

ORIGINAL ARTICLE

Oridonin triggers G2/M cell cycle arrest, cellular apoptosis and autophagy in human gastric cancer cells

Dongliang Zhu¹, Xiaoling Tian², Xiaoping Yin¹, Haidong Yan¹, Weihao Li¹

¹Department of Gastrointestinal Surgery, Xianning Central Hospital, The First Affiliated Hospital of Hubei University of Science and Technology, Xianning 437100, China. ²Department of General Medicine, Xianning Central Hospital, The First Affiliated Hospital of Hubei University of Science and Technology, Xianning 437100, China.

Summary

Purpose: Gastric carcinoma is the fourth principal cause of cancer-related deaths throughout the globe. There are inadequate clinical therapies for gastric cancer due to lack of operational drugs and ambiguity in molecular mechanisms. As such there is a persistent requirement for novel and effective anticancer drugs for gastric cancer. The main purpose of the current study was to investigate the antitumor effects of a plant diterpenoid, namely Oridonin, against SGC-7901 human gastric cancer cells along with examining its effects on cellular apoptosis, cell autophagy and cell cycle phase distribution.

Methods: WST-1 cell proliferation assay was used to evaluate cell viability of SGC-7901 human gastric cancer cells. Apoptosis was evaluated by using DAPI and comet assays using fluorescence microscopy. Autophagy was evaluated by transmission electron microscopy (TEM) and western blot method. Effects on cell cycle phase distribution were studied by flow cytometry.

Results: Oridonin molecule led to considerable and dose-

dependent antiproliferative effects on SGC-7901 human gastric cancer cells exerting only mild cytotoxic effects in normal cells thus exhibiting selective toxicity. The number of gastric cancer cell colonies decreased significantly as oridonin dose increased. DAPI and comet assays revealed that oridonin induced powerful apoptotic effects in these cells, triggering significant DNA damage and both these effects exhibited dose-dependence. TEM indicated that oridonin induced autophagy in SGC-7901 cells by creating autophagosomes and autophagic vacuoles. Oridonin also targeted G2/M phase cell cycle in these gastric cancer cells along with targeting some key cell cycle related proteins including cyclin-B1, cyclin D1 and cyclin E.

Conclusion: In conclusion, the results show that oridonin showed strong anticancer effects in SGC-7901 human gastric cancer cells by triggering apoptosis and autophagy, and targeting cell cycle at G2/M phase.

Key words: gastric cancer, oridonin, electron microscopy, apoptosis, autophagy, cell cycle

Introduction

Gastric cancer is a dangerous malignancy and affects a huge number of people globally [1]. Every year a large human population suffers of this disease and in 2002 alone approximately 0.9 million additional gastric cancer patients and 0.65 million deaths were recorded [2]. The incidence of gastric cancer reveals major geographical variations, but indicates higher dominance in developing countries in contrast to developed countries. Last decade has

been impressive in finding new methodologies, therapeutic targets and agents in fighting cancer still improvements in overall survival rate for gastric cancer lags behind other cancer types [3]. Lesser improvements in overall survival are due to lack of effectual chemotherapeutic drugs with minimum or no side-effects as well as very poor prognosis which impose heavy obstacles in gastric cancer management [4]. Complete resections of the tumour is the

Corresponding author: Xiaoling Tian, MD. Department of General Medicine, Xianning Central Hospital, The First Affiliated Hospital of Hubei University of Science and Technology, 228 Jingui Road, Xianning 437100, China.
Tel/Fax: + 86 0715 8896221, Email: OlenRamseycpv@yahoo.com
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only curative treatment available for gastric cancer, still having a drawback of disease reoccurrence. Chemotherapeutic agents mostly used for gastric cancer treatment are platinum or fluoropyrimidine based have failed to obtain adequate results of improving survival chances [5]. Therefore an emergency for novel potent chemotherapeutic drugs arises to completely eliminate gastric cancer [6]. Natural products serve as a source of natural bank of medicines which assisted in curbing numerous diseases [7]. Especially plants are extremely sophisticated natural chemical manufacturing factories which reveal a tremendous potential to bear and further produce a wide array of chemicals [8-11]. Oridonin is a natural diterpenoid which is isolated from *Rabdosia rubescens* in its pure form. Its anticancer effects are well documented in the published literature [12]. Herein, this research was performed to unveil the anticancer potential of oridonin against SGC-7901 gastric cancer cells along with examining its effects on apoptosis and autophagy as well as G2/M-phase cell cycle arrest

