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ORIGINAL ARTICLE

Eriodictyol inhibits the growth of CNE1 human nasopharyngeal cancer growth by targeting MEK/ERK signalling pathway, inducing cellular autophagy and inhibition of cell migration and invasion

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Summary

Purpose: *Eriodictyol is an active flavonoid present in several vegetables and fruits. Eriodictyol-bearing plants have long been used in folk medicine used to treat different human disorders. It has been reported to exhibit the anticancer, antioxidative and antiinflammatory properties. The current research study was designed to explore the anticancer potential of eriodictyol against CNE1 nasopharyngeal cancer (NP) cells. Additionally, its effects of targeting MEK/ERK signalling pathway, autophagy, cell migration and invasion were also examined.*

Methods: *MTT assay was applied for viability measurements, and clonogenic potency measurements were made by clonogenic assay. Autophagy was monitored by transmission electron microscopy (TEM). Cell migration capability was examined by wound healing assay, and transwell chambers assay was used for estimation of cell invasion. Western blotting assay was performed to examine protein expression levels.*

Results: *The results indicated the proliferation rate of CNE1 cells was reduced in eriodictyol dose-dependently. Cell colonies were also observed to be minimised after eriodictyol exposure. The underlying mechanism of antiproliferative effects of eriodictyol in the current research was found to be autophagy-mediated as suggested by TEM and increased expressions of pro-autophagy proteins. Cell migration and invasion was significantly suppressed by eriodictyol in CNE1 cells. Finally, western blotting assay indicated that eriodictyol blocked MEK/ERK signalling pathway dose-dependently. In conclusion, the results of the currently performed investigation indicated that eriodictyol is a potential anticancer agent against CNE1 nasopharyngeal cancer.*

Conclusions: *Therefore, this molecule may prove a leading agent in nasopharyngeal cancer treatment provided further in vitro and in vivo investigations are performed.*

Key words: *nasopharyngeal cancer, flavonoids, eriodictyol, autophagy*

Introduction

Flavonoids constitute a major class of naturally occurring polyphenolic compounds belonging in heterogeneous plant families. Vegetables and fruits are a rich source of flavonoids, especially tea, apples, and grapes bear high flavonoid concentrations [1,2]. Flavonoids-bearing plants have been used in folk medicine from thousands of years. Being natural products, they show a wide array of medicinal and biological applications such as cancer preventive activity [3,4]. Flavonoids show free radical scavenging, enhance enzymatic activity of anti-carcinogens, limit LDL oxidation, inhibit peroxidation of lipids, suppress transcription of tumor promoters and regulate immune responses in various biological

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systems [5-8]. On application to tumor cells, flavonoids reveal an array of effects including apoptosis initiation, inhibition of kinase activity (protein kinase CK2), limiting the secretion of MMP (matrix metalloproteinase) suppression of angiogenesis and modify the behavior of cancer cell invasion [9-11]. Eriodictyol is an active member of flavonoids and exists in many vegetables and fruits. It has been proved to hold antioxidant and antiinflammatory activities [12,13]. In addition, eriodictyol showed antidiabetic activity (streptozotocin) induced diabetic rats [14]. The anti-oxidative and antiinflammatory effects of eriodictyol were found to be regulated via modulation of Nrf2 pathway [15]. Besides, eriodictyol was observed with anticancer cancer activity against RGCs (retinal ganglial cells) via induction of apoptosis and oxidative stress [16,17]. Nasopharyngeal carcinoma (NC) is a detrimental human disease exhibiting regional, genetic and epidemiological distribution properties. NC shows uniqueness in natural behavior as well therapy. Radiation therapy has revealed good potential in the initial stages of NC treatment but in later stages NC still remains a big challenge for researchers and clinicians [18]. NC has a higher incidence for distant metastasis and local relapse after application of radiotherapy [19]. The treatment strategy for early stage NC involves radiotherapy and for advanced stages chemotherapy and radiotherapy are synergistically implicated [20]. NC chemotherapy involves two major active cytotoxic drugs that is paclitaxel and cisplatin [21]. Despite of strong and aggressive efforts of merging radio and chemotherapy for NC treatment, 30% of NC patients still fall prey of distant metastasis and disease relapse due to poor prognosis [22]. Therefore, there is an immediate need for novel therapeutics that can reduce disease relapse and distant metastasis. Herein, this investigation was formulated to explore the anticancer potential of eriodictyol against CNE1 human nasopharyngeal cancer cells. Its effects on MEK/ERK signalling pathway, inducing cellular autophagy and inhibition of cell migration and invasion were also examined

