Original article

Dihydroartemisinin inhibits colon cancer cell viability by inducing apoptosis through up-regulation of PPARγ expression

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ABSTRACT

The present study was aimed to investigate the effect of dihydroartemisinin on the colon cancer cell proliferation and apoptosis. The results from MTT assay revealed a concentration and time dependent relation between the inhibition of SW 948 cell viability and dihydroartemisinin addition. The viability of SW 948 cells was reduced to 45 and 24% on treatment with 30 and 50 μM, respectively concentrations of dihydroartemisinin after 48 h. Morphological examination of SW 948 cells showed attainment of rounded shape and cluster formation on treatment with dihydroartemisinin. Western blot analysis showed a significant increase in the activation of caspase-3 and expression of cleaved PARP by dihydroartemisinin treatment. The activation of PPARγ was increased significantly in SW 948 cells by treatment with dihydroartemisinin. Compared to control, the migration potential of SW 948 cells was reduced significantly ($p < 0.005$) and the expression levels of MMP-2 and -9 inhibited by dihydroartemisinin at 50 μM concentration. In the dihydroartemisinin treatment group colon tumor formation was significantly inhibited on treatment with 20 mg/kg doses of dihydroartemisinin after 30 days. Therefore, dihydroartemisinin inhibits colon cancer growth by inducing apoptosis and increasing the expression of PPARγ. Thus dihydroartemisinin can be used for the treatment of colon cancer.

1. Introduction

Colorectal cancer, one of the most commonly detected cancer in the world is responsible for more than 10% of cancer-related deaths (Ferlay et al., 2010). Colorectal cancer has very high rate of metastasis and the most common target of distant metastasis is liver. Among the colorectal cancer patients around 15% have been found to possess liver metastases even at the time of diagnosis (Manfredi et al., 2006). It has been observed that liver resection is followed by recurrence in one-third of the liver metastasis

patients (Yamada et al., 2001). Thus the development of the efficient strategy for colon cancer treatment is urgently desired. Peroxisome proliferator-activated receptors γ (PPARγ) present in the various types of cells plays a vital role in the inhibition of inflammatory processes (Bassaganya-Riera et al., 2004). Increase in the expression of PPARγ by various chemotherapeutic agents has been used for the inhibition of inflammation and carcinoma treatment (Bassaganya-Riera et al., 2011). Studies have demonstrated that PPARγ exhibits inhibitory effect on the expression of genes involved in inflammation (8). Its mechanism of action involves interaction with various factors such as NF-κB, p65, p50 or mitogen-activated protein kinase (Ricote and Glass, 2007).

Metabolites recovered from medicinal plants have wide level of biological properties (Antonisamy et al., 2015; Balamurugan, 2015; Rathi et al., 2015; Nandhini and Stella Bai, 2015). Artemisinin isolated from a Chinese herb *Artemisia annua* is a well-known anti-malarial drug which has been used from very long time in China (Meshnick, 2002; O'Neill, 2004). Various other analogs of artemisinin such as dihydroartemisinin, artesunate, etc. have also shown promising anti-malarial activity and are therefore used for the clinical treatment of malaria. Screening of the artemisinin and its

analogs against various types of cancers including, breast and ovarian cancers led to a marked reduction in the rate of carcinoma cell proliferation and metastasis (Chen et al., 2009; Kalaiselvi et al., 2016; Neelamkavil and Thoppil, 2016; Valsan and Raphael, 2016). Use of dihydroartemisinin against various normal cell lines showed no cytotoxicity (Chen et al., 2009). The promising antiproliferative activity of dihydroartemisinin against cancer cell lines and its safety against normal cells makes it a potential candidate for cancer therapy. The present study was performed to investigate the effect of dihydroartemisinin on the colon cancer cell growth. The results demonstrated that dihydroartemisinin inhibited the viability and induced apoptosis through increase in the expression of PPARγ.

