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ORIGINAL ARTICLE

Anticancer activity of Eremanthin against the human cervical cancer cells is due to G2/M phase cell cycle arrest, ROS-mediated necrosis-like cell death and inhibition of PI3K/AKT signalling pathway

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Summary

Purpose: *Cervical cancer is a global health issue in women accounting for huge morbidity and mortality. Lack of effective chemotherapy and harmful side-effects of the current chemotherapeutics creates an urgent need for the development of safer and efficient chemotherapy to curb cervical cancer. Hence, the current study was designed to investigate the in vitro anticancer potency of Eremanthin against human HeLa cervical cancer cells.*

Methods: *Cell cytotoxicity was examined by MTT cell proliferation assay and clonogenic assay was performed for cell colony analysis. Eremanthin-induced necrotic cell death was assessed through phase contrast microscopy. Flow cytometric analysis was performed for estimation of cell cycle phase distributions. Reactive oxygen species (ROS) production was detected via DCF fluorescence and mitochondrial membrane potential (MMP) loss was monitored by flow cytometry. ENLITEN ATP assay system bioluminescence detection kit was used for quantification of complete intracellular ATP. Caspase-3 activity along with PI3K/AKT signalling pathway were analyzed through western blotting analysis.*

Results: *Eremanthin exerted significant cytotoxic effects on HeLa cervival cancer cells. Clonogenic assay revealed that the colony formation of HeLa cells was reduced by eremanthin treatment. Eremanthin induced specific modifications in cellular morphology of HeLa cells, indicating its necrosis-mediated cytotoxicity. Eremanthin treatment led to G2/M-phase cell cycle arrest. Flow cytometric analysis showed that the molecule significantly reduced MMP which was associated with complete intracellular ATP loss and also resulted in enhancement in ROS production. Western blotting revealed that the activity of Caspase-3 remained unchanged on lower Eremanthin concentrations and decreased insignificantly on higher molecule doses. Western blotting also revealed that the activity of PI3K/AKT signalling pathway dependent proteins altered after Eremanthin exposure. After drug exposure PI3K and AKT activity remained unchanged and the activity of phosphorylated PI3K and AKT diminished.*

Conclusion: *Eremanthin molecule is a potent anticancer agent against human cervical cancer and may be included as lead molecule in cervical cancer treatment provided further in vitro and in vivo investigations are performed.*

Key words: *cervical cancer, eremanthin, necrosis, reactive oxygen species, caspase-3*

Introduction

Cervical cancer (CC) is a global life threat that affects a huge number of female populations [1]. World Health Organization (WHO) recognises CC as second largest cancer type frequently prevailing among females with about 0.45 million new cases and nearly 0.27 million deaths each year [2]. CC shows regional variations as the majority of cases are reported in middle- and -low income countries.

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Out of 0.27 million deaths nearly 85% deaths are reported in developing countries. Long-term human papillomavirus (HPV) infection is a leading risk factor for CC development [3-7]. HPV injects its genome into host cell, thereby taking control over it and deregulating the normal cellular processes. These processes include alterations in cell differentiation and growth responses, cell multiplication and DNA synthesis, which ultimately result in CC development and relapse [8]. However, not all HPV infections are associated with the development of oncogenic progression; other factors are also accountable like nitrosative and oxidative stress, higher expressions of protein carbonyls in invasive CC tissues and CIN2/3 (dysplastic cervical tissue lesions) [9]. Current treatment options available for CC management include surgical resection, radiotherapy and chemotherapy [10]. CC is radiosensitive, therefore radiotherapy is an effective treatment strategy, but chemotherapy has been recorded with better results. Hysterectomy is used for stage IA (microinvasive tumours) cervical tumours involving removal of a part of vagina and whole uterus and for stage IA2 uterus, vagina, and lymph nodes are also removed. If biopsy suggests cancer-free tissue surrounding the cervical tumour, then another possible treatment strategy, that is trachelectomy, can be applied. This involves specific tumour removal which leads to retention of woman's fertility but remains more conservative compared to hysterectomy [11-14]. Despite all the above treatment options the overall survival rate for CC is poor. Thus, research has been directed to find novel safe and effective drugs that can be used in chemotherapy with better results. Over the past two decades, natural products are being investigated for their medicinal potential due to their huge diversity and underlying biologically active compounds. Sesquiterpene lactones (natural products) are recognised as biologically active compounds with various medical uses including anticancer, antimicrobial, antiinflammatory, antifeedants and antimigraine [15,16]. Eremanthin - a naturally occurring sesquiterpene lactone isolated from *Eremanthus elaeagnus* - remains a target of studies due to its huge structural complexity and biological activities [17,18]. Several studies have reported that Eremanthin possesses various pharmacological activities including antioxidant, antidiabetic, antilipidemic and immunomodulator inhibition [19].