Methods

Estimation of cell proliferation

Cell proliferation rate was estimated via cytotoxicity assay kit (Beyotime Institute of Biotechnology, China) by performing WST-1 cell viability assay with strictly obeying the manufacturer's instructions. Briefly, normal gastric GES-1 cells and gastric cancer SGC-7901 cells were cultured in 200 μ l of culture media in 96-well plates. Cell lines were incubated in a 5% CO₂ incubator for 24 h at 37°C and harvested after 70% confluence. Afterwards, cells were subjected to oridonin treatment at changing doses (0, 5, 50, 100 and 150 μ M), followed by addition of WST-1 stock solution (10 μ l). Thereafter, cells were again incubated at similar conditions as above for 2 h and finally using spectrophotometer (BioTek, USA) optical density (OD) was measured at 450 nm wavelength.

Clonogenic assay for colony formation analysis

For performing of clonogenic assay, SGC-7901 gastric carcinoma cell line was collected at exponential phase of growth and numbered with hemocytometer. Harvested cells were then transferred to 6-well culture plates with 400 cells per well and incubated for 48 h for attachment. Afterwards, treatment of gastric SGC-7901 carcinoma cells with variant doses of oridonin (0, 5, 100 and 150 μ M), was performed for one week at 37°C followed by phosphate buffered saline washing. Finally, colonies were fixed in methanol and stained with crystal violet for light microscope examination.

DAPI staining and comet assay for analysing apoptosis

The effect of apoptosis induction by oridonin molecule in gastric SGC-7901 carcinoma cells was assessed through DAPI staining. In brief, in 96-well plates cells were seeded with 2×10^3 cells in each well. Seeding was

followed by oridonin administration for 48 h with changing concentrations (0, 5, 100 and 150 μ M). Oridonin-treated cells were DAPI-stained and observations were performed through capturing photographs with fluorescence microscope.

Comet assay was performed for the detection of DNA damage in apoptotic cells. After treatment with variant doses of oridonin (0, 5, 100 and 150 μ M) nearly 200 μ l of cell suspensions (110 cells/ml) were added to 500 μ l of agarose (1% lower melting point) and quickly layered onto custom frosted slides. Solidification of agarose was done on chilled plates followed by lysing bath in RIPA lysing solution for about 1 h at 25°C. For DNA unwinding and to allow settling of salt equilibrium, slides were dipped in electrophoresis alkaline buffer for nearly about half an hour. Electrophoresis was followed by submerging of slides in distilled water bath for 6 min at 25°C for DNA annealing. Finally, glass-slipped-cover was put onto the slides and standard Photonics Camera fixed over Axioscope fluorescent microscope was used for analysis.

Phase distribution of cell cycle through flow cytometry

Gastric SGC-7901 carcinoma cells were cultured in DMEM medium with a number of 1×10^4 cells/ml in 6-well plates. Cancer cells were subjected to oridonin treatment of different doses (0, 5, 100 and 150 μ M), and exposed to RNase A (15 μ g/ml). Oridonin-treated cells were fixed with alcohol and subjected to PBS washing. After washing, cells were stained with PI solution (20 μ g/mL) and finally different cell cycle phases were monitored through FACS Calibur flow cytometer (BD Biosciences, China).