2. Materials and methods

2.1. Chemicals

Dihydroartiminisin and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. (St. Louis, MO).

2.2. Cell culture

The SW 948, human colon cancer cell line was purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium (Gibco, Shanghai, China), supplemented with 10% FBC (Gibco) and penicillin/streptomycin (1:100, Sigma) in an incubator with humid atmosphere of 5% CO_2 at 37 °C.

2.3. Analysis of cell proliferation

The cell viability was determined by suing 3-(4,5-dimethylthia zol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, SW 948 cell suspension was prepared by stirring the cells with PBS after washing. The concentration of cells was determined and the cells were then seeded at a density of 2×10^6 per well into the 96-well plates. The plates were cultured overnight and subsequently treated with various concentrations of the dihydroartiminisin. After incubation for 24, and 48 h, MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each for the well and incubated for 4 h. The medium was removed and DMSO (150 μL) was added to the wells. The absorbance for each well was measured in triplicates at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Western blot analysis

SW 948 cells were seeded at a density of 2×10^6 cells per well 6-well plates and incubated with various concentrations of dihydroartiminisin for 48 h. The cells were harvested and washed twice with phosphate-buffered saline. RIPA lysis buffer kit (Best Bio, shanghai, China) was used for the extraction of total proteins from the cells on ice for 45 min. The cell lysates were collected and then centrifuged at 4 °C for 20 min at 12,000×g to remove the debris. Bicinchoninic acid protein assay kit (Multi sciences, Hangzhou, China) was used for the detection of concentration of proteins. The proteins were separated by electrophoresis using 10% SDS-PAGE gels and transferred to a PVDF membrane (Merck Millipore, Darmstadt, Germany). The membrane blocking was performed using BSA for 1 h followed by incubation with primary polyclonal antibodies overnight 4 °C. The membranes were the washed again with PBS before incubation with goat-anti-rabbit secondary antibodies at room temperature for 1 h. The protein bands were

detected by using enhanced chemiluminescence Western blotting detection kit (Santa Cruz Biotechnology, Inc.).

2.5. Analysis of apoptosis

Apoptosis induction in SW 948 cells by dihydroartiminisin treatment was measured by the Annexin V-FITC Apoptosis Detection kit (BD Bioscience, San Jose, CA, USA). The cells after incubation for 24 h in 6-well plates at a density of 2.5×10^6 cells per ell were treated with various concentrations of dihydroartiminisin for 48 h. Following incubation, the cells were washed two times with ice cold PBS and subsequently re-suspended in 100 μl binding buffer. The cells were treated with 3 μl of Annexin V-FITC (BD Bioscience) and 10 μl propidium iodide (PI; BD Bioscience) at room temperature for 15 min. Flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used for the fluorescent intensity determination three times.

2.6. Wound healing assay

The SW 948 colon cancer cells at a density of 2×10^5 were seeded into 6-well plates and allowed to attain confluent monolayers. The cells were then cultured for 12 h in FBS free RPMI-1640 medium. In the center of each well a line was drawn using a 200 μl pipette tip for producing wound area. The cells were washed with PBS two times to remove the non-adherent cells. Various concentration of dihydroartiminisin were added to each of the well in medium containing 1% FBS. The images were captured using digital photography at a magnification of ×200.

2.7. Migration assay

For migration assay a 24-well Trans-well chamber (Corning Life Sciences, Corning, New York, NY, USA) bearing an 8-μm-pore PET membrane was used. Briefly, into the lower chamber 600 μl RPMI 1640 supplemented with 10% FBS was placed. In the upper chamber, SW 948 colon cancer cells at a density of 2×10^5 cells per mL into the medium containing dihydroartiminisin were added and allowed to migrate. After 24 h, the cells in the upper chamber were cleaned using cotton-tipped swab and the migrated cells in the lower chamber were washed with PBS and subjected to crystal violet staining after fixation. The optical density was recorded at 570 nm for quantification of the migrated cells.