Methods

Cytotoxicity assessment

Cytotoxicity of eriodictyol against human nasopharyngeal CNE1 cancer cells and normal nasopharyngeal cells was determined by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay. Briefly, both CNE1 cancer cells and normal nasopharyngeal cells were separately plated onto 96-well plate at a density of 2×10^5 cells per well and were precultured for 24h. Afterwards, each well was supplemented with different eriodictyol doses (0 to 640 μM). Following drug treatment, cells were added with MTT stock solution and incubated for another 4h. MTT addition resulted in the formation of formazan crystals that are finally dissolved in DMSO (dimethyl sulfoxide). For optical density measurements, absorbance was recorded at 490nm with an ELISA plate reader (Bio-Tek Instruments, Winooski, VT). Cell viability was determined after 0h, 2.5h, 5h, 10h, 20h, 40h, 80h, 160h, 320h and 640h of the molecule exposure.

Determination of clonogenic potential

CNE1 cells were seeded in 6-well plates at a concentration of 1000 cells/well for 24h. Afterwards, varying doses of eriodictyol (0, 10, 20 and 40 μM), were implemented to CNE1 cells for 48h. Thereafter, cells were left untouched for 21days and then collected and fixed for 15min in methanol. Following fixation, cells were crystal-violet stained with incubation for 30min and finally, the number of cell colonies was counted using a light microscope.

Autophagy assessment

For autophagy determination, CNE1 cells were analysed through TEM (Transmission electron microscopy) after eriodictyol treatment. Nasopharyngeal CNE1 cancer cells were plated in 6-well plates and subjected to varying doses (0, 10, 20 and 40 μM) for 24h. After eriodictyol exposure, cells were fixed with 0.05M sodium cacodylate buffer bearing 4% glutaraldehyde. 1.5%. Osmium tetroxide was applied for post fixation and afterwards dehydration was performed by using ethyl alcohol. Thereafter, ordering of treated CNE1 cells was accomplished prior to embedding in Epon 812. Finally, cells were investigated under a Zeiss CEM 902 electron microscope.

Wound healing assay

Nasopharyngeal CNE1 cancer cells were cultured till 90% confluence in 6-well plates. After that, each well plate was supplemented with variant eriodictyol doses (0, 10, 20 and 40 μM). Cells were then washed with PBS followed by scratching a wound in treated as well as in control cells with a sterile pipette tip. Pictures of each well were prerecorded, and after incubation for another 24h at 37°C pictures were again captured by using an inverted microscope.

Cell invasion assay

Transwell chambers assay was implemented to feature the effect of eriodictyol on cell invasive ability of nasopharyngeal CNE1 cancer cells. The upper chambers of the transwell were filled with Dulbecco's Modified Eagle's (DMEM) culture medium (300ml) containing 10% FBS (fetal bovine serum). The lower chambers of the transwell were left only with the medium and FBS. Each well of the transwell chambers was then exposed to varying eriodictyol doses (0, 10, 20 and 40 μM). Thereafter, transwell chambers were left untouched with incubation for 24h. Non-invasive cells were cleaned off using a cotton swab and invasive cells were fixed in methyl alcohol. Next, staining was performed with crystal violet and quantification of invaded cells was performed through inverted microscopy with 200× magnification.