Analyzing autophagy through transmission electron microscopy (TEM)

Cultured gastric SGC-7901 carcinoma cells were exposed to different amounts of oridonin molecule (0, 5, 100 and 150 μ M). Treated cells were fixed in 4% glutaraldehyde in 0.05M sodium cacodylate buffer and later post-fixed in osmium tetroxide (1.5%). Cancer cells were dehydrated in alcohol and evenly ordered. Cells were implanted in Epon 812 and analyzed under Zeiss CEM 902 electron microscope.

Western blotting technique to check protein levels

Oridonin-treated gastric SGC-7901 carcinoma cells were lysed in RIPA lysis buffer. Net 10 μ g protein was loaded to SDS-PAGE gel (8%) and electrophoresis was performed to resolve protein content. Next, primary and secondary antibody treatment was administered to these oridonin-treated cells, respectively. Proteins were transferred to PVDF membranes with visualization of bands under advanced chemiluminescence.

Statistics

All the experiments were performed in triplicate and the data are presented as mean \pm SD. Statistical analysis was performed with Student's t-test with Graph-Pad prism 7. $P < 0.05$ denoted statistically significant difference.

Results

Cell growth and colony inhibitory effects of oridonin in human gastric SGC-7901 carcinoma cells

Cell growth was determined via WST-1 cell proliferation assay. The results revealed significant inhibition of cell growth by oridonin treatment. In controls cell proliferation rate of both gastric SGC-7901 carcinoma and normal GES-1 cells were nearly 100%, but with increasing drug doses the viability of cancer cells decreased and was 80%, 65%, 25% and 10% at 0, 5, 50, 100 and 150 μ M oridonin concentrations, respectively (Figure 1), thus indicating dose-dependent inhibition of cell growth. In the case of normal GES-1 cells the proliferation rate didn't hampered much hence signifying drug selectivity against cancer cells. Furthermore, clo-

nogenic assay performed for evaluation of oridonin treatment effect on colony formation of these cancer cells revealed remarkable inhibition and suppression of colony formation. Clonogenic assay recorded decrease of the number of colonies from 210 to 10 with increasing doses of oridonin from 0 to 150 μ M (Figure 2). Thus both the cell proliferation and clonogenic assay revealed dose-dependent inhibition of cell growth as well as colony formation, respectively.

Analysis of apoptosis induction of oridonin against human gastric cancer SGC-7901 cells

DAPI staining was performed to analyze gastric SGC-7901 carcinoma cells for apoptotic cell

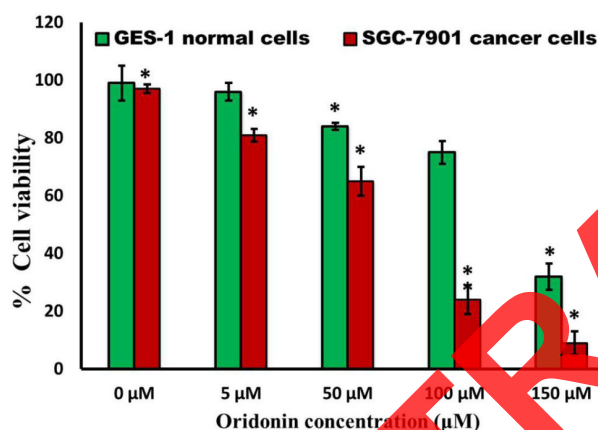


Figure 1. Cell viability at indicated doses of oridonin in human gastric cancer SGC-7901 and normal GES-1 cells was assessed through WST-1 proliferation assay. The experiments were performed in triplicate and the data were presented as mean \pm SD (* p < 0.05).