2.8. In vivo tumorigenicity assay

Twenty pathogen-free male Balbc-nu mice (age, 8 weeks) were obtained from the Animal Institute of the Chinese Academy of Medical Science (Guangzhou, China). All the procedures on animals were performed in accordance with standard protocols approved by the Ethics Committee of Hunan Normal University (Changsha, China). The animals were given colonic carcinogen (Sigma-Aldrich) and DSS (MP Biomedicals, LLC, Aurora, OH) for producing colon cancer mice model. The mice in the treatment group were then given dihydroartiminisin at a dosage of 20 mg/kg body weight daily for one month intra-peritoneally. After completion of the treatment the animals were sacrificed to extract the colon cancer tissues.

2.9. Statistical analysis

The data presented are the mean of ±SD and were analyzed using SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was also used for the data analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Effect of dihydroartemisinin on SW 948 cell viability

The MTT assay revealed a concentration and time dependent relation between the inhibition of SW 948cell viability and dihydroartemisinin addition (Fig. 1A). Treatment with various concentrations of dihydroartemisinin revealed significant inhibition from 10 μM after 48 h. The viability of SW 948cells was reduced to 45 and 24% on treatment with 30 and 50 μM, respectively concentrations of dihydroartemisinin after 48 h. However, the inhibition in viability was 37 and 59% on treatment with 30 and 50 μM, respectively concentrations after 24 h (Fig. 1A). Morphological examination of SW 948 cells showed attainment of rounded shape and cluster formation on treatment with dihydroartemisinin at 50 μM concentration (Fig. 1B).

3.2. Effect of dihydroartemisininon apoptosis induction in SW 948 cells

Hoechst 33342 staining revealed apoptosis induction in SW 948 cells on treatment with various concentrations of dihydroartemisinin for 48 h. Compared to the control cells, dihydroartemisinin treatment increased the population cells with condensation of nuclear material in concentration dependent manner (Fig. 2A). Western blot analysis showed a significant increase in the activation of caspase-3 and expression of cleaved PARP on treatment with dihydroartemisinin for 48 h in SW 948 cells (Fig. 2B).

3.3. Effect of dihydroartemisininon activity of PPARγ

Western blot analysis showed that the activation of PPARγ was increased significantly in SW 948 cells on treatment with dihydroartemisinin for 48 h (Fig. 3). Increase in the concentration of dihydroartemisinin from 30to 50 μM also increased the expression level of PPARγ.

3.4. Effect of dihydroartemisinin on SW 948 cell migration

Analysis of the migration potential of SW 948 cells using Matrigel chamber revealed a significant decrease on treatment with dihydroartemisinin. Compared to control, the migration potential of SW 948 cells was reduced significantly ($p < 0.005$) by 50 μM concentration of dihydroartemisinin (Fig. 4A). Dihydroartemisinin treatment at a concentration of50 μM markedly inhibited the expression levels of MMP-2 and -9 activities in SW 948 cells (Fig. 4B).

3.5. Dihydroartemisinin inhibits tumor formation inmice model

Treatment of the AOM/DSS mice model with dihydroartemisinin inhibited the colon tissue inflammation markedly compared to the control (Fig. 5). In the dihydroartemisinin treatment group colon tumor formation was significantly inhibited on treatment with 20 mg/kg doses of dihydroartemisinin after 30 days (Fig. 5).

