or IIIB gastric cancer. *J Surg Oncol* 2019; 119: 56–63.
11. Lee KE, Hahm E, Bae S, et al. The enhanced tumor inhibitory effects of gefitinib and L-ascorbic acid combination therapy in non-small cell lung cancer cells. *Oncol Lett* 2017; 14: 276–282.
12. Zhu Y, Wang X, Zhang J, et al. Exogenous vitamin C boosts the antitumor efficacy of paclitaxel containing reduction-sensitive shell-sheddable micelles in vivo. *J Control Release* 2017; 250: 9–19.
13. Piska K, Gunia-Krzyżak A, Koczurkiewicz P, et al. Piperlongumine (piplartine) as a lead compound for anticancer agents – Synthesis and properties of analogues: A mini-review. *Eur J Med Chem* 2018; 156: 13–20.
14. Chen D, Ma Y, Guo Z, et al. Two natural alkaloids synergistically induce apoptosis in breast cancer cells by inhibiting STAT3 activation. *Molecules* 2020; 25: 216.
15. Chen Y, Liu JM, Xiong XX, et al. Piperlongumine selectively kills hepatocellular carcinoma cells and preferentially inhibits their invasion via ROS-ER-MAPKs-CHOP. *Oncotarget* 2015; 6: 6406–6421.
16. Tripathi SK and Biswal BK. Piperlongumine, a potent anticancer phytotherapeutic: Perspectives on contemporary status and future possibilities as an anticancer agent. *Pharmacol Res* 2020; 156: 104772.
17. Bezerra DP, Pessoa C, de Moraes MO, et al. Overview of the therapeutic potential of piplartine (piperlongumine). *Eur J Pharm Sci* 2013; 48: 453–63.
18. Zhang C, He LJ, Zhu YB, et al. Piperlongumine inhibits Akt phosphorylation to reverse resistance to cisplatin in

- human non-small cell lung cancer cells via ROS regulation. *Front Pharmacol* 2019; 10: 1178.
19. Chen D, Ma Y, Li P, et al. Piperlongumine induces apoptosis and synergizes with doxorubicin by inhibiting the JAK2-STAT3 pathway in triple-negative breast cancer. *Molecules* 2019; 24: 2338.
 20. Bharadwaj U, Eckols TK, Kolosov M, et al. Drug-repositioning screening identified piperlongumine as a direct STAT3 inhibitor with potent activity against breast cancer. *Oncogene* 2015; 34: 1341–1353.
 21. Wei L, Sun J, Zhang N, et al. Noncoding RNAs in gastric cancer: implications for drug resistance. *Mol Cancer* 2020; 19: 62.
 22. Satheesh NJ, Samuel SM and Büsselberg D. Combination therapy with vitamin C could eradicate cancer stem cells. *Biomolecules* 2020; 10: 79.
 23. Cui Q, Wang JQ, Assaraf YG, et al. Modulating ROS to overcome multidrug resistance in cancer. *Drug Resist Updat* 2018; 41: 1–25.
 24. Rawat L, Hegde H, Hoti SL, et al. Piperlongumine induces ROS mediated cell death and synergizes paclitaxel in human intestinal cancer cells. *Biomed Pharmacother* 2020; 128: 110243.
 25. Su X, Shen Z, Yang Q, et al. Vitamin C kills thyroid cancer cells through ROS-dependent inhibition of MAPK/ERK and PI3K/AKT pathways via distinct mechanisms. *Theranostics* 2019; 9: 4461–4473.
 26. Zhou J, Huang Z, Ni X, et al. Piperlongumine induces apoptosis and G(2)/M phase arrest in human osteosarcoma cells by regulating ROS/PI3K/Akt pathway. *Toxicol In Vitro* 2020; 65: 104775.
 27. Cao Y, Wang J, Tian H, et al. Mitochondrial ROS accumulation inhibiting JAK2/STAT3 pathway is a critical modulator of CYT997-induced autophagy and apoptosis in gastric cancer. *J Exp Clin Cancer Res* 2020; 39: 119.
 28. Toh TB, Lim JJ, Hooi L, et al. Targeting Jak/Stat pathway as a therapeutic strategy against SP/CD44+ tumorigenic cells in Akt/beta-catenin-driven hepatocellular carcinoma. *J Hepatol* 2020; 72: 104–118.
 29. Siersbæk R, Scabia V, Nagarajan S, et al. IL6/STAT3 signaling hijacks estrogen receptor alpha enhancers to drive breast cancer metastasis. *Cancer Cell* 2020; 38: 412–423.
 30. Bai L, Zhou H, Xu R, et al. A potent and selective small-molecule degrader of STAT3 achieves complete tumor regression in vivo. *Cancer Cell* 2019; 36: 498–511.
 31. Lee H, Jeong AJ, Ye SK, et al. Highlighted STAT3 as a potential drug target for cancer therapy. *BMB Rep* 2019; 52: 415–423.
 32. Sun CY, Nie J, Huang JP, et al. Targeting STAT3 inhibition to reverse cisplatin resistance. *Biomed Pharmacother* 2019; 117: 109135.
 33. Song S, Min H, Niu M, et al. SIPR1 predicts patient survival and promotes chemotherapy drug resistance in gastric cancer cells through STAT3 constitutive activation. *EBioMedicine* 2018; 37: 168–176.
 34. Zhang JL, Liu XZ, Wang PY, et al. Targeting HCCR expression resensitizes gastric cancer cells to chemotherapy via down-regulating the activation of STAT3. *Sci Rep* 2016; 6: 24196.
 35. Kim TH, Song J, Kim SH, et al. Piperlongumine treatment inactivates peroxiredoxin 4, exacerbates endoplasmic reticulum stress, and preferentially kills high-grade glioma cells. *Neuro Oncol* 2014; 16: 1354–1364.
 36. Yao Y, Sun Y, Shi M, et al. Piperlongumine induces apoptosis and reduces bortezomib resistance by inhibiting STAT3 in multiple myeloma cells. *Oncotarget* 2016; 7: 73497–73508.
 37. Kim YH, Yoon YJ, Lee YJ, et al. Piperlongumine derivative, CG-06, inhibits STAT3 activity by direct binding to STAT3 and regulating the reactive oxygen species in DU145 prostate carcinoma cells. *Bioorg Med Chem Lett* 2018; 28: 2566–2572.
 38. Song B, Zhan H, Bian Q, et al. Piperlongumine inhibits gastric cancer cells via suppression of the JAK1,2/STAT3 signaling pathway. *Mol Med Rep* 2016; 13: 4475–4480.
 39. Patel K, Chowdhury N, Doddapaneni R, et al. Piperlongumine for enhancing oral bioavailability and cytotoxicity of docetaxel in triple-negative breast cancer. *J Pharm Sci* 2015; 104: 4417–4426.