The current study was thus designed to clarify whether the anticancer activity of Eremanthin against the HeLa human cervical cancer cells is due to G2/M phase cell cycle arrest, ROS-mediated necrosis-like cell death and inhibition of PI3K/AKT signalling pathway.

Methods

Cell cytotoxic evaluation

The cell viability suppressive effects of Eremanthin on human cervical HeLa CC were assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) cell cytotoxicity assay. In brief, HeLa cells (2×10^3 cells per each well with 180 µL RPMI-1640 culture medium) were cultured in 96-well plates. Afterwards, cell culture was exposed to different Eremanthin doses (0, 10, 20, 40 and 120 µM) for 24 h and 48 h, respectively. Treated cells were then stained with MTT (0.5mg/ml) stock solution for 4h at 37°C. Thereafter, all of the RPMI-1640 culture medium was discarded and formazan crystals left out were dissolved in DMSO (dimethyl sulfoxide). Finally, optical density (OD) measurements were performed at 560nm and 630nm with Envision microplate reader (PerkinElmer, Waltham, MA, USA).

Clonogenic analysis

Human HeLa CC cells (3000 cells/well) were seeded in 6-well plates with 10% fetal bovine serum (FBS) and RPMI-1640 medium. After seeding, HeLa cells were subjected to Eremanthin treatment at changing concentrations (0, 10, 40 and 120 µM) for 12 days. Eremanthin treatment was followed by fixation and staining with paraformaldehyde and crystal violet (Beyotime, China), respectively. Cell colonies were photographed with a digital camera (Olympus, Japan) for further investigations.

Necrotic cell morphology determination by phase contrast inverted microscopy

Phase contrast inverted microscopy was performed for morphological analysis of HeLa cells. Briefly, HeLa cells were seeded in 10-well plates with 4×10^3 cells in each well. Culturing was followed by Eremanthin treatment at varying doses (0, 10, 40 and 120 µM) for 24h. Then, RPMI-1640 culture medium was completely removed and cells were washed one time with phosphate buffered saline (PBS). HeLA cell morphology was then analyzed through phase contrast inverted microscope (Leica DMI 3000B, Germany) at a magnification of 200×.

Analysis of different cell cycle phases

Briefly, HeLa cells (1×10^3 cells/well) were exposed to Eremanthin for 24h and with changing concentrations (0, 10, 40 and 120 µM). Afterwards, treated cells were washed with PBS, followed by centrifugation (3,500 rpm) for 5 min. Cells were then fixed in ethanol (70%) and again washed twice with PBS. Next, RNAse 50 µl and propidium iodide (PI) 25 µl were supplemented to cell suspension followed by incubation of 15 min. Finally, Muse flow cytometric analysis (Millipore, Billerica, MA, USA) was carried out for DNA content analysis.

Estimation of mitochondrial membrane potential (Δψm/MMP)

Briefly, human HeLa cervical CC were harvested at 80% confluence and subjected to Eremanthin treatment

(0, 10, 40 and 120 µM). Eremanthin-treated cells were then incubated with 3,3'-dihexyloxacarbocyanine iodide (15 nmol/L) (Molecular Probes, Invitrogen, Eugene, USA) for 20 min. Finally, washing of the cells was accomplished with 500 µl of PBS followed by MMP analysis using FACScan (BD Biosciences, San Jose, CA, USA).

Total intracellular ATP measurement

Human HeLa CC cells were harvested and treated with varying doses of Eremanthin (0, 10, 40 and 120 µM). Further, to estimate the quantity of intracellular ATP, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer. Afterwards, quantification of intracellular ATP was performed using ENLITEN ATP assay system bioluminescence detection kit (strictly following manufacturer's guidelines). ATP concentration was normalized by protein content within lysates.