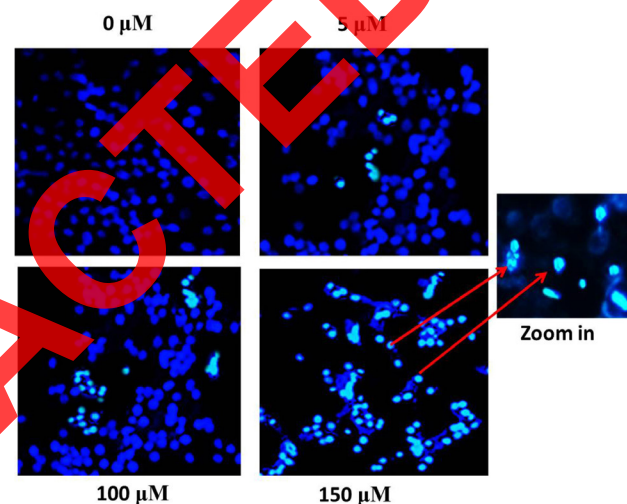


Figure 3. DAPI staining for apoptosis analysis after oridonin treatment of human gastric SGS-7901 carcinoma cells indicating membrane blebbing and formation of apoptotic crops. The arrows show DAPI stained apoptotic cells after cells were subjected to oridonin treatment.

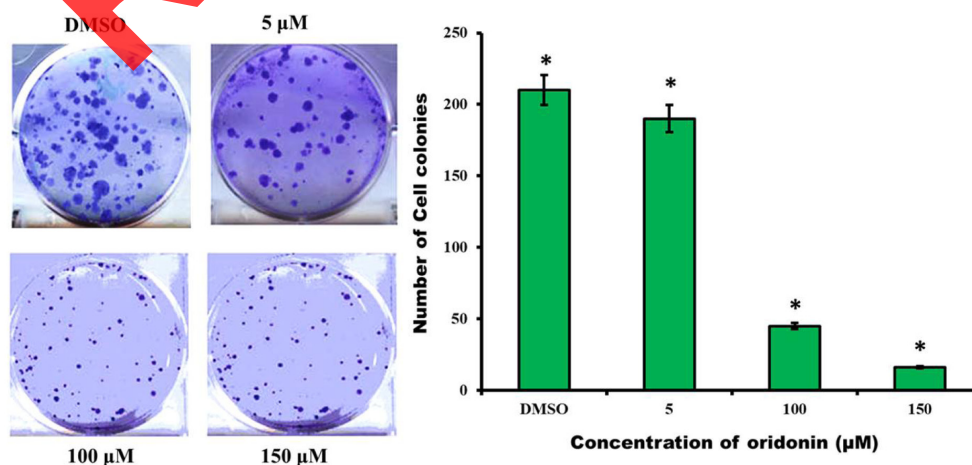


Figure 2. Clonogenic analysis under light microscope after subjecting gastric SGC-7901 carcinoma cells at indicated of oridonin doses. The experiments were performed in triplicate and the data are presented as the mean \pm SD. * p < 0.05 indicates a significant difference from the control group.

death after oridonin treatment. The results revealed major morphological changes indicative of apoptotic cell death like membrane blebbing and appearance of apoptotic crops (Figure 3). Furthermore, the effect of apoptosis induction strengthened with increased amounts of oridonin in parallel with the duration of treatment. The percentage of apoptotic cells recorded at controls after 12 h and 24 h was 4% and 7%, but on treatment it increased abruptly and touched 45% and 55% respectively (Figure 4). Thus oridonin induced anti-proliferative effects dose and time dependently.

Oridonin treatment induced DNA damage mediated apoptosis

Cellular DNA was analyzed by performing comet assay. The results illustrated outstanding effects of DNA damage induction against gastric SGC-7901 carcinoma cells by oridonin treatment with increasing doses. The tail length of cells was directly proportional to DNA damage. At 0 μM concentration of the molecule no DNA damage

was observed as the DNA was of live cells. At 5 μM drug dose no significant effect was seen but some minute pieces of DNA had migrated. At 100 μM DNA damage strengthened but cells were still alive and finally at 150 μM of the molecule dose cellular DNA could be barely seen as the gastric SGC-7901 carcinoma cells died of apoptotic cell death (Figure 5). The percentage tail DNA was observed increasing upon increased oridonin doses and almost touched 60% at 150 μM (Figure 6). As evidenced via comet assay it is clear that oridonin resulted in apoptotic cell death mediated through DNA damage induction.