4. Discussion

Dihydroartemisinin is a potent anti-cancer candidate because of its marked potential to inhibit cancer cell viability without inducing any toxicity in the normal cells (Chen et al., 2009). In the present study effect of dihydroartemisinin on the colon cancer cell viability was investigated. Dihydroartemisinin inhibited the viability and induced apoptosis in colon cancer cells through increase in the expression of PPARγ. Breast and ovary cancer cell proliferation is inhibited significantly by treatment of the cells with dihydroartemisinin (Singh and Lai, 2001; Noorudheen and Chandrasekharan, 2016; Santhosh et al., 2016; Sreeshma et al., 2016). In the present study dihydroartemisinin treatment inhibited the viability of SW 948 colon cancer cells markedly in concentration and time dependent manner compared to the control cells. The inhibition of cell viability was marked by dihydroartemisinin treatment after 48 h. Apoptosis, programmed death of cells is very important for removal of unwanted cells from the body. The mech-
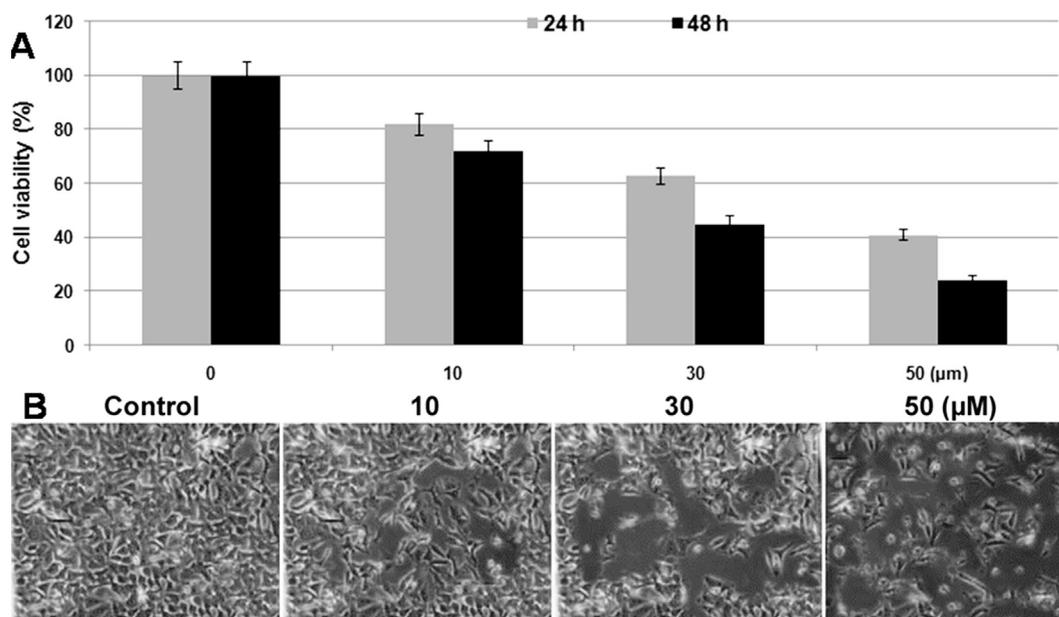