Analysis of ROS production

The intracellular ROS production in HeLa CC cells was monitored by DCF fluorescence staining. Briefly, cells were collected at exponential growth phase followed by Eremanthin treatment of changing concentrations (0, 10, 40 and 120 µM) for 24 h. Afterwards, treated cells were suspended in PBS with DCFH-DA at 10 mM final concentration, followed by incubation for 30 min at 37°C. Stained HeLa cells were then resuspended in PBS for washing. Finally, ROS were analyzed through detection of fluorescence intensity under fluorescence microscope and microplate reader at a wavelength of 500 and 529 nm. Experiments were repeated in triplicate.

Western blotting analysis

RIPA lysis buffer was bought from Sigma-Aldrich and used for HeLa cell lysing. Estimation of protein content within each lysate was performed via bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, China). Afterwards, using SDS-PAGE (10%), uniform protein amounts (35 µg) were separated and loaded over PVDF membranes bought from Millipore, Billerica, MA, USA. Thereafter, the membranes were subjected to primary antibodies against Caspase-3, PI3K and AKT (1:1000; Epitomics, Burlingame, CA, United States). Primary antibody treatment was followed by secondary antibody treatment (anti-rabit IgG conjugated to HRP). Finally, protein bands were visualized through ECL (enhanced chemiluminescence) detection system (Pierce, Rockford, IL, United States).

Statistics

All the data are presented as means ± standard error of the mean. One-Way Analysis of Variance (ANOVA) was used for determination of statistical significance. Multiple comparisons were performed with Tukey's test. Individual experiments were repeated in triplicate. P value <0.05 was considered as statistically significant.

Results

Effect of Eremanthin treatment on cell viability of human HeLa CC cells

HeLa cells were treated with increasing Eremanthin concentrations (0, 10, 20, 40 and 120 µM) for 24 h and 48 h respectively. Cell growth rate was estimated by MTT cell cytotoxicity assay. Eremanthin markedly reduced the viability of HeLa cells in dose-as well as in time-dependent manner (Figure 1). Cell viability at 0, 10, 20, 40 and 120 µM drug doses was 100%, 85%, 65%, 45% and 15% respectively after 24 h of exposure. After 48 h of exposure, cell viability at similar drug doses was 100%, 85%, 87%, 75% and 45%.

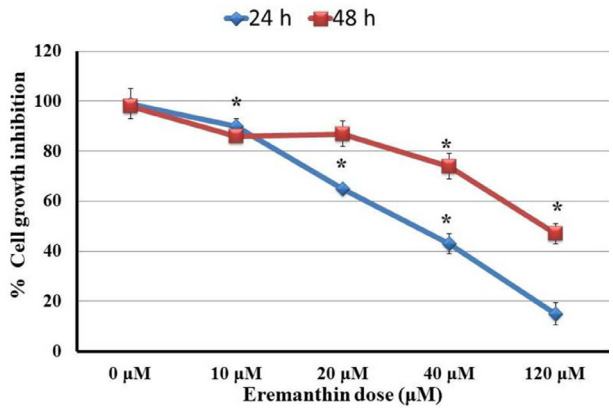

Figure 1. MTT cell viability assay, showing the effect of Eremanthin treatment on cell viability of HeLa cervical cancer cells. All the data are presented as mean±standard error of mean. The experiments were performed in triplicate. *p<0.05.

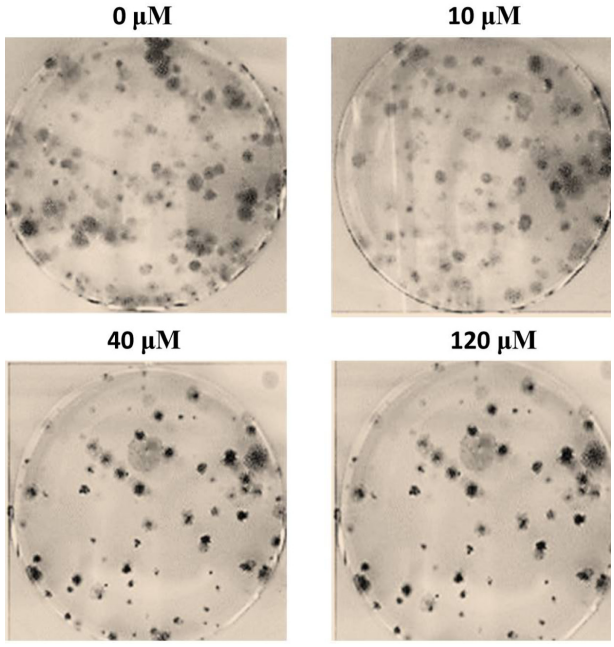

Figure 2. Clonogenic analysis of HeLa cells after Eremanthin treatment at indicated doses. The experiments were performed in triplicate.