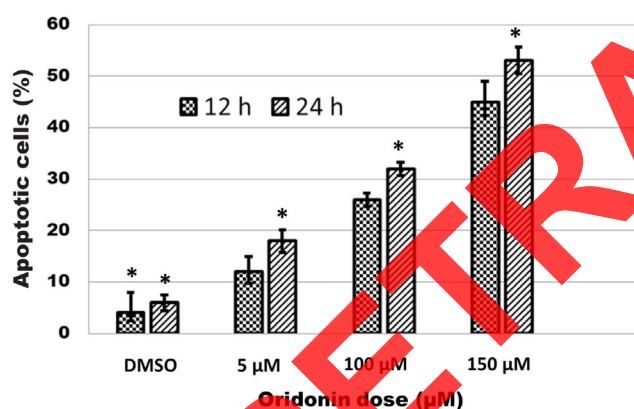


Figure 4. DAPI staining graphical presentation revealing increased frequency of apoptotic cells after oridonin treatment of gastric SGC-7901 carcinoma cells. The experiments were performed in triplicate and the data are presented as mean \pm SD. * $p < 0.05$.

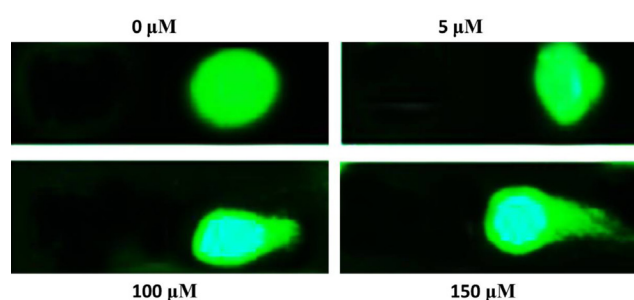


Figure 5. Comet assay results of oridonin treatment to human gastric SGC-7901 carcinoma cells. Comet tail length is directly proportional to DNA damage. The experiments were performed in triplicate. $p < 0.05$.

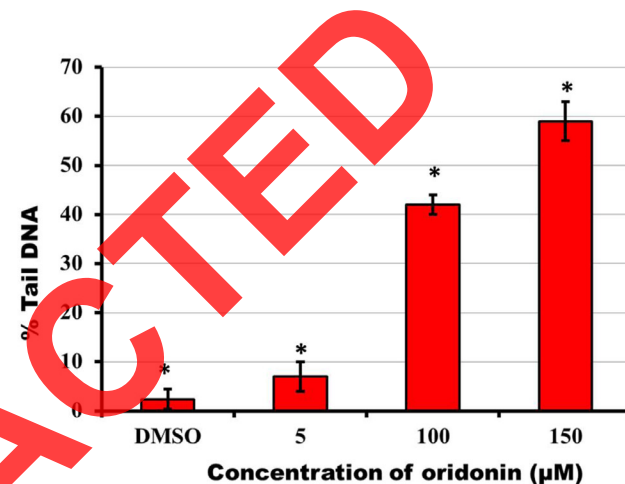


Figure 6. Percentage tail DNA with increased oridonin doses in human gastric SGC-7901 carcinoma cells at indicated doses. All the data are presented as mean \pm SD. * $p < 0.05$.

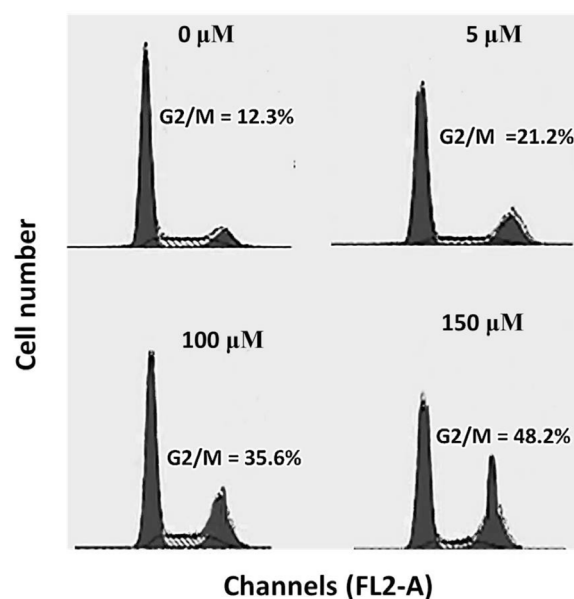


Figure 7. Phase distribution of cell cycle after oridonin treatment at varying doses of human gastric SGC-7901 carcinoma cells. Increased percentage of G2/M-phase cells could be easily recognized. The experiments were performed in triplicate. $p < 0.05$.