Western blotting analysis

Western blotting assay was performed to check the activity of autophagy and MEK/ERK signalling pathway associated proteins. After treatment with varying eriodictyol doses (0, 10, 20 and 40 µM) in 6-well plates, cells were lysed using RIPA lysis buffer. Protein content within each lysate was quantified with bicinchoninic acid (BCA) assay. Afterwards, 40 µg cell lysates were resolved through SDS-PAGE and then electrophoretically transferred to nitrocellulose the membranes (Bio-Rad Laboratories, Hercules, CA, United States). Thereafter, membranes were blotted using primary antibodies against LC3B-I, LC3B-II, p-62, MEK ½ and ERK (Santa Cruz, CA, USA) with 1:1000 dilution. Then, the membranes were subjected to secondary antibodies treatment at 4°C overnight. Finally, enhanced chemiluminescence reagent (ECL) (Amersham, Piscataway, NJ, United States) was utilized to spot the protein signals.

Statistics

Data for all separate experiments are shown as mean ±SE (standard error). Fisher's least significant difference test and one-way ANOVA were implemented to analyse statistical differences. In all tests statistical significance was set at $p<0.05$.

Results

Suppression of cell viability in CNE1 cells by eriodictyol

To examine the antiproliferative effects of eriodictyol (Figure 1A) in nasopharyngeal CNE1

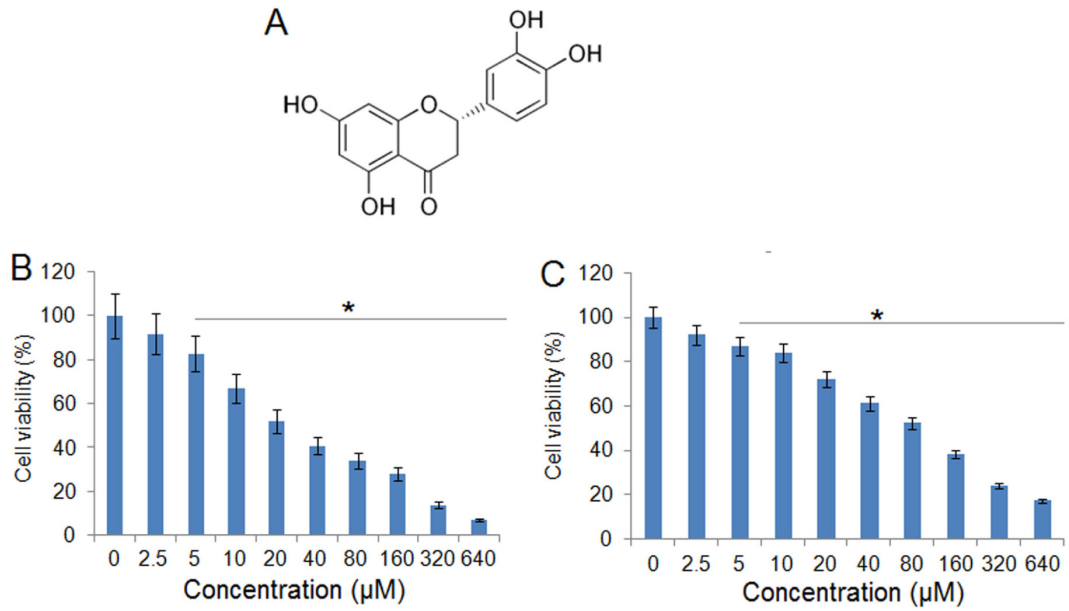

Figure 1. A: Molecular structure of eriodictyol drug. **B:** Viability of cancer CNE1 nasopharyngeal cells after eriodictyol exposure at indicated doses. **C:** Viability of normal nasopharyngeal cells after eriodictyol exposure for varying time intervals at indicated doses. Data from three separate experiments are presented as mean ± SE (*p<0.05).