Eremanthin treatment reduced the clonogenic potential of HeLa CC cells

HeLa colony formation was assessed through clonogenic assay after 12 days of Eremanthin treatment (0, 10, 40 and 120 µM). The results indicated that the clonogenic potential of HeLa cells reduced significantly upon increasing the drug doses (Figure 2).

Effect on cellular morphology of HeLa CC cells after Eremanthin exposure

Cellular morphology of Eremanthin-treated cells (0, 10, 40 and 120 µM) was observed through phase contrast inverted microscopy. The results showed significant morphological changes upon

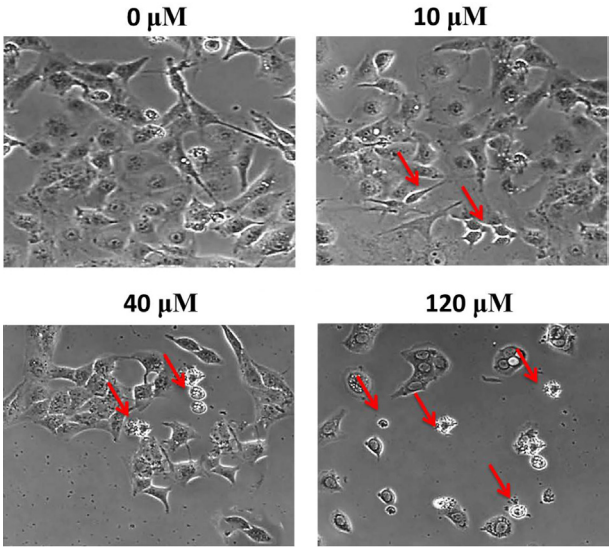

Figure 3. Effect of Eremanthin treatment on the morphology of HeLa cells. Membrane rupture and oncosis as depicted by arrows at indicated doses. The experiments were performed in triplicate.

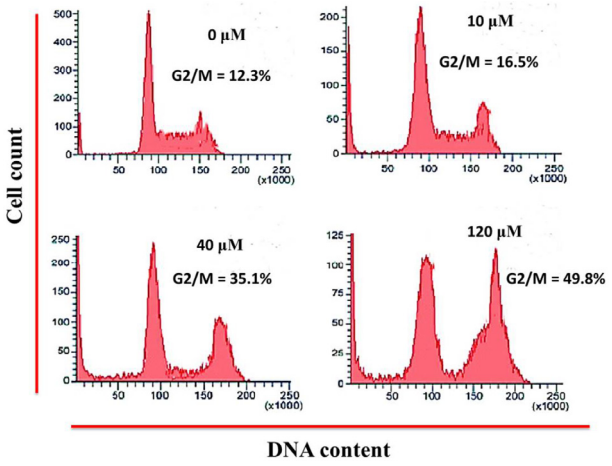

Figure 4. Flow cytometric analysis showing HeLa G2/M-phase cells after Eremanthin exposure. Data are shown as mean±standard error of the mean. Individual experiments were repeated thrice. p<0.05.

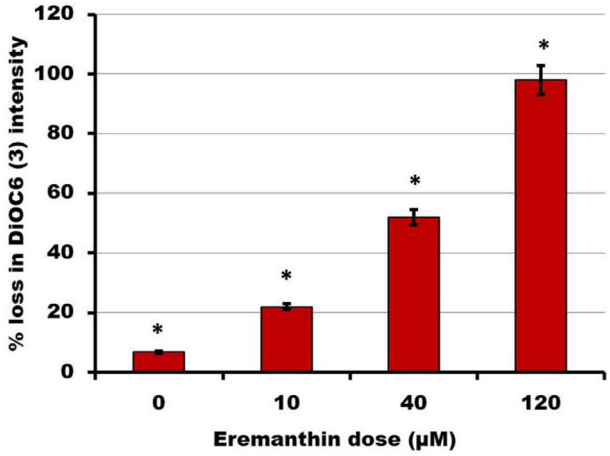

Figure 5. Percentage loss in DiOC6 (3) intensity (MMP) after Eremanthin exposure at presented doses. Data are shown as mean±standard error of the mean. Individual experiments were performed in triplicate. *p<0.05.