Effect of oridonin exposure on cell cycle phase distribution

The gastric SGC-7901 carcinoma cells were treated with different doses of oridonin and the distribution of SGC-7901 cells at each cell cycle phase was analyzed through flow cytometry. The results suggested that the G2/M-phase cells amplified significantly upon oridonin treatment. The percentage of G2/M phase cells was observed to be 12.3%, 21.2%, 35.6% and 48.2% at 0, 5, 100 and 150 μ M, respectively, indicative of G2/M phase cell cycle arrest (Figure 7). Furthermore, the impact of oridonin exposure was measured on the expressions of cell cycle related proteins through western blotting. The observations revealed down-regulation of cyclin-B1, cyclin-D1 and cyclin-E on increasing oridonin doses (Figure 8).

Induction of autophagy in gastric SGC-7901 carcinoma cells by oridonin exposure

Autophagic analysis of oridonin-treated gastric SGC-7901 carcinoma cells was performed via transmission electron microscopy (TEM). Treated cells revealed formation of autophagosomes/autophagic vesicles which completely hallmark the autophagic cell death (Figure 9). Also, to validate the autophagic cell death induction by oridonin western blotting analysis was performed to check the levels of related proteins. The results revealed dose-dependent upregulation of LC3-I, LC3-II and Beclin-1, which are clearly suggestive of autophagy (Figure 10). Thus, along with apoptosis, it is clear that oridonin resulted in induction of autophagic cell death too.

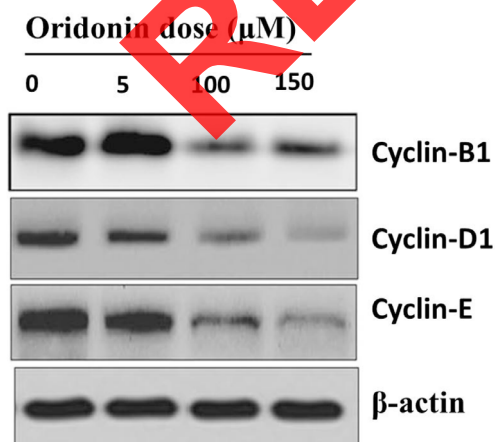


Figure 8. Analysis of cell cycle related protein levels via western blotting analysis. The expressions of cell cycle related proteins Cyclin-1, Cyclin-D1 and Cyclin-E all were reduced in SGC-790 cells after oridonin exposure. The experiments were performed in triplicate with $p < 0.05$ and Actin was used as normalization control.

Discussion

Gastric cancer is a lethal malignancy associated with huge mortality. Its lethality gets enhanced due to very poor prognosis and lack of effective chemotherapeutic drugs. While the overall survival of other cancer types gradually increased, gastric cancer survival remained unchanged. Thus, to curb this dismal situation, the hunt for new methods and therapeutic agents continues. Apoptosis is considered as type-I predominant mode of cell death (programmed) [13]. Drugs and gene-encoding treatments of cancer initiate apoptosis and ultimately cell death [14,15]. Bcl-2 and Bax are the two genes associated with apoptosis, hence they are targeted in attaining current cancer treatment goals. Along with apoptosis, autophagy is type-II predominant mode of cell death characterized by