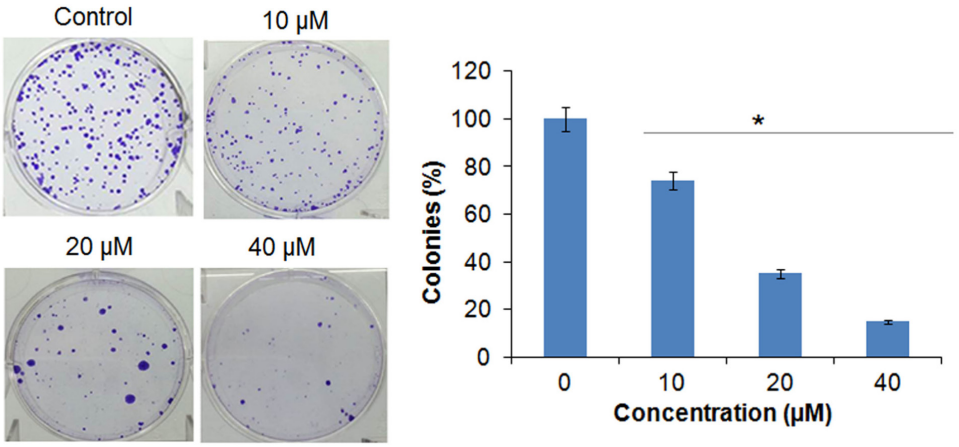

Figure 2. Clonogenic assay results after exposure to varying eriodictyol doses for 21 days. Reduced number of blue stains were observed in 6-well plates as indicated and the number of cell colonies were observed to be reduced to minimum. Data from three distinct experiments are shown as mean±standard deviation (*p<0.05).

cancer cells, MTT assay was implemented. Both normal and cancer CNE1 cells were exposed to variant eriodictyol doses and viability was monitored after various time intervals (0h, 2.5h, 5h, 10h, 20h, 40h, 80h, 160h, 320h and 640h). The results indicated the viability of cancer CNE1 cells was suppressed remarkably in a concentration- as well as time-dependent manner. The viability of controls was taken as 100%. Viability significantly decreased from 100% to about 10% after extending the eriodictyol exposure from 2.5 to 640 μM (Figure 1B). In case of normal nasopharyngeal cells, the viability inhibition by eriodictyol was insignificant after monitoring at different time intervals (Figure 1C). Therefore, MTT assay results showed that this molecule is a potential and selective proliferation inhibitor against nasopharyngeal cancer.

Eriodictyol inhibited the clonogenic potency of CNE1 cells

The clonogenic potential of nasopharyngeal CNE1 cancer cells was evaluated by clonogenic assay. After exposure to variant eriodictyol doses, cells were left for incubation for 3 weeks. The results showed that the clonogenic potential reduced significantly by the application of eriodictyol as evidenced from reduced blue stains in 6-well plates. The number of cell colonies in controls was observed to be 100 % and reduced to almost 15% after eriodictyol treatment (0-40μM) (Figure 2). Therefore, along with proliferation rate of cell the clonogenic potential was also reduced to minimum by eriodictyol.

Eriodictyol induced autophagy in CNE1 cells

In an attempt to unveil the underlying mechanism of anti-proliferative effects of eriodictyol in nasopharyngeal CNE1 cancer cells, autophagy assessment was performed. Autophagic analysis in cancer cells was performed with TEM after exposure to variant doses of eriodictyol. The results indicated formation of autophagosomes in the treated cells, which are characteristic of autophagy (Figure 3A). Thus, it was evidenced that the anti-proliferative effects of eriodictyol are mediated via autophagy induction. To further support this fact, western blotting assay was performed to monitor the expressions of pro-autophagic proteins and the results indicated that the expressions of p-62, LC3B-I and LC3B-II elevated in a dose-dependent manner (Figure 3B).