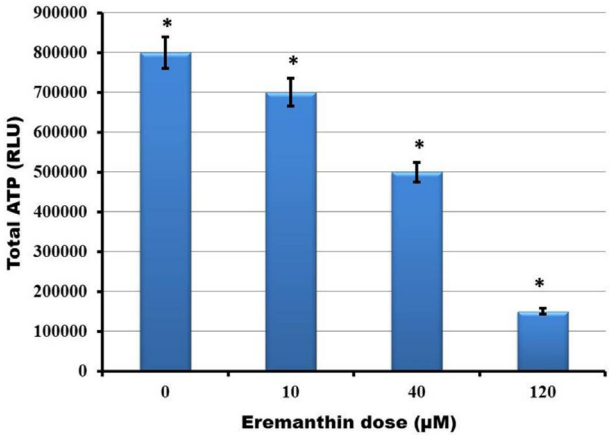

Figure 6. Effect on complete ATP count of HeLa cells by Eremanthin treatment at indicated doses. Data are shown as means±standard error of the mean. Individual experiments were repeated thrice. *p<0.05.

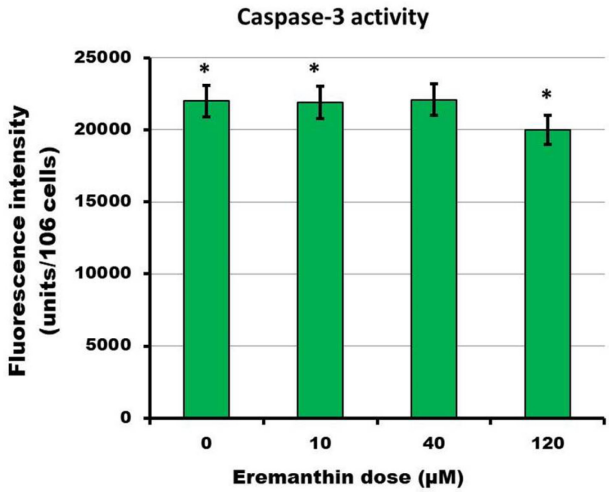

Figure 7. Caspase-3 activity in HeLa cells after Eremanthin treatment. Data are shown as means±standard error of the mean. Individual experiments were repeated thrice. *p<0.05.