Fig. 1. Dihydroartemisinin inhibits SW 948 cell viability in concentration based manner. (A) SW 948cells were incubated for 24 and 48 h with various concentrations of dihydroartemisinin and then analyzed by MTT assay. For each concentration the absorbance was measured in triplicates. (B) Morphological examination of SW 948 cells after treatment with dihydroartemisinin for 48 h showed apoptotic features. Magnification ×220.
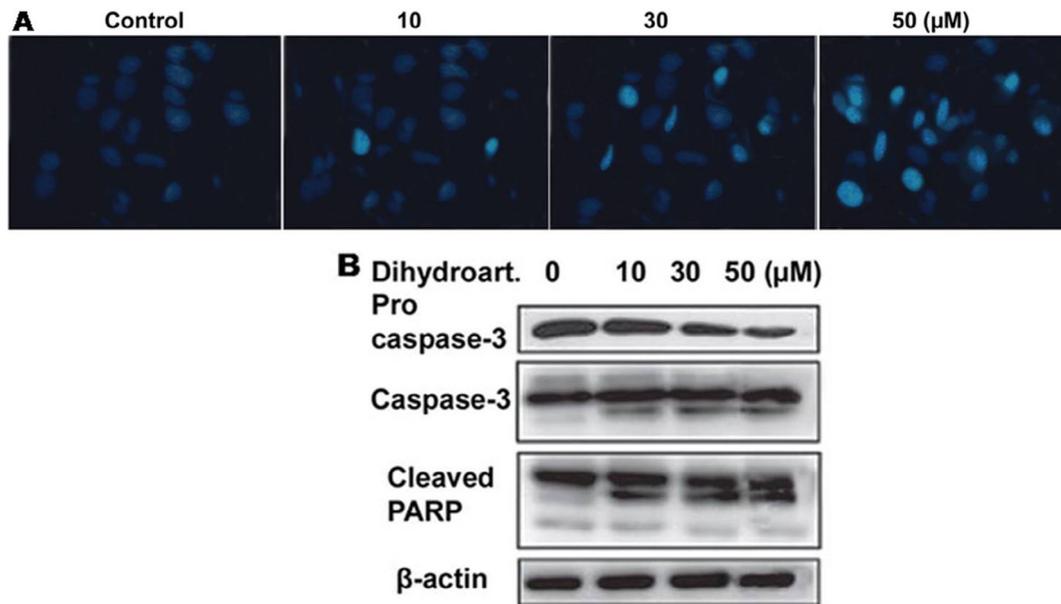
Fig. 2. Dihydroartemisinin induces apoptosis in SW 948 cells. (A) SW 948 cells after incubation for 48 h with dihydroartemisinin were examined by fluorescent microscopy. (B) The cells after treatment with various concentrations of dihydroartemisinin for 48 h were subjected to western blot analysis.
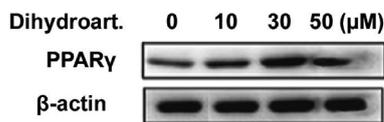

Fig. 3. Dihydroartemisinin treatment increases the activation of PPARγ in SW 948 cells. After incubation for 48 h, the cells were analyzed for the activation of PPARγ using western blot assay. B-actin was used as the internal loading control.

anism of apoptosis can involve either mitochondrial non-mitochondrial pathways (Lorenzo and Susin, 2007; Puthur, 2016). The results from present study demonstrated that dihydroartemisinin treatment induced apoptosis in the SW 948 cells. Treatment of the colon cancer cells with dihydroartemisinin significantly increased the expression of activated caspase-3 and cleaved PARP. The cells became rounded and shrunken in size following

treatment with dihydroartemisinin. PPARγ present in the various types of cells plays a vital role in the inhibition of inflammatory processes. Increase in the expression of PPARγ by various chemotherapeutic agents has been used for the inhibition of inflammation and carcinoma treatment (Serasanambati and Chilakapati, 2016). The colorectal cancer has been suppressed by the use of chemotherapeutic agents activating PPARγ which in turn activate tumor suppressor genes (Yamaguchi et al., 2008). In the current study dihydroartemisinin treatment of SW 948 cells caused a marked increase in the expression of PPARγ. PPARs plays an important role in the suppression of NF-κB which is involved in the generation of reactive oxygen species and tumor necrosis factor (Surh, 2008). In the present study dihydroartemisinin treatment of the mice with colon cancer inhibited the formation of colon inflammation. The migration of cancer cells to adjacent and distant organs if facilitated by the expression of matrix metallopro-
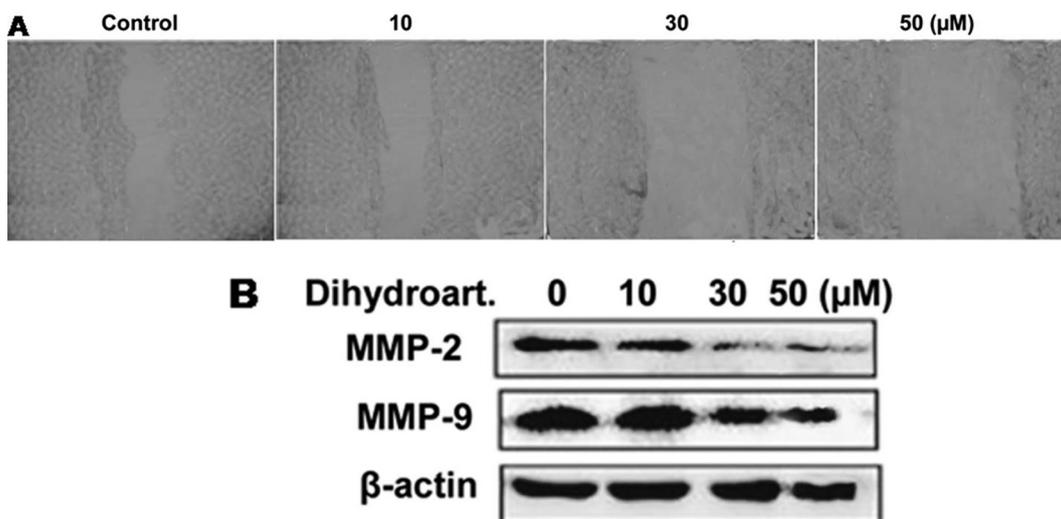