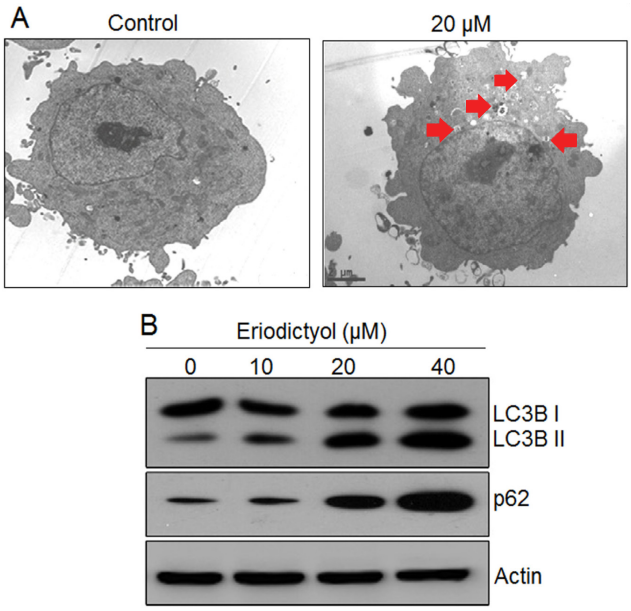

Figure 3. A: TEM pictures presenting the CNE1 cells morphology in controls and after eriodictyol treatment. Arrows show the formation of autophagosomes. **B:** Western blotting assay results presenting expressions of pro-autophagic proteins at indicated eriodictyol doses. Individual experiments were repeated three times.

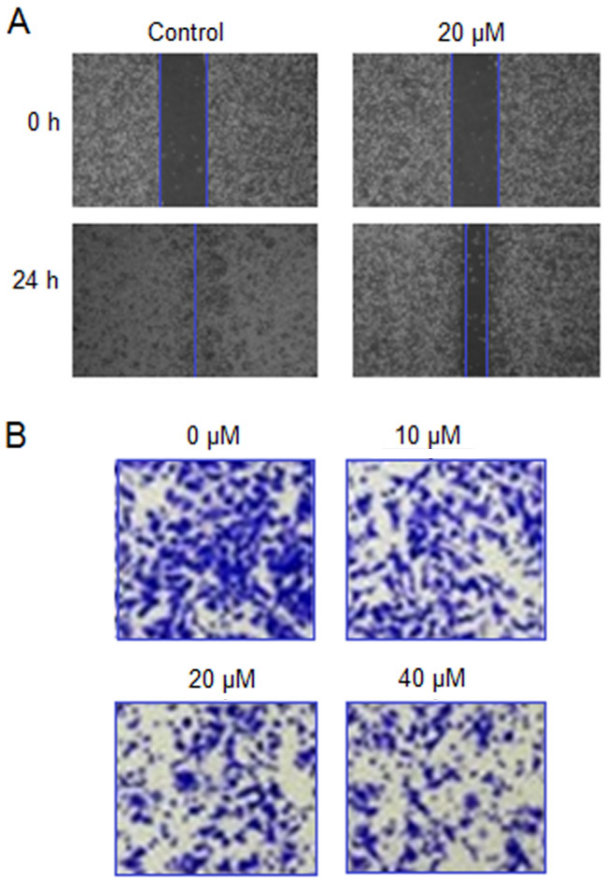

Figure 4. A: Pictures presenting the width of scratched wound in controls and eriodictyol treated cells at indicate doses. **B:** Pictures presenting the invasive CNE1 cells in controls and eriodictyol treated cells at indicate doses. Individual experiments were repeated three times.

Suppression of cell migration and invasion of CNE1 cells by eriodictyol

Cell migration and invasion of cancer cells results in distant cancer metastasis. Herein, the cell migration and invasion was assessed by performing wound healing and transwell chambers assay, respectively. Wound healing assay depicted that the cell migration was reduced remarkably by administering eriodictyol in CNE1 cancer cells. The wound width at in controls was observed to be almost closed while in treated cells the wound width remained unchanged at higher molecule doses (0-40 µM) (Figure 4A). Therefore, wound healing assay evidenced that cell migratory potential was limited to minimum by eriodictyol. Cell invasive potential assessment by transwell chambers assay has shown that eriodictyol application decreased the number of invasive cells in comparison to controls (Figure 4B). Therefore, eriodictyol exposure of CNE1 cells resulted in suppression of nasopharyngeal cancer metastasis in a dose-dependent manner.