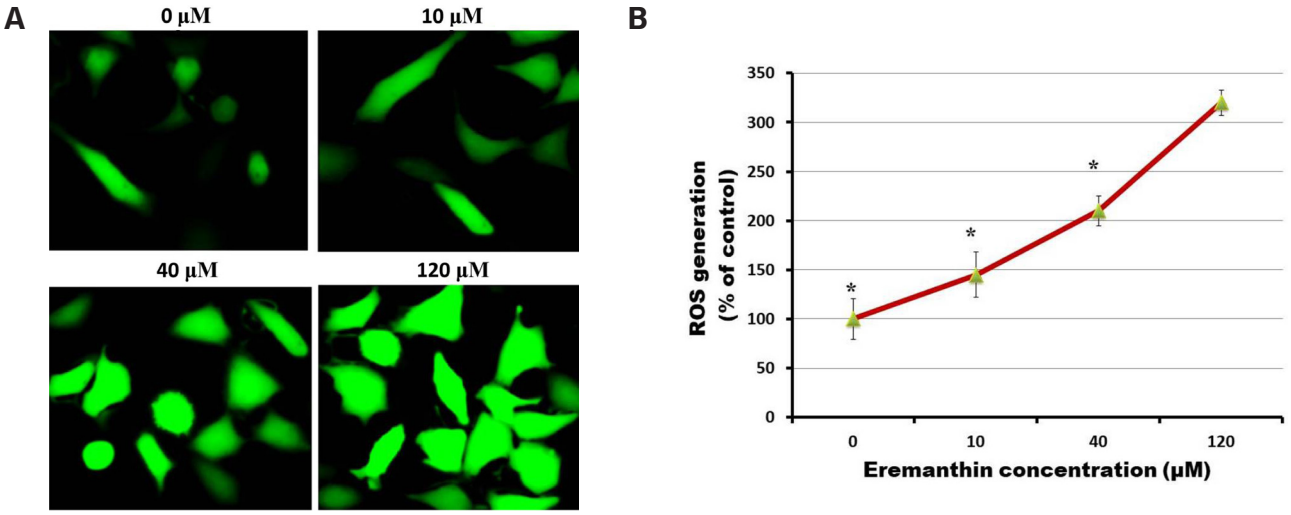

Figure 8. A: Carboxy-H_2 DCFDA intensity in HeLa cells after Eremanthin exposure at indicated doses. Individual experiments were repeated thrice. **B:** Effect of Eremanthin treatment on ROS production in HeLa cells. Data are shown as mean±standard error of the mean. Individual experiments were repeated thrice. *p<0.05.

drug exposure including plasma membrane rupture and oncosis while no apoptotic crops or autophagosomes were observed (Figure 3). Oncosis and plasma membrane rupture represents necrotic cell death. Thus these results indicate that the anti-proliferative effects induced by Eremanthin in HeLa cells might be due to necrosis. Furthermore, it was observed that the number of necrotic cells increased upon increasing drug doses.

Cell cycle phase analysis after Eremanthin exposure of HeLa CC cells

Different cell cycle phases in HeLa cells were analysed through flow cytometry after Eremanthin treatment. The results showed significant rise in the number of G2/M-phase cells with increasing drug concentrations. At concentrations of 0, 10, 40 and 120 µM, the percentage of G2/M-phase cells was 12.3%, 16.5%, 35.1% and 49.8% respectively (Figure 4), thus indicating induction of G2/M-phase cell cycle arrest by Eremanthin treatment against HeLa CC cells.

Effect on mitochondrial membrane potential and complete intracellular ATP of HeLa CC cells by Eremanthin

MMP measurements were performed to check whether the antiproliferative effects were associated with loss in MMP. Flow cytometric analysis of Eremanthin-treated HeLa cells revealed significant loss in MMP. The percentage loss in DiOC6 intensity (indicating Δψm/MMP loss) increased remarkably on increasing drug doses. At drug concentrations of 0, 10, 40 and 120 µM, the percentage loss in DiOC6 intensity was 8%, 21%, 50% and 98%, respectively (Figure 5), thus indicating remarkable dose-dependent MMP loss in HeLa cells.

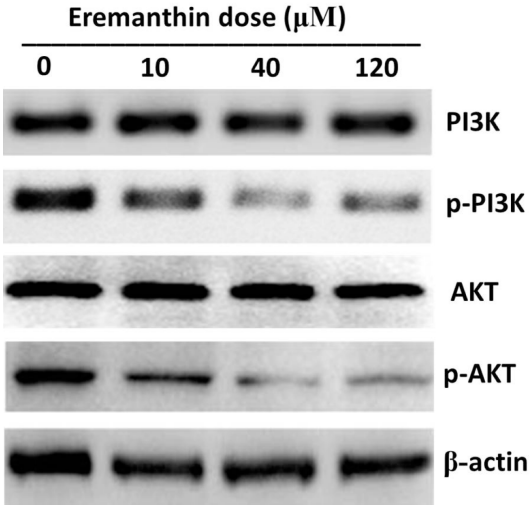

Figure 9. Western blotting analysis presenting the effect of Eremanthin treatment on proteins of PI3K/AKT signalling pathway. The Figure shows that the levels of activities of phosphorylated AKT and PI3K reduced in comparison to untreated controls, while the expression levels of non-phosphorylated AKT and PI3K remained almost unchanged. Individual experiments were repeated thrice.

Loss in MMP takes place both in necrotic as well as in apoptotic cells but their differences lies in ATP consumption. Necrotic does not need ATPs to take place while in the case of apoptosis ATPs take active part and are needed. Thus quantification of intracellular ATPs was performed after Eremanthin treatment was also observed in both the cases of necrotic as well as of apoptotic cell death, but necrosis was associated with complete ATP loss. The results depicted significant loss in cellular ATP and it was observed that on increasing the drug doses from 0 to 120 µM the number of ATP reduced from 800000 to 150000 RLU (Figure 6). Further, western

blotting analysis was performed to check the effects of drug treatment on Caspase-3 activity and the results revealed no significant change in Caspase-3 activity with a negligible decrease on higher concentrations (Figure 7). Thus, the results of cellular ATP analysis and western blotting analysis clearly evidenced the induction of dose-dependent necrotic cell death in HeLa CC cells by Eremanthin.