Fig. 4. Dihydroartemisinin treatment inhibited the migration potential of SW 948 cells. (A) The after attaining confluence were wounded using pipette tip, treated with dihydroartemisinin and then analyzed for migration potential. (B) The cells treated with dihydroartemisinin for 48 h were analyzed for the expression of MMP-2 and -9 using western blot analysis.
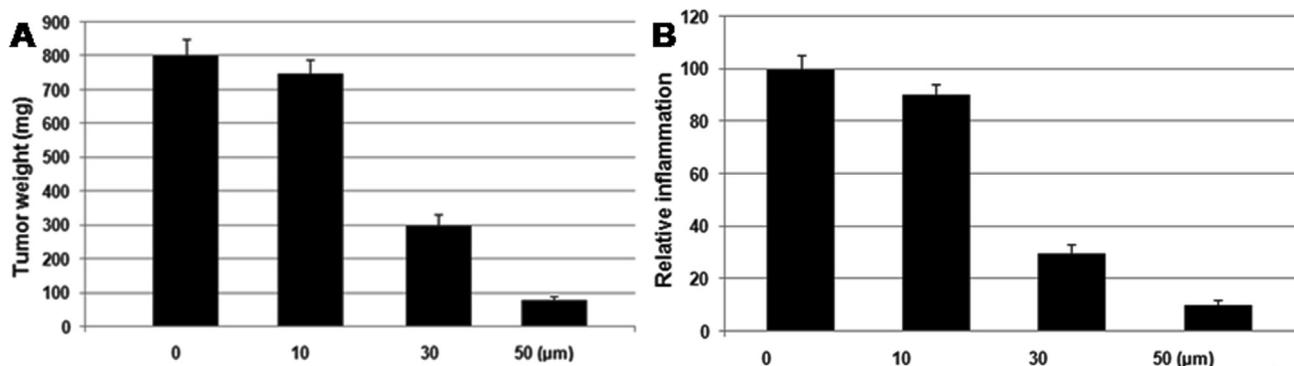
Fig. 5. Dihydroartemisinin treatment for 30 days inhibits the colon cancer growth and inflammation of colon in the mice model. The mice after treatment with 20 mg/kg doses of dihydroartemisinin for 30 days were sacrificed to extract the colon tissues for histopathological examination.

teins (Chiu et al., 2011). In the present study treatment of colon cancer cells with dihydroartemisinin marked inhibited the expression of MMP-2 and MMP-9. In the mice model of colon cancer, dihydroartemisinin treatment reduced tumor growth and inhibited the formation of inflammation.

5. Conclusion

In summary, dihydroartemisinin inhibits colon cancer growth by inducing apoptosis and increasing the expression of PPARγ. Thus dihydroartemisinin can be used for the treatment of colon cancer.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgement

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