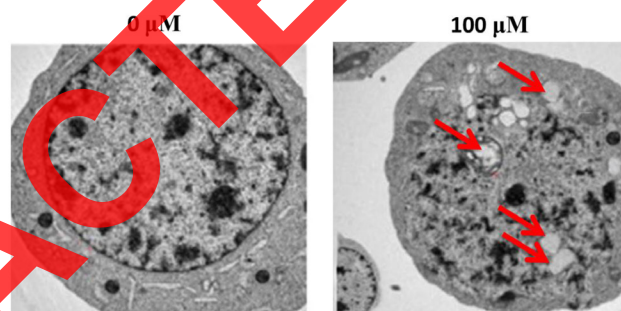


Figure 9. Autophagy induction effect of oridonin against human gastric SGC-7901 carcinoma cells at indicated doses through TEM analysis. Arrows show the formation of autophagosomes. The experiments were performed in triplicate.

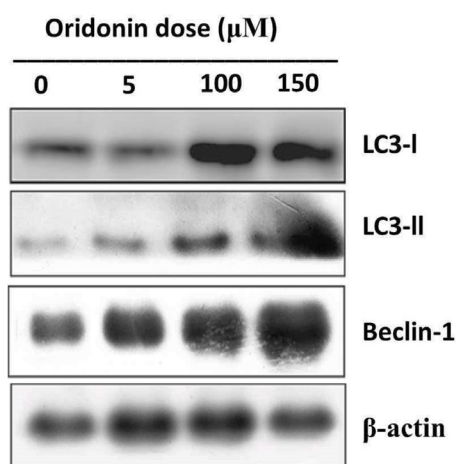


Figure 10. Determination of impact of oridonin treatment on autophagy associated proteins through western blotting analysis. The figure shows increased levels of proautophagic proteins (LC3-I, LC3-II and Beclin-1) in SGC-7901 cells after being subjected to different oridonin doses. The experiments were performed in triplicate with $p < 0.05$ and Actin was used as normalization control.

autophagosomes. Natural products from the last two decades are being explored for their medicinal applications and have been proved fruitful against various diseases, including cancer [16,17]. Natural chemical entities are regarded as potential apoptosis and autophagy inducers. Herein, the anticancer effects of oridonin were unearthed via its ability of autophagy and apoptosis induction. The effect on cell viability of gastric SGC-7901 carcinoma cells and normal GES-1 cells were determined through WST-1 proliferation assay, the results of which revealed tremendous dose-dependent inhibition of cell growth of cancer cells selectively without altering normal cell viability much. Along with cell viability the effect of oridonin treatment on colony formation was determined via clonogenic assay, suggesting dose-dependent and substantial inhibition of colony formation. Next, it was testified that whether the anti-cell growth effects of oridonin were apoptosis-mediated through DAPI staining, the results revealed formation of apoptotic crops and membrane blebbing along with increased number of apoptotic cells following increased drug doses. In addition, DNA damage revealed apoptosis was studied through comet assay, revealing tremendous damage to cellular DNA which was depicted by the comet tail length. DNA damage resulted in induction of cell death at higher oridonin doses. Cell cycle phase distribution was analyzed by flow

cytometry, revealing G2/M-phase cell cycle arrest in a dose-dependent manner. Along with flow cytometry, western blotting analysis was done to check the levels of cell cycle dependent proteins revealing downregulation of cyclin-B1, cyclin-D1 and cyclin-E on increasing oridonin doses. Finally, TEM was performed for autophagy analysis, with the results revealing formation of autophagic vesicles/autophagosomes which are hallmarks of autophagy, followed by western blotting analysis of autophagy-dependent protein expressions, showing dose-dependent upregulation of LC3-I, LC3-II and Beclin-1 and hence proving autophagic cell death.

Conclusions

In conclusion, the data of this study using the above assays revealed clearly that oridonin induced cell death in gastric SGC-7901 carcinoma cells via induction of apoptosis and autophagy. Oridonin treatment also induced cell cycle G2/M-phase arrest. Hence, it may be concluded that this molecule is a potent anticancer therapeutic agent against human gastric carcinoma and can be considered as lead molecule for its management.

Conflict of interests

The authors declare no conflict of interests.

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