Effect of Eremanthin on ROS production of HeLa CC cells

In addition to loss in MMP, necrosis was also associated with increase in intracellular ROS production. Eremanthin-treated HeLa cells were monitored for ROS productions through flow cytometry which revealed that with increasing drug concentration carboxy-H_2 DCFDA intensity increased considerably (Figure 8A). It was observed that ROS percentage at drug concentrations of 0, 10, 40 and 120 µM was 100%, 150%, 200% and 325%, respectively (Figure 8B). Hence, monitoring of ROS production clearly evidenced that the antiproliferative effects of Eremanthin in HeLa CC cells were mediated via ROS production.

Impact of Eremanthin treatment on PI3K/AKT signalling pathway of HeLa CC cells

Western blotting analysis was performed to check the activity of PI3K/AKT signalling pathway associated proteins. The results indicated considerable variations in the expressions of PI3K/AKT signalling pathway associated proteins after Eremanthin treatment. The expressions of PI3K and AKT remained almost unchanged as compared to the expressions of phosphorylated PI3K and AKT, which decreased significantly with increasing Eremanthin concentrations (Figure 9). These results revealed significant inhibition of PI3K/AKT signalling pathway by Eremanthin.

Discussion

Unfortunately, due to the prevalence of drug-resistant tumor, the balance between apoptotic and antiapoptotic proteins gets hampered which often leads to cancer cell survival. In that case nonapoptotic pathways of cell death overcome the shortcomings of apoptotic pathway [20]. Hence, targeting nonapoptotic pathways in drug-resistant cancer serves as an effective treatment strategy [21]. Necrosis is a nonapoptotic type III pathway of naturally occurring programmed cell death and is often termed as normal cell death pathway. Necrosis is hallmarked by plasma membrane rupture and depletion as well as oncosis. It is associated with overproduction of ROS and MMP ($\Delta\psi m$) loss. MMP loss occurs during the course of apoptosis as well, but here it is accompanied with total ATP loss and in case of apoptosis ATP are required [22]. CC is a dangerous malignancy and its incidence and mortality are increasing due to lack of effective treatment strategies and chemotherapeutic agents with minimum side-effects and enhanced overall survival. Cell growth rate of HeLa CC cells was assessed via MTT cytotoxicity assay with results indicating dose - as well as time-dependent inhibitory effects on HeLa cells proliferation by Eremanthin molecule. Colony formation potential of HeLa cells was testified by clonogenic assay after drug treatment and the results revealed potential dose-dependent colony inhibition by Eremanthin. Phase contrast inverted microscopy was performed for cell morphological analysis and the results revealed plasma membrane rupture and oncosis, which indicate induction of necrotic cell death by the test molecule as no formation of autophagosomes was observed. Further, MMP was estimated via flow cytometry, revealing significant loss in MMP after drug exposure. Loss in MMP is observed in both apoptosis as well as necrosis but in the former it is associated with retention of cellular ATP and in the latter complete ATP loss takes place. Thus, quantification of cellular ATP was performed via ENLITEN ATP assay system bioluminescence detection kit revealing remarkable decrease in cellular ATP. Western blotting was performed to check the activity of caspase-3 but insignificant effects were observed. Hence, MMP loss, cellular ATP loss and caspase-3 activity clearly indicated that the antiproliferative effects of Eremanthin are mediated via necrosis. Further, assessment of ROS production indicated that on increasing drug doses ROS increased significantly, supporting the fact that Eremanthin induced ROS-mediated necrosis-like cell death. Finally, Eremanthin treatment on PI3K/AKT signalling pathway of HeLa cells showed that the expressions of PI3K and AKT remained almost unchanged and phosphorylated PI3K and AKT increased with increased drug doses, implying the potency of Eremanthin as potential inhibitor of PI3K/AKT signalling pathway against HeLa cells.

Conclusion

In conclusion, Eremanthin is a potential anticancer agent against human HeLa cervical cancer cells. Eremanthin induces its anticancer effects via G2/M phase cell cycle arrest, ROS-mediated necrosis like cell death and inhibition of PI3K/AKT signalling pathway.

Conflict of interests

The authors declare no conflict of interests.

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