Eriodictyol targeted MEK/ERK signalling pathway in CNE1 cells

Western blotting analysis was performed to monitor the activity of MEK/ERK signalling pathway linked proteins. After exposure to the molecule at varying doses, cells were lysed and proteins were extracted electrophoretically. The results of western blotting assay revealed that the levels of MEK/ERK signalling pathway linked proteins was significantly altered by the application of eriodictyol. The levels of MEK1/2, p-Mek1/2 and p-ERK lowered significantly with enhancing eriodictyol concentrations (0-40 µM). The levels of ERK remained almost unaltered on application of higher molecule doses. Thus, western blotting analysis evidenced that the protein levels of MEK/ERK signalling pathway related proteins was supressed to minimum by the current test molecule which suggested blocking of MEK/ERK signalling pathway in CNE1 cells.

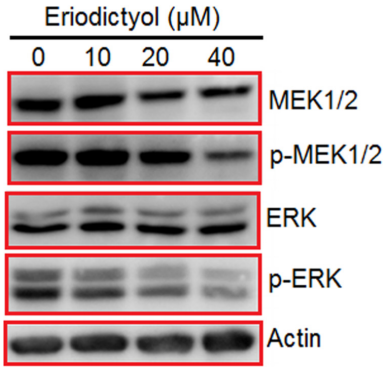

Figure 5. Western blotting assay indicating the expressions of MEK/ERK signalling pathway associated proteins. Individual experiments were repeated three times.

Discussion

Nasopharyngeal cancer (NC) is a malignancy associated with high morbidity as well as mortality. Lack of potential anticancer drugs, poor prognosis and disease metastasis are the major hurdles in NC management. Therefore, an urgent need for novel agents emerges that can overcome these hurdles. Autophagy is one of the important targets in cancer chemotherapy with effective results [23]. It is a self-degradative mechanism and plays a vital role in balancing the energy needs and nutrient stress [24]. Autophagy is responsible for clearing off the damaged organelles (peroxisomes, endoplasmic reticulum and mitochondria), aggregated or misfolded proteins and abolishing intracellular pathogens [25]. Thus, autophagy is also regarded as cell survival passage and deregulation of autophagy is found to be associated with non-apoptotic cell death. Autophagy plays vital role in overcoming different human abnormalities including cardiomyopathy, neurodegenration, diabetes, autoimmune diseases, liver diseases, infections as well as cancer [26]. Autophagy in removal of particular cell organelles and protein aggregates, behaves as either selective or non-selective. p62 (sequestosome 1/SQSTM1), plays a key role in selective autophagy and is universally expressed protein conserved in animals. p62 reacts with microtubule-associated protein light chain 3 (LC3) through LC3-interacting region. Afterwards, p62 gets assimilated into autophagosome and finally degraded [27,28]. The current research was performed for evaluation of the anticancer effects of eriodictyol flavonoid against CNE1 human nasopharyngeal cancer. Eriodictyol was also testified for targeting MEK/ERK signalling pathway, inducing cellular autophagy and inhibition of cell migration and invasion. It was also observed that this molecule induced potent anti-proliferative effects in CNE1 cells in a dose- and time-dependent manner. Clonogenic analysis showed that cell colonies were reduced significantly after eriodictyol exposure. Autophagic analysis through TEM and western blotting assay revealed formation of autophagosomes and enhancement in the levels of pro-autophagic proteins. Thus, it was evidenced that the anti-proliferative effects of eriodictyol were mediated via autophagy induction. Eriodictyol was observed with metastasis suppression through cell migration and invasion inhibition. Finally, western blotting assay indicated that eriodictyol targeted the MEK/ERK signalling pathway.

Conclusion

In conclusion, all the results from the performed assays indicated that eriodictyol induced anticancer effects against CNE1 human nasopharyngeal cancer cells. The anticancer effects of eriodictyol were mediated via targeting MEK/ERK signalling pathway, inducing cellular autophagy and inhibition of cell migration and invasion.

Conflict of interests

The authors declare no conflict of interests.

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